Evaluation of TGF-β, rapamycin, and IL-2 microparticle (TRI MP) treatment for disease prevention in models of type 1 diabetes and arthritis

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Evaluation of TGF-β, rapamycin, and IL-2 microparticle (TRI MP) treatment for disease prevention in models of type 1 diabetes and arthritis

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Autoimmune diseases are disorders in which the immune system attacks the body's own tissues. These include type 1 diabetes (T1D) and rheumatoid arthritis (RA) in which the autoimmune targets are the pancreatic islets and the joints, respectively. T1D can be managed by insulin replacement therapy, but patients still have an elevated death risk and a reduced quality of life. Likewise, less than half of RA patients are able to achieve low disease activity with current therapeutic options.

Animal models of both diseases have found that the administration of antigen-specific regulatory T cells (Treg) suppresses auto-reactive effector T cells (Teff), which contribute to disease, and in turn prevents disease development or treats established autoimmunity. However, obstacles to the translation of antigen-specific Treg cell-therapy include obtaining sufficient cell numbers, and the cost and complexity of cell-therapy. Treatment with auto-antigen and/or immunomodulatory agent therapy has the potential to expand regulatory cell populations inside the body without the costs and challenges associated with cell-therapy. Our group has previously shown that subcutaneous administration of polymeric microparticles (MP), which release TGF-β, rapamycin, and IL-2 (TRI MP), expands Tregs and limits Teff levels, resulting in disease prevention in several preclinical models.

Here we evaluated TRI MP in models of autoimmunity for the first time. Despite a small increase in antigen-specific Tregs by the injection site and the investigation of multiple alterations

to both TRI MP and auto-antigen delivery, TRI MP did not have any added benefit to auto-antigen alone for disease prevention in the T1D model. In contrast, TRI MP significantly reduced the incidence and severity of arthritis in a preventative collagen-induced arthritis model of RA. While this was not associated with an increase in the percentage of Tregs, it was associated with reduced T cell proliferation and an increase in a $FoxP3^{-}CD25^{+}$ T cell population with elevated Treg-associated suppressive markers. Collectively this data suggests that TRI MP will have the greatest utility for models in which auto-antigen alone is not protective and that TRI MP dosing may need to be fine-tuned in each model to ensure increases in Tregs without systemic immunosuppression.

Table of Contents

List of Tables

List of Figures

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1.0 Introduction

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Parts of Section 1.3 and 1.4 are adapted from the following manuscript in accordance with PLOS's creative commons attribution (CC BY) license: Bassin, E. J., Buckley, A. R., Piganelli, J. D. & Little, S. R. TRI microparticles prevent inflammatory arthritis in a collagen-induced arthritis model. *PLoS One* **15**, e0239396 (2020).

1.1 Breakdown of T cell tolerance to self in T1D

1.1.1 Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which T cell mediated destruction of the insulin producing β cells in the pancreatic islets leads to sustained hyperglycemia, or elevated blood glucose[1]. Although formerly known as juvenile diabetes, this debilitating autoimmune disorder can strike both children and adults at any age. T1D is diagnosed based on symptoms of hyperglycemia (polyuria, weight loss, ketoacidosis, etc.) and distinguished from type 2 diabetes by the detection of islet-auto-antibodies and inadequate insulin production[2]. Nearly 1.6 million Americans have T1D and incidence is projected to triple between 2010 and 2050[3,4]. T1D is currently managed with intensive insulin therapy, in which insulin replacement

therapy is coordinated with diet and physical activity to maintain blood glucose levels within a target range[2]. However, insulin replacement is not a cure. Patient failure to appropriately manage blood glucose can lead to a life-threating risk of hypoglycemia, and sustained periods of hyperglycemia over time lead to increased risk (2-8x) of cardiovascular disease and death as well increased rates of microvascular complications including nephropathy and neuropathy[2,5–8].

Although the presence of auto-antibodies are involved in T1D diagnosis and B cells have an antigen presentation role in T1D development, auto-reactive islet-specific $CD4^+$ and $CD8^+$ T are thought to be directly responsible for β cell destruction[9–12]. Conventional islet antigens recognized by auto-antibodies and/or auto-reactive T cells include one or more of the following: insulin (and non-active forms preproinsulin or proinsulin), chromogranin A (ChgA), 65 kDa glutamic acid decarboxylase (GAD65), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), islet amyloid polypeptide (IAPP), 78 kDa glucose-regulated protein (GRP78), zinc transporter 8 (ZNT8), insulinoma antigen 2 (IA-2), islet cell autoantigen 69 (ICA69), and heat shock protein 60 (HSP60)[13,14]. Islet-specific T cells are activated by antigen presenting cells (APCs) such as dendritic cells (DCs) in the pancreatic lymph node (LN, pLN), infiltrate the pancreatic islets (known as insulitis), and are thought to kill β cells through a combination of contact-dependent cytolysis and production of inflammatory cytokines triggering β cell apoptosis[15,16].

A combination of genetic and environmental risk factors influence T1D progression. Over 50 different candidate genes have been identified including both insulin-dependent diabetes mellitus (IDDM) genetic loci and single nucleotide polymorphisms (SNPs)[14,17,18]. The loci encoding the major histocompatibility complex (MHC) region, in humans also known as human leukocyte antigen (HLA) is by far the highest genetic risk factor for T1D, accounting for up to

50% of the familial aggregation of T1D with odds ratios ranging from 6.8 to as high as 16 depending on the high-risk haplotypes considered[19,20]. The non-HLA loci associated with T1D have much more modest contributions to disease risk, with most having odds ratios of 1.3 or less[17]. A number of these non-HLA loci have a connection to immune function while others have connections to metabolic function, apoptosis, or have unknown function[17]. Environmental factors also play a substantial role in T1D risk as apparent from the 65% concordance rate among identical twins and the quickly rising incidence of T1D[20]. These environmental factors include both bacterial and viral exposures, which can either contribute to or protect against disease development depending on the type (microbiota composition or viral strain), timing, and context of the exposure. Mechanisms by which viral and bacterial exposures could promote autoimmunity include bystander activation and molecular mimicry. In bystander activation, pathogen associated molecular patterns (PAMPs) from the virus/bacteria itself or damage associated molecular patterns (DAMPs) from pathogen or immune killing of infected cells leads to presentation of auto-antigen in inflammatory context that results in auto-reactive T cell activation[21,22]. In molecular mimicry, T cells specific to a viral/bacterial epitope are activated in the presence of that pathogen and then cross-react with an auto-antigen of a similar sequence[21]. However, bacteria in the body's microbiome also known as the microbiota and early exposure to pathogens are thought to be necessary for proper development of the immune system including T1D protective regulatory cell populations[23–25].

The non-obese diabetic (NOD) mouse is the mostly commonly used model of T1D, in which mice spontaneously develop T1D. Although the disease progression and incidence vary slightly between different colonies, peri-insulitis and insulitis can begin as early as 3-5 weeks of age, T1D onset occurs as early as 12 weeks of age, and by 30 weeks of age 60-80% of females are

diabetic[9,26]. T1D onset is defined by hyperglycemia, ideally with two consecutive daily readings with a blood glucose cutoff of at least 250 mg/dL, although cutoffs of 200 mg/dL or lower are sometimes used[27]. Environmental factors also play a role in disease development as they do in humans. This is evident by the influence sex and pathogen exposure have on the timing of T1D onset and incidence rates [9]. Similar to humans, T1D is a polygenic disease in NOD mice with over 30 insulin dependent diabetes (Idd) genetic loci with differences (polymorphisms) relative to other strains associated with T1D susceptibility or resistance and the MHC II allele (I-A^{g7}) playing a substantial role[14]. Not all of these Idd loci have candidate genes, and many candidate genes have several different cell types /processes they can affect so the relative contribution of those cell types/processes to the increased T1D risk is often unclear. The role of genetics in T1D development can be studied using congenic mice in which NOD mice have their allele at one (or more) Idd loci replaced with the alleles from a wild type (WT) strain, or WT mice have their alleles at one (or more) Idd loci replaced with the alleles from the NOD strain. Notable examples include B6g7 mice, which have the NOD MHC II allele (Idd1) I-A^{g7} on a B6 (WT strain) background, and non-obese diabetes resistant (NOR) mice, which differ from the NOD background at four Idd loci (Idd4, Idd5, Idd9, and Idd13)[14,28]. Other NOD model variants include NOD.*scid* mice and BDC2.5 TCR mice. NOD.*scid* mice are immunodeficient due to a defect in DNA recombination that leads to a lack of T and B cells, and thus do not develop T1D[29]. BDC2.5 TCR mice, also known as NOD.BDC2.5 mice, are transgenic (tg) mice expressing the t cell receptor (TCR) of the diabetogenic BDC2.5 T cell clone (which recognizes a ChgA-derived antigen)[30,31].

Normally central and peripheral tolerance eliminate auto-reactive T and B cells or limit their function. During the negative selection phase of $CD4^+$ T cell central tolerance, tissue-specific antigen peptides expressed in the thymus due to gene expression of the autoimmune regulator

(AIRE) are presented to T cells on MHC[32]. T cells with the highest affinity interaction between these peptide-MHC (pMHC) complexes and their T cell receptor (TCR) normally either undergo deletion (via apoptosis) or differentiate into thymic regulatory T cells (tTregs)[33]. After central tolerance, peripheral tolerance is the second line of defense against deleting or limiting the function of auto-reactive T cells. Auto-reactive conventional (non-Treg) T cells that escape central tolerance in the thymus, remain naïve until they are activated in the periphery or secondary lymphoid organs (SLOs) (i.e. LN or spleen). The first way peripheral tolerance can be enforced is through ignorance, in which auto-reactive T never encounter their cognate antigen[34]. For those naïve auto-reactive T cells that do encounter their antigen, peripheral tolerance can be enforced at the time of first antigen encounter (priming) or at a later time. Peripheral tolerance can be enforced during auto-reactive T cell priming through deletion, anergy (persistence in an unresponsive state), or peripheral Treg (pTreg) induction of the auto-reactive T cell[35,36]. One of these T cell states occurs instead of auto-reactive T cell differentiation into a pathogenic effector T cell (Teff) if priming occurs in the absence of strong co-stimulation and/or inflammatory cytokines [35]. Co-stimulatory ligands are upregulated and inflammatory cytokines are produced when an APC's pattern recognition receptors (PRRs) encounter PAMPs or DAMPs[22]. Even if auto-reactive T cells are primed into Teff, their pathogenic function can be limited by intrinsic regulatory mechanisms or regulatory cells such as Tregs. Intrinsic regulatory mechanisms include Teff expression of co-stimulatory and co-inhibitory receptors (also known as checkpoint molecules), with the balance in how these receptors are expressed and ligated affecting Teff function with T cell exhaustion occurring if co-inhibitory signaling is favored[36]. Regulatory cells can also suppress auto-reactive Teff proliferation and function. The term Treg (including both tTreg and pTreg) is used to refer to suppressive $CD4^+$ T cells expressing the transcription factor FoxP3 as well as CD25 (and

$CD127^{Low}$ in humans)[33,37]. While the importance of $FoxP3^+$ Tregs to preventing autoimmunity in mice and humans is clear from loss of function mutations[33], other regulatory immune populations have been identified in a number of contexts including some that are not T cells and others that are T cells lacking FoxP3[38–42]. Tregs can suppress Teff function/proliferation both directly and indirectly (via APCs) as well as through both contact dependent and contact independent mechanisms depending on the context[43]. Mechanisms that Tregs can use include production of the immunosuppressive cytokines (such as TGF-β, IL-10, and IL-35), blocking of co-stimulatory signals (such as through CTLA-4), metabolic disruption (such as IL-2 consumption and conversion of ATP into ADP), and direct Teff killing[44]. Some of these same mechanisms are used by other regulatory populations, such as T regulatory type 1 (Tr1) cells, which produce TGF-β and IL-10[38]. Even $CD4^+$ Teff subsets that are involved in pathogen elimination can have regulatory-like function by impairing the differentiation of other Teff subsets involved in autoimmunity, such as Th2 differentiation blocking Th1 driven autoimmunity[9].

In T1D and other autoimmune diseases for disease to arise all of the following must occur: 1) auto-reactive T and/or B cell escape from central tolerance, 2) priming of auto-reactive T cells and/or B cells to produce pathogenic effector T cells (Teff) or auto-antibodies, and 3) insufficient intrinsic regulation and/or an imbalance between Tregs and Teff such that there is a failure to restrain Teff proliferation/activity or auto-antibodies production/activity. Each of these steps can be influenced by genetics and/or environmental exposures. Here the literature surrounding the breakdown of T cell tolerance in T1D will be discussed as it relates to each of these steps, with an emphasis on the NOD mouse model and a more limited discussion on what is known for humans.

The *Escape from central tolerance* section (Section 1.1.2) discusses the role of high-risk MHC alleles in this process, general thymic defects, thymically expressed antigen expressed in a

low amount or different format from the periphery, epitopes that are not expressed or presented in the thymus, and a baseline level of T cell escape from central tolerance without any risk factors.

The *Priming of auto-reactive T cells* section (Section 1.1.3) reviews the possibility of T cell ignorance in T1D; the ways genetic differences (Idd or IDDM loci and SNPs) could directly or indirectly affect the co-stimulation and cytokine interactions between APCs and T cells during priming and thus T cell fate outcomes; how bacteria may influence priming through bystander activation, molecular mimicry, or bacterial products/metabolites; and evidence regarding viral bystander activation, viral molecular mimicry, and viral cytotoxicity/anti-viral immunity in T1D priming and onset.

The *Insufficient intrinsic regulation and/or an imbalance between Tregs and Teff* section (Section 1.1.4) discusses co-stimulatory and co-inhibitory pathways that are altered in T1D, such as CTLA-4 and 4-IBB; reviews the roles of genetic polymorphisms and ongoing inflammation in affecting Treg-Teff balance by altering Treg numbers, Treg function, and/or Teff resistance to suppression; points out the possible contribution of neo-epitope specific Teff to the late upset of Treg-Teff balance, and discusses the hygiene hypothesis that early exposure to certain bacteria and/or viruses could provide T1D protection through promoting the numbers/function of regulatory cells or upregulating co-inhibitory ligands.

Lastly, the *β cell susceptibility to and/or participation in own destruction* section (Section 1.1.5) briefly reviews how some genetic polymorphisms associated with T1D may manifest due to altered gene expression in islets as opposed to immune cells, and how maladaptive apoptosis and cytokine responses by β cells may contribute to T1D.

1.1.2 Escape from central tolerance

1.1.2.1 Role of MHC II in failed central tolerance

MHC II has a major contribution to T1D susceptibility and T1D protection in mice and humans. In NOD mice the MHC II gene I-A^{g7} is necessary for disease as replacement prevents T1D development[14,45]. In humans the MHC region is the largest genetic risk factor for T1D and the highest risk MHC haplotype (DR3-DQ2 / DR4-DQ8), which occurs in 2.3% of the white population, has an odds ratio of approximately 16[17,19]. Notably MHC II alleles can be protective against T1D as well, such as DQB1*0602 in humans[46] or the introduction of I-E alleles into NOD mice[14,47]. The mechanism of this protective effect could theoretically be due to determinant capture (protective MHC out-competing high-risk MHC for islet-antigen binding/presentation), protective MHC restricted Tregs that suppress high-risk MHC restricted Teff, or the deletion of high-risk MHC restricted T cells and/or their differentiation into Tregs due to cross-reactivity with protective MHC, with the most compelling evidence to date supporting the latter[48–50].

Although the aspect of the high-risk T1D alleles which confers risk is known for several alleles, the mechanism by which these high-risk alleles lead to impaired central tolerance is not fully understood. The structural basis for MHC II alleles contributing to T1D risk is actually very similar in both mice and humans. For both I-A^{g7} in mice in the DQ risk alleles in humans (DQ2 and DQ8), T1D risk is driven by the identity of a single amino acid at position 57 which affects the charge of the P9 peptide binding pocket[48,51,52]. This P9 pocket charge is thought to result in fewer self-epitopes binding to the MHC alleles and weaker binding of the self-epitopes that are able to bind[48,53–55]. While this weak binding theory clearly explains how high-risk MHC alleles could lead to a breakdown in central tolerance, a limitation of this theory is how auto-reactive T cells are activated in the periphery if peptide binds MHC as weakly there as it does in the thymus.

Additional possible mechanisms of central tolerance escape are discussed below and shown in Figure 1.1 with particular consideration given to how these mechanisms account for the connection between high-risk MHC II alleles and T1D development.

1.1.2.2 Role of a general thymic defect in failed central tolerance

While a general thymic defect could be responsible for loss of central tolerance in T1D, this is rare in humans and unlikely to be the primary factor in NOD mice. Mutation of AIRE can lead to an autoimmune syndrome in humans that includes T1D development, but this is not a common among T1D patients[56] Although several thymic defects have been associated with the NOD background (including thymic structure and apoptosis)[9,57–60] and the NOD strain has a predisposition to several autoimmune conditions[61,62], similar levels of $CD4^+$ islet-specific (chromagranin A and insulin) T cells in NOD and B6g7 and the role of $I\text{-}A^{g7}$ in T1D susceptibility suggests this MHC allele is the primary factor enabling diabetogenic T cells to escape from central tolerance in NOD mice[9,63,64]. However, it remains possible that non-MHC NOD genetics influence the characteristics of escaping T cells[65] and apoptosis defects may play a role in peripheral tolerance.

1.1.2.3 Failed central tolerance to self-antigens expressed in the thymus

In order to have effective central tolerance to a self-antigen the antigen must be presented in the thymus, so central tolerance to a given self-antigen could be impaired if there is reduced thymic expression or an altered form/sequence (relative to the periphery). Several T1D auto-antigens are known to be expressed in the thymus including Insulin, GAD65/67, and IA-2 in mice and humans[66,67]. Insulin will be focused on as an example due to the amount known about tolerance to this self-antigen. Mice have 2 different insulin genes, Ins1 and Ins2 which differ in their tissue

expression and encoded amino acid (AA) sequence. While both are expressed in β cells only Ins2 is expressed in the thymus, and the encoded preproinsulin proteins differ at 8% of their AA sequence including at 2 AA of active insulin[68–70]. Taken together the following evidence suggests there is partial but incomplete central/peripheral tolerance to insulin in NOD mice: NOD mice have 50% less thymic insulin than Balb/c mice[71], there is a weak proliferative response to insulin in NOD mice[72,73], and NODIns2$^{-/-}$ mice have increased frequency and an accelerated onset of T1D[71,74]. Findings that both NODIns1$^{-/-}$ mice and NODIns2 overexpressing mice do not develop T1D[71,75,76] could be explained if an Ins1-specific epitope existed and Ins2 overexpression provided protection by boosting thymic or peripheral Tregs that act via bystander suppression, however evidence of cross-reactivity generated between preprosinulin1 and preproinsulin2 and a lack of dominant tolerance in Ins2 overexpressing NOD mice makes it difficult to establish a unifying theory that reconciles this data[70].

In humans, T1D is also associated with the level of insulin expression in the thymus, but variability in insulin levels arises from polymorphism of the insulin (INS) gene. Variable number of tandem repeats (VNTR) in the INS promoter act as binding sites for AIRE, with long VNTR alleles (class III VNTR) enabling 2-3 more insulin mRNA expression in the thymus than short VNTR alleles (class I VNTR)[56,77,78]. Peripheral blood mononuclear cells (PBMCs) from T1D patients including those with class I VNTR have weak to non-detectable proliferative responses to insulin/pro-insulin[79–82],suggesting some degree of central and/or peripheral tolerance to insulin. However, proinsulin-specific T cells can be detected in PBMCs by tetramer staining and higher affinity insulin-specific T cells are predominantly found in class I VNTR subjects compared to class III VNTR subjects[83,84]. This association between thymic insulin levels and affinity of insulin-specific T cells which escape central tolerance may suggest that even VNTR class I individuals

have partial protection against insulin reactivity with the deletion of the highest affinity insulin-specific T cells. The INS odds ratio (approximately 2.3) is relatively low compared to the highest risk MHC II haplotypes, and INS polymorphism is much less important to T1D susceptibility for individuals with high-risk HLA than for those without high-risk HLA[17,85,86]. While INS polymorphism substantially contributes to risk of central tolerance breakdown in individuals without high-risk MHC II alleles, the magnitude of this effect may be weaker than that of high-risk MHC haplotypes because some level of INS expression still provides a degree of protection, and INS polymorphism may only have a marginal effect in individuals with high-risk MHC haplotypes because escape of high affinity islet-reactive clones is already enabled.

1.1.2.4 Failed central tolerance to self-antigens not expressed in the thymus

However, self-antigens that are not presented in the thymus bypass central tolerance and rely on peripheral tolerance in preventing autoimmunity. This includes some conventional antigens (ex: IGRP[87,88] as well as unconventional or neo-epitope antigens, which are modified forms of genetically encoded antigens and are not thought to be found in the thymus although this has not conclusively been demonstrated[13,89]. Example neo-epitopes in mice and humans include posttranslational modification (PTM) antigens and hybrid insulin peptides (HIPs). Two relevant PTMs that occur in islets are citrullination, conversion of arginine residues to citrulline, by peptidylarginine deiminase (PAD) and deamidation (or cross-linking) of glutamine residues by tissue transglutaminase 2 (Tgase2)[13]. HIPs are formed by the splicing of insulin peptide with peptides from other auto-antigens such as ChgA or IAPP[89,90].

While it remains unclear if neo-epitopes play a critical causal role in T1D development, if they do the nature of that role likely depends on how β cell stress affects neo-epitope formation and the presence of high-risk MHC alleles. Citrullination and deamidation occur in response to the

calcium flux associated with endoplasmic reticulum (ER) stress, and while normal β cell secretory function may generate sufficient stress to produce some PTMs, production of these PTMs is enhanced by external factors which cause ER stress including inflammation and viral infection[13,91]. While HIP formation is thought to occur in β cell secretory granules due to the high concentrations of conventional auto-antigens and proteases, it is unclear if inflammatory conditions are necessary for HIP formation or enhance HIP formation through upregulation of proteasomal degradation pathways[89,90]. If physiological β cell stress is sufficient to produce these PTMs, then auto-reactive responses to these neo-epitopes could occur alongside or even before those of conventional auto-antigens[89,92]. However, if non-physiological β cell stress is needed to produce a given neo-epitope and high-risk MHC alleles are present, then auto-reactive responses may arise after auto-immunity to conventional auto-antigens generates sufficient inflammation[89]. In this scenario, neo-epitopes could still be key drivers of T1D onset despite their emergence after an initial break of tolerance as discussed in Section 1.1.4.3. If non-physiological β cell stress is needed to produce a given neo-epitope and high-risk MHC alleles are not present, then neo-epitopes could act as initiating auto-antigens with an alternative source of β cell stress such as viral infection.

Although neo-epitopes could play a role to the breakdown of central tolerance in some individuals independent of MHC-allele, for neo-epitopes to have a widespread critical causal role in T1D they must have some connection to the MHC alleles (I-Ag7 in mice or DQ2, DQ8, and DR4 in humans) that are so strongly associated with T1D development. Two of the most likely scenarios for neo-epitopes to elicit an auto-reactive T cell response dependent on high-risk MHC-alleles are: 1) The neoepitope binds preferentially to high-risk MHC alleles relative to how it binds other MHC alleles or how its wild-type (WT) counterpart binds to high-risk MHC alleles, or 2) There is an underlying dependence on failed tolerance to conventional auto-antigens.

Different PTM neo-epitopes belong to each of these categories, although the enhanced binding (relative to WT epitopes) of PTM to high-risk allele MHC alleles (Scenario 1) is likely a more common mechanism for PTM neo-epitopes in T1D and other autoimmune disease. For some PTM neo-epitopes, post-translation modifications lead to the activation of an auto-reactive T cell population that could otherwise not be activated due to an inability to bind to MHC alleles. Failed central tolerance to conventional epitope reactive T cells occurs due to absent/poor binding of WT epitope to high-risk MHCs, however PTMs such as citrullination and deamidation produce negatively charged residues enabling these epitopes to bind to the positively charged pocket of I-Ag7 or DQ2/DQ8 (Scenario 1) and permit the activation of these auto-reactive T cells in the periphery[48,92]. This is thought to be the primary mechanism by which central tolerance is broken in other autoimmune diseases including rheumatoid arthritis and celiac disease[92,93]. Recent evidence indicates that PTM can enhance binding of WT sequence T1D auto-antigens in both mice and humans (DQ2/DQ8 and DR4), including some for which the WT sequence did not detectably bind or elicit a T cell response[94–97]. However, as discussed above it remains unclear what level/sources of stress are necessary to generate PTMs in the periphery, and so even in this scenario there may also be dependence on underlying autoimmunity to conventional auto-antigens (Scenario 2) to generate β cell stress for PTM formation. For other PTM neo-epitopes, their WT counterpart has sufficient MHC binding to stimulate auto-reactive T cells but the PTM leads to a stronger response in this same T cell population. Since these PTMs are stimulating the same population of T cells as a conventional antigen, failed central tolerance to the thymically expressed conventional antigen (Scenario 2) is required for that T cell population to be present in the periphery. In some examples these PTM enhance MHC binding as described above, but in other

examples they are thought to increase antigen uptake by APCs or alter the TCR contact residues of epitopes[31,97–99].

HIPs also may fall into different categories depending on the specific neo-epitope, however the IAPP HIP is an interesting example case of a truly new neo-antigen that likely falls into Scenario 2 if it plays an important role in disease progression. Although HIPs identified to date are formed from the splicing of two different conventional auto-antigens, there are IAPP HIP reactive T cells identified in mice and humans that show no reactivity to either of the epitopes from which the HIP is spliced[90,100,101]. This suggests that (at least some) HIPs are truly new auto-antigens, and that regardless of MHC status an individual would not have any central tolerance to these neo-epitopes. It is entirely possible that HIPs are not a driving auto-antigen of T1D, and that peripheral tolerance differences not central tolerance differences account for auto-immune responses to this auto-antigen in T1D cases and not in controls. However, if HIPs play a critical causal role in T1D development, one explanation that accounts for a high-risk MHC allele connection is that these MHC alleles are necessary to generate autoimmunity to conventional auto-antigens (such as insulin and GAD65), and this in turn leads to β cell stress and the formation of neo-epitopes (Scenario 2) that nobody has central tolerance against and which do not necessarily require binding to a high-risk MHC allele[89]. Notably, this assumes that HIP formation is driven by β cell stress. It is also possible that some HIPs exhibit preferential binding to high-risk MHC alleles relative to other MHC alleles (Scenario 1). While HIPs identified to date are restricted to high-risk MHC alleles as a virtue of being studied in mice/T1D patients with these alleles, it seems unlikely that epitopes from these peptides (or other spliced peptides in β cells) would be unable to bind to MHC if they occurred in mice/humans without high-risk MHC alleles for T1D. In fact spliced

peptides are thought to be common in several cell types and act as important neo-epitopes in some cancers[102,103].

1.1.2.5 Failed central tolerance to Ins B:9-23 as a special neo-epitope-like case

Ins B:9-23, an insulin β chain peptide, is a critical auto-antigen epitope particularly in NOD mice. Ins B:9-23 is thought to be the initiating antigen in the NOD mouse, because knockout of native insulin (Ins1 and Ins2) and replacement with a transgene (Y → A AA substitution at Ins B16) that enables metabolic activity but inhibits Ins B:9-23 T cell responses prevented insulin auto-antibody production, insulitis, and T1D [104]. Its importance to the development of T1D in the NOD mouse has also been supported by findings that T cells recognizing Ins B-9:23 have been found to account for as many as 90% of the insulin-specific T cells in the islets of NOD mice and are capable of causing T1D in adoptive transfer experiments[73,105].

Ins B:9-23 is thought to have such a critical role in T1D development in part due to the ability of reactive T cells to avoid central tolerance and then be activated in the periphery in a manner similar to neo-epitopes. The first evidence supporting this theory was a much stronger proliferative response to Ins B:9-23 than to an equimolar amount of insulin protein in NOD mice[73]. This and subsequent studies found that the Ins B:9-23 epitope tends to be presented in a different (weaker) MHC register when given in the peptide form as opposed to the protein form (and binding in this peptide-associated register can be further enhanced by truncating or modifying the peptide sequence), that a distinct T cell population responds to the peptide-associated register, and that the peptide form is found in islets and the periphery but is not thought to be present in the thymus[73,106–108].

Even though the Ins B:9-23 epitope is not thought to be as critical to human T1D as it is to the NOD mouse model, evidence indicates that a similar mechanism for this epitope may

contribute to escape from central tolerance in human T1D. Similar to NOD mice, human T1D patients and auto-antibody positive individuals with a high risk of T1D have significant proliferative responses (relative to HLA-matched controls) to Ins B:9-23 but not to insulin protein[109]. Later work using tetramer staining confirmed that Ins B:9-23 specific T cells in $DQ8^{+}$ T1D patients did not respond to naturally processed native insulin, and that these Ins B:9-23 specific T cells recognized antigen in a weakly bound register of DQ8 analogous to what had been observed for $I\text{-}A^{g7}$ in NOD mice[110]. Lastly, the study showing that insulin peptide containing β cell granules led to insulin peptide presentation in the SLOs of mice also found these β cell granules in human islets[108]. Notably, this evidence that Ins B:9-23 specific T cells do not respond to insulin protein could explain a seeming paradox between the substantial fraction of T1D patients with insulin auto-reactivity (as assessed by insulin auto-antibodies) and the fact that less than 1% of diabetic patients have allergic or delayed-type hypersensitivity reactions to insulin replacement therapy[111]. A connection between Ins B:9-23 specific T cells and insulin auto-antibody formation has also been supported by recent findings of a correlation between Ins B:9-23 specific T cell frequency and insulin auto-antibody titers[112].

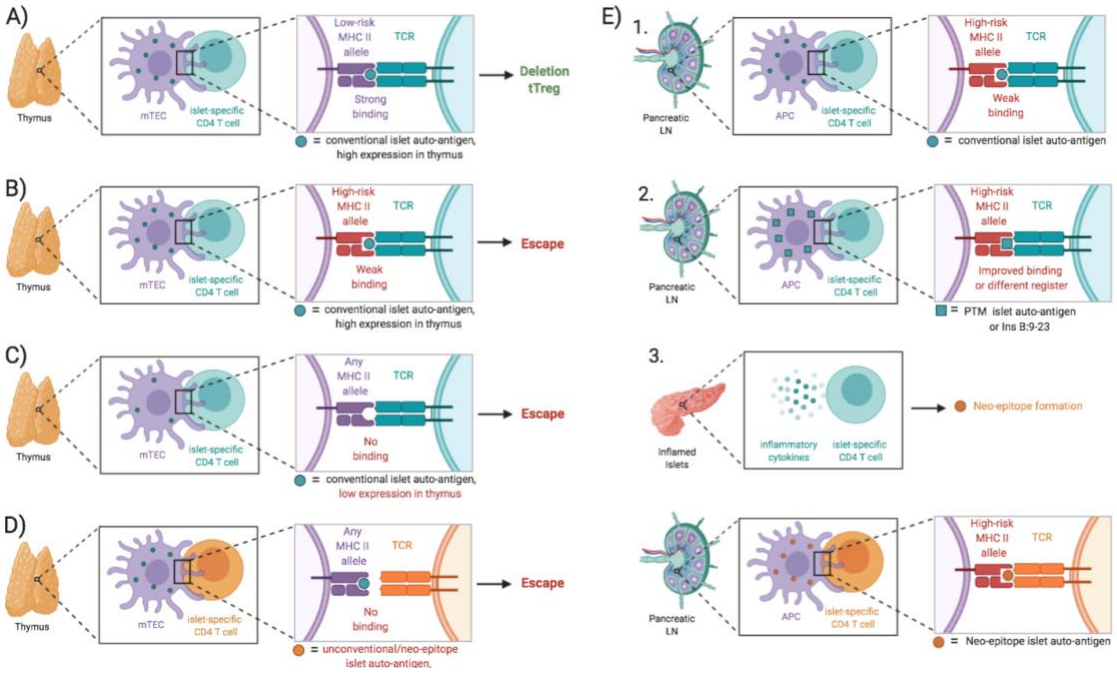

Figure 1.1 T cell escape from central tolerance in the NOD model of T1D. A) Normally self-antigen is presented in the thymus by medullary thymic epithelial cells (mTECs) resulting in the deletion or tTreg conversion of T cells recognizing the corresponding auto-antigen. This depends on self-antigen epitopes, in this case islet antigens, being expressed in the thymus and binding to MHC II alleles. B-E) T1D is enabled if islet-specific T cells escape central tolerance in the thymus which can occur because high-risk MHC II alleles (I-A^{g7} in NOD mice or DQ2, DQ8, DR3, and/or DR4 in humans) bind weakly to islet antigens (B), there is insufficient thymic levels (such as insulin in NOD mice or insulin via VNTR polymorphism in humans) of an islet antigen (C), or the islet antigen is not expressed in the thymus at all, possible because it is a neo-epitope that is only formed in the peripheral immune system (D). E) A high-risk MHC-allele(s) is required for T1D development in NOD mice and the strongest genetic risk factor for T1D in humans, indicating that this pathway is critical for T cell escape from central tolerance. There are 3 different mechanism by which a high-risk MHC II allele with weak binding of a given islet-antigen could lead to sufficient activation of islet-specific T cells in the peripheral immune system for T1D to occur: 1) The high-risk MHC allele weakly binds islet antigen, but there is still sufficient binding to weakly stimulate naïve T cells to become Teff; 2) The high-risk MHC allele has improved binding of the islet antigen in the solid lymphoid organs because there is a post-translationally modified (PTM) form with improved binding or a different form of the antigen that binds in a different register (ex: Ins B:9-23) such that the corresponding T cell population was never presented with antigen in the thymus or is provided with stronger proliferation in the lymph node; 3) If neo-epitopes play a critical role to T1D development one way they could have a dependence on high-risk MHC alleles is if high-risk MHC alleles lead to the escape (as in B) and activation (as in 1) of islet specific T cells to conventional islet antigens, and then the inflammation associated with conventional auto-reactive T cells enables the formation of neo-epitopes that are then able to active auto-reactive neo-epitope specific T cells that would otherwise not be activated.

1.1.3 Priming of auto-reactive T cells

Although auto-reactive T cell escape is necessary for T1D (or other auto-immune disease) to occur, it is not sufficient for disease development. In mice this is illustrated by Ins B:9-23 specific T cells in B6g7 mice which can be found in in the secondary lymphoid organs (SLOs) just like NOD mice, but fail to become activated[64]. Likewise in humans, although some HLA-matched healthy controls to T1D patients have detectable auto-reactive T cell populations, these populations tend to have a naïve, anergic, or regulatory phenotype as opposed to the effector or memory phenotype observed in T1D patients[112–114]. Thus, auto-reactive T cells must be primed into Teff in order to promote autoimmunity.

1.1.3.1 Source of auto-antigen for Teff priming

In order for islet-reactive T cells that escape central tolerance to become activated they must encounter antigen, which normally occurs in the SLOs. One possible mechanism of peripheral tolerance is ignorance in which T cells fail to encounter cognate antigen, such as for antigens found in immunologically privileged sites[34]. Recent studies using model self-antigens (GFP or Cre) restricted to the pancreas through coupling with insulin promoters (Ins1 or Rat Insulin Promoter (RIP)) on a B6 background suggested that these mice had ignorance based tolerance since immunization with model antigen induced antigen-specific T cell expansion comparable to WT mice[115,116]. However, other lines of evidence suggest that islet antigens are presented in the SLOs. Despite the paradigm of steady-state trafficking of migratory DCs between the tissue and draining lymph node (LN), the pancreatic LN (pLN) remains free of β cell derived antigens until after a wave of β cell apoptosis[15]. This wave of β cell apoptosis occurs in rodents regardless of strain at approximately 2 weeks of age, and evidence in NOD and B6g7 mice suggests

these apoptotic cells are a source of antigen that can active islet-reactive T cells in the pLN[117,118]. Additionally, it has been shown that the pLN is critical to Teff priming that occurs in NOD mice around this time, as excision of the pLN at 3 weeks prevented T1D development but had no effect at 10 weeks[119]. A similar wave of neonatal β cell apoptosis has been reported to occur normally in humans as well[120]. Another source of β cell antigens encountered in SLOs could be β cell granules containing insulin peptide identified in NOD, B6g7, and human islets[108]. Insulin peptide secreted from islets and the motility arrest of an insulin peptide-specific T cell clone in the pLN and other LN in NOD and B6g7 was attributed to these granules[108]. One possible explanation for reconciling findings that the immune system has ignorance to islet-restricted model antigens but encounters genuine islet auto-antigens in SLOs, is that β cell granule dependent presentation would require antigen localization to granules that would be unlikely to occur for model antigens.

1.1.3.2 Mechanisms by which genetic factors could contribute to presentation of auto-antigen in an inflammatory context for Teff priming

Normally self-antigens are presented to naïve T cells without co-stimulation and/or in an anti-inflammatory cytokine milieu leading to T cell deletion, anergy, or pTreg induction. However, for T1D to occur there must either be co-stimulation and inflammatory cytokine signals to cause Teff priming or a failure to execute signaling needed for T cell apoptosis, anergy, or pTreg induction as shown in Figure 1.2. Those signals may have either genetic or environmental origins.

β cell apoptosis is thought to act as a source of islet auto-antigen presented in SLOs in all mice strains, but differences in the way apoptotic cells are cleared may lead to priming in NOD mice. Macrophages that phagocytose apoptotic cells produce anti-inflammatory cytokines, but if un-engulfed apoptotic cells persist they can undergo secondary necrosis releasing DAMPs that can

activate APCs via PRRs to upregulate co-stimulation and produce inflammatory cytokines[22,121]. Given that NOD mice have ineffective clearance of apoptotic cells, secondary necrosis of apoptotic β cells is one explanation for the effective priming of islet-specific T cells in this model[122,123]. Specifically, double stranded DNA (dsDNA) from initially apoptotic NOD β cells was shown to act as a DAMP that resulted in DC activation through a complex pathway that involved toll-like receptor (TLR)7/9 recognition[123]. While it remains unclear how to reconcile this theory with findings that treatments of apoptotic β cells or islet antigen-coupled apoptotic cells can promote tolerance (Section 1.2.3.3), the dose of apoptotic cells may play a role [121].

Genetic differences between NOD mice and non-autoimmune strains in pathways directly involved in the processes of APC DAMP/PAMP sensing, APC co-stimulation/cytokine expression, APC migration, and T cell response to antigen presentation can all influence whether tolerance or successful Teff priming occurs. In NOD mice the loci Idd3, Idd4, Idd5, and Idd9 associated with disease susceptibility have been implicated in these processes. Analysis of the Idd4.1 loci recently found a strong candidate gene in Nlrp1b, an inflammasome forming PRR in the NOD-like receptor (NLR) family[28]. The T1D protective Nlrp1b allele in NOR mice encoded a non-functional protein while the NOD allele was capable of inflammasome activation and IL-1β activation[28]. Alleles in the NOD Idd9 loci (including 9.1, 9.2, and 9.3 regions) expressed in $CD4^+$ T cells were found to increase islet-reactive T cell proliferation and Th1/Th17 differentiation relative to the protective B10 Idd9 loci[124]. Differentially expressed genes at these loci included mTOR and several other genes involved in cell growth and development[124]. Idd3 and Idd5 loci together may also play a role in auto-reactive T cell priming. Although NOD congenic mice expressing resistance alleles of Idd3 and Idd5 (Idd3/5 mice) and those expressing resistance alleles of Idd9 (Idd9 mice) are substantially protected from T1D, the mechanism of protection is

different[35,125]. Islet-reactive $CD4^+$ or $CD8^+$ T cells adoptively transferred into Idd3/5 mice had limited proliferation in the pLN and no infiltration of the pancreas while those transferred into Idd9 mice had extensive proliferation in the pLN and infiltration of the pancreas[35,126]. While the Tregs may have played a role in the protection observed in Idd3/5 mice, the rapid nature of this tolerance, the need for protective Idd3 and Idd5 alleles to be expressed in both lymphoid and non-lymphoid compartments for maximum protection, and the resistance of Idd3/5 mice to T1D induced by PD1 blockade suggests that tolerance could be regulated through inhibition of effective islet-reactive T cell priming[127–129]. Select candidate genes for Idd3 and Idd5 that may have a role in T cell priming include IL-21 and NRAMP1. NOD mice have elevated IL-21 expression and IL-21signaling in DCs promotes their migration and is needed for auto-reactive T cell activation[130,131]. The NOD allele of NRAMP1 may alter antigen processing or APC cytokine production[128,132,133].

In addition to the direct impact NOD Idd loci have on antigen presentation/T cell priming, these loci can also indirectly affect priming by influencing DC differentiation. The conventional DC (cDC) subsets cDC1 ($CD8^+$ or $CD103^+$, $DEC205^+$) and cDC2 ($CD11b^+$ and $DCIR2^+$), plasmacytoid DCs (pDCs), and monocyte-derived DCs (moDCs, $CD11b^+$ and $DCIR2^-$) can all promote tolerance or active Teff and the roles that these subsets play depends on context and timing in the T1D disease course[134]. However, these DC subsets have different mechanisms of promoting tolerance (pTreg induction, Treg expansion, or Tr1 induction) and different locations, so NOD genetics influencing DC differentiation to alter the numbers/ratios of these subsets may contribute to priming of islet-reactive T cells likely in conjunction with other NOD defects[134,135]. One example of this abnormal DC differentiation is that NOD mice have altered ratios of splenic DCs with more pDCs and fewer cDC1s than non-autoimmune strains[136,137]. This altered ratio is likely due to a reduction in IL-2 that is associated with the NOD Idd3 locus because IL-2 inhibits Flt3

ligand, a critical regulator of DC development[136–138]. Another example of abnormal DC differentiation in NOD mice is that NOD mice have an elevated level of merocytic DCs (mcDCs, $CD8^-$ and $CD11b^-$) that is due to the Idd13 locus, although the candidate gene is unclear[139]. The recently identified mcDC subset has been shown to present normally tolerogenic apoptotic cells in an inflammatory fashion for both tumor cells and islets, and this thought to be due to their unusual antigen internalization and processing resulting in sustained antigen presentation and elevated type 1 IFN induction[140,141]. Therefore, elevated levels of mcDC in NOD mice could lead to preferential presentation of apoptotic β cell antigens by this inflammatory prone subset instead of tolerance prone DC subsets thereby enabling priming of islet-reactive T cells.

Other phenotypic differences related to antigen presentation/T cell priming that do not directly relate to Idd loci have been observed between NOD mice and non-autoimmune strains, including those involving IL-12, CD40, and CCL2, and are likely indirectly the consequence of differences at Idd loci. NOD macrophages and monocyte-derived DCs have elevated production of IL-12 in response to various stimulation signals relative to control strains even in young mice (3-4 weeks), which was attributed to a defect in NFκB regulation[142,143]. Experiments using anti-CD40L Ab administered at different time points and CD40L knockout TCR tg NOD mice have demonstrated that the co-stimulatory interaction between CD40 on APCs and CD40L on T cells is required for priming of islet-reactive $CD4^+$ T cells[144,145]. A NOD specific alteration in this pathway was recently identified; NOD cDC1 cells have elevated CD40 expression that results in a failure to delete islet-reactive $CD4^+$ T cells during priming and deletion of this population was restored by CD40L blockade[146]. Interestingly, this defect was restricted to the cDC1 population as NOD cDC2 cells, which had CD40 expression that was higher than that of cDC2 cells from non-autoimmune mice but lower than that of NOD cDC1 cells, were able to promote deletional

tolerance of islet-reactive $CD4^+$ T cells[147]. NOD mice also have impaired APC recruitment in response to the chemokine CCL2 and tg expression of CCL2 in the islets prevented T1D by promoting the migration of a tolerogenic cDC2 subset[148,149].

Genetic polymorphisms also likely contribute to auto-reactive T cell priming in human T1D. Many of the genes with loci that confer TID susceptibility (IDDM) or single nucleotide polymorphisms (SNPs) associated with T1D susceptibility are expressed in DCs and/or T cells[150]. However, some of these genes have more plausible mechanisms than others about how their associated T1D susceptibility could be due to effects on T cell priming as opposed to other immunological processes and/or cell types. Some of these affect the activation/proliferation of DCs and/or T cells. This includes the non-receptor tyrosine phosphatase PTPN22 for which a T1D associated SNP (odds ratio 1.3-1.9) may lead to altered TCR signaling and/or DC activation[150–152], and the adaptor protein SH2B3 (IDDM 20) for which PBMCs expressing the high-risk allele (odds ratio 1.52) results in greater T cell proliferation[150,153]. Others could affect $CD4^+$ T cell differentiation. This includes the transcription factor STAT4 for which a T1D associated SNP (odds ratio 1.94) may alter IL-12 signaling and thus Th1 differentiation[150,154], and the vitamin D metabolizing enzyme CYP27B1 for which a T1D associated SNP is associated with reduced expression in patient mo-DCs and could lead to less of the tolerogenic form of vitamin D3[150,155]. Other phenotypic differences related to antigen presentation/T cell priming that do not directly relate to IDDM loci or SNPs include dysregulated NFκB signaling and an inflammatory cytokine predisposition. T1D patient derived mo-DCs had dysregulated NFκB signaling, and the absence of this phenotype in rheumatoid arthritis patients as well as similar findings in young NOD mice suggest it could be a T1D-specific occurrence that precedes the onset of inflammation as opposed to a consequence of chronic inflammation[156]. Another study found an innate inflammatory state

characterized by elevated IL-1 and IL-12 in T1D families that was independent of MHC type, auto-antibody status, or disease progression[157]. The presence of a similar inflammatory state in patients with onset T1D and auto-antibody negative relatives suggests that this inflammation could be due to upstream genetic polymorphisms.

1.1.3.3 Mechanisms by which bacterial exposures could contribute to presentation of auto-antigen in an inflammatory context for Teff priming

Exposure to the environmental factors of bacteria and viruses can have either a protective effect or contribute to disease development, depending in part on the timing, strain, and context of these exposures. The protective effects of these environmental factors are discussed in Section 1.1.4.4.

Bacteria are an environmental factor that can enable islet-reactive Teff cell priming through bystander activation, molecular mimicry, or by bacterial products/metabolites influencing APC co-stimulation levels and/or Th subset differentiation (including pTregs). Although bacteria are not required for Teff priming in NOD mice as evident from the fact that germ-free NOD mice still develop T1D[158], bacteria and the microbiota composition can still have either harmful or protective effects on T1D progression by influencing T cell activation and differentiation. The most direct evidence of bacteria associated bystander activation is under conditions of enhanced gut permeability where bacterial translocation have been to shown to occur. In models treating with dextran-sulfate sodium (DSS) or streptozotocin (STZ), the translocation of gut bacteria was shown to activate islet-reactive T cells and to cause T1D[159–161]. This priming may occur either through translocation of bacteria to the pLN, or with translocated bacteria remaining in the gut mucosa but with APCs encountering these bacteria migrating from the gut to the pLN and/or islet-reactive T cells migrating to the gut mucosa due to a gut-pLN axis[159,160,162]. The degree of bacterial

translocation in NOD mice without treatments to enhance gut permeability is not entirely clear, nor is it clear that bacterial translocation would be necessary for bystander activation if APC are sampling microbiota in the gut lumen. However, it is important to note that NOD mice and the diabetes-prone biobreeding (BB) rat have increased gut permeability that precedes the onset of T1D or hyperglycemia[159,163,164], so a role of bacterial translocation contributing to T1D progression through bystander activation is certainly plausible.

Particular strains of bacteria may also enable priming of islet-reactive T cells through molecular mimicry. In the first direct evidence of this, microbial peptides from a strain of Fusobacteria that are similar to peptides of the islet auto-antigen IGRP were shown to active IGRP-specific T cells. Oral gavage of the Fusobacteria in TCR transgenic (tg) NOD mice accelerated T1D[165]. Particular strains of bacteria or the microbiota composition could also either lead to or prevent islet-reactive Teff priming by influencing the level of co-stimulation on APCs or altering Th subset differentiation of islet-reactive T cells. In an example of the former, treatment of pregnant NOD mice with the antibiotic neomycin reduced the incidence of T1D in offspring due to an altered microbiota composition which led to less CD80 and CD86 expression on APCs in the mesenteric LN and spleen[166]. A bacterial strain that can effect Th subset polarization is segmented filamentous bacteria (SFB), which was associated with T1D protection in NOD mice due to its induction of a Th17 response thought to inhibit a Th1 response[167]. Short chain fatty acids (SCFAs) are microbial metabolites that were shown to affect both co-stimulation levels and Th subset differentiation. NOD mice that lack the TLR adaptor protein MyD88 are protected from T1D under specific pathogen-free conditions and this was attributed to an altered microbiota composition with higher systemic levels of the SCFAs butyrate and acetate[158,168]. Special fiber diets that released acetate and/or butyrate when fermented by gut microbiota protected against T1D due in part to the

effects of acetate inhibiting expansion of islet-reactive T cells through reduced co-stimulation on APCs, and butyrate promoting pTreg differentiation[168]. Interestingly, the protective effects of acetate and butyrate may also be due in part to their roles in enhancing gut barrier function[168–171]. Thus, the leaky gut and T1D prone microbiota composition of NOD mice may both be due to a lack of SCFA producing strains in their gut bacteria. The underlying cause(s) of these features is unclear, but it is possible that the T cell repertoire and/or defects related to bacterial sensing play a role[158,165].

As in rodents, a leaky gut has also been identified in T1D patients and auto-antibody positive individuals prior to the onset of hyperglycemia[172–174]. Changes to the gut microbiota composition are also associated with T1D development in humans, however it remains unclear if these changes are a cause or a consequence of disease due to a limited number of randomized controlled interventional studies[25,175]. Some of the most common changes observed include diminished microbial diversity and reduced abundance of butyrate producing or other SCFA producing bacteria[25,175,176].

1.1.3.4 Mechanisms by which viral exposures could contribute to presentation of auto-antigen in an inflammatory context for Teff priming

Viral exposure is another environmental factor that theoretically could affect T cell priming in T1D. Although the majority of the research in this area has explored the theory of bystander activation over that of molecular mimicry, neither of these mechanisms seems to play a driving role in priming islet-reactive T cells in NOD mice. Viral exposure has a more substantiated role of causing rapid onset of T1D in mice with existing insulitis, but the relative contributions of viral cytotoxicity/anti-viral immune response, bystander activation of islet-reactive T cells, and other mechanisms remains unclear. The enterovirus coxsackievirus B (CVB) has been commonly used

to study viral exposure in NOD mice due to initial case reports connecting it to human T1D, its pancreas tropism, and the ability of multiple CVB strains to infect mice[177]. The most compelling evidence that viruses do not contribute to islet-reactive T cell priming in NOD mice comes from comparing the timing of spontaneous autoimmunity to the timing of T1D onset in viral exposure studies. Infection of young NOD mice (< 8 weeks old) with CVB does not accelerate T1D onset and can even protect against T1D development[178,179], while priming of islet-reactive T cells begins occurring in NOD mice as early as 3 weeks of age[118]. Studies directly evaluating molecular mimicry and bystander activation in the context of T1D in mice have found that these phenomena due occur but are not sufficient to prime autoimmunity. A role for molecular mimicry in T1D priming was initially suspected because of similar peptide sequences in the islet auto-antigen GAD65 and the P2-C protein of CVB and because NOD mice immunized with one of these peptides/proteins exhibited a cross-reactive response to the other peptide/protein[180]. However, infection of NOD or B10g7 mice with CVB virus did not generate this cross-reactive response or enhance insulitis/T1D, suggesting that an immune response to the P2-C epitope was not part of natural viral clearance and molecular mimicry was not occurring[179]. While molecular mimicry was able to accelerate ongoing insulitis in a RIP- lymphocytic choriomeningitis virus (LCMV) mouse model, it was not able to initiate T1D in this model[181]. Similarly, although non-specific bystander activation occurred with LCMV infection it was not sufficient to cause T1D in a transgenic mouse model[182]. Viral infection does cause rapid onset T1D though if NOD mice have existing insulitis. Infection of NOD mice with virulent CVB strains led to sudden onset of T1D in zero young mice (4 weeks old), in some 8-week-old mice, and in the majority of older mice (15-17 weeks old)[178,183,184]. Even a poorly pathogenic CVB strain triggered T1D onset in older NOD mice given a sufficient viral dose[185]. The role of insulitis in enabling CVB induced T1D remains unclear and

may have to do with the localization of APCs and auto-reactive T cells within the pancreas, an altered cytokine milieu that enables CVB replication in the islets instead of just in acinar tissue, and/or pre-existing stress on or depletion of β cell mass[179,184,186]. In turn, the relative contribution of direct viral cytotoxicity to islets, anti-viral immune response, and bystander activation islet-reactive T cells to rapid onset T1D following viral infection in NOD mice remains unclear[177,186,187].

In humans, viral infection can play a role either in the early pathogenesis of T1D or in T1D onset, however much of the corresponding mechanisms remains unclear. A few studies have demonstrated that infections with CVB1 in children with high-risk MHC haplotypes increases the odds later developing auto-antibodies, with one study finding an odds ratio of 2.4 for the development of insulin-autoantibodies[188,189]. While this demonstrates the role viruses can play in the early pathogenesis of T1D, it is unclear if CVB is involved in islet-reactive T cell priming or is providing stimulation to already ongoing autoimmune process as in NOD mice. The mechanisms by which virus could be contributing to early pathogenesis include molecular mimicry and bystander activation. Several putative cross-reactive epitopes shared between islet auto-antigens and viruses have been identified including GAD65 with CVB, GAD65 with cytomegalovirus, and I-A2 with rotavirus[190–193]. However, a later study exploring GAD65-CVB molecular mimicry used T cell clones instead of PBMCs and found that different T cells were recognizing the different epitopes instead of individual T cells recognizing both epitopes[194]. Bystander activation due to viral infection has also been theorized to play a role in the initiation of T1D in humans including chronic/recurrent infection of β cells or infection of adjacent exocrine/neuronal cells, however, direct evidence remains elusive[195]. In addition to bystander activation to conventional auto-antigens, ER stress of β cells caused by viral infection could result in generation of neo-epitopes and bystander activation of corresponding islet-reactive T cells[13,196,197]. Viral infection may also

play a role in T1D onset, with different mechanisms at play in acute and chronic viral infections. Acute viral infection of the pancreas with widespread β cell cytotoxicity may occur in rare cases of fulminant T1D[198,199]. However, low-grade chronic/recurrent infection of the pancreas is thought to be the predominant form of enterovirus infection in autoimmune T1D, in part because pancreas biopsies of recent-onset T1D enterovirus positive patients had detectable enterovirus in less than a third of residual insulin containing islets[196]. Instead, viral infection of the pancreas may contribute to late pathogenesis/T1D onset in humans by causing β cells to be more susceptible to auto-reactive T cell killing due to anti-viral responses such as upregulated MHC I expression and reduced expression of anti-apoptotic proteins[196,200,201].

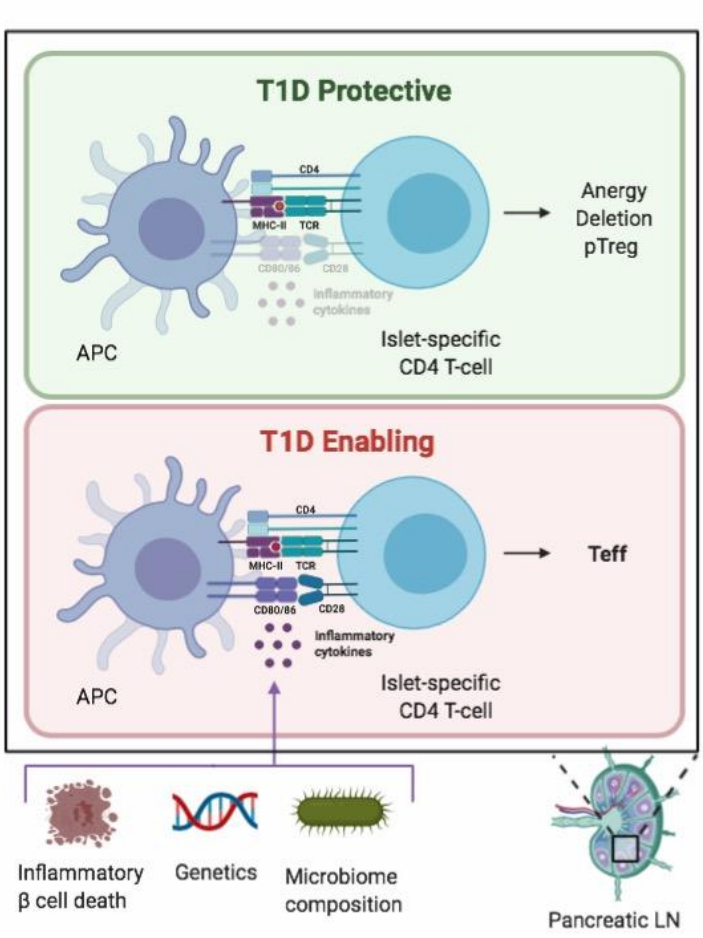

Figure 1.2 Priming of auto-reactive T cells in the NOD mouse model of T1D. **Normally self-antigens are presented to naïve T cells without co-stimulation and/or without an inflammatory cytokine milieu leading to T cell deletion, anergy, or pTreg induction. Even if islet-specific T cells escape central tolerance, presentation under these conditions protects against the development of T1D (Top). For T1D to occur there must either be co-stimulation and inflammatory cytokine signals to cause islet-specific Teff priming or a failure to execute signaling needed for T cell apoptosis, anergy, or pTreg induction when islet auto-antigens are presented in the pancreatic lymph node (LN) (Bottom). In NOD mice evidence suggests that these elevated co-stimulation and inflammatory cytokine levels could be due to inflammatory β cell death, genetic polymorphism, or the microbiome composition. Specifically, inflammatory β cell death can cause Teff priming due ineffective clearance of apoptotic debris leading to secondary necrosis; genetics can influence Teff priming with Idd loci affecting APC DAMP/PAMP sensing, APC co-stimulation/cytokine expression, APC differentiation, APC migration, or T cell response to antigen presentation (Idd3, Idd4, Idd5, Idd9, and Idd13) as well as phenotypic differences affecting these same processes that are not directly associated with Idd loci but are presumably connected downstream (ex: NF-κB, IL-12, CD40, CCL2); and microbiome composition can contribute to Teff priming through bacteria translocation/bystander activation, molecular mimicry (ex: Fusobacteria and IGRP), or the effect (or lack thereof) of bacterial products/metabolites on T cell differentiation (ex: SFB and SCFA).**

1.1.4 Insufficient intrinsic regulation and/or an imbalance between Tregs and Teff

In both NOD mice and humans not all individuals who develop a break in tolerance to islet auto-antigens develop T1D, and often there is a substantial delay between a break in tolerance and disease onset. It was originally speculated that the duration of this delay was a reflection of the time needed for auto-reactive Teff to expand and cause sufficient β cell death. However, later work has demonstrated that the presence of regulatory cells and critical co-inhibitory signals for Teff cells substantially delay disease incidence and/or the timing of disease onset, and these regulatory mechanisms act in large part after Teff activation[202,203]. Therefore, the progression from islet reactive T cell priming to T1D onset reveals an inadequate balance of co-stimulatory and co-inhibitory checkpoint molecules or inadequate levels/function of regulatory cells. This inadequate balance could be due to genetic defects in these checkpoint or regulatory pathways, or it could reflect the strength of the auto-reactive Teff response which either overpowers physiological levels of these pathways or compromises their function via chronic inflammation as shown in Figure 1.3.

1.1.4.1 Impaired co-inhibitory pathways in Teff

Although manipulation of various co-stimulatory or co-inhibitory pathways can affect disease progression in NOD mice, only a few of these pathways including CTLA-4 and 4-IBB are thought to have impaired function due to NOD genetics. In NOD mice knockout and/or antibody based blockade or stimulation studies have shown that the CD28/CD80/CD86/CTLA-4, ICOS/ICOSL, CD40/CD40L (as discussed above), OX40/OX40L, 4-1BB/4-1BBL, PD-1/PD-L1, LAG3, and TIM3 pathways all can influence T1D disease progression[204]. These different pathways act at different stages of disease development, for example PD-1 blockade led to rapid onset T1D in both young and old mice while CTLA-4 blockade only led to T1D onset when administered

prior to insulitis[36,204–206]. However, only a couple of these pathways including CTLA-4 and 4-IBB are known to be naturally perturbed in NOD mice[9]. A SNP in the NOD Idd5.1 loci results in a reduction of the production of the ligand-independent form of CTLA-4 (li-CTLA-4), a transmembrane isoform lacking the CD80/86 binding domain, by 70% relative to B6 mice[207]. Li-CTLA-4 is expressed in activated/effector T cells and intrinsically limits their proliferation and IFN-γ production[208]. However, the role of li-CTLA-4 is not redundant to that of full length CTLA-4 because congenic or transgenic NOD mice with elevated li-CTLA-4 expression have reduced T1D incidence[208,209]. It has also been suggested that NOD mice have reduced expression of full length CTLA-4 on activated T cells relative to other strains, and this was attributed to weak T cell stimulation due to a lower CD86 expression on APCs[210]. This finding is consistent with the weak MHC binding model, which suggests that some auto-reactive T cells expand because they are not stimulated strongly enough to engage intrinsic (deletion, anergy, or pTreg induction) peripheral tolerance mechanisms. The NOD allele encoding the co-stimulatory molecule 4-1BB is also associated with T1D (Idd9.3), however current evidence suggests that the NOD allele promotes disease due its impact on Tregs as opposed to overexpression or hyper-responsiveness of Teff following 4-1BB ligation[211,212]. Therefore, the role of 4-IBB will be discussed in more detail in the following section focused on impaired Treg function.

Similar to NOD mice, in humans a SNP in CTLA-4 is also associated with T1D risk and the PD-1/PD-L1 pathway is critical to preventing T1D in a population of at-risk patients. A CTLA-4 SNP had an odds ratio of ~1.15 and was associated with less mRNA for a soluble form of CTLA-4[207]. A SNP related to PD-1 has been suggested to be associated with T1D (odds ratio of 1.92) with a proposed mechanism related to transcription factor binding[213]. Co-inhibitory checkpoint molecule polymorphisms are not only associated with T1D risk, but the growing use of checkpoint

inhibitor immunotherapy for cancer has led to the emergence of a new type of diabetes referred to as Checkpoint Inhibitor -induced Diabetes Mellitus (CPI-DM). It is estimated that CPI-DM occurs in 0.2-1.4% of patients receiving checkpoint immunotherapy with a median onset of 7-17 weeks after beginning immunotherapy[214]. However, the risk is particular high for patients with the T1D high-risk MHC allele DR4, as one study found that 76% of patients diagnosed with CPI-DM had this allele[36,215]. Taken together with the findings that ~95% of CPI-DM patients were receiving a PD-1 or PD-L1 inhibitor and ~90% of CPI-DM patients who were islet auto-antibody positive had anti-GAD65 antibodies[214], these results suggest that the PD-1/PL-1 is critical to restraining the activation of auto-reactive T cells in a subset of individuals with a high risk of T1D.

1.1.4.2 Impaired Treg numbers/ function and Teff resistance to Treg suppression

An inability of regulatory cells to sufficiently suppress islet-reactive Teff activity and prevent T1D onset could be theoretically due to inadequate regulatory cell numbers, defective regulatory cell function, or Teff resistance to regulatory cell suppression, and all of these factors contribute in varying degrees in T1D[216]. While the most well studied regulatory population is FoxP3$^+$ Tregs, multiple other regulatory populations have been defined in various contexts [38–42]. Defects in several of these regulatory populations (particularly NKT cells) have been identified in the context of T1D and reviewed elsewhere[9,35]. Here, we focus on defects related to FoxP3$^+$ Tregs and Teff.

While NOD mice have a normal level of overall Tregs in SLOs there is evidence for impaired pTreg induction and reduced Treg levels in the islets. NOD mice have a normal level of tTreg generation in the thymus, and although initial studies relying on CD25 as a Treg marker suggested that NOD mice had reduced Treg levels, most later studies using FoxP3 as a Treg marker found that NOD mice had normal levels of Tregs in the SLOs over time through T1D onset[217–221].

This discrepant finding was likely due to increased proportion of FoxP3^{+} cells that are CD25$^{lo/-}$ in NOD mice[222]. It is also worth noting that some increases in Treg levels over time has also been reported in NOD pLN or spleen and this is compensatory to ongoing inflammation but insufficient to control autoimmunity[223,224].

However, it is possible that evaluating Treg levels in locations where tTregs predominate over pTregs conceals impaired pTreg generation in NOD mice. NOD mice had reduced colonic Tregs, which is consistent with the reduced level of butyrate in NOD mice (relative to T1D protected MyD88 KO NOD mice) and the role of butyrate in inducing colonic pTregs[159,168,225,226]. The cDC1 and cDC2 subsets also had impaired pTreg induction in NOD mice when using antibodies against antigen-uptake receptors to target an islet peptide to one of these subsets, but splenic DCs from NOD effectively induced pTregs *in vitro*[146,147,227]. While it is possible that impaired *in vivo* pTreg generation in NOD is due to a genetic defect in particular DC subset(s), it is more likely that is instead a reflection of the inflammatory milieu that triggers Teff priming and/or from ongoing autoimmunity. Two recent studies aimed to elucidate the role of pTregs in TID through knockout of a conserved non-coding sequence (CNS) upstream of the FoxP3 promoter (CNS1) that affects pTreg generation without affecting tTreg generation due to the presence of binding motifs for the TGF-β pathway[228]. However, these studies had contradicting findings on whether pTregs played a role in restraining T1D development[229,230]. This discrepancy could be due to differing locations/degrees to which pTregs were eliminated, slightly different sizes of CNS1 deletion, and/or gut microbiome differences[230].

Despite the normal level of tTregs in the SLOs in NOD mice, a loss of Tregs has been observed in the islets and is thought to be due in part to the NOD Idd3 allele that results in less IL-2 production. A decline in the Treg to Teff ratio in the pancreas was correlated with T1D

development in NOD mice, and islet Tregs (and Tregs from other sites of inflammation such as inflamed salivary and lacrimal glands) had reduced expression of markers of IL-2 driven T cell survival in CD25 and Bcl-2[223]. Exogenous IL-2 administration was able to increase Treg levels in islets (and other locations) and restore expression of these markers[223]. One explanation for a reduction in Treg levels at sites of inflammation in NOD mice is that NOD mice have reduced IL-2 production associated with the NOD Idd3 allele[138]. NOD Idd3 mice with a protective B6 Id33 allele have restored IL-2 production in Teff and increased Tregs in the pancreas, but no changes to systemic Treg levels[231]. This is consistent with NOD mice having a defect in Treg levels in the islets but not the periphery due to a greater dependence on IL-2 for Treg survival/expansion in the context of inflammation than in steady-state conditions.

NOD mice also exhibit defects in Treg function and Teff resistance to Treg function, and some of these are innate due to genetic differences. Suppression assays using splenic $CD4^{+}CD25^{-}$ conventional T cells (Tconv) and Tregs from young (6 week old) NOD and B6g7 mice in all four possible combinations of Tconv and Tregs (criss-cross) found that NOD Tconv are inherently more proliferative and resistant to Treg suppression than B6g7 Tconv[220]. Additional experiments using 3 week old NOD mice and NOD mice protected from T1D by MHC II I-E expression confirmed that this Tconv proliferative effect was due to the NOD background and not ongoing autoimmunity[220]. The origin of this hyperproliferative response was not identified, but it was not due to an Idd3 gene (IL-2 or IL-21), and it was transferrable in a contact-dependent fashion[220]. Although D'Alise et al. found minimal deficiency in the *in vitro* suppressive function of splenic NOD Tregs relative to those of B6g7 mice, other groups have found NOD Tregs to be less suppressive than Tregs from congenic strains with protective Idd3 or Idd9 alleles[35,138,231,232]. However, given that these alleles provide T1D protection and Tregs were taken from WT NOD

mice after the onset of insulitis, the relative contribution of Treg intrinsic defects vs. ongoing autoimmunity to this impaired Treg function remains unclear. The Idd9.3 loci was shown to influence Treg function by influencing levels of Treg subsets. Tregs from NOD Idd9.3 mice, of which 4-IBB is a candidate gene, had more 4-IBB^{+} Tregs than NOD mice and this subset shown to more suppressive than 4-IBB^{-} Tregs[212]. Although CTLA-4 is a candidate gene for Idd5, an association between protective Idd5 alleles and Treg function has not been reported[35].

Other defects in Treg function and Teff resistance develop as a consequence of ongoing autoimmunity. Criss-cross suppression experiments using splenic Tconv and CD4^{+}CD25^{+} Tregs from young (6-8 week) and older/diabetic NOD mice found that impaired Treg suppression over time/disease course was primarily due to Tconv resistance to suppression as opposed to defective Treg function[233,234]. However, this Tconv or Teff (activated Tconv) resistance to Treg suppression is likely a feature of chronic autoimmunity as opposed to a NOD specific defect because similar Tconv resistance to Treg suppression occurred in a RIP-OVA model of T1D[235]. This change may be due to higher levels of inflammatory cytokines, such as IL-21, or co-stimulation[233,235]. Whether Tregs are unstable under inflammatory conditions and can lose FoxP3 expression and produce inflammatory cytokines in the context of T1D remains controversial[236,237]. These conflicting findings about Treg instability in the context of autoimmunity could be due to transient FoxP3 expression by activated cells that due not develop a true Treg phenotype or due to lymphopenia in adoptive transfer models[238–240]. A lack of IL-2 at sites of inflammation may also enable deterioration of Treg function over time. Exogenous IL-2 boosts the level of molecules associated with Treg function, including CTLA-4, GITR, and ICOS on intra-islet Tregs[224,241]. IL-2 was also found to reduce Treg instability in an EAE model[240].

Assessment of impaired Treg-Teff balance in T1D patients has likewise identified functional impairments as opposed to reduced systemic Treg levels .There have been some conflicting findings about whether there is a reduction in Tregs in the PBMC of patients with T1D, but as in NOD mice, most studies incorporating FoxP3 (and CD127) into the panel used to identify Tregs have found that Treg levels remain normal[216,242–244]. However, it remains largely unknown if there is a sufficient ratio of Tregs to Teff in the islets of T1D patients, and the reduction of intra-islet Tregs that occurs in NOD mice from an IL-2 deficit may likewise occur in the islets/pLN of some T1D patients due to IL-2 pathway polymorphisms[216,245]. Specifically, SNPs in PTPN2 (a non-receptor tyrosine phosphatase that regulates IL-2 signaling) and CD25 (the IL-2 receptor α chain) that are associated with T1D are also associated with reduced IL-2 signaling[246–250]. In terms of Treg -Teff interaction, evidence of both impaired Treg suppressive function and Teff resistance to suppression has observed in some individuals using PBMC from T1D patients[216,251,252]. In addition to defective Treg suppression, other Treg functional defects have been associated with T1D including unstable FoxP3 expression, increased Treg apoptosis, and increased Treg production of inflammatory cytokines which may be due in part to IL-2 signaling alterations[247]. However, it remains unclear the relative contributions of genetics and ongoing inflammation to these defects since even though Treg dysfunction has been identified prior to T1D onset, existing islet inflammation could still be playing a role[247,253,254].

1.1.4.3 Possible role of neo-epitope-specific Teff emergence in shifting Treg-Teff balance

In addition to and/or instead of a defect in regulatory cell number, regulatory function, or Teff resistance to suppression, autoimmunity (insulitis) may convert to overt autoimmune disease (T1D onset) due to the emergence of high affinity Teff without sufficient corresponding Tregs to maintain balance. Specifically, this has been theorized to occur with neo-epitope-specific Teff and

may occur as part of the first wave of auto-reactive T cell priming, or as a second wave of auto-reactive T cell priming that corresponds to the conversion from insulitis to T1D onset with these neo-epitope auto-antigens formed as a result of inflammation-induced of β cell stress[89]. While this is a relatively new area of research in T1D and comprehensive evidence for this theory is lacking, there is some convincing evidence that supports pieces of this theory. While there is normally a range in TCR affinities for a given auto-antigen pMHC complex, auto-reactive naïve T cells specific for thymically presented antigens tend to be of lower affinity than foreign antigens because the highest affinity cells are deleted or converted to tTregs in the thymus[255,256]. However, if central tolerance fails either because antigen is not expressed or doesn't bind to MHC in the format expressed in thymus as is thought to be the case with PTM and HIP auto-antigens, then theoretically there will be more higher affinity naïve T cells. While a number of studies in NOD mice and human T1D patients have shown that there is a stronger response to PTM and HIP auto-antigens than WT antigens[31,94,95,101], none have compared the affinity of the pMHC - TCR interaction for these unconventional T1D auto-antigens to that of conventional T1D auto-antigens. In celiac disease patients, such an experiment demonstrated that the pMHC-TCR affinity for some T cell clones recognizing a PTM auto-antigen was comparable to that of foreign antigens[89,257]. If neo-epitope specific T cells are of higher affinity, then they likely would be more diabetogenic as several groups have shown T cell clones/populations with higher affinity for a given pMHC complex lead to more rapid T1D onset when analyzed independently from their corresponding Treg population[88,255,258]. The last piece of this theory is a lack of Tregs specific to these neo-epitopes, which could be due to a lack of tTreg induction in the thymus as described above and/or because if these auto-antigens are formed by β cell stress then they will be first presented by APCs under inflammatory conditions not conducive to peripheral tolerance. While the levels of Tregs

specific to PTM and other neo-epitopes has not been widely studied in T1D, a recent paper examining HIP-specific T cells in NOD mice found the percentage of these that were FoxP3$^+$ was significantly lower than that of Ins B:9-23-specific T cells[259]. PTM epitopes could also promote stronger stimulation of previously activated Teff (by enhancing APC uptake or altering the TCR contact site as discussed in Section 1.1.2.4) and thereby upset the Treg-Teff balance. Therefore while additional research is needed, the preliminary research suggests that activation of T cells specific to neo-epitope auto-antigens could play a role in disturbing Treg-Teff balance in T1D.

1.1.4.4 Protective environmental exposures can enhance co-inhibitory molecule expression or promote regulatory cell development/expansion

While bacterial and viral exposures have the potential to promote (or inhibit in the case of Th17 or pTreg promoting microbiota) T1D development through influencing islet-reactive T cell priming (or other mechanisms in the case of viruses) as discussed in Sections 1.1.3.3 and 1.1.3.4, these environmental exposures can also protect against T1D by boosting levels of polyclonal Tregs and/or upregulating checkpoint molecules.

The microbiota composition and associated metabolites such as SCFAs have the potential to influence the differentiation of islet-specific auto-reactive T cells (particularly due to the gut-pancreas axis) which would not only effect priming (as discussed in Section 1.1.3.3), but also affect the level of islet-specific pTregs. However, in addition to these possible antigen-specific Tregs, microbiota composition is critical to the development of polyclonal pTreg and other regulatory cell populations[225,226,260]. Single administration of the complete Freund's adjuvant (CFA), which contains heat-killed mycobacteria in an oil emulsion for sustained delivery, or weekly administration of bacterial lipopolysaccharide (LPS) provides protection against T1D in NOD mice by boosting levels of non-specific Tregs or other regulatory populations[261–264]. While

CFA and LPS are typically used as Th1 priming adjuvants, the sustained or repeated delivery may be important for TLR desensitization, in which low sustained TLR stimulation leads to a temporary refractory period in which the pathway is unable to be activated and thus leads to tolerogenic Treg inducing antigen presentation as opposed to auto-reactive Teff activation[265]. The timing of bacterial exposure may also be important, as encounter of gut microbiota during a defined early-life window was critical for tolerance induction[24].

Early exposure to viruses may be likewise important for T1D protection. Although viral exposure in older NOD mice precipitates T1D onset (as discussed in Section 1.1.3.4), viral exposure in younger NOD mice protects against T1D by increasing Treg levels and upregulating PD-L1. Infection of young (4-8 weeks old) NOD mice with diverse CVB strains provided T1D protection with virulent strains being the most protective[178]. An inability of CVB to infect islets in 4 week old mice (which was due to the cytokine milieu not the absence of the receptor needed for viral entry) may have contributed to the lack of a pathogenic effect but did not explain the protective effect[178,184]. A later study found that the mechanism behind the protective effects of viral exposure (either CVB or LCMV which lacks a pancreatic tropism) in young NOD mice involved a temporary increase in PD-L1 expression in the pLN and spleen as well as increase in Tregs[177,266].

In humans the incidence of T1D has been rising in recent decades at a rate (~3-4% per year in Europe) that cannot be explained by genetics, indicating the involvement of environmental factors in T1D development[20,267,268]. Given that viral exposures and bacteria can either have either pathogenic or protective effects depending on the strain and the timing of exposure, rising incidence could theoretically be due to either more pathogenic effects or less protective effects. The hygiene hypothesis asserts the latter, that the rising incidence of autoimmune and allergic

diseases including T1D in industrialized countries is due to improved sanitation and public health which has reduced the frequency/onset of exposure to protective infectious agents (including viruses, bacteria, and/or parasites)[20,23,269]. In the context of viruses, the lack of emergence of new pathogenic enterovirus or other pancreatropic viruses, falling frequency of exposure to enteroviruses and infectious diseases in general in countries with rising T1D incidence, and a presumably later first exposure to enteroviruses or infectious agents are all consistent with the hygiene hypothesis[23,177,270]. The protective effects of viruses that is dependent on early exposure could work through one or more of the following mechanisms: 1) Early exposure needed to help "educate" the immune system and induce or stimulate regulatory cell populations thus preventing T1D (especially for non-pancreatropic viruses), 2) Early exposure during pre-insulitis age and/or to a non-diabetogenic viral strain (such that islets are not infected by the virus) provides anti-viral immunity which prevents islets from being infected when same/similar virus is encountered a later time and/or in the presence of insulitis (only for enteroviruses or other pancreatropic viruses), 3) Antigenic competition in which higher affinity viral reactive T cells outcompete lower affinity auto-reactive T cells for APC presentation and cytokines including IL-2 and IL-7, or 4) TLR desensitization (described above)[177,265]. This second mechanism is supported by a recent paper which found not only were certain CVB serotypes (CVB2/3/4/6) protective against insulin auto-antibody development, but also that the timing of CVB exposures was important and that individuals who were exposed to the auto-antibody promoting CVB serotypes (CVB 1/5) had the best outcomes if they had prior (as opposed to concurrent or subsequent) exposure to a protective CVB serotype[188].

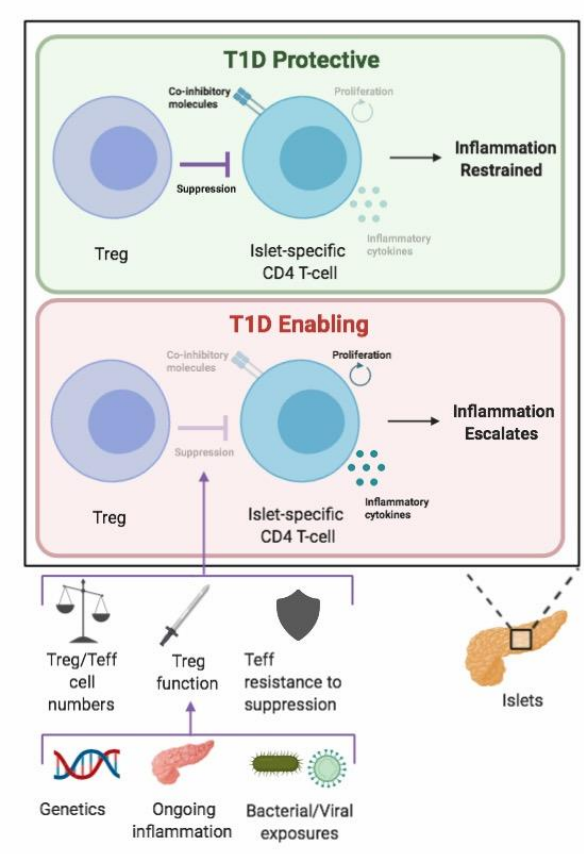

Figure 1.3 Insufficient intrinsic regulation and Treg/Teff imbalance in the NOD mouse model of T1D. **Normally auto-reactive Teff cells can be restrained by regulatory populations such as Tregs and/or by sufficient co-inhibitory molecule levels and stimulation relative to the amount of co-stimulatory molecule activity (Top). Progression from islet-specific T cell priming to T1D onset can occur when there is an inadequate balance of co-stimulatory and co-inhibitory checkpoint molecules or inadequate Treg suppression that enables Teff proliferation and inflammatory cytokine production in the islets (Bottom). Although manipulation of various co-stimulatory or co-inhibitory pathways can affect disease progression in NOD mice, only a few of these pathways have impaired function, and several of these defects have been attributed to processes or cell populations other than regulation of activated Teff. However, one example is reduced levels of li-CTLA-4 in NOD mice due to a polymorphism at Idd5.1. Defective Treg suppression in NOD mice is due to a combination of insufficient Treg numbers, impaired Treg function, and Teff resistance to Treg suppression. The origins of these shortcomings include genetics, ongoing inflammation, and insufficient bacterial/viral exposures. Specifically, although there is not a systemic loss of Tregs in NOD mice a loss of intra-islet Tregs is thought to be due to the combination of ongoing inflammation and insufficient IL-2 (Idd 3). A decrease in Treg function and Teff resistance to suppression have been shown to occur over time in NOD mice using criss-cross suppression assays. While some of this, especially for Teff resistance to suppression (with a potential role of IL-21) is due to ongoing inflammation, genetics also play a role as illustrated by the innate hyperproliferative nature of NOD T cells and impaired Treg function attributed to Idd loci (ex: IL-2 and 4-1BB). Lastly, exposing NOD mice to protective gut bacteria, CFA, or viruses at early age can boost levels of Tregs and/or co-inhibitory ligand expression and protect against T1D development.**

1.1.5 β cell susceptibility to and/or participation in their own destruction

Lastly, in addition to the steps of central tolerance, auto-reactive T cell priming, and intrinsic regulation & imbalance between regulatory cells and effectors, β cell susceptibility to killing is the last stage at which genetic and environmental factors can influence disease progression. Over 60% of genes associated with T1D in humans are expressed in the islets, and for several of these genes there are plausible mechanisms for T1D susceptibility being due to altered expression in β cells as opposed to altered expression in immune cells[271]. In particular, human T1D associated alleles including IFIH1, STX4, and CTSH may increase β cell susceptibility to apoptosis and production of inflammatory cytokines or chemokines[271].

In NOD mice, several groups have examined how β cells play a role in their own destruction in T1D is through a maladaptive apoptosis and cytokine responses. It was recently discovered that the NOD background is associated with a defect in the unfolded protein stress response (UPR) pathway, and this led to immune-independent β cell apoptosis in islets from congenic NOD I-A^K (NOD^k) mice (which are protected from autoimmune T1D due to the MHC substitution) challenged with ER stress *in vivo* (tg HEL-INS) or *in vitro* (high glucose or chemical stress)[272]. However, the fact that NOD^k mice required added stress to develop spontaneous diabetes suggests that this genetic defect is more likely to contribute to rapid/widespread β cell death in the presence of the stress (cytokines and eventually high glucose) from an existing autoimmune response than it is to contribute to the β cell apoptosis that initially primes auto-reactive T cells (although the latter is possible in the case of exposure to virus or other environmental stress). This idea that the degree of β cell death/dysfunction in response to islet-reactive T cell attack depends on the β cells response to stress/inflammation is further supported by the findings that a subset of

NOD β cells develop an inflammatory phenotype known as senescence-associated secretory phenotype (SASP), and that preventative treatment of NOD mice with Bcl-2 inhibitors that caused apoptosis of this population without affecting immune cells reduced T1D incidence[273]. Although the relative contribution of NOD genetics and ER stress from islet immune infiltration to the SASP phenotype is unclear (B6 mice were used as a control instead of NOD.*scid* mice), the finding that SASP was primarily found in 14-16 week old euglycemic mice and not 8 week old mice suggests that if there is a genetic component it only manifests in the presence of ER stress/inflammation as with the NOD^k mice.

1.1.6 Conclusions

T1D arises in both NOD mice and humans because auto-reactive T cells escape central tolerance, become primed into Teff in the periphery, and their expansion and function cannot be adequately restrained by intrinsic coinhibitory molecule expression or regulatory cell populations.

The probability of T cell escape from central tolerance is strongly influenced by high-risk MHC II alleles in both NOD mice and humans. This could be due to high-risk MHC alleles having generally weak peptide binding and a reduced peptide repertoire, and while this weak binding theory does not have the most satisfying explanation of how auto-antigen binds MHC in the periphery to active T cells, it is possible that auto-antigen binding strength is at a sweet spot where it does not bind strong enough for central tolerance but it can still bind to a sufficient degree to cause weak T cell activation. Alternatively, high-risk MHC alleles may be critical due their interaction with PTM epitopes (and Ins B:9-23). These epitopes fail to bind strongly (or bind in a different register) in the WT form found in the thymus, but do bind strongly in the PTM (or peptide) form found in the periphery. This PTM theory is supported by the fact that Ins B:9-23 is

required for T1D development in NOD mice and many PTM auto-antigens have recently been identified in mice and humans with enhanced MHC binding, however the critical causal role of most of these epitopes remains unproven. It also worth considering that the weak binding theory and PTM theory are not mutually exclusive, but likely coexist since weak WT epitope binding is a prerequisite for the PTM theory. A degree of general weak binding also likely accounts for the large number of T1D auto-antigens, some of which may not contain relevant PTM epitopes. In addition to the MHC II alleles, central tolerance is also dependent on the amount of auto-antigen expressed in the thymus. Low levels of thymic insulin in NOD mice and some humans (those with short VNTR alleles) may lead to incomplete central tolerance to insulin-specific T cells. However, the odds ratio in humans and the need for I-A^{g7} and Ins B:9-23 for T1D development in NOD mice suggest that the thymic insulin level is less critical than high-risk MHC II alleles. Auto-reactive T cells for which the corresponding auto-antigen is not found in the thymus are only regulated by peripheral tolerance. PTM epitopes and Ins B:9-23 behave in a similar manner because the form recognized are not found in the thymus. In some cases an MHC connection to PTM and HIP epitopes not found in the thymus could be due to the need for failed (central) tolerance to conventional auto-antigens in order to generate stress/inflammation to form these neo-epitopes in the periphery.

Teff priming occurs in NOD mice and human T1D patients not only because β cell antigen is presented in SLOs (since it also presented in WT mice and healthy humans), but because it is presented in an inflammatory context. Genetic factors that may contribute to auto-antigen being presented in an inflammatory context in NOD mice include defects/alterations in the clearance of apoptotic cells, APC DAMP/PAMP sensing, APC co-stimulation/cytokine expression, T cell response to antigen presentation, or DC differentiation. Some of these factors have been attributed

to Idd loci (ex: Idd3, Idd5) with the candidate genes still not entirely clear, while other of these factors have been shown to be dysregulated in NOD mice although a direct connection to Idd loci has not been established (ex: IL-12, CD40). Similarly in humans, genetic polymorphisms in some patients likely contributed to these same processes (ex: PTPN22 and STAT4), but additional phenotypic differences of dysregulated NF-κB signaling and an inflammatory cytokine predisposition have also been found. Bacterial and viral exposures may also contribute to auto-antigen being presented in an inflammatory context. In NOD mice bacteria can influence priming through translocation and bystander activation, molecular mimicry (ex: Fusobacteria and IGRP), or through the e. A leaky gut (which may be associated with bacterial translocation) and a reduced abundance of SCFA producing bacteria have been found in T1D patients but the casual role of these factors remains to be proven. Viral exposure has a more substantiated role of causing rapid onset of T1D in NOD mice with existing insulitis than it does in auto-reactive Teff priming, but the increased frequency of auto-antibodies in high-risk children exposed to CVB suggests that viral exposure may contribute to Teff priming or early pathogenesis in human T1D.

In order for T1D to progress from auto-reactive Teff priming to disease onset, intrinsic regulatory mechanisms as well as regulatory cell levels or function must be inadequate to control Teff. Polymorphisms affecting expression of unconventional forms of CTL-A4 may play a role in impaired intrinsic regulation of auto-reactive Teff in both NOD mice and humans. Regulatory cells could theoretical be deficient in numbers or function, but Teff could also be resistant to suppression. Although NOD mice do not have systemic deficiencies in Treg numbers, evidence suggests that may have inadequate levels of pTregs and insufficient Treg levels in the islets due to a lack of IL-2. They also have impaired Treg function and Teff resistance to Treg suppression arising both from innate defects (Idd3 and Idd9 affecting IL-2 and 4-1BB respectively) and from

the impact of ongoing inflammation. Similarly, in humans the general consensus is that there is not a systemic shortage of Treg numbers (although intra-islet Treg levels are not known), and defects in both Treg function and Teff resistance to suppression have been observed. However, the relative contribution of genetic polymorphisms and ongoing inflammation to these defects remains unclear. Other ways that imbalance could develop between auto-reactive Teff and regulatory cells (or co-stimulatory ligand expression) include the late emergence of neo-epitope-specific Teff without a corresponding Treg population or the lack of early exposure to protective bacteria and viruses that promote regulatory cell development/expansion and co-inhibitory molecule upregulation. The latter (i.e. the hygiene hypothesis) is particularly compelling in explaining the rapid rise in human T1D incidence in regions with improved sanitation and public health.

Thus, even though much is known about the mechanism of each of these three steps, they are multifactorial and different mechanisms are likely responsible in different individuals. Human T1D patients are diverse in both their genetics and environmental exposures, so different sub-populations of T1D patients will have a different route by which tolerance is broken at each of the three steps (i.e. high-risk HLA alleles vs. short insulin VNTR alleles for central tolerance, or innate inflammatory state due to genetic polymorphism vs. permissive gut microbiome for Teff priming). Although it is satisfying to attribute a distinct cause to each of the three steps, it also worth considering that multiple mechanisms likely contribute to a given step for a given individual (particularly in NOD mice) and that there is also a degree of randomness and snowballing of previous steps. For example, recent evidence suggests central tolerance is not as stringent of a process as initially thought and WT mice and humans without high-risk HLA-alleles contain a surprising number of auto-reactive T cells – these cells are just held in check by peripheral tolerance mechanisms[274–276]. Likewise, in addition to the mechanisms described above by which

intrinsic pathways and regulatory cells fail to control Teff, the size and the strength of activation of auto-reactive Teff may simply overcome physiological levels of regulatory cells in some cases. Perhaps the strongest evidence of the role of random or stochastic factors is that even though NOD mice are genetically identical and have relatively homogenous environmental exposures, there is typically not 100% T1D penetrance in a colony and there is a relatively large degree of heterogeneity in the timing of T1D onset.

Understanding the steps and mechanisms by which tolerance is broken in the development of T1D offers insight into approaches to treat T1D. An understanding of the role of environmental exposures in T1D development has led to unconventional potential treatment approaches such as diet/microbiome modification or vaccination. Additionally, an understanding of the inflammatory context in which auto-antigen is presented imply that auto-antigen immunotherapy may need to take extra effort to promote presentation in a tolerogenic context. Lastly, the defects associated with regulatory cell function suggest a possible need for not only providing/generating regulatory cells, but also supporting continued regulatory cell function.

1.2 Antigen-specific approaches to restore tolerance in T1D

1.2.1 Introduction

Although there is a substantial loss of β cell mass at the time of T1D diagnosis, both immune-based preventative treatment and therapeutic treatment of (recent) onset T1D are potentially viable for patients. Preventative treatments are categorized as either primary prevention if treating patients prior to islet auto-antibody appearance (typically based on high-risk HLA

genotype and/or first-degree family member with T1D), or secondary prevention if treating patients after islet auto-antibody appearance (but prior to T1D onset)[2]. The patient population (Stage 1) eligible for secondary prevention is at considerable risk of developing T1D as up to 85% of children with two or more islet auto-antibodies develop T1D within 15 years[277,278]. Despite the presence of hyperglycemia at the time of diabetes diagnosis, several lines of evidence suggest that recent-onset T1D patients still have some β cell mass with functional potential remaining. Although an early estimate suggested that ~90% of β cell mass was lost at time of T1D diagnosis[279], more recent evidence found that β cell mass lost at T1D diagnosis is highly variable and dependent on age with an average of 40% of β cell mass lost at T1D diagnosis for a 20 yr old patient[280]. Instead of (near) complete β cell loss, the onset of hyperglycemia in T1D is likely due to partial β cell loss and dysfunction of a substantial portion of the remaining β cells. While this is still not fully understood, it is thought that the dysfunction of remaining β cells is caused by elevated insulin demand, glucose toxicity, and/or inflammation induced stress and this dysfunction may be temporarily reversible before permanent β cell dysfunction or loss occurs[16,20,281–284]. This idea of remaining functional β cell mass is supported by findings that metabolically stabilized recent-onset T1D patients have approximately 50% of the normal insulin secretion response (as measured by C-peptide, cleavage byproduct active insulin that can be used to distinguish endogenous insulin production from insulin therapy)[282], and many of these patients have a 'honeymoon' phase in which they are temporarily able to reduce or even stop insulin therapy[20,285]. While the ability to reverse T1D and re-establish euglycemia without insulin-therapy is the ultimate goal for immune-based treatments of T1D, preservation of β cell function is still a goal with considerable benefit as even modest stimulated C-peptide levels are associated with reduced diabetic complications[7,286].

Likewise both T1D prevention and reversal can be evaluated in NOD mice models, with the rigor of difficulty depending on the timing of intervention and model type. Prevention models include spontaneous T1D and adoptive transfer of Teff into NOD.*scid* mice. While the number of islet-specific Tregs to significantly delay T1D onset in a spontaneous T1D model can be 10x or more that needed in an adoptive transfer T1D model[287,288], the difficultly in preventing T1D in these models also varies depending on the timing of intervention in the spontaneous model (ex: early is normally at 4 weeks old vs. late is normally at 12 weeks old or later) as well as the number, source, and activation status of Teff used in the adoptive transfer model (ex: diabetic spleen, naïve TCR tg T cells, or activated TCR tg T cells)[134,287,289,290]. In NOD mice T1D reversal models, mice are treated after the onset of hyperglycemia with the aim of restoring euglycemia (true reversal), prolonging re-established euglycemia, or prolonging survival[291–293]. Protocols also vary in terms of the blood glucose cutoff used to denote T1D onset, the time after T1D onset that the first treatment is given, whether insulin (pellets) is used to provide metabolic stabilization before/during treatment, and the degree/duration of blood glucose reduction to qualify as reversal[27,288,291,292,294–297].

The broadest categorization of new interventions for the treatment of T1D distinguishes approaches that interfere with destruction of existing β cells and those that seek to restore or replace β cells. Approaches that aim to restore or replace β cells such as islet transplantation, microencapsulation devices for β cells, or conversion of other islet cells into β cells will still need to prevent immune destruction of the replaced β cells as well as overcome other approach-specific challenges which include inadequate vascularization and a foreign body response[298–301]. Interventions for treating T1D that interfere with destruction of existing β cells can be categorized both by their ability to produce tolerance and their antigen-specificity. Operational tolerance, in

the context of treatment for autoimmunity and transplantation, generally means sustained (indefinite or at least long-term) protection of the self (or transplanted) tissue of interest from immune attack without continued treatment[302]. Antigen-specificity indicates that only the immune response against the desired self (or foreign) tissue is inhibited as opposed to non-specific effects which could impair the ability of the immune system to fight pathogens or cancer. Although some non-specific approaches for treating recent-onset T1D have had modest effects on preserving β cell function[281], these approaches are not used in clinical practice in part due to the risk-benefit ratio relative to the standard of care of insulin replacement. The ideal treatment for T1D would induce tolerance in an antigen-specific manner.

It is theoretically possible that this could be achieved either by providing/generating an islet-specific regulatory cell population or by antigen-specific T cell depletion[303,304]. However the generation of islet-specific regulatory cells is the more frequently pursued approach for antigen-specific tolerance, as ongoing thymic output and the need for the complete elimination of islet-reactive T cells of multiple specificities make antigen-specific T cell depletion unfeasible in practice[305]. Different regulatory immune populations that could be generated include $FoxP3^+$ regulatory T cells (Tregs), T regulatory type 1 (Tr1) cells, as well as a variety of other T cell and non T cell populations[33,39–42,44]. Even $CD4^+$ effector T cell (Teff) subsets that are involved in pathogen elimination can have regulatory-like function by impairing the differentiation of other Teff subsets involved in autoimmunity, such as Th2 differentiation blocking Th1 driven autoimmunity[9].

These antigen-specific regulatory cell populations can either be provided, such as through islet-specific Treg cell-therapy, or be generated *in vivo* through approaches which can be broadly categorized by the use of auto-antigen and/or immunomodulatory agents. Immunomodulatory

agents or properties restrict/modify an immune response. In the case of antigen-specific tolerance, immunomodulation aims to promote tolerance and may involve suppressive cytokines, cytokines supporting proliferation/differentiation, and/or select immunosuppressive drugs. However, there are several notable differences between immunosuppression and immunomodulation. Conventional immunosuppression is given in large (systemic) doses so it non-specifically suppresses the immune system. It is also given long-term with continued treatment needed to maintain immune system non-responsiveness. Although immunomodulation may use some of the same drugs, it can suppress the immune system in an antigen-specific manner if immunomodulatory agents are given in small doses localized to either the endogenous antigen of interest or co-localized with administered antigen. Immunomodulatory agents can also have long-term effects (i.e. tolerance) arising from short-term treatment by eliciting a regulatory cell response. Delivery systems can also play a role in promoting the presentation of antigen in a tolerogenic context without the use of other immunomodulatory agents. This immunomodulatory ability can either be due to the delivery system targeting a particular tissue or cell population, or due to the composition of the delivery system itself (i.e. phospholipid or polymer composition). Commonly used delivery systems for antigen-specific tolerance that can have immunomodulatory properties include nanoparticles (NP) and microparticles (MP). NP (and MP up to a few μm in diameter) can be intravenously (i.v.) administered allowing for distribution throughout the blood and interactions with immune populations that may otherwise be inaccessible (ex: splenic APCs). In contrast, larger MP cannot be i.v. administered without causing embolization, but they can be injected s.c. and remain at the injection site providing sustained release of encapsulated drug without being phagocytosed[306].

Antigen-specific tolerance for T1D has the potential to be achieved with DC or Treg cell-therapy as well as with auto-antigen and/or immunomodulatory agent therapy and the testing of these approaches is reviewed here. An emphasis is placed on newer approaches in NOD mice and a brief review of clinical testing to date includes possible explanations for thus far disappointing results for auto-antigen immunotherapy.

1.2.2 DC or Treg cell-therapy

1.2.2.1 DC cell-therapy

DC cell-therapy has had success with T1D prevention and in one case reversal in the NOD model, however there have been inconsistent findings regarding the benefit of exposing DCs to islet auto-antigens. Tolerogenic DCs (tolDCs), generated through culture with GM-CSF and IL-4 alone (i.e. conventional bone marrow derived DCs BMDCs) or with additional immunomodulatory agents to promote a tolerogenic state, have been successful in preventing T1D in NOD mice without auto-antigen to provide specificity. The mechanism of action has typically been attributed to Th2 skewing or Treg expansion[307–311]. Trafficking of these injected DCs to the pLN or pancreas has been observed in several studies, suggesting that they may be presenting endogenous auto-antigen. However, even though added benefit to pulsing tolDCs with auto-antigen has been seen in models of arthritis and MS[312–315] and in some T1D studies[316,317], several T1D studies have found the addition of auto-antigen impairs tolDC T1D prevention[307,309,310]. The mechanism remains unclear, but it may be because the antigen dose used was too high[310]. Most studies examining DC cell-therapy in NOD mice have only published data on prevention models, however one group that previously showed T1D prevention did find that multiple (8) injections of tolDC was able to stably reverse T1D onset (onset defined at BG > 300 mg/dL and stable reversal was BG < 280 mg/dL for

~ 20 weeks) in over 50% of mice[318]. This same group was also the first to investigate tolDC in a clinical trial for T1D. DC cell-therapy was well tolerated, and although the Phase I trial was very small (n=1 total) and thus not powered to assess efficacy, promising signs included detectable C-peptide levels in patients for which C-peptide was undetectable prior to treatment and temporary increase in a potentially beneficial B cell population[319]. The latter finding was consistent with preclinical data in which tolDCs were shown to not only increase Tregs, but also induce/expand a population of IL-10 producing B regulatory cells[309,318].

1.2.2.2 Treg cell-therapy in NOD mice

Despite evidence of Teff resistance to suppression and impaired Treg function in NOD mice due to a combination of intrinsic NOD genetics and ongoing inflammation associated with autoimmunity, Treg cell-therapy is able to prevent T1D or reverse recent-onset T1D in NOD mice if administered Tregs are of a sufficient number and potency. Initial studies evaluating Treg cell-therapy in NOD mice used *ex vivo* expanded Tregs from BDC2.5 mice and found that these islet(ChgA)-specific Tregs were able to completely prevent T1D in T cell adoptive transfer models into immunocompromised mice with a NOD background (NOD.*scid* or NOD.RAG$^{-/-}$), to completely prevent or substantially reduce the incidence of spontaneous T1D in NOD mice, and to reverse recent-onset T1D (0-2 weeks after onset) in up to 60% of NOD mice[287,288,291]. These studies also demonstrated the importance of antigen-specificity to Treg potency and that a therapy based on a single auto-antigen can be effective if it elicits infectious tolerance. Activated islet-specific Tregs were able to reduce the incidence of T1D with as few as 5,000 cells in an adoptive transfer prevention model and as few as 50,000 cells in a spontaneous prevention model[287,288]. However, polyclonal Tregs had no effect in these same models at equivalent cell numbers and even at up to 20-30 times the cell numbers[287,288,291]. Despite the ability of BDC2.5 Treg cell-

therapy to provide long-term (or indefinite) protection from T1D, using congenic Thy1.1 and Thy1.2 mice it was demonstrated that the administered Tregs do not survive long-term but result in increased levels of endogenous Tregs in the pLN and pancreas that were not BDC2.5 specific[288]. Presumably this occurs through the creation of a tolerogenic milieu which promotes Treg induction/expansion in a process known as infectious tolerance[320], and in this case results in Tregs specific to a variety of islet antigens given that intra-islet T cells are exclusively specific to islet antigens[321]. More recently, it was demonstrated that the natural repertoire of intra-islet Tregs, but not more polyclonal Treg populations from various lymphoid tissues, was as effective as BDC2.5 Tregs in preventing spontaneous T1D in NOD.CD28$^{-/-}$ mice (which have a greater T1D incidence and faster onset due to Treg defects)[322]. Many of these intra-islet Tregs were specific to insulin epitopes[322].

In addition to antigen specificity, other factors that affect Treg potency in T1D prevention include expression of markers that affect localization and the strength of TCR stimulation. The above studies found that BDC2.5 Treg subset expressing CD62L, which is involved in LN homing and marks a central memory phenotype as opposed to an effector memory type in activated T cells, was responsible for T1D protection[288,291]. Additionally expression of the Th1 associated transcription factor Tbet and chemokine receptor CXCR3 by a subset of Tregs is important for T1D protection, as Treg specific deletion of Tbet resulted in accelerated T1D onset[323]. Although the mechanism of the protection provided by these Th1-Tregs was not clear and did not involve increased trafficking to the islets, it may improve co-localization with Th1 Teff within the islets resulting in better Teff suppression[323]. Just as Teff with higher T cell affinity are more diabetogenic, Tregs that receive stronger TCR stimulation, as defined by elevated expression of the marker CD5, are more suppressive. Intra-islet CD5^{hi} Tregs were significantly more effective

than intra-islet $CD5^{lo}$ Tregs at preventing T1D in an adoptive transfer model[324]. The mechanism of this improved suppressive function by $CD5^{hi}$ intra-islet could either be due to elevated expression of suppressive Treg markers or expression of T-bet[324]. Together, these results suggest that even Tregs recognizing the same antigen are not necessarily of equal potency, and the strength of their TCR-pMHC interaction as well as their localization affect their ability to suppress Teff.

1.2.2.3 Translation of Treg cell-therapy

Initial Treg cell-therapy clinical trials in autoimmunity and transplantation, including two trials in T1D, have almost exclusively used polyclonal Tregs due to limitations in the yield of Tregs that can be isolated from PBMCS and their expansion capacity[325,326]. These phase I T1D polyclonal Treg trials each treated 12-14 recently diagnosed T1D patients with different doses of expanded autologous Tregs ranging from 5 million cells to nearly 300 million cells and found no serious adverse events attributed to cell-therapy[326–328]. Prolonged maintenance of C-peptide levels and long-term persistence of administered Tregs (up to 25% of peak levels at 1 year) in the blood was observed in some patients[326–328]. However these trials were not designed to evaluate efficacy, and larger phase II studies with an appropriate control group are underway[326]. Potential issues with polyclonal Treg cell-therapy for T1D include the blood volume needed (for pre-expansion Treg yields) being prohibitive for younger children[329], remaining concerns about non-specific immunosuppression[330], and lower potency than antigen-specific Tregs.

Translational approaches for the generation of antigen-specific Tregs include chimeric antigen receptors (CAR) Tregs, however identification of an appropriate antigenic target for CAR Treg in T1D may be a challenge. CARs are engineered to combine an extracellular antibody domain and intracellular T cell signaling and co-stimulation domains. This enables T cells to mount a strong response to a cell-surface or extracellular antigen regardless of the MHC allele or

level of co-stimulation expressed by APCs. Given the success of CAR T cells in the treatment of hematologic cancers[331], CAR receptors have also been studied in other contexts including for CAR Tregs in autoimmunity and transplantation. CAR Tregs have demonstrated pre-clinical effectiveness in models of colitis, multiple sclerosis, graft vs. host disease, and skin transplantation[332–336]. However, there are antigen target limitations for CAR Tregs in that a high density of antigen is likely needed to trigger activation and antigen targets are limited since this approach is not compatible with intracellular antigens normally processed and presented by APC[337,338]. Thus while transplantation remains an attractive application for CAR Treg due to an ability to target donor MHC alleles[332], identifying suitable antigen targets in T1D may be more challenging. For example, an insulin-specific CAR Treg was unable to prevent T1D in NOD mice[339]. Additionally, there are concerns about CAR Treg cytotoxicity towards target cells[335,337,340], and Treg instability leading to the formation of pathogenic ex-Tregs[236] would be more problematic with an antigen-specific population.

Polyclonal Treg cell-therapy limitations of low Treg yields and lack of antigen-specificity could also be addressed by enforced expression of FoxP3 in conventional (antigen-specific) $CD4^+$ T cells, however the ability of these cells to fully mimic Tregs remains a concern. Approaches for forced FoxP3 expression in both mouse and human cells include retroviral gene expression of Foxp3 itself in conventional polyclonal or antigen-specific $CD4^+$ T cells[341–343], as well as a recent gene editing approach using TALEN cleavage and homology directed repair to insert a strong promoter at the FoxP3 locus leading to a controlled insertion site and more sustained FoxP3 expression[344]. However, while these studies found these FoxP3 expressing T cells to be suppressive *in vitro* and *in vivo*[341–344], other studies have suggested that both FoxP3 expression and

independent epigenetic changes are needed to encompass the Treg phenotype, raising concerns about the long-term stability and function of these approaches[337,345–347].

Regardless of the type of DC or Treg cell-therapy approach used, remaining challenges include the cost and complexity of good manufacturing practice (GMP) isolation combined with cell expansion and/or gene editing, the lack of an off-the-shelf product, and concerns about DC or Treg instability[238,337,348,349].

1.2.3 Auto-antigen and/or immunomodulatory agent therapy

1.2.3.1 Treatment attributes to consider for auto-antigen immunotherapy

Auto-antigen immunotherapy has the promise of activating endogenous T cells to expand/induce auto-antigen specific Tregs or other regulatory / Th1-antagonistic population using an off-the-shelf product without the cost and complexities associated with administering cell-therapy. Theoretically, immunotherapy with a single auto-antigen can generate a strong enough response to produce linked-recognition/infectious tolerance to suppress Teff recognizing multiple islet antigens and provide disease protection akin to BDC2.5 Treg cell-therapy. However, to do so presumably requires the generation of a regulatory population of sufficient size and potency. When administering auto-antigen immunotherapy with the aim of inducing tolerance, important treatment attributes include dosing, route of administration, and the selected auto-antigen. In addition to attribute-specific considerations, decisions for all of these parameters would ideally also be influenced by the inflammatory milieu.

A low dose of auto-antigen is critical for Treg induction and/or expansion in the absence of other immunomodulatory drugs, however antigen dose is less critical if a tolerogenic microenvironment is enforced with additional therapeutic agents. In the absence of exogenous

cytokines, the percentage of expanded/induced murine T cells that are FoxP3$^+$ Tregs is highest when a relatively low dose of auto-antigen or concentration of anti-CD3 is used[350,351]. This was attributed to inhibition of endogenous TGF-β production and higher antigen dose causing stronger TCR stimulation which activates the Akt/mammalian target of rapamycin (mTOR) pathway[350], a metabolic pathway necessary for Teff differentiation[350–352]. Importantly, what constitutes a "low" auto-antigen dose versus a "high" antigen dose is dependent on the strength of pMHC-TCR interaction, with Treg promoting "low" auto-antigen *in vitro* doses for two different BDC2.5 peptide mimotopes (0.8 nM and 0.4 μM) found to be of a comparable magnitude to their EC_{50} concentrations[350]. While more of an antigen with low pMHC-TCR affinity is needed to achieve a Treg inducing/expanding dose, this is not necessarily the same or as effective as less of an antigen with high pMHC-TCR affinity as discussed below. Low antigen-dose was also found to promote Treg induction using human cells, with the greater frequency of FoxP3$^+$ Tregs observed attributed at low antigen doses attributed to reduced Teff expansion[353]. The importance of low auto-antigen levels for Treg induction has also been shown *in vivo*. When the dose of an insulin peptide mimotope administered increased above 5μg/day, Treg conversion decreased in insulin-specific TCR tg mice[354]. Likewise, T1D prevention studies administering insulin in NOD mice have found a bell-shaped dose-efficacy curve, where too low or too high a dose was ineffective [355–357]. Although low antigen/anti-CD3 dose still plays a role in Treg induction/expansion the presence of immunomodulatory agents[351,353,358], antigen dose is less of a concern in this situation since rapamycin (mTOR inhibitor) enables Treg expansion and TGF-β enables Treg induction even in the presence of strong T cell stimulation (anti-CD3/CD28)[359,360]. In addition to the frequency of Tregs (or Treg:Teff ratio) that many studies looking at antigen dose have focused on, the number of antigen-specific Treg cells matters as well as shown by Treg cell-therapy studies.[287,288,291]. The

combined use of auto-antigen and immunomodulatory agents *in vivo* can further improve Treg numbers while maintaining a favorable Treg/Teff ratio either through improved Treg conversion/inhibition of Teff expansion[361], or possibly enabling a higher antigen dose that elicits greater Treg numbers while maintaining a comparable Treg:Teff ratio analogous to strong *in vitro* stimulation of Treg induction/expansion in the presence of immunomodulatory agents. Unlike low dose auto-antigen alone which is impaired in its ability to induce/expand Tregs in the presence of inflammatory cytokines[350], the combination of immunomodulatory agents and auto-antigen increases the likelihood that antigen is presented in a tolerogenic context for Treg induction/expansion through multi-faceted effects on co-stimulation levels and cytokine production[362,363]. However outcomes still may vary depending on the specific immunomodulatory agent and inflammatory signals present (ex: TGF-β and IL-6 promoting Th17 polarization)[364]. Therefore, while auto-antigen alone can induce Tregs at low antigen doses, combining auto-antigen with immunomodulatory agents has the potential advantages of a greater range of antigen dosage that promotes Tregs, a greater number of Tregs while producing a similar or better Treg:Teff ratio, and greater resiliency in the face of inflammatory conditions.

The route of auto-antigen administration can influence the degree to which auto-antigen is presented to the immune system and whether that presentation occurs in a tolerogenic context. For example, oral administration of antigens is generally tolerogenic due to the default tolerogenic environment of gut lymphoid tissue[365,366], but degradative (pH and enzymes) and absorption barriers (gut epithelium and mucus) associated with this route limit the fraction of administered antigen that is presented[367].

Although a variety of different auto-antigens and auto-antigen formats have been successful at preventing T1D in NOD mice as discussed below, some auto-antigens and/or formats

could be better than others and this may have implications for T1D reversal in NOD mice or clinical translation of T1D treatments. The selection of auto-antigen includes not only the antigen itself (i.e. insulin, GAD65, ChgA, etc.), but also its format such as if a peptide is used instead of a whole protein and if so, which peptide epitopes and whether a WT, PTM, or artificial mimotope sequence is used. This may be important because different auto-antigens may elicit different strengths of T cell stimulation and/or have a different pre-existing state of the antigen-specific T cell repertoire which in turn could affect the T cell response. The pre-existing state, including the antigen-specific T cell population size and fraction of effector and memory cells, may vary between different auto-antigens and affect the response to auto-antigen immunotherapy. The strength of the T cell response is important not only for ensuring a relatively large expansion of Tregs while maintaining a favorable Treg:Teff ratio, but also for Treg stability and function. The strength of T cell stimulation depends on several variables including not only the dose of antigen and strength of peptide-MHC binding which together influence the amount of a given peptide presented at a single time by an APC, but also on the strength of interaction between a pMHC complex and a T cell TCR. Notably, experiments using TCR tg T cells have shown that while a high antigen dose can compensate for low pHMC-TCR affinity in terms of T cell proliferation, it is not equivalent to a low dose of antigen with a high affinity pHMC-TCR interaction in terms of IL-2 and IL-2R upregulation and generation of stable Tregs[368,369]. Even though these findings were with TCR tg systems, there may be some applicability to antigen selection in the context of natural TCR repertoires as discussed below.

The format of auto-antigen used can influence the strength of MHC binding, and in turn the strength of T cell stimulation and/or Treg population generated. For example, in both mice and humans a number of T1D auto-antigens elicit stronger T cell (Teff) proliferation using a PTM

epitope than the corresponding WT epitope [31,94,95,101]. In many of these cases this is attributed to improved MHC binding of the PTM epitope. An example of improved MHC binding that has been shown to impact Tregs is an R22E substitution in the Ins B:9-23 peptide improves peptide binding to I-A^{g7} MHC allele[354]. Using insulin-specific TCR tg mice, one group found the R22E substitution led to greater T cell proliferation and Treg induction than WT peptide at the same dose[354], while another group found that the Treg level was not altered but more Tregs were $Tbet^{+}$ due to the stronger stimulation[324], which has functional implications for their ability to suppress diabetogenic Teff[323].

Although there is a range of natural TCR affinities for a given pMHC complex[255], the selection of an auto-antigen for which there is no central tolerance may enable a stronger pMHC-TCR interaction on average, and thus greater Treg expansion and improved Treg function if a Treg response is elicited. Naïve T cells recognizing antigens not presented in the thymus (including foreign antigens and auto-antigen neoepitopes) will theoretically on average have higher affinity pMHC-TCR interactions since the highest affinity cells have not been deleted, become anergic, or been converted into tTreg[256]. This has been directly shown in celiac disease patients, the pMHC-TCR affinity for some T cell clones recognizing a PTM auto-antigen was comparable to that of foreign antigens[89,257]. Likewise a lack of central tolerance is also thought to be responsible for the stronger proliferative response to Ins B:9-23 peptide compared to insulin protein[73]. Given that Tregs with a stronger pMHC-TCR interaction (as defined by high expression of CD5) are more potent in suppressing T1D development[324], the use of neoepitopes in auto-antigen therapy whose mechanism of action involves Treg induction may lead to more potent Tregs.

The fraction of the T cell population specific to an administered auto-antigen that has a naïve phenotype could also influence outcomes for antigen-immunotherapy aiming to induce

Tregs. In NOD mice by 5 weeks of age on average approximately 30% of Ins B:9-23 specific T cells in non-draining secondary lymphoid organs (SLO) already have an activated phenotype and on average nearly 20% of Ins B:9-23 specific T cells in SLOs (including the pancreatic lymph node) are producing IFN-γ[64]. Likewise, the fraction of Ins B:9-23 specific T cells with an effector or memory phenotype in the blood HLA-DQ8^{+} patients with T1D was 64% compared to 14% in HLA-matched controls without T1D[112]. These pre-existing high levels of auto-antigen specific T cells with effector and memory phenotypes could limit the ability of auto-antigen immunotherapy to induce Tregs. Since some PTM neoepitopes are thought to arise from β cell ER stress which could be caused by inflammation associated with autoimmunity to conventional auto-antigens[13,89], the T cells recognizing some neoepitope antigens may have a naïve phenotype later into disease progression.

Thus, for an auto-antigen immunotherapy that aims to induce Tregs, the use of a neoepitope could be advantageous due to the ability to target a strong affinity naïve T cell population. This approach remains mostly speculative, and care must be taken to ensure the strong T cell stimulation is skewed towards a Treg phenotype instead of a Teff phenotype. However for an auto-antigen immunotherapy that aims to expand existing Tregs, the use of a conventional auto-antigen may be better as there will likely be larger existing Treg population than for neoepitopes[259], some of which likely correspond to high affinity tTregs.

1.2.3.2 Auto-antigen (without other immunomodulatory agents) therapy – free or sustained release delivery

Approaches using auto-antigen and/or immunomodulatory agents are organized according to three categories that generally move from those that are less complex, but have greater risk of

presenting auto-antigen in an inflammatory context, to those of greater complexity, but have less risk of presenting auto-antigen in an inflammatory context. These categories are: 1) Auto-antigen (without other immunomodulatory agents) therapy using freely administered or sustained release of auto-antigen, 2) Auto-antigen (without other immunomodulatory agents) therapy using delivery systems that promote presentation in a tolerogenic context, and 3) Immunomodulatory agent (with or without auto-antigen) therapy (Figure 1.4). This second category is further broken down into approaches that: A) target tolerogenic sites, B) target tolerogenic APC subsets, or C) the delivery system itself has tolerogenic properties, and the third category is further broken down by approaches that: A) localize immunomodulatory agents to the site of endogenous auto-antigen and those that B) co-deliver immunomodulatory agents and auto-antigen (Figure 1.4).

Originally, auto-antigen immunotherapy was administered in NOD mice as a bolus of free protein/peptide or with simple delivery systems used to provide sustained release. Peptides include altered peptide ligands (APLs) and T cell mimotopes. The peptide sequence of APLs is modified from the natural sequence, while mimotopes are APLs in which modifications are designed or experimentally determined to elicit stronger responses, in the case of BDC2.5 and insulin mimotopes to specific T cell receptor (TCR) transgenic (tg) cells or T cell clone(s). While approaches using free auto-antigen or sustained auto-antigen release have been successful to various degrees in NOD mice, these approaches lack a component ensuring a tolerogenic context of antigen presentation and thus may be more prone to failure. Some of the first prevention studies gave repeated subcutaneous (s.c). or oral administration of insulin protein, repeated (s.c. or intranasal) administration of the insulin peptide Ins B:9-23 (10-100 μg/injection), or a single s.c. injection of Ins B:9-23 peptide (or APL) (100 μg) with sustained release provided through emulsion in incomplete Freund's adjuvant (IFA). These studies assessed early prevention (NOD

mice 4 weeks old at initiation of treatment) and observed significantly reduced T1D incidence (incidence as low as 10% relative to 70-90% in controls) with attributed mechanisms of reducing antigen-specific proliferation and/or Th2 polarization[356,370–374].[356,370–374]. Similar results were achieved using GAD65 and HSP60 as auto-antigens[355,375–378]. Notably, T1D reversal in NOD mice using free auto-antigen has not been incontrovertibly demonstrated. The only reversal reported for insulin was when using viral vector delivery of an insulin analogue which was later retracted and conflicting findings were reported regarding the ability of HSP60 to provide disease protection in diabetic mice[293,379–382]. The success of HSP60 in this regard, compared to other islet-antigens, could be due to its additional role in promoting regulatory cells via TLR2 signaling[383,384].

Osmotic minipumps have also been used instead of IFA for sustained delivery of auto-antigen, but conflicting findings have been reported. Osmotic minipumps are implantable devices that continuously deliver drug solution at a fixed rate over a period of days to weeks by using the diffusion of water across a permeable outer membrane to squeeze an impermeable but flexible drug reservoir. One group found that sustained s.c. administration of an Ins B: 9-23 mimotope (containing an R22E substitution) over 14 days 5 μg/day) using an osmotic minipump completely blocked T1D onset for both early prevention (4 week old) and late prevention (12 week old) studies in NOD mice with moderate insulin auto-antibody levels (~95% of mice at 4 weeks of age)[354]. This therapeutic effect was due to Tregs as the authors demonstrated substantial increases in $FoxP3^+$ Tregs (presumably insulin-specific) in the pancreas and pancreatic lymph node (pLN) (3-4x increases in Treg numbers and % of $CD4^+$ cells) and dominant tolerance of insulin mimotope treated mice in response to challenge with stimulated TCR tg insulin-specific T cells[354]. The same approach was also shown to expand Tregs in humanized mice[385]. However, because this approach does not ensure that auto-antigen is presented in a tolerogenic context, there is a greater risk of

expanding auto-antigen specific Teff. Interestingly, another group found that osmotic minipump delivery of the same insulin peptide mimotope appears to either have no effect on disease progression or increased T1D incidence depending on the timing of intervention[386]. In particular, the finding of increased T1D incidence suggests that insulin mimotope was encountered in an inflammatory context instead of a tolerogenic context in the latter study, which could either be due to microbiota differences or differing timing of osmotic minipump removal between the studies [386,387]

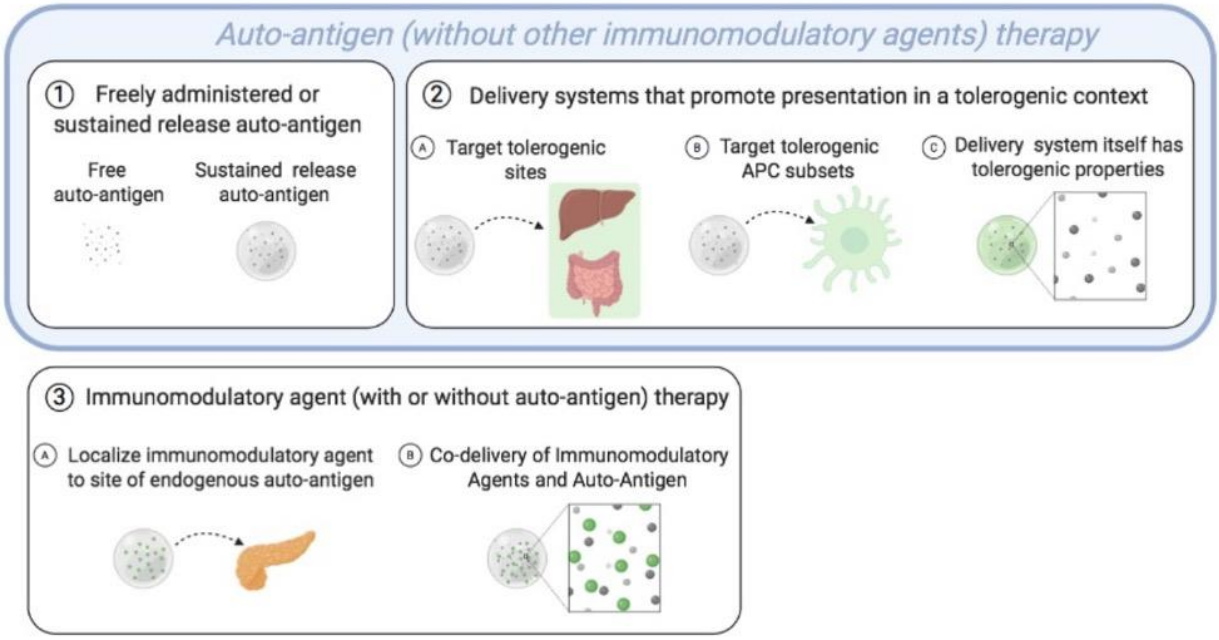

Figure 1.4 Categorization of auto-antigen and immunomodulatory agent based approaches for antigen-specific tolerance in NOD mice. **Three major categories are shown which correspond to Sections 1.2.3.2, 1.2.3.3, and 1.2.3.4 respectively. The first two of these categories are approaches that use auto-antigen without other immunomodulatory agents (as indicated by the blue box). These categories generally go from approaches that are less complex but have a greater risk of presenting auto-antigen in an inflammatory context to approaches of greater complexity but with less risk of presenting auto-antigen in an inflammatory context. Sub-categories are indicated by letters (A-C). For the sake of simplicity a single auto-antigen type is shown as small gray spheres and a particle (large gray sphere) is depicted as a delivery system throughout although the auto-antigen can vary and other delivery systems such as cells and antibodies can also be used. A green color is used to indicate the feature promoting tolerance, and in the third category green spheres indicate immunomodulatory agents other than antigen.**

1.2.3.3 Auto-antigen (without other immunomodulatory agents) therapy – delivery systems that promote presentation in a tolerogenic context

To ensure presentation of auto-antigen in a regulatory cell supportive context, delivery systems which target tolerance-prone tissues, target tolerance-prone antigen presenting cell (APC) populations, or are themselves tolerogenic via drug-free immunomodulatory effects have been tested in NOD mice. Tolerance-prone tissues that have been targeted for auto-antigen delivery in pre-clinical models include the gut and the liver, with their tolerogenic environment due to constitutive expression of immunosuppressive factors including TGF-β and IL-10[366,388]. In one liver targeting approach, BDC2.5 peptide mimotope was synthetically glycosylated to target C-type lectin receptor expressing hepatic antigen presenting cells[290]. Several i.v. administrations of this glycosylated mimotope was able to prevent T1D in an adoptive transfer model through a Treg-based mechanism[290]. Another liver targeting approach i.v. administered a lentiviral vector containing a hepatocyte specific promoter and encoding Ins B:9-23 as an auto-antigen[296]. This lentiviral Ins B:9-23 approach was able to prevent spontaneous T1D due to increased Treg levels and was also able to reverse recent-onset T1D in 75% of mice when combined with a sub-optimal dose of anti-CD3[296]. In order to target the tolerogenic environment of the gut while overcoming the limitations of antigen degradation and limited uptake associated with oral delivery, the auto-antigen heat shock protein 60 (HSP60) was encapsulated in chitosan NP with RGD and mannose incorporated to enhanced gut lymphoid tissue uptake and antigen presentation respectively[389]. Oral gavage (i.g.) of these HSP60 NP led to significant prevention of spontaneous T1D in a mouse model (relative to controls including s.c. and i.g. delivery of HSP60) an effect that was attributed to increased Tregs and decreased Teff in the pLN[389].

Instead of targeting tolerogenic tissues, delivery systems can also be used to target auto-antigen to tolerogenic APC populations which also serves to enhance auto-antigen presentation. Relative to other APCs, different conventional DC (cDC) subsets are particularly efficient at Treg induction (cDC1 = $CD8^+$ or $CD103^+$, $DEC205^+$) and Treg expansion (cDC2 = $CD11b^+$ and $DCIR2^+$) as well as deletional tolerance[134,390]. Antigen can be targeted to these different cDC subsets using chimeric antibodies that combine an antibody targeting a lectin differentially expressed by the subset (DEC205 or DCIR2) with a peptide antigen[134]. A chimeric antibody of anti-DCIR2 (αDCIR2) and BDC2.5 mimotope (αDCIR2-BDC) given intraperitoneally (i.p.) delayed T1D onset in an adoptive transfer model due to T cell deletion, however although Treg expansion had been previously observed using αDCIR2 coupled to conventional antigens in WT mice, it was not observed in this autoimmune setting[147]. The combination of αDCIR2-BDC and low-dose IL-2 led to a synergistic expansion of BDC2.5 Tregs, but failed to improve T1D prevention relative to αDCIR2-BDC alone due to activation and expansion of Teff[391]. Another approach that works by targeting a tolerogenic APC population is the i.v. administration of negatively charged nanoparticles (NP) with auto-antigen (Ag) cross-linked to the NP surface or encapsulated in the NP (Ag-NP). The negative charge of these Ag-NP enables binding to the macrophage receptor with collagenase structure (MARCO) expressed on tolerogenic APCs in the spleen and liver, and experiments with $MARCO^{-/-}$ mice show this interaction is essential for tolerance induction.[392,393]. A single dose of BDC2.5-NP (cross-linked or encapsulated Ag) or IGRP- NP (encapsulated Ag) given i.v. significantly reduced T1D incidence in adoptive transfer models using activated TCR tg T cells of corresponding specificity (CD4 or CD8 respectively) as Teff[289]. The mechanism involved Teff that remain in spleen instead of trafficking to pancreas due to an upregulation of PD-1 and CTLA-4 and an increase in Tregs[289]. This same technology was

also used to deliver a hybrid insulin peptide (in this case a splicing of insulin and ChgA) in a BDC2.5 adoptive transfer model which inhibited T1D incidence by a similar mechanism, representing the first therapeutic use of a neoepitope in a model of T1D[394]. Although i.v. administration of unencapsulated HIP did delay the onset of T1D relative to untreated control at the dose tested, all mice in this group eventually developed T1D[394]. Notably, although daily administration of antigen-free negatively charged NP in other models can have temporary anti-inflammatory effects due to induction of inflammatory macrophage apoptosis[393,395], the use of an irrelevant peptide Ag-NP controls suggests that this is not occurring in these T1D models. Ag-NP were ineffective when administered s.c. or i.p. as well as when they were fabricated from poly(lactide-co-glycolide) (PLG) without the addition of 2-ethyl-2-phenylmalonamide monohydrate (PEMA) to produce a negative charge[289]. These findings are consistent with the need to bind the MARCO+ APC in the spleen for this Ag-NP approach to be effective, and the lack of sufficient negative charge and/or the use of s.c. administration (in addition to dosing and model differences) could explain why antigen nanoparticle systems used as controls by other groups were ineffective[292,295,396]

Tolerogenic NP delivery of auto-antigen can also be achieved using peptide-major histocompatibility complex (pMHC) coated on the particle surface (pMHC-NP). However, the mechanism of regulatory cell induction observed is different from that of Ag-NP targeting tolerogenic APCs. Instead, the large number of identical pMHC complexes enables a strong avidity interaction with a T cell, with the number of pMHC molecules per NP (valency) acting as another controllable parameter in addition to the conventional parameters of (NP) dose and pMHC-TCR affinity that can affect T cell phenotype[397]. The high pMHC valency of these pMHC NP relative to APCs simultaneously enables the deletion of high avidity T cells and regulatory induction of

lower avidity T cells[305,398,399]. This approach has been successful pre-clinically in models of T1D and other autoimmune conditions using both pMHC-NP with MHC I for $CD8^+$ T cells and MHC II for $CD4^+$ T cells[295,399]. Administration of these iron oxide pMHC-NP (MHC II) i.v. converted activated Teff (but not naive) antigen-specific $CD4^+$ T cells into IL-10 and TGF-β producing Tr1 cells, which in turn led to local (pLN) increases in regulatory B cells (Breg) and tolerogenic DCs, but not impaired systemic immunity[295]. In a T1D reversal model, over 90% of diabetic mice (blood glucose (BG) > 200 mg/dL) treated 2x per week with BDC2.5 mimotope pMHC-NP or one of two different IGRP epitope pMHC-NP had disease reversal with stable normoglycemia (BG < 200 mg/dL) compared to no mice given controls of peptide alone or peptide-NP without NP. However, 25-60% of mice reverted to hyperglycemia when treatment was stopped[295]. This suggests that true tolerance may not have been established, possibly because of the instability of Tr1 cells without continued treatment. Alternatively, the generated regulatory populations (Tr1, Breg, and DCs) on their own may not be sufficient to fully control an established Teff population without continued pMHC-NP administration to directly blunt Teff expansion. However, the demonstrated success in a reversal model and ability to convert Teff and/or memory populations to regulatory populations suggests that pMHC-NP may be more suited than other approaches to address clinical concerns of an inflammatory environment and substantial effector/memory auto-antigen populations. The remarkable performance of pMHC-NP using a variety of different auto-antigen peptides (including sub-dominant epitopes) in stark contrast with the lack of effect observed for peptide-NP and pMHC monomer illustrate the importance of pMHC valency and thus pMHC-TCR avidity to regulatory cell induction in this system.

Presentation of auto-antigen in a tolerogenic context can also be enforced in a drug-free manner using apoptotic or pre-apoptotic cells as a delivery system. Phagocytosis of apoptotic cell

membranes containing external phosphatidylserine in the absence of other inflammatory ligands results in tolerogenic antigen presentation by APC due to signaling that inhibits co-stimulation upregulation and perpetuates anti-inflammatory cytokine production[400,401]. The most straight forward method of utilizing apoptotic cells to deliver auto-antigen in T1D is by administering apoptotic β cells, and 3 transfusions of irradiated NIT-1 (β cell line) was sufficient to significantly reduce T1D onset in a spontaneous prevention model through Th2 and Tr1 induction[402]. Another approach is to chemically cross-link auto-antigen to splenocytes (Ag-Sp) using the same chemistry that can be used to cross-link auto-antigen to NP. This cross-linking chemically fixes the splenocytes and induces their apoptosis, which is primarily responsible for the tolerogenic effect of this approach (although direct auto-antigen presentation to T cells by Ag-Sp may also play a role)[403,404]. A single i.v. administration of Ag-SP resulted in a significant reduction in the incidence of spontaneous T1D in early prevention (4-6 weeks old) when using insulin or Ins B:9-23 as Ag, but only for insulin in late prevention (19-21 weeks old)[405]. This was attributed to Treg expansion, but T cell anergy, demonstrated in other models, also likely played a role[405]. Antigen has also been attached to red blood cells (RBCs), in part due to their relatively rapid apoptotic turnover and because they are a more translationally relevant population [406]. In a recent example, RBC were treated with viral vectors to encode membrane protein motifs that could be used to enzymatically attach large amounts of auto-antigen in a controlled manner without non-native materials[406]. Administration of Ins B:9-23 attached to RBCs using this system in a late (10 week old) spontaneous T1D prevention study kept 80% of mice T1D-free[406]

1.2.3.4 Immunomodulatory agent (with or without auto-antigen) therapy

Although drug delivery approaches that target auto-antigen to tolerance-prone tissues or tolerance-prone APCs have had success in NOD mouse models, findings that the tolerogenic

nature of some of these tissues and APC subsets may be compromised in NOD mice and humans due to genetic polymorphisms and/or ongoing autoimmunity is a concern for the translation of these approaches[25,134,147,156,168,176,407]. Therefore, the added complexity of combining auto-antigen with immunomodulatory agents instead of, or in addition to, drug-free tolerogenic delivery systems may be necessary as an added precaution.

Local delivery of immunomodulatory agents without exogenous antigen can induce antigen-specific tolerance and could have advantages related to the breadth of auto-antigens and Treg stability, but appropriate localization will be more difficult for T1D than for applications with more accessible tissues. Our group has previously shown that local administration of a MP system releasing TGF-β, rapamycin, and IL-2 (TRI MP) can promote tolerance in models of contact dermatitis, dry eye disease, and transplantation by promoting Treg induction/expansion and suppressing Teff[408–410]. These studies also demonstrated that TRI MP confined drug activity to a local area resulting in antigen-specific tolerance. Theoretical advantages of this approach relative to other cell-free antigen-specific approaches include the ability to act on multiple immune cell populations simultaneously to increase the Treg/Teff ratio (including naïve T cells, APCs, Tregs, and Teff), the ability to induce/expand Tregs to a diverse array of endogenously presented antigens, and an ability to sustain a tolerogenic local environment. As discussed above, although eliciting Tregs to a single auto-antigen can prevent or reverse T1D in NOD mice if the Treg are of sufficient number and potency, the ability to expand Treg of multiple specificities may be clinically advantageous given individual variability in the number, affinity, and phenotype of the T cell repertoire to a given antigen[112]; this may make it difficult to control islet autoimmunity in most patients by expanding Treg cells to a single auto-antigen. Since Treg instability remains a concern at sites of inflammation due to inflammatory cytokines as well as the T1D-specific issue of

insufficient IL-2[223,238,247], an approach which not only generates Tregs but supports their continued function by directly inhibiting inflammatory cytokines and/or providing IL-2 may be needed clinically. An approach based on the local delivery of immunomodulatory agents without auto-antigen that has been effective in NOD mice is MP (small/phagocytosable) delivery of anti-sense nucleotides (ASN) to co-stimulatory molecules CD80, CD86, and CD40. Administration of these ASN MP s.c. 1-2 times per week for 4-8 weeks was able to completely prevent spontaneous T1D (at 30 weeks) as well as reverse recent-onset T1D in nearly 50% of mice[294]. This protection was attributed to APC uptake of MP and presentation of antigen without co-stimulation, which resulted in an increase in overall polyclonal Tregs in the spleen and dominant tolerance as shown by an adoptive transfer experiment[294]. Even though Tregs were increased in the spleen, this approach demonstrated antigen specific tolerance (to β cell lysate but not alloantigen) attributed to the fact that ASN MP were injected s.c. proximal to the pancreas and accumulated in the pancreas[294]. A later paper by the same group confirmed that the addition of exogenous antigen to their ASN MP system, either in a T1D reversal model, or using Ova and TCR tg T cells as a model system, did not improve tolerance induction[411]. Although this group was able to have success in targeting MP to the pancreas, targeting NP/MP or their released cargo to the pancreas/pLN clinically and verifying successful delivery will be a challenge for approaches that rely on endogenous antigen.

Immunomodulatory agents can also be co-delivered with exogenous auto-antigen, in which case antigen-specific regulatory cells can be expanded by various routes of administration without needing to target the pancreas. One approach used a gold NP system that delivered an auto-antigen (pro-insulin or BDC2.5 mimotope) and an aryl hydrocarbon receptor (AhR) ligand as an immunomodulatory agent in the same NP. AhR activation and this NP system had previously been shown to generate a tolerogenic DC population and Treg expansion[412,413]. In a spontaneous model

of T1D, weekly AhR ligand -Ag NP i.p. administration (weeks 8-12) led to a significant reduction in T1D incidence relative to unloaded NP[414]. The mechanism involved AhR ligand -Ag NP inhibiting splenic DC production of IL-6 and IL-12, and in turn, reductions in Th1 and Th17 cells but increased Tregs relative to unloaded NP[414]. To illustrate the translational potential of this work, the authors demonstrated that a GAD65-specific human T cell clone produced less IFN-γ when co-cultured with immature or mature human monocyte-derived DCs if the DCs had been exposed to AhR ligand - GAD65 NP as compared to GAD65 NP[414]. Another approach in this area relied on "artificial lymph nodes", which are porous scaffolds recruiting DCs through the release of granulocyte macrophage colony stimulating factor (GM-CSF) that had been previously shown to induce an anti-tumor T cell response when also delivering a tumor antigen and a DC activation [415]. Using a similar delivery system consisting of s.c. injected hydrogel incorporating cleavable BDC2.5 mimotope peptide and GM-CSF NP, the authors sought to induce a tolerogenic T cell response for T1D. Although the gels did expand antigen-specific Treg with the majority (60%) of (BDC) tetramer$^+$ cells in the gel being FoxP3$^+$, three doses of the gel did not significantly affect disease incidence in spontaneous T1D model (treatment initiated at 8 weeks)[416]. This could be because the number of these cells in the gel was relatively low for a spontaneous model (5,000 cells) and/or because although the majority of tetramer$^+$ cells in the gel were Treg, the vast majority of the increased tetramer$^+$ cells in the islets were FoxP3$^-$ [416].This could reflect trafficking differences or Treg instability. A different MP system likewise delivered antigen (BDC2.5 mimotope or Ins B:9-23) and GM-CSF for DC recruitment, but also delivered vitamin D3 and TGF-β as immunomodulatory agents to produce tolerogenic DCs[396]. The MP system was dual sized, with smaller phagocytosable MP for enhanced APC uptake and intracellular delivery of antigen or vitamin D3, and larger non-phagocytosable MP for the sustained extracellular delivery

of otherwise relatively short half-life cytokines in GM-CSF and TGF-β[396]. When this MP system (Ins B:9-23 as the antigen) was administered s.c. twice in an early prevention study of spontaneous T1D, it led to a significant reduction in T1D incidence (40% T1D-free) relative to untreated controls (10% T1D-free)[396]. The mechanism was supported by *in vitro* data showing dual-size MP treatment reduced DC promotion of T cell proliferation by reducing MHC II and co-stimulation levels, and *in vivo* with an observation of an increase in polyclonal splenic Tregs presumably expanded by tolerogenic DCs[396]. T1D prevention experiments in a more recent study by the same group (in which Ins B:9-23 was replaced with denatured insulin) found that drug encapsulation was needed and given the dose, frequency, and route of administration used, insulin MP was a necessary component but not sufficient on its own for T1D prevention[292]. In a reversal experiment in which diabetic mice (BG > 240 mg/dL) had blood glucose temporarily controlled by an insulin pellet, 6 s.c. injections of dual sized MP over 4 weeks significantly extended the period of euglycemia relative to controls, but all mice reverted to hyperglycemia[292]. Immunomodulatory agents and auto-antigen can also be co-localized for T1D treatment using biological delivery systems. This was recently demonstrated when attenuated Salmonella expressing plasmids for preproinsulin, TGF-β, and IL-10 to prevented spontaneous T1D and reversed T1D (when combined with low-dose anti-CD3) in nearly 60% of mice through induction/expansion of Tregs and Tr1 cells[297,417].

1.2.3.5 Translation of auto-antigen immunotherapy

Clinical trials evaluating free peptide/protein or using alum as an adjuvant have been conducted for several auto-antigens with the aim of delaying T1D onset in high-risk individuals or preserving β cell function in recent-onset T1D patients. The auto-antigens tested include insulin

protein for T1D prevention or β cell function preservation (oral and nasal administration), insulin peptides for β cell function preservation (s.c. administration), GAD65 protein-alum (s.c.) for β cell function preservation, and HSP60 peptide (s.c.) for β cell function preservation[355,418]. While approaches tested to date have consistently demonstrated safety and tolerability, those that have reached larger trials (insulin protein, Ins B:9-23 APL, GAD65-alum, and HSP60 Diapep277) have failed to consistently show efficacy.[419–427].

Initial T1D prevention attempts with oral insulin did not assess tolerance associated immunological changes and did not utilize preclinical findings to inform dosing. Several secondary prevention studies using oral insulin at a dose of 7.5 mg/day that failed to delay T1D onset were conducted without assessing the immunological effects of this approach[420,421]. The dose used in these trials was substantially lower on a per weight basis than the oral dose that had been effective for T1D prevention in NOD mice[355,356,379]. The theory that the insulin dose was too low for these studies was supported by a small trial testing several oral insulin doses from 7.5 mg/day to 67.5 mg/day which found the 67.5 mg/day dose had the largest frequency of patients with increased antibody or T cell responses to insulin, insulin responding cells had a Treg associated gene signature, and insulin responding cells from patients treated with the 67.5 mg/day dose differentially clustered from other treatment groups by principal component analysis[428]. The efficacy of T1D prevention using 67.5 mg/day oral insulin is being evaluated by the TrialNet TN20 (NCT02580877) study.

Evaluated approaches for preservation of β cell function in recent-onset T1D did achieve favorable immunological changes in small trials prior to performing efficacy trials, but failed to evaluate immunological effects in larger trials. These changes include increased auto-antibody levels and increased antigen-specific T cell proliferation for oral insulin, an increased antigen-

specific Th2 response to Ins B:9-23 APL or GAD65, and an increased antigen-specific Treg response to GAD65, HSP60 peptide, or proinsulin C19-A3 peptide[383,429–433]. Proinsulin C19-A3 peptide showed promising data for C-peptide preservation in a Phase 1b trial for safety, but has not yet been evaluated for efficacy[433]. However, in these other instances immunological changes were assessed in pilot or small trials, and larger trials powered to assess efficacy either had no immunological assessment or only assessed auto-antibody levels[422–427]. Therefore, it remains largely unclear if the failed efficacy of these treatment is due to an inability of tested auto-antigen immunotherapies to have the desired immunological effects in a large heterogenous population, or is due to an inability of immunological effects to translate into clinical endpoints of delayed T1D onset or preservation of C-peptide levels. If the issue is a failure to induce tolerance associated immunological changes, this could be due to properties of the auto-antigen immunotherapy such as dose, route, selected auto-antigen (and format). In the case of GAD65, the clinically evaluated immunotherapy used a different format (protein vs. peptide) and adjuvant (alum vs. none) than what was effective for T1D prevention preclinically[375,376,425]. A failure to induce tolerance associated immunological changes could also be due to the existing phenotype or inflammatory context when auto-antigen is encountered by the responding T cell population. A recent analysis of cryopreserved PBMCs from a Phase II study of GAD65-alum in recent-onset T1D found that while GAD65-alum led to an increase in GAD65-specific production of Th2 cytokines relative to baseline, the majority of clones derived from these patients were shown to have bi-functional Th1/Th2 phenotype based on transcription factor expression and cytokine production[434]. While the origin and functional significance of these bi-functional GAD65-specific T cells is unclear, these cells may be derived from the Th2 promoting immunotherapy activating Th1 memory cells, and could be less effective than GAD65-specific Th2 cells at suppressing diabetogenic T cells[434]. If

the failure is due to inability of tolerance promoting immunological changes to translate into preservation of C-peptide, this could be due to the Th2/Treg response generated in the blood not being indicative of changes in the pancreas, the Th2/Treg response not being of sufficient magnitude or durability to overcome existing Teff, and/or the intervention being too late to reverse β cell death or dysfunction. Notably, with the exception of HSP60 (for which there was conflicting findings), none of the auto-antigen immunotherapies tested clinically in recent-onset T1D were shown to reverse T1D onset in NOD mice[379]. Therefore these approaches may be better suited to clinical T1D prevention trials given that is the intervention time for which they have demonstrated preclinical effectiveness. Moving forward auto-antigen immunotherapy clinical trials would benefit from designing trials consistent with preclinical insights and greater collection of mechanistic data on immunological changes in large trials.

A number of the discussed antigen-specific delivery systems (including pMHC NP, pre-apoptotic cells, antigen NP or MP with or without immunomodulators, and engineered bacteria) are in clinical development for T1D and/or other inflammatory conditions[435]. A few of these approaches that are represented in recently initiated clinical trials for autoimmune diseases include Cour Pharmaceuticals Ag-NP for Celiac disease (NCT03738475) and the Precigen ActoBiotic platform of engineered bacteria delivering proinsulin and IL-10 for T1D treatment (NCT03751007). However, late-stage clinical trials to test efficacy have yet to be conducted for any of these approaches.

1.2.4 Conclusions

Antigen-specific immunotherapy using Treg cell-therapy or auto-antigen to induce/expand endogenous regulatory cells has been effective in NOD mouse models using a single antigen

specificity. Furthermore, auto-antigen and/or immunomodulatory agent approaches have been successful using several different single antigen specificities including insulin, ChgA/BDC2.5, GAD65, HSP50, and IGRP (Table 1-1). Thus, in NOD mice what appears to be important for influencing T1D progression in an antigen-specific manner is not the chosen auto-antigen or Treg specificity per se, but the ability to administer that antigen or cell-therapy with an appropriate dose/frequency, route, delivery system, and/or immunomodulatory drugs to elicit a regulatory population of sufficient size and potency for bystander suppression and/or infectious tolerance to suppress Teff specific to a variety of islet auto-antigens. However, it remains to be seen whether approaches based on a single auto-antigen are capable of generating a regulatory cell population of sufficient size and potency to alter T1D development in humans. The selection of appropriate auto-antigen(s) will be more challenging and may require different auto-antigen(s) for different patient populations. Furthermore, additional unknowns in humans include the appropriate timing of intervention and the stability of immunotherapy generated regulatory cells in the presence of an inflammatory milieu.

While a number of antigen-specific approaches have had success in NOD prevention models, there have been conflicting findings about the ability of an approach to prevent disease and/or whether certain delivery system attributes or additional tolerogenic factors are needed for prevention. An important caveat is that often the prevention models used among similar therapies may differ, not just in terms of whether a spontaneous or adoptive transfer model is used but also in the timing of intervention (spontaneous model) or the number/type of Teff used (adoptive transfer model). Excluding model differences, conflicting findings about T1D prevention in NOD mice among studies using similar (or the same) antigen-specific approach could be attributed to a number of factors including the dose/frequency of antigen used, the route of administration, the

properties of delivery systems used, and the inflammatory context/microbiome composition (Table 1-2). The inability of one approach to be effective in a context that works for another approach (ex: s.c. delivery for different NP/MP approaches[289,294] illustrates that while many of the above approaches administer auto-antigen and elicit a regulatory population, there are often mechanistic differences in terms of the antigen presenting subsets involved, the type(s) of regulatory populations produced, and the extent of Teff anergy/deletion shown.

Although systems of varying complexity from free auto-antigen, to auto-antigen & drug-free tolerance mechanism (ex: Ag-NP and pMHC-NP), and lastly to NP/MP or bacterial systems delivering immunomodulatory agents with or without antigen, have prevented T1D in an antigen-specific manner in NOD mice, it remains to be seen if added layer(s) of complexity are beneficial for clinical T1D prevention. Other than insulin protein, which was tested clinically at a dose/kg substantially lower than what was necessary for T1D prevention in NOD mice, few antigen-specific approaches have been evaluated for T1D prevention clinically. Although there have been safety issues with auto-antigen immunotherapy in some trials for MS[436,437], given the track-record of safety of auto-antigen immunotherapy in T1D and reduced risk of Teff expansion in approaches that use local immunomodulation, hopefully more clinical prevention studies can be performed assessing both free auto-antigens and newer approaches with more complex delivery systems.

Based on evidence in NOD models and trials to date for recent-onset T1D, more complex auto-antigen delivery systems may be necessary to reverse T1D onset (or less ambitiously preserve β cell function). The only free auto-antigen that was able to reverse T1D onset in NOD had questionable reproducibility[293,382]. In contrast, several recent approaches using auto-antigen and/or local immunomodulation (although some also used low-dose anti-CD3) have been able to reverse T1D onset or at least prolong euglycemia after T1D onset[292,294–297] (Table 1-1). Thus, these

approaches have promising potential for an ability to preserve β cell function in recent-onset T1D patients clinically. Other approaches in clinical development for T1D have not yet demonstrated an ability to reverse T1D in NOD mice, and these approaches should be evaluated in this model prior to being tested in recent-onset T1D clinically.

Regardless of the antigen-specific immunotherapy approach for T1D being investigated, future research would benefit from clinical trial design that takes preclinical testing into account and greater clinical collection of mechanistic data. Clinical trials should use an auto-antigen format, dosage, route, and intervention time analogous to what has been effective in preclinical models. Greater mechanistic insight into clinical trials will be extremely valuable in understanding why a given auto-antigen immunotherapy is effective or ineffective for a particular patient population. First, there is an evaluation of whether an immunotherapy is having the desired the immunological effect in the periphery and the factors that can be used to distinguish responders from non-responders in this regard. For example, this could involve taking blood before, during, and after immunotherapy to perform tetramer staining to understand the level and phenotype of auto-antigen-specific T cells, flow cytometry to assess the numbers and phenotype of other immune cell populations involved in the approach's mechanism of action, and ELISpots and/or serum ELISAs to understand both the auto-antigen specific and polyclonal cytokine responses. Additional factors that could be considered include assessment of antigen-specific Treg function using *in vitro* suppression assays following immunotherapy given the impaired Treg function observed in T1D patient PBMCs[216,251,252] and examining patient SNPs such as those related to CTLA-4 and IL-2 signaling[246–250] to see if there is any connection between polymorphisms that may impair Treg function and subjects who fail to mount the desired immunological response. Second, is whether this desired immunological response in the blood translates into the desired

immunological and functional responses in the pancreas. Given the inaccessibility of the pancreas, these are currently difficult to assess with reliance on T1D onset in T1D prevention and C-peptide levels and/or glucose tolerance tests in the case of β cell function preservation. The development of additional biomarkers of β cell stress/death as well as imaging modalities to image the pancreas in live patients could provide additional mechanistic insight to bridge the gap between immunological changes in the blood and treatment outcomes[281,438]. Auto-antigen immunotherapy in other indications where the target tissue is more accessible, such as rheumatoid arthritis, may help to provide some insight in this regard. With a number of promising antigen-specific approaches to treat T1D in clinical development, there will likely be several opportunities in the near future to improve outcomes for those at risk or recently diagnosed with T1D, and at minimum improve understanding of the utility of different auto-antigen delivery system approaches.

Table 1.1 Representative auto-antigen and immunomodulatory agent based approaches for preventing and reversing T1D in NOD mice.

Treatment	Auto-antigen	Immuno modulatory agent	Delivery system	Route	Prevention model	Reversal model	Ref
Auto-antigen (without other immunomodulatory agents) therapy – freely administered or sustained release							
Insulin	Insulin protein			s.c., oral	Successful (Spontaneous)		356,370,372
Insulin peptide emulsion	Insulin peptide/APL		IFA (Sustained release)	s.c.	Successful (Spontaneous)		371,373,374
GAD65	GAD65 protein/peptide			i.n., i.p.	Successful (Spontaneous)		375,376
HSP60	HSP60 peptide		IFA (Sustained release)	i.p., s.c.	Successful (Spontaneous)	Conflicting (Prolong survival)	293,377,378,382
Osmotic minipump delivery	Insulin mimotope		Osmotic minipump (Sustained release)	s.c.	Conflicting (Spontaneous)		354,386,387
Auto-antigen (without other immunomodulatory agents) therapy – delivery systems that promote presentation in a tolerogenic context							
Synthetically glycosylated antigen	BDC2.5 mimotope		Binds receptor on hepatic APCs (target tolerogenic site)	i.v.	Successful (Adoptive transfer)		290
Hepatocyte-specific auto-antigen production	Insulin peptide		Lentiviral vector (target tolerogenic site)	i.v.	Successful (Spontaneous)	Successful (With low-dose anti-CD3)	296
NP to target the gut with improved uptake	HSP60		NP with mannose & RGD (target tolerogenic site)	oral	Successful (Spontaneous)		389
Chimeric antibody targeting dendritic cell (DC) subset	BDC2.5 mimotope	(IL-2 in one study)	anti-DCIR2 (target tolerogenic APC subset)	i.p.	Successful (Adoptive transfer)		147,391
Antigen nanoparticles (Ag-NP)	BDC2.5/IGRP mimotope & Neoepitope		Negatively charged NP (target tolerogenic APC subset)	i.v.	Successful (Adoptive transfer)		289,394
Peptide-MHC (pMHC) NP	BDC2.5/IGRP mimotope		NP with strong avidity (Delivery system itself has tolerogenic properties)	i.v.	Successful (Spontaneous)	Successful	295,399
Apoptotic β cell line			Apoptotic cell (Delivery system itself has tolerogenic properties)	i.v.	Successful (Spontaneous)		402
Antigen cross-linked splenocyte (Ag-Sp)	Insulin protein/peptide		Pre-apoptotic cell (Delivery system itself has tolerogenic properties)	i.v.	Successful (Spontaneous)		405
RBC with covalently linked antigen	Insulin peptide		Pre-apoptotic cell (Delivery system itself has tolerogenic properties)	i.v.	Successful (Spontaneous)		406
Immunomodulatory agents (IMA) with or without auto-antigen							
Costimulation anti-sense nucleotide (ASN) microparticles (MP)		ASN for CD80, CD86, and CD40	MP (localize IMA to the site of endogenous auto-antigen)	s.c.	Successful (Spontaneous)	Successful	294,411
Aryl hydrocarbon receptor (AhR) ligand and Ag NP	Proinsulin	AhR ligand	NP (Co-delivery of IMA + Ag)	i.p.	Successful (Spontaneous)		414
DC recruiting hydrogel	BDC2.5 mimotope	GM-CSF	Hydrogel (Ag) and NP (GM-CSF) (Co-delivery of IMA + Ag)	s.c.	Unsuccessful (Spontaneous)		416
Dual sized MP with Ag and IMA	Insulin protein/peptide	GM-CSF, TGF-β, and Vitamin D3	MP (Co-delivery of IMA + Ag)	s.c.	Successful (Spontaneous)	Successful (Prolong euglycemia)	292,396
Engineered bacteria with Ag and IMA	Preproinsulin	TGF-β and IL-10	Salmonella with Ag/IMA plasmids (Co-delivery of IMA + Ag)	oral	Successful (Spontaneous)	Successful (With low-dose anti-CD3)	297,417

Table 1.2 Possible explanations of conflicting findings for antigen-specific tolerogenic approaches in NOD mice. *An important caveat in these comparisons is that different prevention models (spontaneous vs. adoptive transfer) or prevention and reversal models are being compared and these model differences may also explain some conflicting findings

Category	First set of findings	Second set of findings	Possible explanations
Dose/frequency/delivery system	• A single administration of Ins B:9-23 (100 μg) with sustained released provided by IFA successfully prevented T1D when administered s.c. at 4 weeks of age[371]	• Osmotic minipump delivery of Ins B:9-23 as a control treatment (5 μg/day for 14 days) did not prevent T1D when administered s.c. at 4 weeks of age[354] • Microparticle delivery of Ins B:9-23 (~ 12.5 μg/injection) did not prevent T1D when administered as s.c. injections at 4 and 5 weeks of age[396]	• Small differences in antigen dose, frequency of administration, or system used for sustained release and/or enhanced APC uptake may affect strength of stimulation and thus T cell fate (ex: amount of Teff priming/expansion, deletion, anergy, and Treg induction influencing the Treg:Teff ratio) as well as the degree of regulatory cell expansion
Route of administration*	• Ag-NP were successful for T1D prevention when administered i.v. but not s.c.[289]	• Ag-MP alone were unsuccessful for T1D prevention when administered s.c, but were successful when co-administered with immunomodulatory MP s.c.[396] • Costimulation anti-sense nucleotide MP were successful for T1D prevention when administered s.c.[294]	• Route of administration for a particular approach to be effective depends on its mechanism of action • s.c. delivery may require use of immunomodulatory agents, because may be unable to target tolerogenic tissues/APC subsets as in other routes
Delivery system properties*	• PLG/PEMA Ag-NP administered i.v. were more effective than PLG Ag-NP (with reduced negative charge) for T1D prevention[289]	• Iron oxide Ag-NP control treatment administered i.v. were unsuccessful in T1D reversal[295]	• Although the model by Clemente et al. was more rigorous, the lack of sufficient negative charge on their Ag-NP control could play a role in ineffectiveness since a negatively charged NP is important to the mechanism of action for Prasad et al[392,393]
Inflammatory context	• Osmotic minipump delivery of insulin mimotope successful for T1D prevention at several intervention timings[354]	• Reproducing studies of Daniel et al. as close as possible resulted in no significant effect on T1D onset, or an acceleration of T1D onset depending on the intervention timing[386]	• These conflicting findings may be due to differences in the cytokine milieu/APC activation state in which auto-antigen is encountered due to differences in the microbiome or in inflammation associated with pump implantation[386,387]

1.3 Breakdown of T cell tolerance to self in RA and CIA

1.3.1 Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of chronic joint inflammation affecting 0.5-1% of the population in Western countries, and approximately 1.5 million people in the U.S.[439,440]. RA joint inflammation leads to irreversible damage to cartilage and bone. This can

be debilitating for patients and the corresponding decreased work capacity is the main driver of the estimated $46 billion societal burden of RA in the U.S.[441]. Tremendous progress has been made over the past few decades in optimizing treatment with conventional synthetic disease-modifying antirheumatic drugs (DMARDs), developing biologic DMARDs including TNF-α inhibitors, and the recent introduction of Janus kinase (JAK) inhibitors. However, none of these therapies have been able to achieve low disease activity in even 50% of methotrexate-naive patients, and with each line of further therapy there is a diminishing return of patients who adequately respond[442,443]. It has been suggested that a similar maximum efficacy has been observed across RA drug types because regardless of the direct target, all of these drugs ultimately act by blocking TNF-α and/or IL-6[444]. Thus, a substantial population of RA patients remain underserved by existing treatments, and there is a need to develop new treatments with a different mechanism of action

RA pathogenesis differs from that of T1D in that auto-antibodies play a dominant role in tissue damage. Seropositive RA, which accounts for two-thirds of RA cases[445], is characterized by the production of anti-citrullinated protein antibodies (ACPAs) as well as rheumatoid factor (antibodies to self IgG-Fc)[446–448]. While disease onset is still not well understood, it is known that ACPAs ultimately bind to citrullinated proteins on articular cartilage and/or on osteoclasts in adjacent bone which activates osteoclasts and triggers an inflammatory cascade resulting in joint erosion and pain[445]. However, joint inflammation may not occur until years after ACPAs first appear in the and is thought to require an "additional hit" such as inflammation from minor physical trauma or infection[449,450].

Although there is not a natural spontaneous animal model of RA, collagen-induced arthritis (CIA) is a widely used mouse model that has many similarities to RA. This model involves immunizing DBA/1 mice with collagen II (CII), normally of bovine origin (bCII), emulsified in

complete Freund's adjuvant (CFA). CIA resembles RA in some important histological and radiographic measures including fibrin deposition, synovial hyperplasia, mononuclear infiltration, and bone erosion[451–453]. While CII is the initiating antigen in CIA, the defining antibodies of seropositive RA - rheumatoid factor ACPAs - have been detected in CIA with the latter shown to contribute to disease pathogenesis[454,455]. Both auto-antibodies and T cells contribute to CIA pathogenesis. Anti-CII antibody administration is sufficient to transfer CIA[456], assuming it is of appropriate dose, avidity, and isotype[454]. In particular, the level of anti-CII antibody with the Th1-associated IgG2a isotype is thought to be particular important because of its role in complement system activation[454,457]. $CD4^+$ T cells play an important role in generation of anti-CII antibodies in CIA[458–460], and CII or citrullinated protein specific $CD4^+$ T cells can also exacerbate disease by trafficking to the joints and producing inflammatory cytokines[454,456,461].Together, the complement activation by auto-antibodies and $CD4^+$ T cell production of IFN-γ and/or IL-17 is thought to lead to recruitment and activation of innate immune cells which in turn produce TNF-α and IL-1β leading to tissue swelling and destruction[454,462,463]. This differs from RA where an "additional hit" is thought to be needed.

1.3.2 Escape from central tolerance

The generation of ACPAs in seropositive RA depends on help from auto-reactive T cells in addition to requiring auto-reactive B cells, and research to date has primarily focused on T cell tolerance to citrullinated proteins. Thus, auto-reactive T cell escape from central tolerance in RA is likely similar to a proposed mechanism in T1D that involves PTM. The role auto-reactive $CD4^+$ T cells play in RA is apparent from the fact that the predominant genetic risk factor for seropositive RA is a group of MHC II alleles (in the HLA-DRB1 family) known as the shared epitope (SE)

alleles with an odds ratio of 5.8 - 12[464,465]. The SE alleles are characterized by a positively charged position 4 (P4) pocket in the peptide binding groove[445,466]. This SE allele P4 pocket does not easily bind positively charged arginine at P4 in a peptide, but will bind if this amino acid has undergone the PTM of citrullination and been converted to neutrally charged citrulline, for example in aggrecan and vimentin epitopes[467]. Thus, in individuals with the SE allele, an SE restricted T cell recognizing a peptide that has arginine at the MHC contacting site P4 escapes central tolerance. This occurs because the SE allele fails to bind the un-citrullinated form of the cognate epitope found in the thymus, but does bind the citrullinated form found in the periphery (Figure 1.5). Citrullination can also to lead to neo-epitopes in RA in a SE allele-independent manner, and these mechanisms likely play a dominant role in T cell escape from central tolerance in seropositive RA individuals that are SE allele negative. This SE allele-independent citrullination occurs at amino acids that are not at the P4 position of an SE allele restricted epitope such as modifying peptide residues in contact with the TCR[468–470], or changing protease susceptibility resulting in alternatively cleaved self- peptides[467].There is also some evidence of auto-reactive T cells recognizing non-citrullinated epitopes that could still provide help to ACPA producing auto-reactive B cells[445], but the mechanisms of central tolerance evasion by this population has not been identified.

The ability of CII immunization to cause arthritis in DBA/1 suggests that this strain does not have complete central tolerance to CII and the MHC II allele likely plays a role in this failed central tolerance as it does in RA. The I-A^q MHC II allele found in the DBA/1 strain is important to susceptibility to CIA[471] and mice transgenic for a SE allele (DRB1*0401) immunized with CII developed arthritis that was similar in severity and histological presentation to that of DBA/1 mice[472]. The ability of autologous CII to induce CIA in DBA/1 mice (albeit of lesser severity)

indicates there is incomplete central tolerance to the native CII sequence[454,473]. Other forms of CII, including bCII, which elicit more severe CIA in DBA/1 mice have a D266E substitution in the dominant I-A^{q} restricted epitope of CII(256-270) leading to more extensive T cell proliferation[454,474].

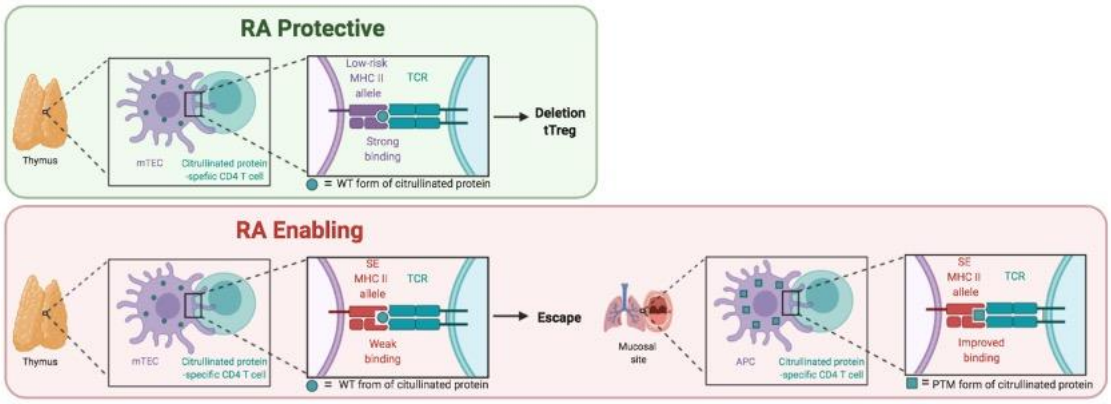

Figure 1.5 T cell escape from central tolerance in RA. **Normally self-antigen is presented in the thymus by medullary thymic epithelial cells (mTECs) resulting in the deletion or tTreg conversion of T cells recognizing the corresponding auto-antigen. This depends on self-antigen epitopes, in this case the wild type (WT) form of proteins such as aggrecan and vimentin that can be citrullinated, strongly binding to MHC alleles so that they can be presented (Top). RA is enabled in individuals with high-risk shared epitope (SE) MHC II alleles because these alleles fail to strongly bind the WT form of citrullinated proteins found in the thymus, leading to the escape of the corresponding auto-reactive T cells (Bottom-left). However, when these citrullinated proteins are converted from their WT form to their post-translationally modified (PTM) citrullinated form at mucosal sites they have improved binding to SE MHC II alleles and can activate citrullinated-protein specific T cell in the secondary lymphoid organs (Bottom-right).**

1.3.3 Priming of auto-reactive T cells

There is considerable evidence for the role of environmental exposures in RA acting not only as a source of PAMPs /DAMP release leading to APC expression of co-stimulation and inflammatory cytokines, but also leading to the generation of (or providing) citrullinated proteins that are the antigens for auto-reactive T cells and B cells (Figure 1.6). The environmental factors of smoking or silica exposure have been shown to increase risk for seropositive RA[475,476]. This

increased risk is thought to be due to the tissue damage and cellular stress these irritants cause in the lungs, which may lead to the local release of DAMPs as well as activation of the PAD enzyme that performs citrullination[445]. Furthermore, because cytosolic PAD is largely inactive until a cell experiences calcium flux due to cellular stress[13], citrullinated antigens may be initially presented by APCs under inflammatory conditions and therefore lack pre-existing peripheral tolerance.

Exposure to pathogenic bacteria or a particular gut microbiome composition could also theoretically be involved in auto-reactive Teff priming in RA through bystander activation, molecularly mimicry, or through the influence of bacterial products/metabolites. However, there is limited evidence in this area to date with the most compelling data implicating a molecular mimicry response to P. gingivalis[445,477–479]. This pathogen, which infects the gums and is associated with periodontitis, may play a role in seropositive RA cases without lung inflammation or smoke/silica exposure. P. gingivalis contains its own citrullinated proteins that could lead to the production of ACPAs and the activation of citrullinated protein specific T cells capable of cross-reacting to citrullinated self-antigens in the joints[480]. Although the role of the gut microbiome in RA pathogenesis remains largely unexplored, there is some evidence of gut microbiome dysbiosis in RA patients[481]. While it is unclear if this altered microbiome composition is a cause or consequence of RA, a few animal models of arthritis have found that microbiome composition influences T cell priming and arthritis development[482,483]. It is also possible that the microbiota influences RA progression through effects on regulatory cell development/function.

In addition to environmental factors, genetics may also affect auto-reactive T cell priming in RA (Figure 1.6). There are approximately one hundred non-HLA genetic loci associated with RA, and these are enriched for genes with immune function[484]. Of these non-HLA loci, the loci with the largest risk association is the non-receptor tyrosine phosphatase PTPN22[485]. Specifically,

the R620W risk allele of PTPN22 is associated with both RA and T1D as this allele is implicated in defective central and peripheral tolerance of T and B cells due to insufficient inhibition of lymphocyte antigen receptor (TCR/BCR) signaling[56,152]. This risk allele of PTPN22 has an odds ratio of 1.7-2.6 for seropositive RA and has an interaction with the SE alleles, indicating that the risk of both alleles is more than the risks of their additive sums[465].

CIA is not a spontaneous model, so APC upregulation of co-stimulation and cytokines needed for Teff priming is not due to genetics or natural environmental exposure, but is caused by the use of CFA as an adjuvant[457,486].

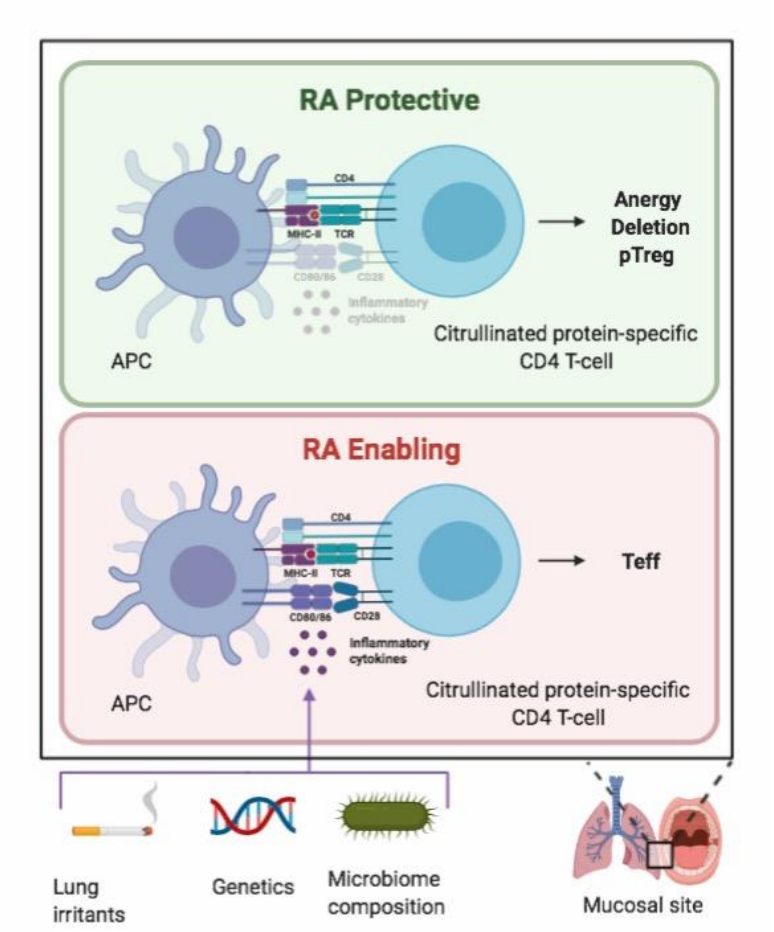

Figure 1.6 Priming of auto-reactive T cells in RA. **Normally self-antigens are presented to naïve T cells without co-stimulation and/or without an inflammatory cytokine milieu leading to T cell deletion, anergy, or pTreg induction. Even if citrullinated protein-specific T cells escape central tolerance, presentation under these conditions protects against the development of RA (Top). For RA to occur there must either be co-stimulation and inflammatory cytokine signals to cause citrullinated protein-specific specific Teff priming or a failure to execute signaling needed for T cell apoptosis, anergy, or pTreg induction (Bottom). Although citrullinated protein-specific specific Teff priming is thought to occur at a mucosal site, the location may vary with the individual based on the source of DAMPs/PAMPs with possible locations including the lungs and gums. Evidence suggests that these elevated co-stimulation and inflammatory cytokine levels could be due to lung irritants, genetic polymorphism, or the microbiome composition. Specifically, the lung irritants of smoking or silica exposure can cause Teff priming by causing stress/damage that releases DAMPs and activates PAD which produces citrullinated proteins; the microbiome composition can contribute to Teff priming through molecular mimicry if P. gingivalis is present in the oral microbiome because it has PAMPs and its own citrullinated epitopes; and genetics can contribute to Teff priming through polymorphism in PTPN22 through insufficient inhibition of TCR signaling.**

1.3.4 Insufficient intrinsic regulation and/or an imbalance between Tregs and Teff

An inability of regulatory cells to sufficiently suppress auto-reactive Teff and other inflammatory immune populations in RA has been predominately attributed to defective Treg function and Teff resistance to suppression as opposed to insufficient Treg levels (Figure 1.7). Most studies have found normal levels of Tregs in the peripheral blood of RA patients, although higher or lower levels have also been reported and these inconsistent findings could be in part due to different markers used to identify Tregs or differences in RA duration[216,487–490]. Unlike in the NOD model of T1D in which Treg levels are reduced in inflamed islets, Treg levels in the joint synovial fluid of RA were consistently higher than of control patients presumably in compensation for elevated levels of inflammatory cells[216,487–489].

Defective Treg function in RA has been shown to both have a genetic basis and be due to the presence of inflammatory cytokines, while Teff resistance to suppression may also occur due to inflammatory cytokines (Figure 1.7). Tregs from PBMCs of RA patients were found to suppress Teff proliferation but were not able to induce pTregs (newly generated $CD62L^-$ Tregs) or suppress inflammatory cytokine production[491]. This defect in infectious tolerance (pTreg induction by Tregs) was due to the inflammatory milieu as anti-TNF-α immunotherapy increased the level of pTregs which exhibited potent ability to suppress inflammatory cytokine production through the production of TGF-β and IL-10[492]. However, the impaired inflammatory cytokine suppression by the initial Treg population ($CD62L^+$) was attributed to a CTLA-4 defect since reduced Treg expression of CTLA-4 was observed in RA patients and stimulation of Tregs from these patients led to upregulation of CTLA-4 expression and restored capacity to suppress inflammatory cytokine production[493]. This CTLA-4 defect was attributed to a genetic polymorphism of CTLA-4 (A49G)

associated with RA instead of the inflammatory environment in part because reduced CTLA-4 expression was not resolved with anti-TNF-α immunotherapy[493]. While the RA susceptible G allele of CTLA-4 only has an odds ratio of 1.11, it is expressed by a relatively large percentage of RA patients (~30-70%) and may have a higher odds ratio within the SE allele positive population making this a plausible explanation[494,495]. Despite the fact that Ehrenstein and colleagues did not observe impaired Treg suppression of Teff proliferation in RA patients, another group suggested that Treg function may be impaired and/or Teff may be resistant to suppression because inflammatory cytokines levels were elevated in the synovial fluid of RA patients and *in vitro* Treg suppression of Teff was impaired in the presence of inflammatory cytokines[496].

It is unlikely that there is a genetic defect related to checkpoint molecules or Treg numbers/function in DBA/1 mice, and the failure of these mechanisms to control auto-reactive T cells is presumably due solely to the strong immune response artificially generated by immunization with CII. However, Treg-Teff balance does play a role in CIA pathogenesis as Treg depletion accelerates the onset of disease[497] and approaches that boost Treg levels can prevent or reverse CIA as discussed below.

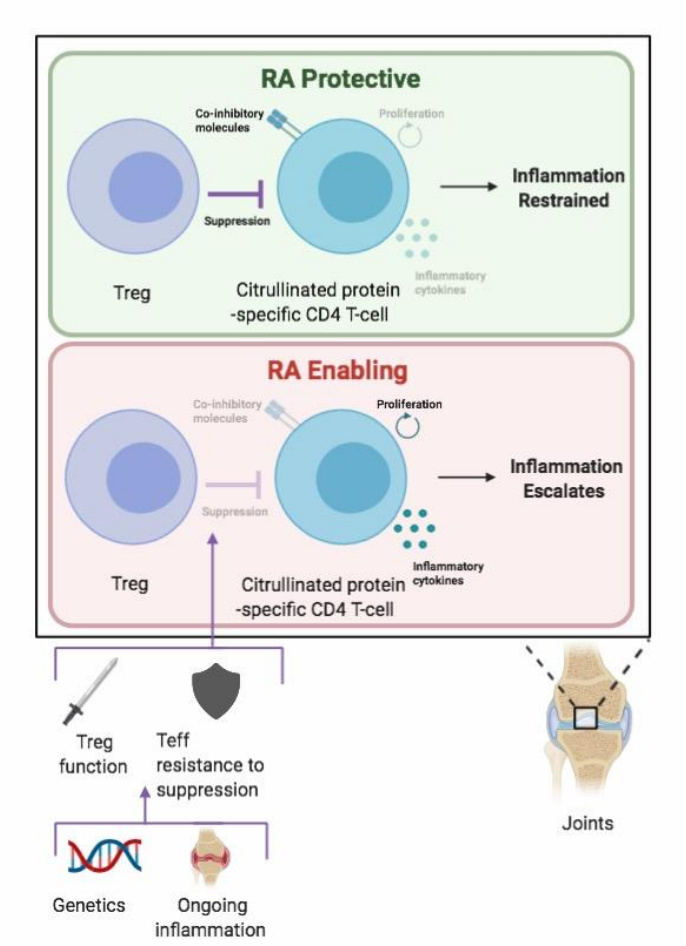

Figure 1.7 Insufficient intrinsic regulation and Treg/Teff imbalance in RA. **Normally auto-reactive Teff cells can be restrained by regulatory populations such as Tregs and/or by sufficient co-inhibitory molecule levels and stimulation relative to the amount of co-stimulatory molecule activity (Top). Progression from citrullinated protein-specific T cell priming to RA onset can occur when there is an inadequate balance of co-stimulatory and co-inhibitory checkpoint molecules or inadequate Treg suppression that enables Teff proliferation and inflammatory cytokine production in the joints (Bottom). Defective Treg suppression in RA has been attributed to impaired Treg function and Teff resistance to suppression as Treg levels even in the synovial fluid of the joints have been largely found to be adequate. The origins of these Treg suppression defects include genetics and ongoing inflammation. Specifically, the Treg function of infectious tolerance (Treg induction) is thought to compromised due to inflammation while the Treg function of suppressing Teff inflammatory cytokine production is thought to be impaired due to a genetic polymorphism reducing CTLA-4 expression. Teff resistance to Treg suppression has also been suggested to be due to ongoing inflammation.**

1.4 Antigen-specific approaches to restore tolerance in RA and CIA

1.4.1 DC and Treg cell-therapy

A treatment capable of re-establishing Treg-Teff balance in RA may be able to restore tolerance and protect against disease progression. Antigen pulsed DCs and antigen-specific Tregs are two antigen-specific cell-therapy based approaches that have had success in animal models of arthritis but clinical testing has been limited. Three different groups found that antigen pulsed bone marrow-derived DC (BMDCs) rendered tolerogenic with tumor necrosis factor (TNF), an irreversible NFκB inhibitor, or dexamethasone and vitamin D3 respectively, reduced arthritis severity in preventative and/or therapeutic models of CIA or methylated bovine serum albumin (mBSA) antigen-induced arthritis (AIA)[312–314]. These studies found that treatment was only effective when BMDCs had been exposed to the relevant antigen, and arthritis suppression was attributed to increased IL-10 production, a shift away from Th1/Th17 polarization and/or towards Th2 T cell polarization, and/or a shift in the isotypes of antigen-specific antibodies to have reduced Th1 (IgG2a or IgG2b/c) titers[312–314]. One of these approaches was later evaluated in a Phase I clinical trial. Specifically, $ACPA^+$ SE $allele^+$ RA patients were treated with one of two doses (n=9 per group) of autologous DCs that had been treated with an irreversible NF-κB inhibitor to promote a tolerogenic phenotype and pulsed with citrullinated peptides[498]. This study demonstrated that treatment was well tolerated, and although it was not powered to assess efficacy it found evidence that PBMCs had increased Treg/Teff ratio and reduced citrullinated protein-specific IL-6 production 1 month after DC therapy[498]. Antigen-specific Treg cell-therapy, using iTregs generated with CII-specific TCR tg mice or with CII-stimulated naïve T cells from mice with CIA, was also effective in reducing arthritis severity in preventative and/or therapeutic models of

CIA[499,500]. Disease protection was associated with reduced anti-CII antibody titers as well as an increased Treg/Th17 ratio in the former study, and reduced TNF-α production in the latter study[499,500]. Clinical trials evaluating Treg cell-therapy in several autoimmune and transplant indications have primarily used polyclonal Tregs due to difficulty obtaining/expanding sufficient quantities of antigen-specific Tregs [325,326]. Although Treg cell-therapy has not yet been evaluated clinically in RA, trials in other indications have demonstrated safety while efficacy has not yet been proven[325,326].

Despite the promise of antigen-specific DC and Treg cell-therapy approaches, considerable challenges and concerns remain. While antigen-specificity is easier to achieve for tolerogenic DCs than for Tregs, there is still some concern about the stability of this DC phenotype in the face of inflammatory conditions[349,501,502]. Polyclonal Treg cell-therapy faces concerns about potency[287,348] and non-specific immunosuppression[330] while obtaining sufficient quantities of antigen-specific Tregs in a clinically relevant manner is challenging. Furthermore, both cell-therapy approaches come with the high cost and complexity of good manufacturing practice (GMP) isolation and cell expansion, particularly if using autologous cells[348].

1.4.2 Auto-antigen and/or immunomodulatory agent therapy

Approaches to restore Treg-Teff balance that use auto-antigen and/or localized immunomodulatory agents in an antigen-specific manner could avoid some of the issues associated with cell-therapy. The simplest approach to achieve this is freely administered auto-antigen, and oral, i.v., or s.c. administration of CII or citrullinated peptide was able to reduce arthritis severity in preventative rodent models of CIA and AIA[455,503–505]. Alternatively, another groups had similar findings using auto-antigen peptide in complex with MHC II in both preventative and therapeutic

CIA models[506]. In many of these studies the proposed mechanism involved an increase in FoxP3$^+$ Tregs or other regulatory population[503–506]. However, clinical trials evaluating oral auto-antigen or auto-antigen peptide-mHC complex failed to show consistent efficacy (possibly because of CII dose, species, or formulation differences) and/or have found only modest clinical effects relative to existing treatments[507–512]. The inability to induce tolerance associated immunological changes in some trials could be due to properties of the immunotherapy such as the selected auto-antigen and dose administered, or because of the existing phenotype or inflammatory context when auto-antigen is encountered by the responding T cell population[513]. In trials that observed the desired immunological changes but not substantially improved disease outcomes[511], the regulatory cell response may not be of sufficient magnitude or durability to overcome existing Teff.

Therefore, approaches that ensure the presentation of auto-antigen (delivered or endogenous) in a tolerogenic context, and/or induce more robust regulatory cell expansion may be more effective in the treatment of RA. One such approach used liposomes delivering auto-antigen and a NF-κB inhibitor as an immunomodulatory agent to ensure a tolerogenic context. This resulted in increased levels of FoxP3$^+$ Tregs, decreased antigen-specific T cell proliferation, and reduced arthritis severity in preventive and therapeutic models of AIA[514]. The same pMHC MP technology used in T1D (discussed in Section 1.2.3.3), was also used to ensure tolerogenic presentation in a therapeutic CIA model of RA where it reduced arthritis severity[295]. As discussed previously, even though this approach does not use immunomodulatory agents, the large number of pMHC complexes (in this case CII peptide on and HLA DR4 MHC in DR4 tg mice) on a NP leads to strong signaling in cells with corresponding TCR specificities that enforces a tolerogenic context and converts activated Teff into regulatory Tr1 cells[295,397]. It remains to be determined if these approaches for ensuring a tolerogenic context will fare better than auto-antigen alone in

treating RA clinically, and additional research will likely be needed to determine which auto-antigen(s) are most effective. Instead of administering an auto-antigen, local delivery of immunomodulatory agents may be effective for antigen-specific tolerance in RA. In particular, injection of immunomodulatory microparticles into inflamed joints could be a viable clinical approach as intra-articular injection of corticosteroid containing microparticles is already FDA-approved for osteoarthritis pain management[515].

2.0 TRI MP for the prevention of T1D

Sections 2.2.1 and 2.2.2 are adapted from the following manuscript in accordance with PLOS's creative commons attribution (CC BY) license: Bassin, E. J., Buckley, A. R., Piganelli, J. D. & Little, S. R. TRI microparticles prevent inflammatory arthritis in a collagen-induced arthritis model. *PLoS One* **15**, e0239396 (2020).

2.1 Introduction

If it were possible to selectively increase the number of endogenous islet-specific Tregs in a patient without *ex vivo* live cell-therapy, the potent effect of islet-specific Tregs[287,288,291] could be harnessed for the treatment of T1D without the safety and feasibility issues associated with cell therapy[348]. Accordingly, we reported the use of polymeric microparticles (MP) which release TGF-β, rapamycin, and IL-2 (TRI MP)[516] so that endogenous antigen can be presented in a tolerance-promoting local immunological microenvironment. TGF-β and rapamycin (Rapa) can have a variety of suppressive effects on the immune system, including directly suppressing Teff cell proliferation, enabling continued Treg expansion, and promoting naïve T cell differentiation into Tregs (which is in part achieved by effects on APCs)[362,363,517]. IL-2 is needed for T cell differentiation/proliferation, and low doses expand Tregs[518]. Subcutaneous TRI MP administration at the site of inflammation has previously demonstrated an ability to expand Tregs and limit Teff levels, resulting in disease prevention or therapeutic treatment in several preclinical models[408–410]. These studies have also shown the combination of all three drugs is more effective than any drug

alone or pair of two drugs, that TRI MP can confine drug activity to a local area resulting in antigen-specific tolerance, and that sustained release of drug from TRI MP is more potent than equivalent unencapsulated doses. This antigen-specific tolerance was presumably obtained because TRI MP was localized to the site of inflammation where the antigen(s) of interest were present. However, the site of inflammation in T1D (the pancreas) is not easily accessible to MP, so TRI MP was administered s.c. with insulin peptide instead of relying on endogenous antigen presentation. We hypothesized that co-administration of TRI MP and insulin peptide would result in the induction/expansion of insulin-specific Tregs, and these islet-specific Tregs would traffic to the pancreas where they would be capable of preventing T1D onset.

Here we evaluate the ability of TRI MP and insulin peptide to delay the onset of spontaneous T1D in NOD mice and investigate how delivery systems attributes and experimental model attributes may be affecting *in vivo* Treg generation.

2.2 Materials and methods

2.2.1 Microparticle fabrication

TRI MP ("Original Formulation") were fabricated using an emulsion-solvent evaporation method as previously described[409]. A 5% w/v polymer solution was prepared by dissolving 200 mg of Poly (lactic-co-glycolic) acid (PLGA) in 4 mL of dichloromethane (DCM) (Sigma Aldrich, St. Louis, MO). For IL-2 and Rapamycin, 200 mg of acid terminated PLGA (50:50 lactide:glycolide, MW:7-17 kDa, Sigma Aldrich) was used for polymer MP. For TGF-β, 170 mg of ester terminated PLGA (50:50 lactide:glycolide, MW: 7-17 kDa) (Sigma Aldrich) and 30 mg

of mPEG-PLGA (50:50 lactide:glycolide, 5-20 kDa, PolySciTech, West Lafayette, IN) were used for polymer MP.

For TGF-β and IL-2, primary emulsions were formed by adding 5 μg of recombinant protein (hTGF-β from PeproTech, Rocky Hill, NJ) (mIL-2 from R&D Systems, Minneapolis, MN), dissolved in 200 μL of deionized (DI) water or phosphate buffered saline (PBS) respectively, to the organic polymer phase, and sonicating at 25% amplitude for 10 s (Vibra-Cell, Newton, CT or Active Motif, Carlsbad, CA). For Rapamycin, 1 mg of rapamycin (Alfa Aesar, Ward Hill, MA) dissolved in 100 μL of dimethyl sulfoxide was added to the polymer solution without sonication. Blank MP was made for each type of MP using vehicle control solution.

The resulting primary emulsion or polymer-drug solution was poured into 60 mL of 2% w/v poly(vinyl alcohol) (PVA, MW ~25 kDa, 98% hydrolyzed, Polysciences, Warrington, PA) in DI water (or 51.6 mM NaCl for IL-2) and homogenized (L4RT-1, Silverson, East Longmeadow MA) at 3,000 rpm for 1 min. The resulting double or single emulsion was then poured into 80 mL of 1% w/v PVA in DI water or (51.6 mM NaCl for IL-2) and stirred (600 rpm) for 3 h to allow DCM to evaporate. TGF-β and IL-2 emulsions were homogenized and stirred on ice (for the "Original Formulation-Higher Dose" group). After stirring, MP were collected by centrifugation (200 g = 1,000 rpm, 5 min, 4 °C) and washed 4 times with DI water before lyophilizing for 48 hours.

For the "Longer IL-2 release" TRI MP formulation, particles were fabricated as previously described[408]. This method is similar to the "Original Formulation" with the following changes: a 2.5% w/v polymer solution; all MP were made with 160 mg of ester-terminated PLGA (7-17 kDa for IL-2 and Rapa, 40kDa for TGF-β) and 40 mg of mPEG-PLGA; IL-2 was dissolved in DI water and PVA without salt was used; 150 μL of a 10mg/mL solution was used for Rapa loading; primary

emulsions or polymer-drug solution was homogenized in PVA on ice at 10,000 rpm for 1 min; and MP were collected by centrifugation at 3,000 rpm for 8 min.

For the original formulation of Peptide MP, insulin Ins B:9-23 peptide (Genscript, Piscataway, NJ) was formulated like the "Original Formulation" IL-2 MP (acid terminated PLGA, double emulsion, salt in PVA) with 100 µL of a 10 mg/mL Ins B:9-23 peptide solution used for loading. Several formulation variations were tested as described in Figure 2.4. Ins B:9-23 R22E peptide (Custom Peptide Synthesis service, Thermo Fisher) was also used for MP made with the original formulation of Peptide MP.

2.2.2 Microparticle characterization

Total drug loading of MP was assessed as previously described[408,519]. For TGF-β and IL-2, drug was extracted using DCM and PBS with 0.1% sodium dodecyl sulfate (SDS) as a surfactant in a two-phase extraction. 5 mg of MP was dissolved in 500 µL DCM, mixed with 250 µL of PBS + SDS using a vortex mixer, and centrifuged (5,000 g, 10 min , 4 °C) to separate the phases. The aqueous phase was collected (200 µL), and the extraction process was repeated 2 more times, with 250 µL of PBS + SDS collected for the third extraction. TGF-β and IL-2 concentrations were measured using enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (R&D Systems) and used to calculate drug loading (nanograms of drug per mg of microparticles). For rapamycin, drug was extracted by dissolving MP (5 mg) in acetonitrile (500 µL). Drug concentration and subsequently drug loading was calculated by measuring absorbance (278 nm) using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) and comparing values to a standard curve of rapamycin in acetonitrile. For insulin peptide, drug was extracted by dissolving Peptide MP (5 mg) in DMSO (500 µL). The peptide solution was diluted

2x DI water and peptide concentration was determined using a quantitative fluorometric peptide assay kit (Thermo Fisher) according to manufacturer's instructions. A standard was prepared from insulin peptide and fluorescence (Ex/Em at 390nm/475nm) was measured using a microplate reader (SpectraMax M5, Molecular Devices)

MP release kinetics were assessed by dissolving 10 mg of MP in 1 mL of release solution, incubating at 37 °C with end-over-end rotation, and collecting samples with solution replacement at indicated time points. PBS with 1% w/v bovine serum albumin (BSA) was used as release solution for TGF-β and IL-2, and PBS with 0.02% v/v Tween-80 was used as release solution for rapamycin and insulin peptide. TGF-β and IL-2 concentrations were assessed by ELISA, rapamycin concentration was assessed by microplate reader (absorbance 278 nm), and insulin peptide concentration was assessed with fluorometric peptide assay kit (Thermo Fisher) as described above. These concentrations were then used to calculate cumulative release (ng drug/mg MP).

2.2.3 Mice

NOD mice, C57Bl/6 (B6) mice, NOD.scid mice, NOD FoxP3-EGFP mice, and C57BL/6.H2g7 (B6g7) mice were housed and bred under specific pathogen-free conditions at the University of Pittsburgh Children's Hospital Rangos Research Center animal facility. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. For spontaneous T1D prevention studies and an adoptive transfer prevention study, NOD mice or NOD.scid mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimated for at least 72 hrs before treatment. Only female NOD mice were used for prevention

studies due to sex difference in T1D onset and incidence[9,26]. For other studies pre-diabetic mice were used with age and gender matching between treatment groups.

2.2.4 *In vitro* Treg induction assay using soluble TRI and comparing B6 and NOD mice

Female NOD FoxP3-EGFP mice (6-10 week old) or B6 mice were sacrificed and spleens were removed then ground to single cell suspensions using 70 μm filters. RBC lysis was performed on spleens with RBC lysis buffer (eBioscience, San Diego, CA) and cells were counted. CD4 negative enrichment was performed by staining cells (approximately 10 million/mL) with biotinylated antibodies - CD8a, CD11b, CD11c, CD19,CD45R/B220, and TCR γ/δ, (BioLegend, San Diego, CA) - followed by streptavidin magnetic beads (Thermo Fisher Scientific, Waltham, MA), and the removal of bound cells using a DynaMag magnet (Thermo Fisher Scientific). CD4 enriched cells were stained with Fc block (eBioscience) and for CD4 (RM4-5;BD) and CD25 (PC61;BD) and naïve T cells ($CD4^+CD25^-$ and also FoxP3-$EGFP^-$ for NOD mice) were FACS sorted (AriaII, BD).

Naïve T cells were cultured in 96 well round bottom plates at 100,000 cells/well in complete DMEM media and stimulated with CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a ratio of 2 dyanabeads:1 cell. In some conditions cells were cultured with 5 ng/mL TGF-β alone or 5 ng/mL TGF-β, 10 ng/mL IL-2, and 10 ng/mL rapamycin. After 4 days, cells were stained with Fc block, fixable viability dye (eBioscience) and for CD4 (RM4-5; BD),then fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience), stained for FoxP3 (FJK-16s; eBioscience), and run on a flow cytometer (LSRII, BD) and analyzed using FlowJo software (Tree Star, Ashland, OR) with gates based on isotype and single-color controls.

2.2.5 *In vitro* Treg induction and suppression assays using TRI MP and comparing pre-diabetic and diabetic NOD mice

Pre-diabetic female (6-10 week old) NOD FoxP3-EGFP mice or diabetic (blood glucose, BG > 300 mg/dL) NOD FoxP3-EGFP mice were sacrificed and naïve T cells were isolated as described above. Natural Tregs (nTregs were also sorted as defined by the markers $CD4^+CD25^+EGFP^+$.

Naïve T cells or nTregs were cultured in 24 well flat bottom transwell plates at 1 million cells/well in complete DMEM media and stimulated with CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a ratio of 1 dyanabead:1 cell. Naïve T cells were cultured with 5 ng/mL TGF-β, 10 ng/mL IL-2, and 10 ng/mL rapamycin (Soluble TRI); a dose of TRI MP (2.85 mg TGF-β MP/well, 1.05 mg IL-2/well, 1.4 x 10^{-3} mg Rapa/well) with roughly equivalent loading to Soluble TRI, or with a dose of Blank MP corresponding to that of TRI MP.

After 4 days, cells were stained with Fc block (eBioscience), fixable viability dye (eBioscience) and for CD4 (RM4-5; BD) and *in vitro* induced Tregs (iTregs) or activated nTregs were FACS sorted (AriaII, BD) to get a pure population of $CD4^+CD25^+EGFP^+$ Tregs. An additional pre-diabetic or diabetic (matching that of the Tregs) mouse was sacrificed and conventional T cells (Tconv) were isolated as described above for naïve T cells, except FACS sorting for $CD4^+EGFP^-$ T cells.

To distinguish Tconv from unstable Tregs in a suppression assay, Tconv were stained with the proliferative dye VPD450 (BD) and Tregs were stained with CellTrace Red (Thermo Fisher Scientific). For the suppression assay, 96 well round bottom plates were plated with 50,000 Tconv and no Tregs or Tregs at Tconv:Treg ratios of 2:1, 4:1, or 8:1, cultured in complete DMEM media,

and stimulated with CD3/CD28 Dynabeads at a ratio of 1 dyanabead:4 cells. After 3 days, cells were stained fixable viability dye (eBioscience), run on a flow cytometer (LSRII, BD) and analyzed using FlowJo software (Tree Star) with gates based on isotype and single-color controls. The percentage of proliferating Tconv was determined by dilution of VPD450 among CellTrace Red negative cells. Proliferation index was used to normalize between different experiments and was calculated as % proliferating Tconv at a particular Tconv:Treg ratio divided by % proliferating Tconv in the absence of Tregs. Treg stability was assessed by measuring the % of CellTrace Red cells retaining FoxP3-EGFP expression.

2.2.6 NOD spontaneous prevention model

For the pilot prevention study (Figure 2.3 A), female NOD mice were injected s.c. with 200 μL of Blank MP, Peptide MP, TRI MP, or TRIpeptide MP on both sides above the hind limb at 6 weeks of age and again 10 days later. Each injection contained 3.4 mg of all four MP types or the corresponding Blank MP dissolved in PBS (17 mg/mL per MP type or 64 mg/mL of total MP). Peptide MP and TRIpeptide MP injections also included soluble Ins B:9-23 peptide at a concentration of 250 μg/mL (50 μg per side per time point). Blood glucose (BG) was checked weekly beginning at 12 weeks of age, and mice were considered diabetic and euthanized if BG was greater than 300 mg/dL and this reading was confirmed the following day. Mice that remained T1D-free at 35 weeks of age (long-term survivors) were administered an i.p. glucose tolerance test (IPGTT) and then sacrificed in order to assess Treg levels in the spleen and pancreatic lymph node (pLN) by flow cytometry (as described above), and to assess regulatory cell function in an adoptive transfer prevention study. Mice were also similarly treated with PBS, Blank MP, or UV-irradiated Blank MP (cell culture hood for 1 hr) in a mock prevention study (Figure 2.3 G).

For the later prevention study (Figure 2.14 A), female NOD mice were injected s.c. on both sides above the hind limb with 200 μL per side of PBS, Blank MP, or TRI MP and for some groups this was co-administered with 50 μL per side of an emulsion of incomplete Freund's adjuvant (IFA) and insulin peptide (Ins B:9-23 R22E). These injections were administered at 4 weeks of age and again at 6 weeks of age, with half the dose of MP or insulin peptide given in the second set of injections. The first set of injections contained 9.6 mg TGF-β MP, 7.5 mg IL-2 MP, and 4 mg of Rapa MP (or corresponding Blank MP) in PBS per 200 μL injection. Peptide emulsions were prepared 1:1 with peptide solution (1-2 mg/mL dissolved in 5-10% DMSO in PBS) and IFA, and the first set of injections contained 50 μg peptide per 50 μL injection. BG was monitored as described above.

2.2.7 NOD adoptive transfer model

NOD.scid recipients were given 5 million pooled splenocytes from diabetic NOD mice alone or with an additional 5 million lymphocytes from MP treated NOD mice through retro orbital injection. For the pilot adoptive transfer prevention study (Figure 2.3 F), these 5 million lymphocytes were splenocytes from long-term survivors treated with TRI MP (n=3) or Blank MP (n=2) and 3 NOD.scid recipients were used per NOD donor (n=6-9). For the later adoptive transfer prevention study (Figure 2.14 B), these 5 million lymphocytes were from the iLN of NOD mice treated with IFA/R22E Ins. peptide and either TRI MP or Blank MP (at the doses described above) for 10 days and iLN from all NOD donors of the same treatment group (n=5 per group) were pooled before transferring to NOD.scid recipients (n=8 per group). BG was checked weekly

beginning at 3 weeks after adoptive transfer, and mice were considered diabetic and euthanized if BG was greater than 300 mg/dL and this reading was confirmed the following day.

2.2.8 i.p. glucose tolerance test (IPGTT)

Mice were fasted overnight (16-18 hours) and given glucose i.p. at a dose of 1.5 mg/kg using a 20% glucose solution. Blood glucose was measured prior to injection and at intervals of 15, 30, and 60 minutes post injection. The area under the curve (AUC) was calculated with GraphPad Prism v7 (San Diego, CA).

2.2.9 *In vivo* Treg induction

NOD FoxP3-EGFP mice were injected s.c. with 200 μL of PBS, Blank MP, or TRI MP and 50 μL of an emulsion of insulin peptide (50 μg) per side. Mice were sacrificed after 10 days (or 21-24 days in indicated groups), the iLN, pLN, and sometimes islets were isolated and single cell suspensions were prepared. Cells were counted and stained with 10 μg/mL insulin tetramer (I-A(g7) mouse InsB p8G var), hen egg lysozyme (HEL) tetramer (I-A(g7) chicken HEL 11-25), or CLIP tetramer control (I-A(g7) human CLIP 87-101) (NIH tetramer core) at a concentration of approximately 10 million cells/mL for 1 hr at 37°C. Cells were then washed, stained with Fc block (eBioscience), fixable viability dye (eBioscience) and for CD4 (RM4-5; BD) and run on a flow cytometer (LSRII, BD) where these stains and FoxP3-EGFP expression were measured. For B6g7 mice, cells were fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience) and stained for FoxP3 (FJK-16s; eBioscience) prior to flow cytometry. Data was

analyzed using FlowJo software (Tree Star) with gates based on CLIP/HEL tetramer controls and single color or isotype controls.

Initially TRI MP dosing was the same as the pilot prevention study (3.4 mg of each MP type per 200 μL) and complete Freud's adjuvant (CFA) was used for insulin peptide delivery (Figure 2.6). Several additional MP doses and/or formulations were also tested (Figure 2.8) as described in Table 2-1. Afterwards, the "Higher Dose" of the original TRI MP formulation was used for *in vivo* Treg induction experiments, which consisted of 6 mg TGF-β MP, 7.5 mg IL-2 MP, and 4 mg of Rapa MP per 200 μL. Variations to insulin peptide delivery were tested (Figures 2.9 and 2.10) including the use of IFA or soluble peptide instead of CFA, R22E substituted Ins B:9-23 peptide, MP encapsulated R22E Ins. peptide, and osmotic pump delivery of R22E Ins. peptide. Alzet Osmotic pumps (Durect, Cupertino, CA) were filled with ~ 1 mg/mL R22E Ins. peptide based on the pump rate in order to release 5 μg/day. A small incision was made, osmotic pumps were aseptically implanted s.c. on the lower back of NOD FoxP3-EGFP mice, and the incision was closed with a surgical staple. Mice with implanted osmotic pumps were sacrificed after 3 weeks. In some mice a single 200 μL of TRI MP with soluble of MP encapsulated R22E Ins. peptide was administered i.p. (Figure 2.13 D-G).

Several variations to this experimental procedure were performed where mice were immunized with HEL protein or HEL peptide. HEL protein (100 μg in single injection) in CFA without MP was given at various s.c. locations and HEL-specific T cell expansion in the pLN was assessed by HEL tetramer staining (Figure 2.13 A-C). NOD FoxP3-EGFP mice or B6g7 mice were immunized with HEL peptide (HEL 11-25; 50 μg per side; Custom Peptide Synthesis service, Thermo Fisher) in CFA and TRI MP or Blank MP in order to compare antigen-specific Tregs and

polyclonal Tregs expansion by TRI MP for these antigen-mouse strain combinations to that of the insulin peptide – NOD mouse combination (Figure 2.7).

2.2.10 Islet isolation

The pancreas was inflated with a collagenase (Sigma) solution (1.95 mg/mL in HBSS Buffer with 20mM HEPES) by clamping the duodenum where it intersects the common bile duct and injecting the collagenase solution into the common bile duct using a 30G needle. The inflated pancreas was kept on ice, and then digested in additional collagenase solution at 37°C for 20 min. Digested pancreas was washed 4 times with excess HBSS-HEPES-BSA buffer (0.5 % BSA) and given a quick spin (1,000 rpm until up to speed) to pellet tissue. Islets were picked under a dissecting microscope and broken up by incubating at 37°C in enzyme-free cell dissociation buffer (Thermo Fisher) for 15 min with vortexing every 5 min. Digested islet were passed through a 70 µm filter and then stained with tetramer and antibodies and analyzed by flow cytometry as described above.

2.2.11 Statistics

Statistical analyses were performed with GraphPad Prism v7. Data are presented as mean ± SEM and the following cutoffs were used for significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For iTreg suppression assay curves, a two-way ANOVA (treatment group and Tconv:Treg ratio) with Tukey post-hoc analysis to compare all pre-diabetic groups to each other and to compare all diabetic groups to each other. For T1D incidence curves, a Log-rank (Mantel-Cox) test was ran comparing all curves. If this was significant, Long-rank (Mantel-Cox) test were

performed for each individual comparison, and p values were multiplied by the number of comparisons made (3-6). All other statistical analysis used a one-way ANOVA to compare multiple groups. When the mean of every group was compared to the mean of every other group (often done when there were 3 groups), Tukey post-hoc analysis was used. When the mean of every group was compared to the mean of a reference group (often done when there was more than 3 groups), Dunnett post-hoc analysis was used.

2.3 Results

2.3.1 TRI MP capable of *in vitro* induction of functional Tregs

Although TRI MP had previously been shown to induce Tregs capable of conventional T cell (Tconv) suppression *in vitro* using cells from C57Bl/6 (B6) mice[516], literature evidence suggested that NOD Tconv may be inherently more resistant to Treg suppression than those of a B6 background and that Treg suppressive capacity and/or Tconv susceptibility to suppression in NOD mice may decrease with age/ T1D progression[220,233,234]. An ability for TRI MP to induce functional Tregs in diabetic mice would be essential for TRI MP to be able to reverse onset T1D. Therefore, the ability of TRI MP to induce Tregs from pre-diabetic or diabetic NOD naïve T cells, and the suppressive function of those T cells was evaluated *in vitro*. TRI MP morphology, size, and drug release kinetics (Figure 2.1) were similar to those previously reported[409]. In order to compare Treg induction for B6 and NOD FoxP3-EGFP mice, naïve $CD4^+CD25^-$ (FoxP3-EGFP $^-$ for NOD) were sorted from spleens and cultured with CD3/CD28 dynabeads in the presence of media only, soluble TGF-β, or soluble TRI for 4 days and then FoxP3 expression was analyzed by

flow cytometry. Soluble TRI led to a larger percentage of cells expressing FoxP3 than TGF-β alone as expected and similar Treg induction was observed for both B6 and NOD mice (Figure 2.2 A). A similar *in vitro* Treg induction assay was performed to compare the ability of soluble TRI and an equivalent dose of TRI MP to induce Tregs in pre-diabetic (6-8 week old) and diabetic NOD mice. Similar Treg induction was observed between TRI soluble and TRI MP (Figure 2.2 B), indicating that the TRI drugs remained functional after encapsulation and release from MP. A slight reduction in Treg induction for TRI MP relative to TRI Soluble likely reflects that "equivalent doses" were based on TRI MP drug loading, which is more than the amount of drug that is actually released over a 4 day period. A slight decrease in Treg induction for soluble TRI in this induction assay (Figure 2.2 B) relative to the former Treg induction assay (Figure 2.2 A) likely reflects cell culture differences, as round bottom 96 well plates were found to result in greater T cell viability and Treg induction than the flat bottom 24 well plates with transwells used to accommodate MP (data not shown). Importantly, similar Treg induction levels were observed in diabetic mice compared to pre-diabetic mice (Figure 2.2 B).

To evaluate the suppressive function of these *in vitro* induced Tregs (iTregs), a pure iTreg population was sorted after TRI MP or TRI soluble culture and cultured in different ratios with freshly sorted $CD4^+$ FoxP3-EGFP $^-$ Tconv population in the presence of CD3/CD28 dynabead stimulation. As a control for comparison to iTreg, natural Tregs (nTreg, $CD4^+$ FoxP3-EGFP $^+$) were also sorted and activated *in vitro*. Tconv from pre-diabetic mice were used with iTreg generated from pre-diabetic mice and Tconv from diabetic mice were used iTreg generated from diabetic mice. The proliferation index, the percentage of proliferating Tconv at a particular Tconv:Treg ratio normalized to the percentage of proliferating Tconv in the absence of Tregs, was comparable regardless of the source of Treg or diabetes status at a Tconv:Treg ratio of 2:1 (Figure

2.2 C). However, there were significant differences for the pre-diabatic mouse derived cells between activated nTregs, iTreg – TRI MP, and iTreg-TRI Soluble at ratios with less Tregs (8:1 and 4:1, Two-way ANOVA, Tukey post-hoc for treatment group, $p < 0.05$) (Figure 2.2 C). Interestingly, Treg suppression of Tconv at high Tconv:Treg ratios (8:1 or 4:1) was uniformly reduced when using diabetic Treg and diabetic Tconv regardless of Treg source (Figure 2.2 C). As expected[345], iTregs were highly unstable compared to nTregs and there is a trend towards greater instability at higher Tconv:Treg ratios (Figure 2.2 D), which could be due to greater concentrations of inflammatory cytokines. Although there were minor differences between TRI MP and TRI Soluble and between pre-diabetic NOD mice and diabetic NOD mice, overall this data shows that TRI MP was cable of inducing suppressive NOD Tregs *in vitro* in both pre-diabetic and diabetic mice.

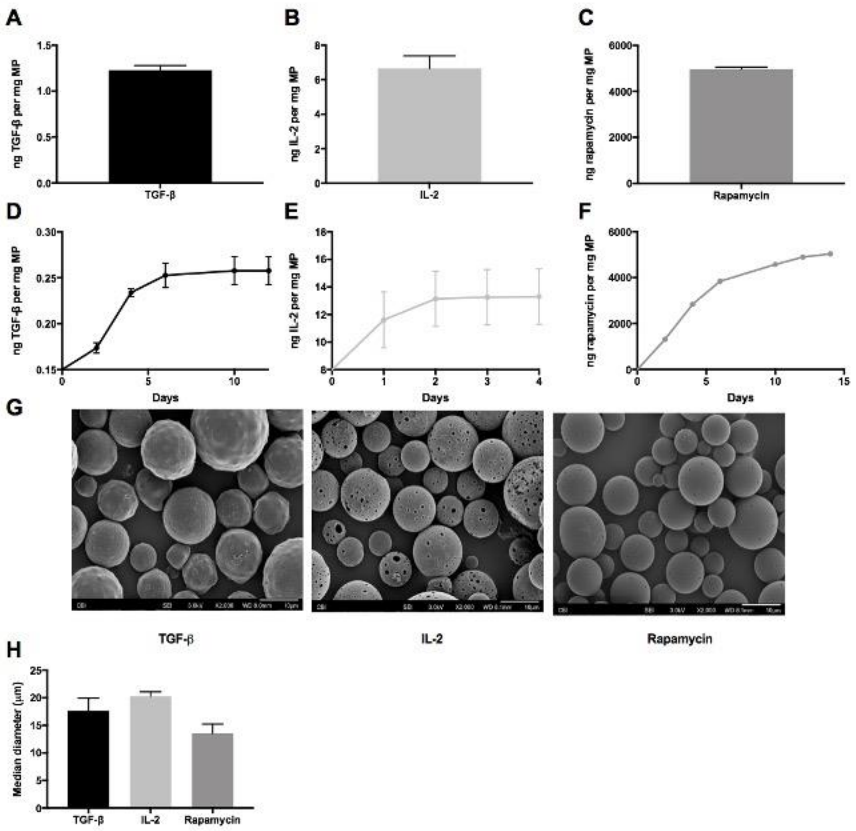

Figure 2.1 TRI MP characterization. **A-C) Drug loading (ng/mg) for TGF-β microparticles (MP), IL-2 MP, and rapamycin MP respectively. n = 6 batches of MP per group, data presented as mean ± SEM. D-F) *In vitro r*elease kinetics for TGF-β MP, IL-2 MP, and rapamycin MP respectively. Representative batch of MP shown with release samples performed in triplicate and presented as mean ± SEM. G) SEM images showing surface morphology of TGF-β MP, IL-2 MP, and rapamycin MP with 10 μm scale bar shown for reference. H) Average MP diameter measured by Coulter Counter, presented as mean ± SEM.**

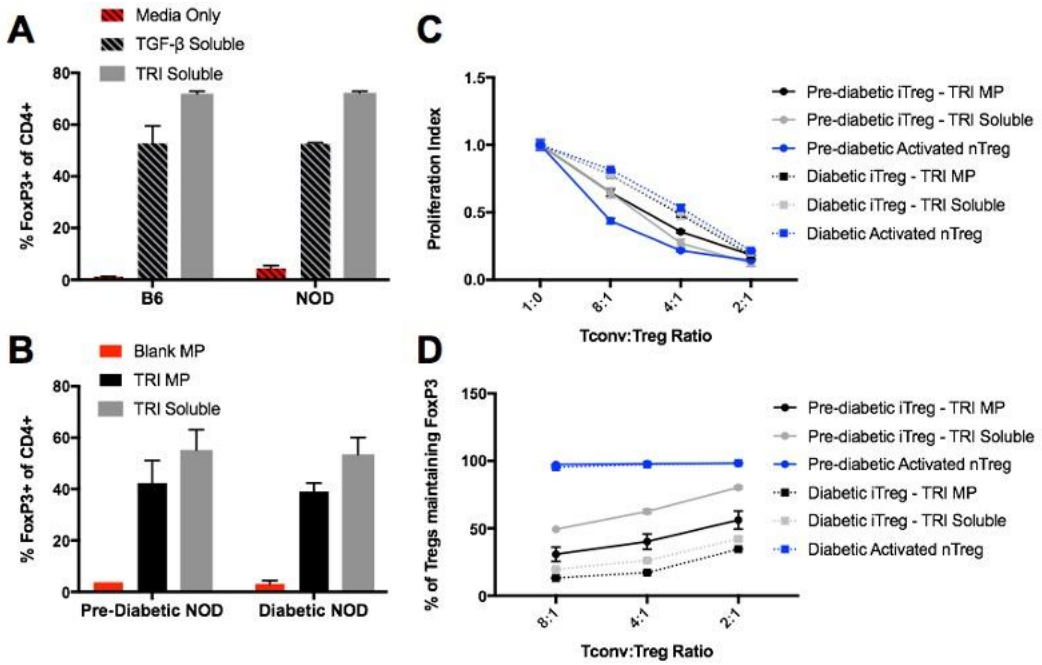

Figure 2.2 TRI MP induces suppressive NOD Tregs in vitro in both pre-diabetic and diabetic mice. A-B) In vitro Treg induction assay indicating percentage of sorted naïve CD4+ cells that are FoxP3+ after 4 days of culture in the indicated media condition. A) On the left are cells from B6 mice and on the right are cells from NOD mice. n = 1 independent experiment in duplicate. B) On the left are cells from pre-diabetic NOD mice and on the right are cells from diabetic NOD mice. n = 2-3 independent experiments. TRI = TGF-β, rapamycin, and IL-2. MP = microparticles, Blank MP = vehicle control MP. Statistics were not run as there were less than 3 samples for some groups, additional samples and statical analysis would be needed for publication quality data to confirm these findings. C-D) In vitro Treg suppression assay using sorted in vitro induced Tregs (iTregs) generated in B) by providing TRI MP or TRI Soluble treatment to pre-diabetic or diabetic NOD T cells. Sorted and activated natural Tregs (nTreg) were also used as a control. Conventional T cells (Tconv) were sorted from mice of corresponding diabetes status. C) Treg suppressive potency for Tregs generated using T cell population and treatment indicated as a function of Tconv:Treg ratio and proliferation index (% of proliferating cells at a Tconv:Treg ratio normalized to % proliferating in absence of Tregs). D) Percentage of sorted Tregs that retained FoxP3 expression after 3 days of culture in a suppression assay as a function of Tconv:Treg ratio. n = 1-2 independent experiments in triplicate. All data presented as mean ± SEM. A two-way ANOVA (treatment group and Tconv:Treg ratio) with Tukey post-hoc analysis to compare all pre-diabetic groups to each other and to compare all diabetic groups to each other. Pre-diabetic iTreg-TRI MP and Pre-diabetic iTreg-TRI Soluble had significantly greater proliferation index ($p < 0.05$ to $p < 0.0001$) at Tcon:Treg ratios of 8:1 and 4:1 but differences were non-significant at other ratios. No significant difference for proliferation index was observed between diabetic treatment groups at any Tconv:Treg ratio. For both pre-diabetic conditions and diabetic conditions, iTreg-TRI MP and iTreg-TRI Soluble had significantly reduced FoxP3 maintenance relative to the corresponding (pre-diabetic or diabetic) nTreg at all Tconv:Treg ratios ($p < 0.05$ to $p < 0.0001$.

2.3.2 Pilot T1D prevention study and follow up to assess β cell function and Treg levels

Since TRI MP was functioning as expected *in vitro*, the ability of TRI MP to prevent spontaneous T1D in NOD mice was assessed. NOD mice were given two s.c. injections of a combination of TRI MP and insulin B:9-23 peptide (Peptide MP), together referred to as TRIpeptide MP, or controls of TRI MP alone, Peptide MP alone, or Blank MP. While an initial group size of n=6 was chosen for this pilot study, several mice died from unknown causes prior to the onset of T1D resulting in an n of 3-5 per group. All the treatment groups had a similar fraction (~50%) of mice remaining T1D-free at an age of 35 weeks, with no significant differences observed (Figure 2.3 A). However, this was likely in part a reflection of the small group sizes. In order to see if long-term survivors (mice T1D-free at 35 weeks of age) treated with TRIpeptide MP differed from long-term survivors given control treatments, analysis was performed to assess β cell function and Treg levels. An i.p. glucose tolerance test (IPGTT) showed comparable β cell function in all long-term survivors with the exception of one Blank MP treated mouse (26-RL) that failed to quickly control blood glucose leading to an elevated area under the curve (Figure 2.3 B and C). Likewise, there were no substantial differences in the amount of $FoxP3^+$ Tregs in the spleen (Figure 2.3 D) or pancreatic lymph node (pLN) (Figure 2.3 E) of long-term survivors treated with TRIpeptide MP relative to long-term survivors treated with controls. Splenocytes from long-term survivors treated with TRIpeptide MP or Blank MP did not significantly delay T1D onset when adoptively transferred with diabetic splenocytes into NOD.scid mice (Figure 2.3 F). This suggest that long-term survivors did not have an elevated level and/or function of regulatory cells needed for dominant tolerance. Since < 30% of female NOD mice normally remain T1D-free by 30 weeks[9,26] and control treatments had more T1D-free mice than this, Blank MP was compared

to PBS in a mock T1D prevention study to see if Blank MP was having any significant effect. Irradiation of Blank MP was also studied in a separate group to see if a lack of sterility could be influencing T1D progression, as pathogen exposures can reduce T1D incidence[23]. There was no significant difference in the T1D incidence of PBS, Blank MP, and irradiated Blank MP treated mice and all incidences fell in expected range (Figure 2.3 G). Therefore the dosing of Blank MP administered in this model did not appear to be significantly influencing disease incidence. Although the results of this pilot study were largely inconclusive due to the small sample size, the failure to see differences between TRIpeptide MP and other treatment groups suggested that additional optimization of the TRI MP drug delivery system for this model may be needed.

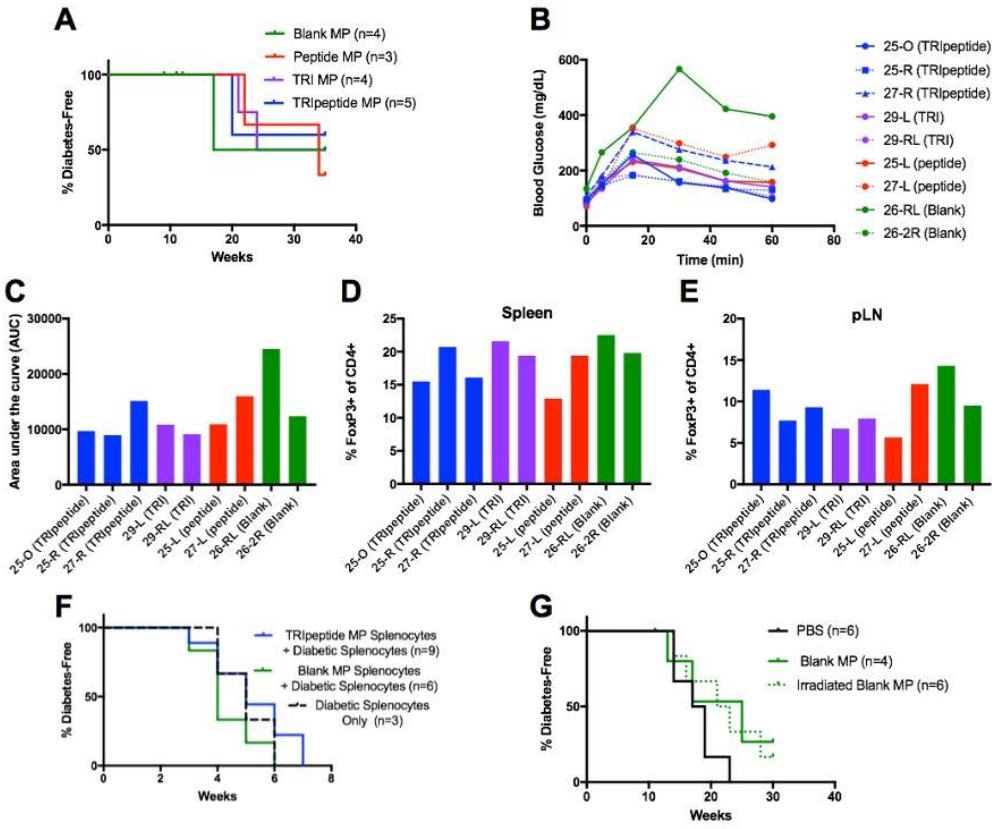

Figure 2.3 Pilot T1D prevention study fails to show differences between treatment groups, and follow up results suggests that T1D-free mice from TRI MP treatment groups do not have substantially greater β cell function or Treg levels than controls. **A) Spontaneous T1D prevention study in which NOD mice were given the indicated treatment at 6 weeks-old and again 10 days later. Groups include TRIpeptide MP consisting of microparticles (MP) with TRI factors and insulin B:9-23 (Peptide) MP, TRI MP, Peptide MP, and vehicle control Blank MP. n=6 per group initially, however several mice died prior to T1D onset leading to an n of 3-5 as indicated. B) i.p. glucose tolerance test (IPGTT) administered in long-term survivors (35 weeks old) of T1D prevention study. Each curve indicates a single mouse, color coded by treatment condition. C) Area under the curve (AUC) of IPGTT performed in B). Each bar indicates a single mouse, color coded by treatment condition. D-E) Percentage of $FoxP3^+$ Tregs among $CD4^+$ T cells in spleen (D) and pancreatic lymph node (pLN) (E) of long-term survivors. F) Adoptive transfer T1D prevention study in which NOD.scid mice received splenocytes from TRIpeptide MP or Blank MP treated long-term survivors and/or diabetic splenocytes in order to assess regulatory cell function. n=3-9 NOD.scid mice per group. G) Mock T1D prevention study to evaluate whether Blank MP was having a protective effect and any role of irradiation in mitigating such an effect. n=4-6 per group as indicated. The low sample size used in prevention studies reflects the fact that these were exploratory studies with an uncertain treatment effect size. Differences between groups for prevention studies (A, F, and G) were assessed by a Log-rank (Mantel-Cox) test and no significant differences were observed.**

2.3.3 TRI MP and insulin peptide do not elicit the desired magnitude of antigen-specific Treg expansion *in vivo*

Since TRI MP could not be easily localized to the pancreas to directly suppress ongoing Teff priming and proliferation[118,119], it was thought that a sufficient population of islet-specific Tregs needed to be generated in order to prevent T1D onset. To assess the ability of TRI MP and insulin peptide to increase the levels of insulin-specific Tregs *in vivo*, NOD FoxP3-EGFP mice were administered MP treatments and then sacrificed 10 days later with detection of insulin tetramer staining and FoxP3-EGFP by flow cytometry used to measure levels of insulin-specific Tregs in the draining lymph node (inguinal, iLN) and pLN. Although Peptide MP containing Ins B:9-23 (and additional soluble Ins B:9-23) was used in the pilot prevention study, a low percentage of encapsulated Ins B:9-23 was released in several tested MP formulations (Figure 2.4). Additionally, the use of complete Freund's adjuvant (CFA) /insulin (Ins.) peptide emulsion was considered as an alternative to Peptide MP due to the possibility of this approach providing sustained delivery of a large amount of peptide as well inflammatory signaling that could elicit greater T cell proliferation. If CFA caused more proliferation, then there would be a larger pool of antigen-specific T cells that could be converted to Tregs (mimicking how TRI MP is provided at the site of inflammation in other models). An initial exploratory trial suggested that TRI MP with insulin peptide delivered in CFA as opposed to a MP could lead to a larger Treg response in the iLN (Figure 2.5), so CFA/Ins. Peptide + TRI MP was compared to controls in a larger cohort.

As expected, CFA/Ins. peptide led to a significant increase in the level of insulin-specific T cells in the iLN regardless of whether it was co-administered with Blank MP or TRI MP (Figure 2.6 A and B). However, only when TRI MP was combined with CFA/Ins. peptide was the percentage of insulin-specific Tregs among $CD4^+$ T cells in the iLN significantly higher than that

of the PBS control (Figure 2.6 C). This was because TRI MP led to a significantly higher fraction of FoxP3$^+$ Tregs among insulin-specific cells than Blank MP (Figure 2.6 D). Interestingly although the use of a CFA emulsion led to a significant increase in the overall level of (polyclonal) Tregs in the iLN, this was not further promoted by the addition of TRI MP (Figure 2.6 E).

The effect of MP and insulin peptide treatment on insulin-specific Tregs in the pLN was also assessed, as this is a site more relevant for influencing T1D progression. While CFA/Ins. peptide led to a significant increase in the level of insulin-specific T cells in the pLN (Figure 2.6 F), the magnitude of this increase was less than that observed in the iLN. Presumably this was because only a fraction of insulin-specific T cells activated in the iLN trafficked to the pLN, and those that did reach the pLN did not proliferate sufficiently in this timeframe to make up the difference. Although CFA/Ins. peptide + TRI MP had a significantly higher level of insulin-specific Tregs than PBS (Figure 2.6 G), this was because of the increase in overall insulin-specific T cells as the fraction of insulin-specific cells that were FoxP3$^+$ was comparable across all treatment groups (Figure 2.6 H). Likewise, the level of polyclonal Tregs in the pLN was equivalent across all treatment groups (Figure 2.6 I).

In summary, while the administration of TRI MP with insulin peptide enabled expansion of insulin-specific T cells while increasing the fraction of these that are Tregs in the iLN, it had no effect on the fraction of insulin-specific T cells that were Tregs in the pLN. This latter point is critical because increasing the amount of insulin-specific T cells in the pLN without improving the Treg fraction above baseline would not be expected to be protective against T1D and could be harmful if Tconv or Teff were being expanded. Notably, the data suggest that the baseline level (PBS treated mice) of FoxP3$^+$ expression among insulin specific T cells is higher in the pLN (~30%) than the iLN (~18%) (Figure 2.6 D and F), so it makes sense that the existing TRI MP

formulation which led to ~ 30% FoxP3$^+$ population among insulin-specific T cells in the iLN was unable to improve this measure in the pLN.

A

Name	PLGA Polymer type	Emulsion type	Porous (Salt in PVA)
1) Formulation 1 (Original)	Acid terminated	Double	Yes
2) Formulation 2	Acid terminated	Single	No
3) Formulation 3	Ester terminated + PEG-PLGA	Double	No
4) Formulation 4	Ester terminated + PEG-PLGA	Single	No

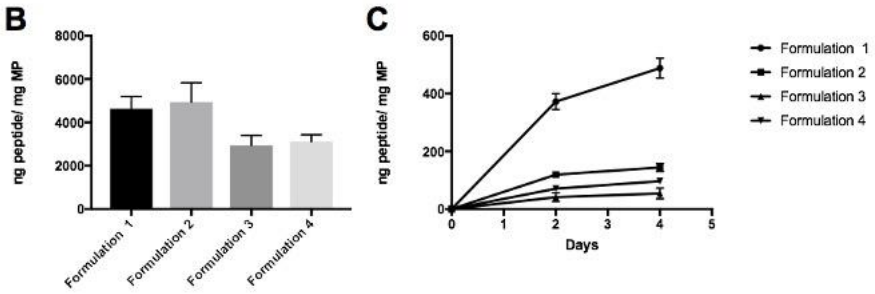

Figure 2.4 Peptide MP characterization. A) Description of Peptide MP formulations for the delivery of insulin peptide (Ins B:9-23) evaluated *in vitro* including the original formulation used in the pilot T1D prevention study (Formulation 1) and three additional formulations. B) Drug loading (ng/mg) for various Peptide MP formulations with samples run in triplicate and presented as mean ± SEM. C) *In vitro* release kinetics for Peptide MP respectively with samples run in triplicate and presented as mean ± SEM.

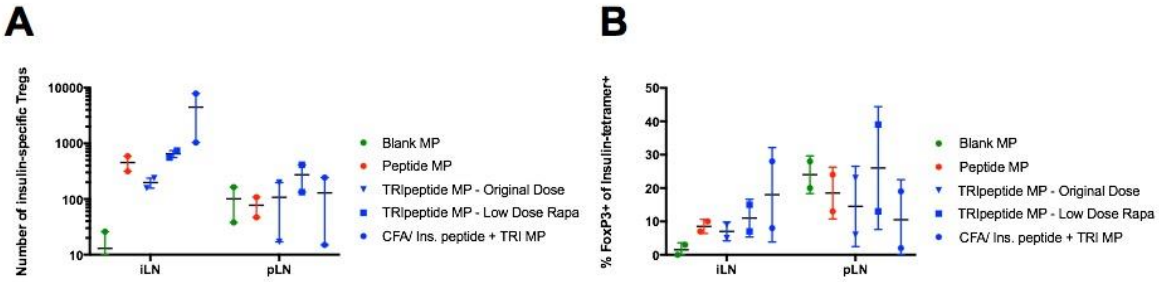

Figure 2.5 Initial exploratory assessment of insulin-specific Treg levels after in vivo TRI MP administration.

A) Number of CD4$^+$ cells that are insulin tetramer$^+$ and FoxP3$^+$ in the iLN or pancreatic LN (pLN) for mice treated 10 days previously with the indicated treatment. Dose explanations can be found in Table 1. B) Percentage of insulin tetramer$^+$ cells that FoxP3$^+$ in the iLN or pLN respectively for each treatment group. n = 2 mice per group and data presented as mean ± SEM. The low sample size used and lack of statical analysis performed reflect the fact that this was an exploratory study. Select groups (CFA/Ins. peptide + TRI MP) were evaluated in follow-up studies, but additional experiments to increase the sample size were not performed for groups in which it was not probable that the magnitude fold change observed would be consistent with the hypothesis.

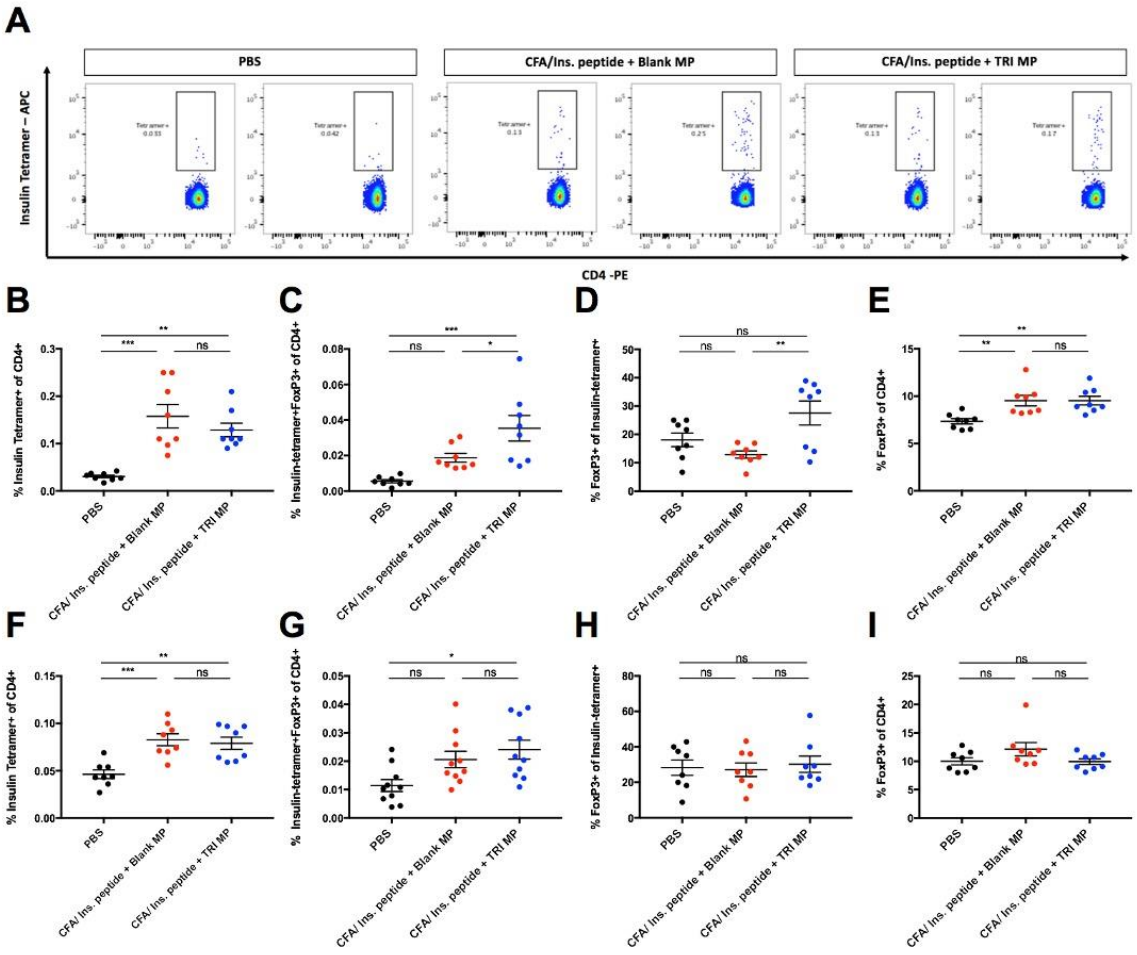

Figure 2.6 TRI MP co-administered with insulin peptide increases insulin-specific Treg frequency in draining lymph node, but does not affect fraction of insulin-specific cells that are Tregs in pancreatic lymph node.

A) Representative pseudocolor plots of CD4 expression versus insulin tetramer expression for $CD4^+$ cells from the draining (inguinal) lymph node (iLN) with two representative plots for mice treated 10 days previously with PBS (left), a CFA and insulin peptide emulsion (CFA/Ins. peptide) + Blank MP (middle), or CFA/Ins. peptide + TRI MP (right). B) Quantification of plots from A, showing percentage of $CD4^+$ cells that are insulin $tetramer^+$ in the iLN. C) Percentage of $CD4^+$ cells that are both insulin $tetramer^+$ and $FoxP3^+$ in the iLN. D) Percentage of insulin $tetramer^+$ cells that $FoxP3^+$ in the iLN. E) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the iLN. F-I) Same as B-E) but for the pancreatic lymph node (pLN) instead of iLN. n = 8 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group and the following cutoffs were used for significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Since it was not probable that the magnitude fold change observed would be consistent with the hypothesis, treatment modifications were made instead of further increasing the sample size.

2.3.4 Limited Treg expansion in this model is not due to use of a self-antigen or defect associated with the NOD strain

Since the antigen-specific and polyclonal Treg expansion for TRI MP observed was less than expected for this model, the role of the antigen type and mouse strain in these results was explored. T cells specific to a self-antigen that have previously encountered antigen in the periphery prior to TRI MP treatment may have an anergic and/or Teff phenotype instead of naïve phenotypes, and therefore be less amenable to Treg induction. In particular, 30% of Ins B:9-23 specific T cells in non-draining secondary lymphoid organs (i.e. LN other than the pLN) already have an activated phenotype in 5 week-old NOD mice[64]. In order to assess if using a self-antigen was limiting antigen-specific Treg induction/expansion, NOD mice were immunized with CFA and the nominal foreign antigen hen egg lysozyme (HEL) peptide instead of insulin peptide in combination with Blank MP or TRI MP (Figure 2.7 A and B). It has also been suggested that an approach for delivering antigen that normally leads to peripheral Treg (pTreg) induction when using foreign antigen in B6 mice, fails to induce pTregs when using self-antigen in NOD mice due to altered CD40L expression by a dendritic cell subset[146]. Notably, this is a defect that wouldn't be detected by *in vitro* Treg induction using CD3/CD28 stimulation and is still compatible with the success of approaches that expand instead of induce Tregs in the NOD model. In order to assess if the NOD mouse background was limiting antigen-specific Treg induction/expansion, B6g7 mice were immunized with CFA and HEL peptide combination with Blank MP or TRI MP (Figure 2.7 C and D). Since the baseline level of antigen-specific Tregs differed between insulin and HEL, the fold change between the CFA/antigen emulsion + TRI MP and CFA/antigen emulsion + Blank MP was compared for NOD mice treated with insulin peptide (Figure 2.6), NOD mice treated with HEL peptide (Figure 2.7 A and B), and B6g7 mice treated with HEL peptide (Figure 2.7 C and

D). TRI MP induced Treg expansion was not substantially higher for either antigen-specific Treg or polyclonal Tregs when using a nominal antigen (HEL) instead of a self-antigen or when using B6g7 mice instead of NOD mice (Figure 2.7 E). These findings suggested that the success of TRI MP in this model was not hindered by the use of self-antigen or the NOD background, and that alterations to the TRI MP and antigen delivery systems itself to improve Treg expansion in this model was worth investigation.

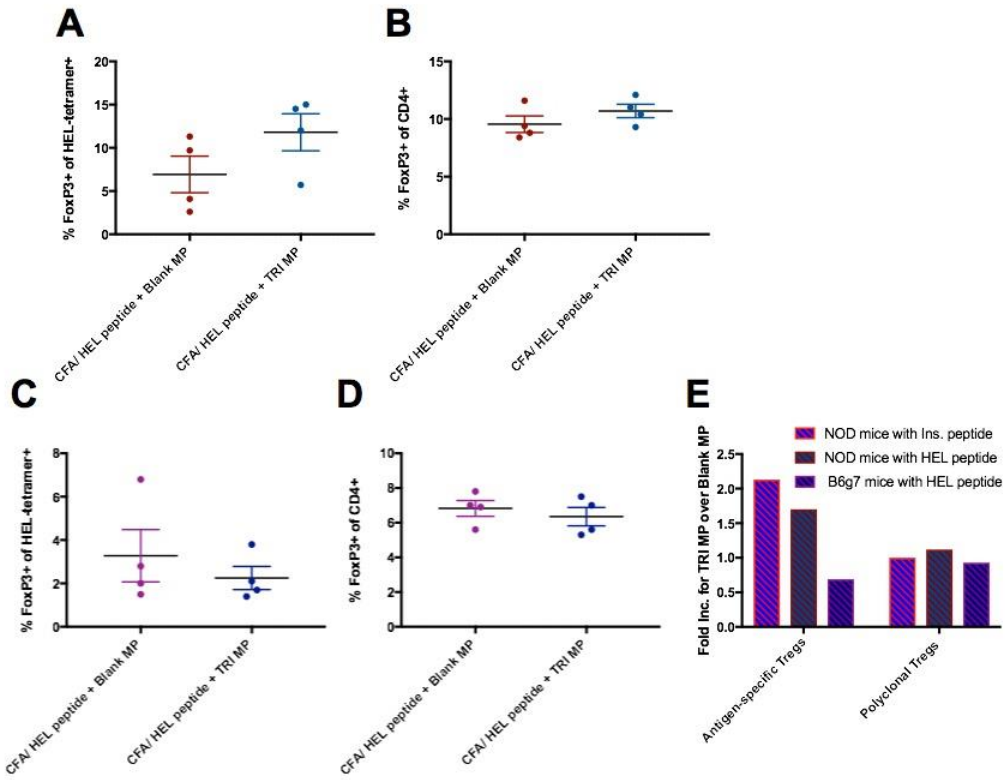

Figure 2.7 Antigen-specific and polyclonal Treg induction not improved by using alternative mouse strain and/or antigen. **A) Percentage of $CD4^+$ cells that are hen egg lysozyme (HEL) $tetramer^+$ in the iLN at day 10 after treatment of NOD mice with CFA/HEL Peptide + Blank MP or CFA/HEL Peptide + TRI MP. B) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the iLN. C-D) Same as A-B) but using B6g7 mice instead of NOD mice. n = 4 mice per group and data presented as mean ± SEM. E) Comparison of the average fold increase in antigen-specific Tregs (left) or polyclonal Tregs (right) CFA/peptide + TRI MP treatment relate to CFA/peptide + TRI MP treatment for the indicated mouse strain and antigen as indicated by red/blue color coded bars. The low sample size used and lack of statical analysis performed reflect the fact that this was an exploratory study. Since it was not probable that the magnitude fold change observed would be consistent with the hypothesis, this experiment was not performed on a larger sample size.**

2.3.5 Explored changes to TRI MP dosing, release kinetics, and evaluated time point do not substantially improve fraction of insulin-specific T cells that are Tregs

A number of alterations to the TRI MP formulation and insulin peptide delivery were considered in an effort to expand insulin-specific T cells while further increasing the fraction of insulin-specific T cells that were FoxP3$^+$. The rationale for testing these different TRI MP doses and formulations as well as the specific MP and drug amounts can be found in Table 2-1. All these formulations were tested in conjugation with CFA/Ins. peptide. The only TRI formulation alteration that led to a significant increase in the level of insulin-specific T cells in the iLN relative to the Original Formulation was the Low Dose Rapa group (Figure 2.8 A), however this group also had a 2-fold reduction in the average percentage of these insulin-specific T cells that were FoxP3$^+$ (Figure 2.8 B) although that difference was not significantly different. These findings suggest that the dose of rapamycin in the Low Dose Rapa group was too low to prevent the induction/expansion of Teff. Although the differences were not statistically significant, the Original Formulation – High Dose group had a higher average than the Original Formulation for both the level of insulin-specific T cells and the fraction of these that were FoxP3$^+$ (Figure 2.8 A and B). No significant differences from the Original Formulation were seen for the level of polyclonal Tregs in the iLN (Figure 2.8 C), the level of insulin-specific T cells in the pLN (Figure 2.8 D), or the fraction of insulin-specific T cells in the pLN that were FoxP3$^+$ (Figure 2.8 E). Although the level of polyclonal Tregs in the pLN for the Original Formulation – High Dose group was less than that of the Original Formulation (Figure 2.8 F), this was likely an anomaly given that no differences in insulin-specific Tregs were seen and that the group sizes were relatively small with groups performed at different times so treatment was not randomized among mice from the same litter. Although none of the tested TRI MP alterations led to substantial improvement in

insulin-specific Treg expansion, the Original Formulation – High Dose was chosen as the TRI MP formulation and dose moving forward due to trends observed in the iLN.

Table 2.1 Rationale and description for tested TRI MP formulations and doses.

Group	Rationale	Amount of MP per injection	Estimated drug dose per injection (based on loading)
Original Formulation	Initial formulation/dose that student who started project was using. *In vivo* dose used for previous figures.	T = 3.4 mg R = 3.4 mg I = 3.4 mg	T = 4 ng R = 17,000 ng I = 23 ng
Original Formulation - Day 24	Extended end point from Day 10 to Day 24 to provide longer time for insulin-specific T cells activated in iLN to traffic to pLN and proliferate further	(Same as above)	(Same as above)
Original Formulation – Low Dose Rapa	Adjusted TRI doses to mimic the ratio used for *in vitro* Treg induction (5 ng/mL of T and 10 ng/mL of R and I). In addition to more T and I, possible that dose of R used in original dose was limiting the degree of Treg induction/expansion	T = 14.25 mg R = 0.007 mg I = 5.25 mg	T = 17.5 ng R = 35 ng I = 35 ng
Original Formulation – Higher Dose	Increased dose of all TRI factors from original dose. Greatest emphasis placed on T, which had improved loading due to new fabrication protocol (kept cold throughout fabrication).	T = 9.6 mg R = 4 mg I = 7.5 mg	T = 75 ng R = 20,000 ng I = 50 ng
Longer IL-2 release formulation	Formulation/dose used in dermatitis model, tested due to success in that model as well as longer IL-2 release than original formulation (however, MP also smaller and both R and I have different PLGA type)	T = 4.3 mg R = 1.5 mg I = 2.2 mg	T = 19 ng R = 8,250 ng I = 37 ng
Longer IL-2 release formulation – Higher Dose	Doubled the dose used in the above formulation	T = 8.6 mg R = 3 mg I = 4.4 mg	T = 38 ng R = 16,500 ng I = 75 ng

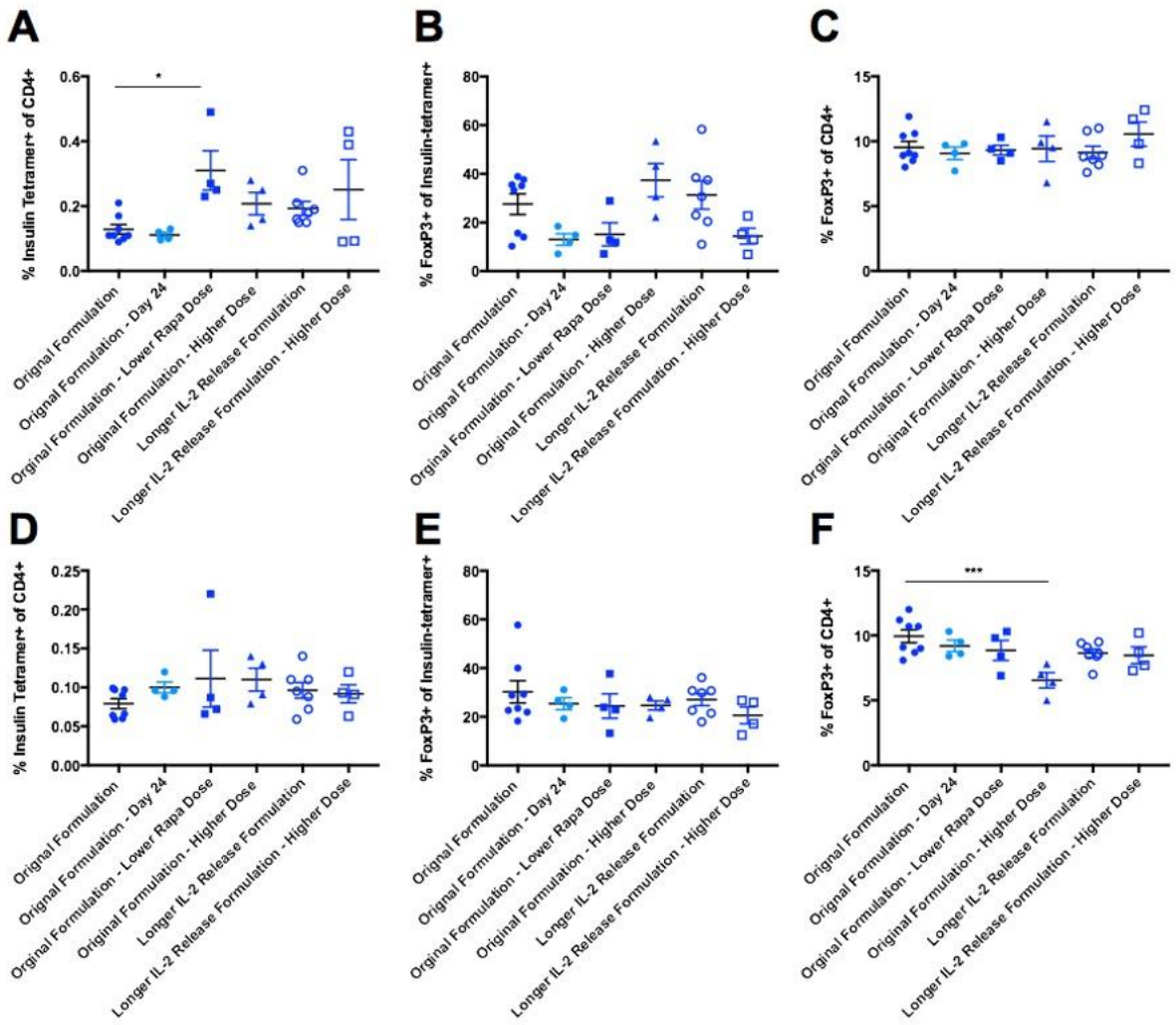

Figure 2.8 TRI MP dose and formulation alterations do not substantially affect in vivo level of insulin-specific and polyclonal Tregs relative to original TRI MP formulation. **A) Percentage of $CD4^+$ cells that are insulin $tetramer^+$ in the iLN at day 10 or day 24 (light blue circles) after treatment of mice with CFA/Ins. peptide and the original TRI MP formulation or a formulation modified as indicated. B) Percentage of insulin $tetramer^+$ cells that $FoxP3^+$ in the iLN. C) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the iLN. D-F) Same as B-E) but for the pancreatic lymph node (pLN) instead of iLN. n = 4-8 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Dunnett post-hoc analysis in order to compare the mean of every group with the mean of the Original Formulation group and the following cutoffs were used for significance: * $p < 0.05$, *** $p < 0.001$. All other comparisons were non-significant (ns).**

2.3.6 Explored changes to antigen delivery including adjuvant type/delivery system and insulin peptide sequence further improve insulin-specific Treg levels in the iLN but not the pLN

Since the level of insulin-specific T cells that were Tregs was not substantially increased by altering TRI MP dose, formulation, or the experimental endpoint, changes to the delivery of insulin peptide were investigated. First, the role of the adjuvant being used was considered. While CFA was used in initial trials in effort to boost inflammation and overall T cell proliferation in order to have more insulin-specific T cells that could possibly be induced into Tregs, CFA on its own can delay T1D onset in NOD mice[261,262]. Therefore, CFA was compared to incomplete Freund's adjuvant (IFA) and to no adjuvant (Soluble) for the delivery of insulin peptide in conjunction with TRI MP. Surprisingly, there were no significant differences between any of the groups (Figure 2.9). IFA was chosen as the adjuvant moving forward because of its role in providing sustained delivery of insulin peptide, even if a difference was not observed relative to soluble peptide at the tested time point.

A sequence modification to insulin peptide and alternatives to IFA for insulin peptide delivery were investigated next. The Ins B:9-23 R22E modification has previously been shown to improve peptide binding to the I-A^{g7} MHC allele in a better recognized register and elicit stronger T cell proliferation and improved Treg induction[107,354]. In addition to evaluating R22E Ins. peptide delivered in IFA, MP delivery, and osmotic pump delivery were also explored. Osmotic pump delivery of an R22E insulin mimotope without any additional tolerogenic factors had been shown by one group to robustly prevent T1D onset due to Treg expansion, while another group trying to replicate this study found that R22E insulin mimotope actually accelerated T1D onset when given to NOD mice at 6 weeks of age[354,386]. Although these results were conflicting, one interpretation

is that osmotic pump delivery of R22E insulin peptide elicited a strong insulin-specific T cell response and that the disease outcome depended on whether antigen was encounter under an inflammatory context or a tolerogenic context[387]. If this were the case, then TRI MP could have value in enforcing a tolerogenic context for more consistent T1D prevention. However, in our study neither replacement of wild-type (WT) Ins. peptide with R22E nor use of osmotic pump further increased the level of insulin-specific T cells in the iLN (Figure 2.10 A). In fact, osmotic pump delivery of R22E Ins. peptide actually led to less insulin-specific T cells in the iLN (only significant relative to IFA/R22E + TRI MP based on the number of statistical comparisons made), which could signify that the osmotic pump (implanted s.c. on the back) was draining to a LN other than the iLN. Substituting R22E Ins. peptide for WT Ins. peptide did however lead to an increase in the fraction of insulin-specific T cells that were Tregs in the iLN (Figure 2.10 B). While this difference between IFA/R22E + TRI MP and either the corresponding Blank MP group or the osmotic pump group was statistically significant, this difference was not statistically significant in comparison to the prior lead TRI MP delivery system (IFA/WT Ins. +TRI MP) or the MP-based approach for R22E Ins. peptide delivery + TRI MP (Figure 2.10 B). No significant differences between groups were seen in terms of polyclonal Tregs in the iLN (Figure 2.10 C), the level (Treg fraction) of insulin-specific T cells in the pLN (Figure 2.10 D and E), or the level of polyclonal Tregs in the pLN (Figure 2.10 F). The lack of an increase in insulin tetramer staining in the pLN for the osmotic pump treatment group (Figure 2.10 D) cannot be explained by draining LN differences, and this finding suggests that the osmotic pump approach did not elicit a strong expansion of insulin-specific T cells as expected. Comparison of the IFA/R22E + TRI MP and corresponding Blank MP groups to the original PBS treated mice group (from Figure 2.6) similarly showed that this latest delivery system for TRI MP and insulin peptide led to significant increases

in Treg levels in the iLN but not the pLN (Figure 2.11). Although tetramer staining of intra-islet T cells was only explored in certain treatment groups and definitive conclusions cannot be drawn based on the small sample sizes, the results suggest that tested TRI MP formulations did not substantially expand insulin-specific T cells in the islets (Figure 2.12).

After exploring a number of changes to both TRI MP delivery and insulin peptide delivery, the IFA/R22E + TRI MP (Original Formulation – Higher Dose) system was chosen as the best approach of co-administering insulin peptide and TRI MP. While this system led to a higher fraction of insulin-specific Tregs among insulin-specific T cells in the draining lymph node, it did not accomplish the original goal of increasing the level of insulin-specific and/or polyclonal Tregs in the pancreatic lymph node.

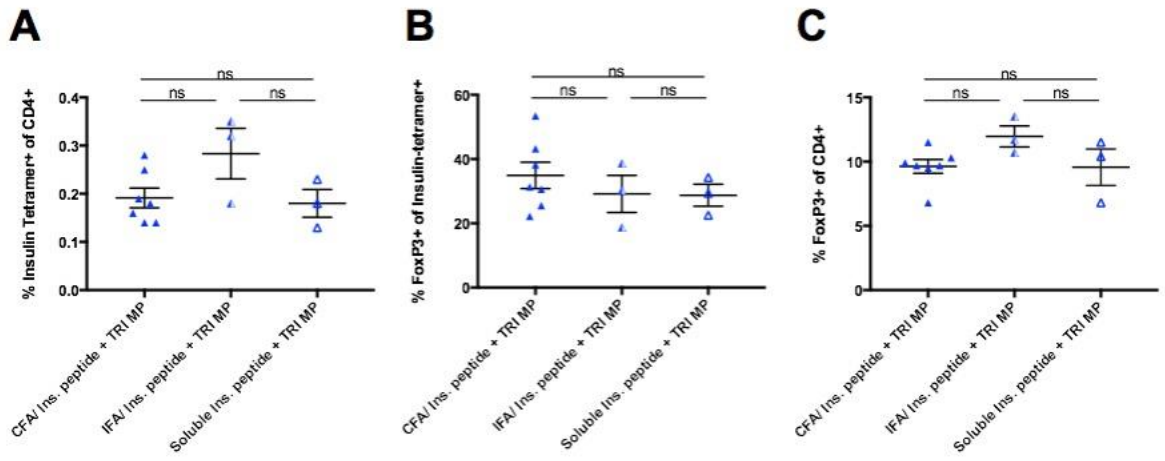

Figure 2.9 Alteration of adjuvant used with TRI MP did not effect in vivo insulin-specific or polyclonal Treg levels. **A) Percentage of $CD4^+$ cells that are insulin $tetramer^+$ in the iLN at day 10 after treatment of mice with CFA/Ins. Peptide + TRI MP, IFA/Ins. Peptide + TRI MP, or Soluble Ins. peptide + TRI MP. B) Percentage of insulin $tetramer^+$ cells that $FoxP3^+$ in the iLN. C) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the iLN. n = 3-7 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group. . The low sample size used reflect the fact that this was an exploratory study and the use of IFA was further evaluated in the following experiment.**

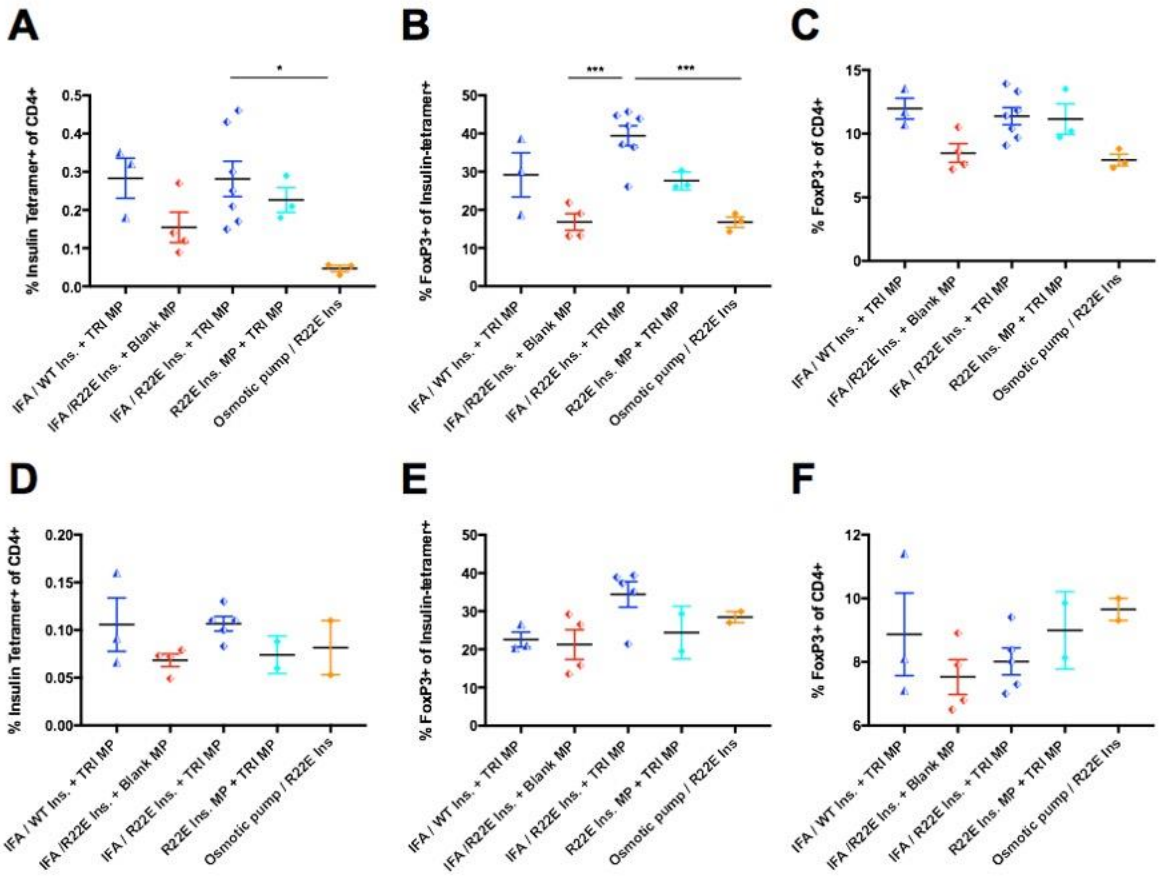

Figure 2.10 Insulin peptide sequence alteration, but not alternative insulin peptide delivery systems, improves in vivo level of insulin-specific Tregs in iLN but not pLN. **A) Percentage of $CD4^+$ cells that are insulin $tetramer^+$ in the iLN at day 10 (or Day 21 for Osmotic pump) for mice treated with an emulsion of incomplete Freund's adjuvant (IFA) / wild type (WT) Ins. peptide + TRI MP, IFA / R22E substituted Ins. peptide + Blank MP, IFA / R22E Ins. peptide + TRI MP, R22E Ins. MP + TRI MP, or osmotic pump delivery of R22E Ins. peptide. B) Percentage of insulin $tetramer^+$ cells that $FoxP3^+$ in the iLN. C) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the iLN. D-F) Same as B-E) but for the pancreatic lymph node (pLN) instead of iLN. n = 2-7 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group and the following cutoffs were used for significance: * $p < 0.05$, *** $p < 0.001$. All other comparisons were non-significant. The low sample size used reflect the fact that this was an exploratory study. Additional experiments to increase the sample size were not performed for groups in which it was not probable that the magnitude fold change observed would be consistent with the hypothesis.**

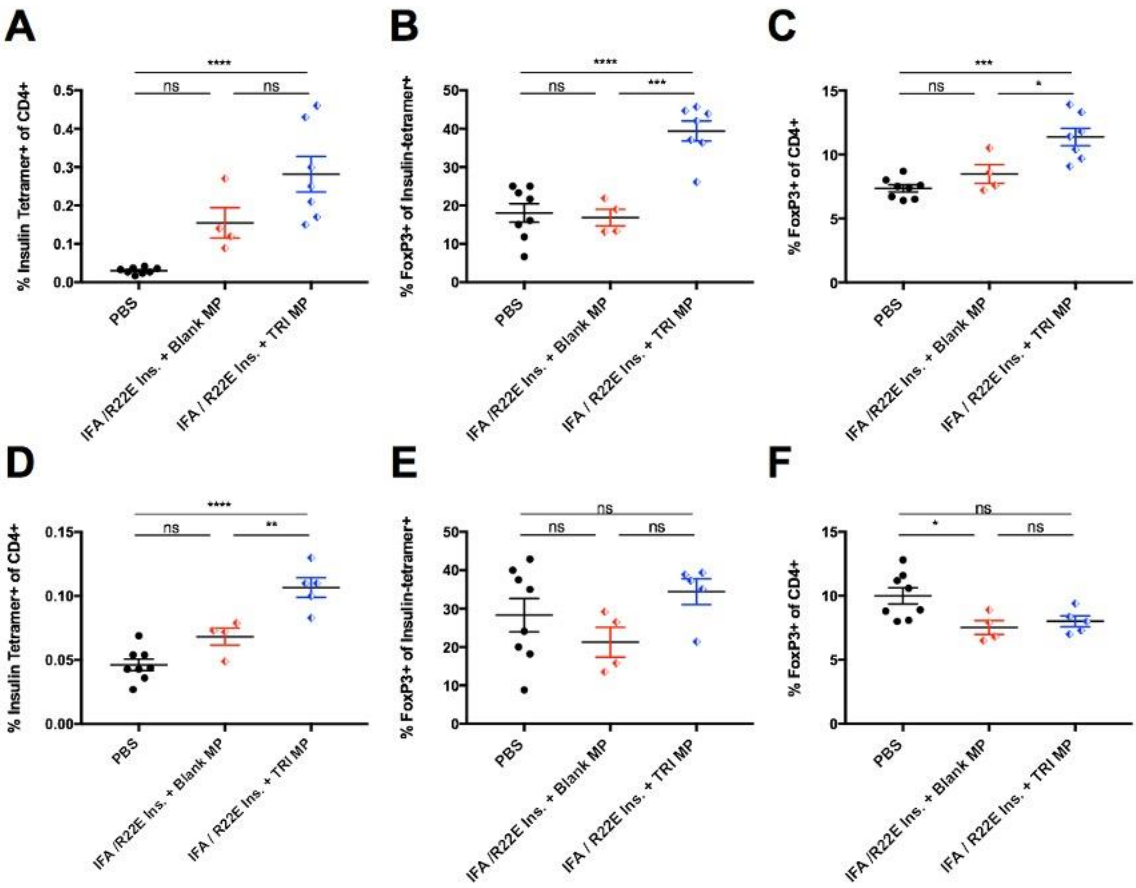

Figure 2.11 Comparison of final Blank MP and TRI MP formulations to original PBS treated group still shows elevated in vivo level of insulin-specific and polyclonal Tregs in iLN but not pLN. **A) Percentage of CD4$^+$ cells that are insulin tetramer$^+$ in the iLN at day 10 for PBS treated mice (data from Figure 2.6) and final formulations of Blank MP with Ins. Peptide and TRI MP + Ins. Peptide (data from Figure 2.10). B) Percentage of insulin tetramer$^+$ cells that FoxP3$^+$ in the iLN. C) Percentage of overall CD4$^+$ (polyclonal) cells that are FoxP3$^+$ in the iLN. D-F) Same as B-E) but for the pancreatic lymph node (pLN) instead of iLN. n = 4-8 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group and the following cutoffs were used for significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.**

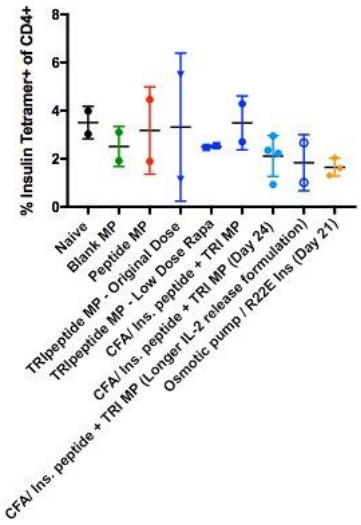

Figure 2.12 Assessment of insulin-specific T cell levels in the islets of select treatment groups. **Percentage of CD4$^+$ cells that are insulin tetramer$^+$ from recovered intra-islet infiltrate at day 10 (or Day 21-24 as indicated) after treatment. n = 2-4 mice per group and data presented as mean ± SEM. The low sample size used and lack of statical analysis performed reflect the fact that this was not a primary endpoint as intra-islet T cells were not systemically collected in all experimental groups. Additionally, a sufficient islet mass was not recovered in all mice for experimental groups in which insulin-specific T cell levels in the islets was assessed.**

2.3.7 Explored changes to TRI MP injection site and route do not improve antigen-specific T cell or Treg levels in the pLN

Lastly, alterations to the injection site and route of administration were considered for the co-administration of TRI MP and insulin peptide. A previous report had suggested that s.c. injections could have drainage to the pancreas/pLN if injected close to the pancreas[294]. In order to evaluate drainage to the pLN, mice were injected s.c. by the hind limb (the site TRI MP and insulin antigen had been administered previously) or at several abdominal sites (Figure 2.13 A). HEL (in a CFA emulsion) was used for this assay instead of insulin, because the baseline level of HEL-specific T cells in the pLN was expected to be very low as opposed to that of insulin-specific T cells which was expected to be higher and more variable making changes more difficult to detect.

Injection s.c. by the hind limb or abdominal locations led to increased levels of HEL-specific T cells in the iLN (Figure 2.13 B) relative to untreated Naïve mice. However, there was no difference between the hind limb injection site and the other injection sites in the iLN (Figure 2.13 B) and no difference between the hind limb injection site and any other group for the pLN (Figure 2.13 C). This lack of HEL-specific T cell expansion in the pLN suggests that none of the tested injection sites led to HEL antigen being presented in the pLN. An i.p. administration route was also considered as a possible way to get TRI MP and insulin peptide closer to the pLN. Mice were either injected with Blank MP (n=2) or different formulations of TRI MP and R22E insulin peptide using soluble peptide or peptide encapsulated in MP (n=4 total). Surprisingly, TRI MP treated mice but not Blank MP treated mice all had liver discoloration/damage (Figure 2.13 D). The fact that this damage was not seen with Blank MP suggests that it was not due to the injection or the microparticles themselves, but instead due to the drugs being released. This is somewhat surprising because no signs of liver damage had been seen in any mice given a comparable dose of s.c. TRI MP. Liver damage observed for i.p. TRI MP administration likely reflects the fact that the drugs, in particular the cytokines TGF-β and IL-2, remain relatively localized to the injection site due to the low doses administered and short half-lives[408,410,520,521]. In particular, higher local concentrations of TGF-β by the liver could contribute to liver fibrosis[522]. Furthermore, i.p. injection of TRI MP and insulin peptide did not achieve the intended goals of boosting levels of insulin-specific or polyclonal Tregs in the pLN when compared to s.c. administration of IFA/R22E + TRI MP (Figure 2.13 E-G). Therefore, the original route and administration site of TRI MP was continued moving forward.

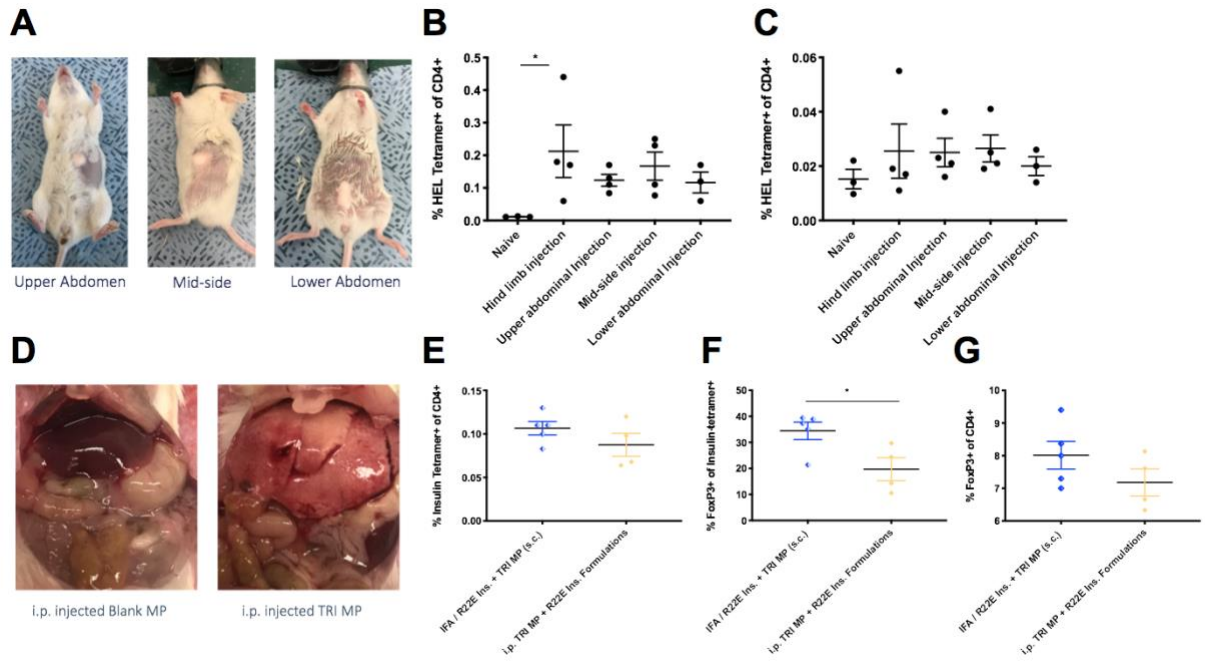

Figure 2.13 Alternative injection sites and administration routes fail to improve antigen-specific T cell expansion in the pLN. **A-C) Subcutaneous (s.c.) immunization of NOD mice with CFA/nominal antigen (HEL) emulsion at different sites to check ability of each site to enable pLN draining and expansion of antigen-specific T cells. A) Representative images showing emulsion location for tested sites (hind limb not shown). B) Percentage of $CD4^+$ cells that are HEL $tetramer^+$ in the iLN for mice treated 10 days previously with CFA/HEL at the normal site of TRI MP (hind limb) or the alternative sites of upper abdomen, side, or lower abdomen. C) Same as B) but for the pLN. n = 3-4 mice per group and data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Dunnett post-hoc analysis in order to compare the mean of every group with the mean of the Hind limb injection group and the following cutoffs were used for significance: * $p < 0.05$. All other comparisons were non-significant (ns). D-G) NOD mice administered Blank MP or TRI MP + R22E Ins. by i.p. injection and pLN insulin-specific T cell and Treg levels compared to latest s.c. TRI MP Formulation (IFA/R22E Ins. + TRI MP from Figure 2.10). D) Representative images of the liver showing healthy liver for i.p. Blank MP (n=2) and discolored/damaged liver for i.p. injected TRI MP + R22E Ins. (n=4). E) Percentage of $CD4^+$ cells that are insulin $tetramer^+$ in the pLN. F) Percentage of insulin $tetramer^+$ cells that $FoxP3^+$ in the pLN. G) Percentage of overall $CD4^+$ (polyclonal) cells that are $FoxP3^+$ in the pLN. n = 4-5 mice per group and data presented as mean ± SEM. Differences between groups were assessed by an unpaired t test and the following cutoffs were used for significance: * $p < 0.05$. All other comparisons were non-significant (ns). Since it was not probable that the magnitude fold change observed would be consistent with the hypothesis, this experiment was not performed on a larger sample size.**

2.3.8 TRI MP provides no additional benefit to insulin peptide emulsion treatment for T1D prevention in spontaneous and adoptive transfer NOD mouse models

The ability of TRI MP co-administered with insulin peptide to prevent spontaneous T1D in NOD mice was revisited after making alterations and/or investigating multiple parameters of the TRI MP + insulin peptide treatment including TRI MP formulation/dose, insulin peptide delivery system and peptide sequence. Female NOD mice were given 2 s.c. injections of IFA/R22E + TRI MP or controls at 4 weeks of age and 6 weeks of age (with half the original dose given in the 2nd injection) and blood glucose was checked weekly beginning at 12 weeks. TRI MP co-administered with insulin peptide (IFA/R22E + TRI MP) significantly reduced T1D onset relative to the PBS and Blank MP control but was not significantly different from the Blank MP co-administered with insulin peptide (IFA/R22E + Blank MP) group (Figure 2.14 A). These results also indicate that Blank MP had no effect on T1D progression (Figure 2.14 A). Together these findings suggest that TRI MP had no added benefit to the insulin peptide emulsion component of the Blank MP + insulin peptide group in preventing T1D.

In order to assess whether the protective effect of insulin peptide emulsion was due to an effect on insulin-specific T cell deletion/anergy as opposed to regulatory cell numbers/function and whether TRI MP led to any meaningful enhancements to regulatory cell number/function, NOD mice were co-transferred with diabetic splenocytes into NOD.scid mice. This study design enables the distinction between T1D protection due to deletion/anergy of insulin-specific T cells and T1D protection due to increased numbers and/or function of antigen-specific or polyclonal regulatory cells. The former would not be expected to have dominant tolerance in the ability to suppress Teff among diabetic splenocytes from causing T1D, but the latter would be expected to suppress Teff and thereby delay T1D onset or reduce incidence. Relative to the NOD.scid mice

that received diabetic splenocytes only, NOD.scid mice that received diabetic splenocytes and iLN lymphocytes from mice treated with IFA/R22E + TRI MP or IFA/R22E + Blank MP had significantly delayed T1D onset (Figure 2.14 B). However, similar to the spontaneous T1D prevention study, there was no significant difference in T1D incidence between NOD.scid mice treated with iLN lymphocytes from IFA/R22E + TRI MP treated mice and those treated with iLN lymphocytes from IFA/R22E + Blank MP treated mice (Figure 2.14 B). These results suggest that insulin peptide emulsion led to a local increase in regulatory cell numbers and/or function, but that TRI MP did not meaningfully further enhance regulatory cell numbers or function.

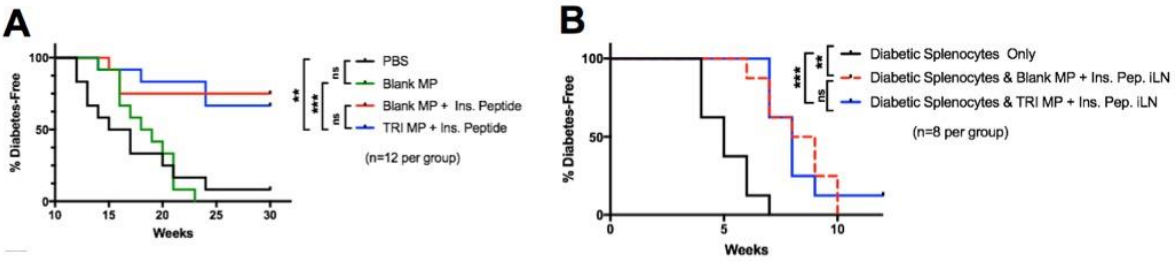

Figure 2.14 TRI MP co-administered with insulin peptide does not further delay T1D onset relative to Blank MP co-administered with insulin peptide in spontaneous and adoptive transfer NOD mouse models.

A) Spontaneous T1D prevention study in which NOD mice were given the indicated treatment at 4 weeks-old and again 2 weeks later. Groups include PBS, Blank MP, Blank MP + Ins. peptide (IFA/R22E) and TRI MP + Ins. peptide (IFA/R22E). n = 12 mice per group. B) Adoptive transfer T1D prevention study in which NOD.scid mice received iLN lymphocytes from TRI MP + Ins. peptide or Blank MP + Ins. peptide treated NOD mice and/or diabetic splenocytes in order to assess regulatory cell function. iLN lymphocytes from MP treated NOD mice pooled (n=5 per group) with n=8 NOD.scid recipients per group. Differences between groups were assessed by a Log-rank (Mantel-Cox) test was ran comparing all curves. Since this was significant, Long-rank (Mantel-Cox) tests were performed for each individual comparison, and p values were multiplied by the number of comparisons made (6 for 4 treatment groups or 3 for 3 treatment groups). The following cutoffs were used for significance after accounting for multiple comparisons: ** $p < 0.01$, *** $p < 0.001$.

2.4 Discussion

T1D can be managed by insulin replacement therapy, but this is not a cure and T1D is still associated with elevated death risk, increased risk of both microvascular and cardiovascular complications, as well as a reduced quality of life[2,5–8]. However, due in large part to the option of insulin replacement therapy, the risk-benefit ratio for conventional/non-specific immunosuppressive treatments is not justified in T1D despite some of these approaches having a modest effect on preserving β cell function[281]. Instead, there is considerable interest in an antigen-specific approach such as those based on islet-specific regulatory cells[305]. Our group has previously developed the TRI MP delivery system in order to promote local *in vivo* Treg induction/expansion in an antigen-specific manner and demonstrated that this approach can successfully prevent disease in several preclinical inflammatory models[408–410,516]. Here, we evaluated the ability of TRI MP co-administered with insulin to increase levels of insulin specific Tregs and prevent T1D onset. TRI MP induced Tregs from NOD mouse naïve T cells with comparable potency to soluble TRI factors, and the suppressive capacity of these Tregs was demonstrated (Figure 2.2). When the ability of TRI MP co-administered with insulin peptide to induce/expand insulin-specific and polyclonal Tregs *in vivo* was first assessed, this treatment increased the fraction of insulin-specific T cells that were Tregs in the iLN but not the pLN and did not impact polyclonal Treg levels in either the iLN or pLN relative to the corresponding Blank MP control (Figure 2.6). This limited Treg expansion was not due to a defect in the NOD model or the use of self-antigen (Figure 2.7), so alterations to the formulation/dose of TRI MP, the delivery system and peptide sequence used for insulin peptide, and the injection site/route of administration were investigated. A higher dose of the original TRI MP formulation modestly improved the level of Tregs among insulin-specific T cells in the iLN, and was used moving

forward since it had better results than any of the other tested TRI MP formulations and doses (Figure 2.8). Likewise, administration of a modified sequence of insulin peptide (R22E) with IFA as a delivery system for antigen together with TRI MP further enhanced the level of Tregs among insulin-specific T cells in the iLN and was the most promising of all tested antigen delivery methods (Figure 2.10). This treatment may have also significantly increased polyclonal Tregs in the iLN relative to the corresponding Blank MP control (Figure 2.11), but still had no effect on insulin-specific T cell or polyclonal Treg levels in the pLN (Figures 2.10 and 2.11). Experiments evaluating s.c. injections at various abdominal sites and i.p. TRI MP injections suggested that neither alternative injection sites or injection routes were likely to be superior to the existing s.c. injection site of TRI MP and insulin peptide (Figure 2.13). Based on these experiments, the IFA/R22E + TRI MP (Original Formulation – Higher Dose) system was chosen as the best approach of co-administering TRI MP and insulin peptide and evaluated for its ability to prevent spontaneous T1D in NOD mice. While TRI MP + insulin peptide significantly delayed T1D onset relative to PBS, it was not significantly different from Blank MP + insulin peptide (Figure 2.14). While it was possible that deletion or anergy of insulin-specific T cells due to insulin peptide emulsion masked the effects of TRI MP on regulatory cells, a follow up adoptive transfer study using iLN lymphocytes from MP + insulin peptide treated mice and diabetic splenocytes suggested that insulin peptide emulsion had an impact on the number or function of regulatory cells and this was not meaningfully enhanced by TRI MP treatment (Figure 2.14).

For most of this study the level of insulin-specific Tregs (and polyclonal Tregs) in the pLN was used a proxy for measuring whether a particular formulation of TRI MP + insulin peptide was likely to be capable of preventing spontaneous T1D. A proxy measure was needed to assess whether a given TRI MP + insulin peptide dose, formulation, and route of administration was

likely to be effective due to the long timeline of spontaneous T1D prevention studies (~7 months). The level of Tregs in the pLN was chosen as a primary parameter to focus on because this lymph node and the islets are the major sites of Treg suppression of diabetogenic T cells[202,523,524] and because multiple previous approaches that were effective at preventing spontaneous T1D through a Treg (antigen-specific or non-specific) based mechanism found treatment led to a significant increase in the overall Treg level in the pLN[223,288,292,354,389,414]. In contrast, an approach which led to a local increase in islet-specific Tregs but failed to substantially increase the level of islet-specific T cells that were Tregs in the pLN or islets had no effect on T1D progression[416]. Although insulin-specific T cell levels in the islets were analyzed in some mice (Figure 2.12), the pLN was focused on due to an ability to more consistently isolate lymphocytes and perform larger cohorts at a time than would have been possible with islets. Even though it is possible that some activated islet-specific T cells can go directly from the circulation to the islets bypassing the pLN[119], appreciable trafficking to and proliferation of at least some of these Tregs in the pLN would still be expected[287,288,291]. While a regulatory cell-based treatment that increases pLN Tregs may have a high likelihood of T1D prevention success, in retrospect the level of Tregs in the pLN may not be the best proxy as our findings suggest that the absence of an increase in pLN Tregs does not necessarily indicate an inability to prevent T1D onset.

Although our data showed minimal Treg increases in mice given Blank MP + Insulin peptide, our adoptive transfer study data suggest that the protective effect of this treatment was at least in part due to its action on regulatory cells. While it was not surprising that administration of 100 μg of Ins B:9-23 mimotope in IFA led to T1D protection[371,373,374], we were not sure how reproducible/strong this protective effect would be given that a similar approach of using osmotic pump delivery was not reproducible[354,386]. These early studies suggested several different

mechanisms of protection including inhibition of insulin-specific T cell proliferation, Th2 skewing, and the induction/expansion of CD4/CD8 regulatory populations[371,373,374]. In our experiments, Blank MP + Insulin peptide did not increase the percentage of insulin-specific T cells that were FoxP3^{+} Tregs in either the iLN or the pLN (Figures 2.6, 2.10, and 2.11). However, spontaneous and adoptive transfer prevention study suggested that Blank MP + Insulin peptide was T1D protective at least in part through an effect on regulatory cells (Figure 2.14). This seeming discrepancy could be explained by the effects of Blank MP + Insulin peptide on insulin-specific Treg function, on polyclonal Treg numbers/function, or on the number/function of other regulatory cell populations. While as few as 500 activated islet-specific Tregs can influence T1D onset in an adoptive transfer model[287], it possible that the difference in the number of islet specific Tregs between the TRI MP + insulin peptide and Blank MP + insulin peptide treatment groups (~2,000 insulin specific Tregs) was too small to see a difference between these groups in spontaneous and adoptive transfer prevention studies, or that such a difference was overshadowed by the fact that insulin-specific Tregs were activated in both groups and thus had enhanced function. Alternatively it is possible that the IFA had an effect on polyclonal Treg levels, and this expansion was of sufficient magnitude to make up for the reduced potency of polyclonal Tregs to islet-specific Tregs in suppressing T1D development[287,291]. Although it is possible that IFA could be having non-specific effects on regulatory cell levels/function such as the increase observed with CFA (Figure 2.6), insulin peptide in IFA did not appear to elicit a significant increase in polyclonal Tregs in the iLN based on the small cohort tested with IFA/R22E + Blank MP (Figures 2.10 and 2.11) and it has been previously demonstrated that IFA does not have non-specific T1D preventative effects[373]. Lastly, it is possible that both IFA/R22E + Blank MP and IFA/R22E + TRI MP led to similar increases in a Th1 inhibitory population (ex: Th2 skewing) or a regulatory population (either T

cell or non-T cell) that is not $CD4^+FoxP3^+$ [9,33,39–42,44]. An increase in such a population could have overshadowed differences observed for insulin-specific Treg number.

While the inability of TRI MP to provide additional benefit in T1D prevention relative to insulin peptide may have been in part due to the strong protective effects of insulin peptide based on how it was administered, comparison of the data reported here with that of TRI MP in other models and that of similar technologies successful in NOD T1D models suggests that further changes to how the TRI drugs are delivered may be needed to impact T1D disease progression in an antigen-specific manner. There would be more room for improvement compared to the Blank MP + insulin peptide control if insulin peptide was given in a reduced dose/frequency (such as doing a couple s.c. injections of antigen MP)[289,396], or if treatments were evaluated in a NOD mouse T1D reversal study for which sustained delivery of insulin auto-antigen without additional tolerance enforcing mechanisms has not been consistently successful (Chapter 1). However, the lack of any trend towards improvement for TRI MP + insulin peptide compared to Blank MP + insulin peptide in spontaneous and adoptive transfer prevention studies (Figure 2.14), suggests that creating additional room for improvement would be insufficient and further changes to how the TRI drugs or antigen are delivered are necessary. This is perplexing given the success of TRI MP in other models, as well as the success of other antigen-specific Treg based approaches in NOD prevention models. Compared to other models in which TRI MP has been effective in causing a substantial increase in polyclonal Tregs and/or having a significant impact on disease prevention[408–410], there are both delivery system and model differences from TRI MP in the T1D model. Delivery system differences include differences in TRI MP formulation/properties, drug dose/release kinetics, and MP dose (as discussed further in Chapter 4), as well as the administration at a distant site from the site of inflammation and the inclusion of exogenous antigen. Model

differences include the fact that it is a spontaneous model, a model of autoimmunity involving self-antigen, possible defects associated with Treg generation and function in the NOD strain, and the use of a single antigen (insulin peptide) as opposed to influencing polyclonal responses. However, all of these model difference as well as the delivery difference of administering exogenous antigen at a site away from the source of inflammation were overcome by similar approaches that have been effective in NOD models[289,295,389,414] suggesting that these factors themselves are not incompatible with antigen-specific Treg induction. Instead, conflicting findings for antigen-specific tolerance approaches in the NOD model suggests that relatively similar approaches that use different delivery systems, drugs, and/or routes of administration can get different outcomes because they involve different mechanisms with different APC and regulatory cell subsets, and may also depend on T cell deletion/anergy to varying degrees (Chapter 1). In particular, several similar approaches to TRI MP that were successful in NOD prevention models gave immunomodulatory drugs and/or antigen through a route with more systemic exposure (ex: i.v. or i.p.)[289,295]. Approaches that administered MP s.c. either reported draining to the pancreas/pLN[294] where they likely impact a large population of islet-specific T cells, or they included GM-CSF[292,396,416], which likely led to greater DC recruitment and thus greater antigen-specific T cell expansion. Therefore, the combination of administering TRI MP at a distant site from inflammation and the lack of sufficient exposure to insulin-specific T cells due to the site and route of administration together could explain why TRI MP was not as successful as similar approaches in the literature in the NOD model. It is important to emphasize that this interpretation, which is based on IFA/insulin peptide failing to sufficiently expand insulin-specific T cells for TRI MP to convert into Tregs, is still consistent with the regulatory cell based T1D protective effects of IFA/insulin peptide if (as discussed in the last paragraph) the mechanism of action was

due to enhanced function of existing insulin-specific Tregs through activation or polarization of insulin-specific T cells into FoxP3$^-$ regulatory populations.

A few approaches to overcoming this localization/exposure issue for TRI MP have already been explored while others are not practical or would too closely replicate existing approaches. Identification of a s.c. site that drains to the pancreas and i.p. injection were already explored (Figure 2.13). In order to administer TRI MP i.v., much smaller particles would be needed[306] and all factors would likely need to be included in a single particle to ensure they colocalized, which would negatively impact the ability to maximize and control drug loading and release kinetics. Such an approach (or the addition of GM-CSF) would also make TRI MP more similar to approaches that have been successful using fewer drugs[292,396,414], which would not be ideal from a translational perspective. Instead, future work could focus on local delivery of the TRI MP factors to the pancreas, such as through the use of an implanted osmotic pump and catheter. This would be more similar to how the TRI drugs are given in a sustained release fashion to the site of inflammation in other models. While the dosing of rapamycin would have to be chosen carefully to avoid islet toxicity when given in such a local manner[525], this approach would have the advantages of directly affecting APCs and Teff at the inflamed tissue and dLN as well as providing a sustained tolerogenic environment that could help with Treg stability.

3.0 TRI MP prevents inflammatory arthritis in the CIA model

Sections 3.2 through 3.4 are reprinted from the following manuscript in accordance with PLOS's creative commons attribution (CC BY) license: Bassin, E. J., Buckley, A. R., Piganelli, J. D. & Little, S. R. TRI microparticles prevent inflammatory arthritis in a collagen-induced arthritis model. *PLoS One* **15**, e0239396 (2020).

3.1 Introduction

Even though TRI MP failed to achieve the desired outcomes in T1D, we thought that TRI MP may be more effective in a model of collagen induced arthritis (CIA) due to the ability to inject MP closer to the site of inflammation in this model and because the exogenous antigen provided in this model is used for disease induction and would not have a protective effect. Part of the reason TRI MP was thought to be ineffective in the T1D model was that it failed to alter Tregs levels at the draining LN for the site of inflammation (the pLN), however in the CIA model TRI MP was able to be injected by the draining LN (iLN) of both the antigen used to induce disease as well as of the tissue of interest itself (paws). In the T1D model, an insulin peptide/IFA emulsion used in attempt to elicit an antigen-specific Treg response in conjunction with TRI MP, but delivery of insulin peptide itself provided substantial disease protection making any additional benefit of TRI MP harder to detect. Since antigen (bCII) is provided in a CFA emulsion in order to induce disease in the CIA model, there was no concern about distinguishing the protective effects of TRI MP from that of antigen alone in a prevention model.

Here we demonstrate the ability of TRI MP to prevent arthritic inflammation and bone erosion of the paws in a CIA model of arthritis. The proposed mechanism of this protective effect involves reduced T cell proliferation and the expansion of a regulatory cell population which together ultimately resulted in less immune infiltration of the paws. Anti-CII IgG antibodies were also reduced by TRI MP administration, but not found to contribute to the arthritis prevention provided by this treatment.

3.2 Materials and methods

3.2.1 Microparticle fabrication and characterization

TRI MP were fabricated and characterized as previously described in Sections 2.2.1 and 2.2.2.

3.2.2 Mice

Male DBA/1J mice were purchased from The Jackson Laboratory, Bar Harbor, ME), and used at 8-10 weeks of age. A single gender of mice (male) was used due to gender differences in arthritis severity in the CIA model[526,527]. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (Protocol Number: 18103788) and all methods were performed in accordance with the relevant guidelines and regulations. Animal pain and distress were assessed by checking for lethargy, weight loss (20% or more), and a scruffy coat. However, as no mice exhibited these symptoms, euthanasia was never performed

prior to experimental endpoints. Mice sacrificed at experimental endpoints were euthanized using carbon dioxide followed by cervical dislocation.

3.2.3 Collagen-induced arthritis (CIA) initiation, treatment, and clinical scoring

CIA was initiated as previously described[455,526]. Mice were immunized subcutaneously (s.c.) at the base of the tail on Day 0 and again on Day 21 with 100 µL of a 1:1 emulsion prepared from 4 mg/mL bovine collagen II (bCII, Chondrex, Redmond, WA) dissolved in 0.1 M acetic acid, and complete Freund's adjuvant (CFA) consisting of incomplete Freund's adjuvant (BD, Franklin Lakes, NJ) and 4 mg/mL of M. tuberculosis H37 RA (BD). Mice were shaved and anesthetized with isoflurane for immunizations and MP treatment to facilitate injection.

Mice were injected s.c. with 300 µL of PBS, Blank MP, or TRI MP on each flank above the hind limb on Day 0 and every 4 days through Day 12. For groups receiving MP, each injection contained 15 mg of TGF-β MP and 5 mg of IL-2 MP (or corresponding Blank MP) dissolved in PBS. Injections on Days 0 and 8 also contained 15 mg of rapamycin MP (or corresponding Blank MP). In a pilot prevention study, mice (n=6 per group) were given daily injections (Day 0-13) on each flank above the hind limb with 100 µL of PBS, TRI Low Dose (2 ng TGF-β, 1 µg rapamycin, and 2 ng IL-2), or TRI High Dose (20 ng TGF-β, 10 µg rapamycin, and 20 ng IL-2) instead of MP.

For CIA prevention studies, mice (n=24 per group for MP or n=6 per group for soluble factor pilot study) were anesthetized and paws were imaged at the indicated time points between Day 26 and Day 40 so that they could be scored by a blinded individual. A clinical scoring similar to the one previously described[455,461] was used. Each paw was scored from 0-4 based on the following scale: 0 – no redness or swelling; 1 – a single digit swollen, 2 – two or more digits swollen, but no footpad/palm or ankle/wrist swelling; 3 – two or more digits swollen, and some

footpad/palm or ankle/wrist swelling; 4 – all digits swollen, and severe footpad/palm and ankle/wrist swelling. The scores for each paw were summed, giving a maximum score of 16 per mouse.

3.2.4 Microcomputed tomography (micro-CT) imaging and analysis

On Day 52-60, mice (n=12 per group) selected prior to study initiation for imaging were sacrificed and hind paws were fixed in 4% formaldehyde (Thermo Fisher Scientific, Waltham, MA). The endpoint for this experiment was chosen to provide a sufficient duration of paw inflammation for bone erosion to occur[453]. Micro-CT scanning was performed using an Inveon multimodal scanner (Siemens, Washington, D.C.) at 23 µm isotropic voxel size, with 360 projections, voltage of 80 kV, and current of 500 µA. The open source program ITK-SNAP[528] (www.itksnap.org) was used to reconstruct three-dimensional images and to calculate the bone volume within an arbitrary distance of the metatarsophalangeal (MTP) joints (40 voxels or 920 µm on either side of the joint) similar to a previously described method[453]. Joint bone volume for each hind paw was calculated by summing the 5 MTP volumes. Surface meshes from the three-dimensional images made in ITK-SNAP were exported and surface area was calculated using the Meshmixer program.

3.2.5 Measurement of CII antibody titer

Between Day 40 - 42, mice selected prior to study initiation (n=12 per group) for serum collection were anesthetized with isoflurane and blood was collected via the retro-orbital vein.

Serum was obtained by allowing blood to clot for a minimum of 30 minutes followed by centrifugation (1,000 g, 10 min) and collection of the supernatant.

ELISAs were performed as previously described[526], 96 well plates were coated overnight at 4 °C with 5 µg/mL bCII in Tris-HCl (0.05 M)-NaCl (0.2 M) buffer (pH 7.4). Plates were washed with 0.05% v/v Tween-20 in PBS between all steps prior to the use of stop solution. Plates were blocked with 2% w/v BSA for 1 hr, and serum or a monoclonal anti-CII antibody used as standard (clone 2B1.5, Invitrogen, Carlsbad, CA) were serially diluted in steps of 5x from 500 fold to ~1.5 x 10^6 fold and added in duplicate for 2 hrs. Horseradish peroxidase (HRP) conjugated goat anti-mouse-IgG (Invitrogen) at 1 µg/mL or HRP conjugated goat anti-mouse-IgG2a at 0.25 µg/mL was added for 1 hr, followed by TMB substrate (substrate reagent pack, R&D systems) for 20 min, and sulfuric acid stop solution (R&D systems). Absorbance was measured using a microplate reader (450 nm, subtracting background absorbance at 540 nm).

Antibody titer was defined as the dilution corresponding to the half-maximal absorbance in the linear section of the dilution curve[497], which was calculated as the IC50 value using a non-linear four parameter regression. Normalized titer was calculated by dividing the titer by that of the 2B1.5 antibody standard for a given plate.

3.2.6 Measurement of regulatory T cell levels and phenotype in lymphoid tissue

To characterize regulatory T cells in the lymph node (LN) and spleen, mice (n=6 per group) were immunized with bCII and injected with PBS, Blank MP, or TRI MP as described above. An endpoint of Day 15 was used for assessing draining LN (inguinal LN, iLN) T cells in order to understand the initial response immediately following MP administration, as CII-specific cells initially responding to immunization may traffic elsewhere by later timepoints. On Day 15 mice

were sacrificed and the iLN and spleen were removed and ground to single cell suspensions using 70 µm filters. RBC lysis was performed on spleens with RBC lysis buffer (eBioscience, San Diego, CA), and representative samples of iLN and spleen were counted. Cells (approximately 10 million/mL) were stained with dump channel biotinylated antibodies - CD8a, CD11b, CD11c, CD19, CD45R/B220, TCR γ/δ, and F4/80 (BioLegend, San Diego, CA) - followed by BV786-Streptavidin (BD), Fc block (eBioscience), fixable viability dye (eBioscience), and for CD4 (RM4-5; BD), CD25 (PC61; BD), CD73 (TY/11.8; eBioscience), LAP (TW7-16B4; eBioscience), and CTLA-4 (UC10-4F10-1; BD). Cells were then fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience), stained for FoxP3 (FJK-16s; eBioscience) and Tbet (4B10; BD), and run on a flow cytometer (Aurora, Cytek Biosciences, Barboursville, VA) and analyzed using FlowJo software (Tree Star, Ashland, OR) with gates based on isotype and single-color controls.

3.2.7 Localization of inhibited T cell proliferation

To assess the effects of TRI MP on T cell proliferation and the localization of those effects, mice (n=6 per group) were immunized with a non-arthritic antigen on one flank and immunized with collagen and TRI MP on the opposite flank. Specifically, mice were immunized s.c. on Day 0 on the left flank with 100 µL of a 1:1 emulsion prepared from 2 mg/mL Keyhole limpet hemocyanin (KLH, Sigma Aldrich) and CFA prepared as described above. Mice were also immunized on the right side on Day 0 by the base of the tail with bCII and given injections of PBS, Blank MP, or TRI MP every 4 days through Day 12 as described above. On Day 15, mice were sacrificed and the left and right iLN were removed and separately ground to single cell suspensions using 70 µm filters. Cells were stained with Fc block, fixable viability dye, and for CD4, CD25, fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience), and then

stained for Ki67 (SolA15; eBioscience) and Tbet (O4-06; BD). Counting beads (Thermo Fisher Scientific) were added, then samples were run on a flow cytometer (LSRII, BD) and analyzed using FlowJo (Tree Star) with gates based on isotype and single-color controls.

3.2.8 Assessment of immune infiltrate in arthritic paws

Between Day 40 - 42, mice selected prior to study initiation for immune cell extraction from the paws were sacrificed. Paws were collected and immune cells were isolated as previously described[529]. Digits were removed and bone marrow was flushed with media, then paws (including digits) were chopped up and incubated in digestion media, cDMEM with 1 mg/mL collagenase (Sigma Aldrich) and 2.4 mg/mL hyaluronidase (Sigma Aldrich), at 37 °C for 1 hr with shaking. Digested paws were then mashed and washed in 70 µm filters to create single cell suspensions. Two different staining panels were performed. In one panel (n=12 per group), cells were stained with Fc block, fixable viability dye, and for CD45 (30-F11; eBioscience), CD4, CD25, fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience), and then stained for FoxP3. In a second panel (n=6 per group), cells were stimulated with 5 ng/mL PMA (Sigma Aldrich) and 500 ng/mL Ionomycin (Sigma Aldrich) with Golgi-Plug protein transport inhibitor (BD) for 4 hrs at 37 °C. Cells were then stained with Fc block, fixable viability dye, and for CD45, CD3e (145-2C11; BD), CD19 (1D3; BD),CD11b (M1/70; eBioscience), Ly-6G (1A8-Ly6g; eBioscience), Ly-6C, (HK1.4; eBioscience), fixed/permeabilized (FoxP3/Transcription Factor Staining Buffer Set, eBioscience), and then stained for TNF-alpha (MP6-XT22; eBioscience). Counting beads (Thermo Fisher Scientific) were added, then samples were run on a flow cytometer (LSRII or Fortessa, BD) and analyzed using FlowJo (Tree Star) with gates based on isotype and single-color controls.

3.2.9 Statistical analysis

Statistical analyses were performed with GraphPad Prism v7 (San Diego, CA). Data are presented as mean ± SEM and the following cutoffs were used for significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For arthritis incidence curves, a Log-rank (Mantel-Cox) test was ran comparing all curves. Since this was significant, Long-rank (Mantel-Cox) test were performed for each individual comparison, and p values were multiplied by the number of comparisons made (3).For arthritis clinical score curves, a two-way mixed effects ANOVA (for time as a repeated measure and treatment group) was performed, followed by Tukey post-hoc analysis to compare the mean of every group with the mean of every other group at each time point. The ROUT outlier test with the most stringent threshold for outlier removal (Q = 0.1%) was used to remove outliers from the graph of normalized antibody titers. For all plots assessing a correlation with arthritis scores, the Spearman r correlation coefficient was calculated and a two-tailed p value was used to determine the significance of the correlation. All other graphs had 3 treatment groups and were analyzed by one-way ANOVA, followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group.

3.3 Results

3.3.1 TRI MP treatment prevents induction of arthritis

TRI MP morphology, size, and drug release kinetics (Figure 3.1) were similar to those previously reported[409]. The dose of MP administered for CIA prevention was chosen based on MP

release (Figure 3.1) in order to approximate the effect observed in a pilot CIA prevention study using daily local injection of TRI factors that were not encapsulated in MP (Figure 3.2). An experimental timeline illustrating the different cohorts of mice and associated end points for MP treatment studies can be found in Figure 3.3. In the MP CIA prevention study, PBS treated mice had less than 50% of mice remaining arthritis free by Day 28 and all mice had developed arthritis by Day 36 (Figure 3.4 A). Blank MP, or MP made with vehicle control instead of drug, treated mice had less than 50% of mice remaining arthritis free by Day 30, and 25% of mice remining arthritis free at the study endpoint (Figure 3.4 A). In comparison to these groups, TRI MP had a significantly improved survival curve (Mantel-Cox, $p < 0.0001$ and $p < 0.05$ respectively), with 62.5% of mice remaining arthritis free at the study endpoint (Figure 3.4 A). When the clinical arthritis score was assessed, TRI MP significantly prevented the development of disease relative to both PBS (Two-way ANOVA, Tukey post-hoc, $p < 0.0001$) and Blank MP (Two-way ANOVA, Tukey post-hoc for treatment group, $p < 0.01$) treatment at all timepoints past Day 32 (Figure 3.4 B). These differences were 2-3x in magnitude with TRI MP treatment resulting in an average arthritis score of 2.5 at Day 40, while PBS and Blank MP treatment led to average arthritis scores of 7.5 and 5.8 respectively (Figure 3.4 B). To demonstrate how TRI MP treatment influenced the number and severity of inflamed paws, results were also presented in terms of number of affected paws per mouse. Relative to PBS treatment, TRI MP treatment significantly reduced the number of paws per mouse with arthritis (Figure 3.4 C), as well as the number of paws per mouse with severe arthritis (Figure 3.4 D). Where severe arthritis (arthritis score ≥ 3 per paw) was defined by the involvement of footpad/ankle swelling. Taken together, these data show that TRI MP was able to significantly inhibit the incidence and severity of arthritis in a prevention model.

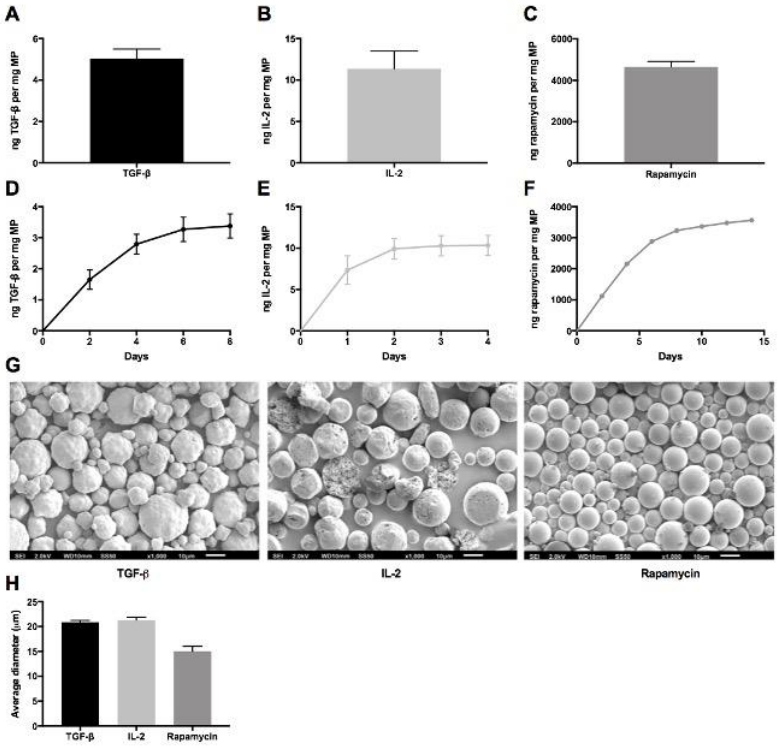

Figure 3.1 Microparticle characterization. **A-C) Drug loading (ng/mg) for TGF-β microparticles (MP) (A), IL-2 MP (B), and rapamycin MP (C) respectively. n = 6-12 batches of MP per group, data presented as mean ± SEM. D-F) *In vitro* release kinetics for TGF-β MP (D), IL-2 MP (E), and rapamycin MP (F) respectively. Representative batch of MP shown with release samples performed in triplicate and presented as mean ± SEM. G) SEM images showing surface morphology of TGF-β MP, IL-2 MP, and rapamycin MP with 10 μm scale bar shown for reference. H) Average MP diameter measured by Coulter Counter, presented as mean ± SEM.**

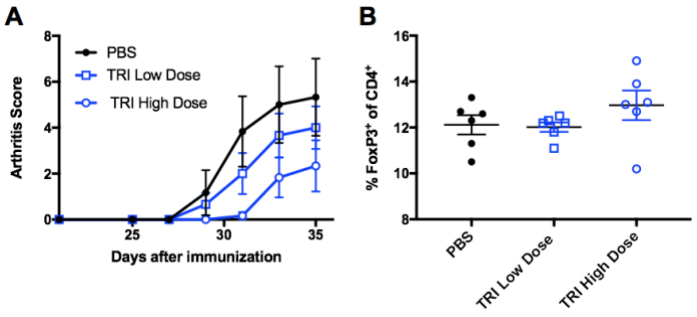

Figure 3.2 Pilot study using unencapsulated TRI factors. **A) Arthritis scores over time for mice given daily injections (Day 0-13) on each flank above the hind limb with 100 µL of PBS, TRI Low Dose (2 ng TGF-β, 1 µg rapamycin, and 2 ng IL-2), or TRI High Dose (20 ng TGF-β, 10 µg rapamycin, and 20 ng IL-2. n = 6 mice per group, data presented as mean ± SEM. A two-way ANOVA was performed, followed by Tukey post-hoc analysis to compare the mean of every group with the mean of every other group at each time point. The treatment effect was non-significant. B) Quantification of the percentage of $CD4^+$ T cells that are $FoxP3^+$ in the draining (inguinal) lymph node on Day 35. n = 6 mice per group, data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group. All comparisons were non-significant.**

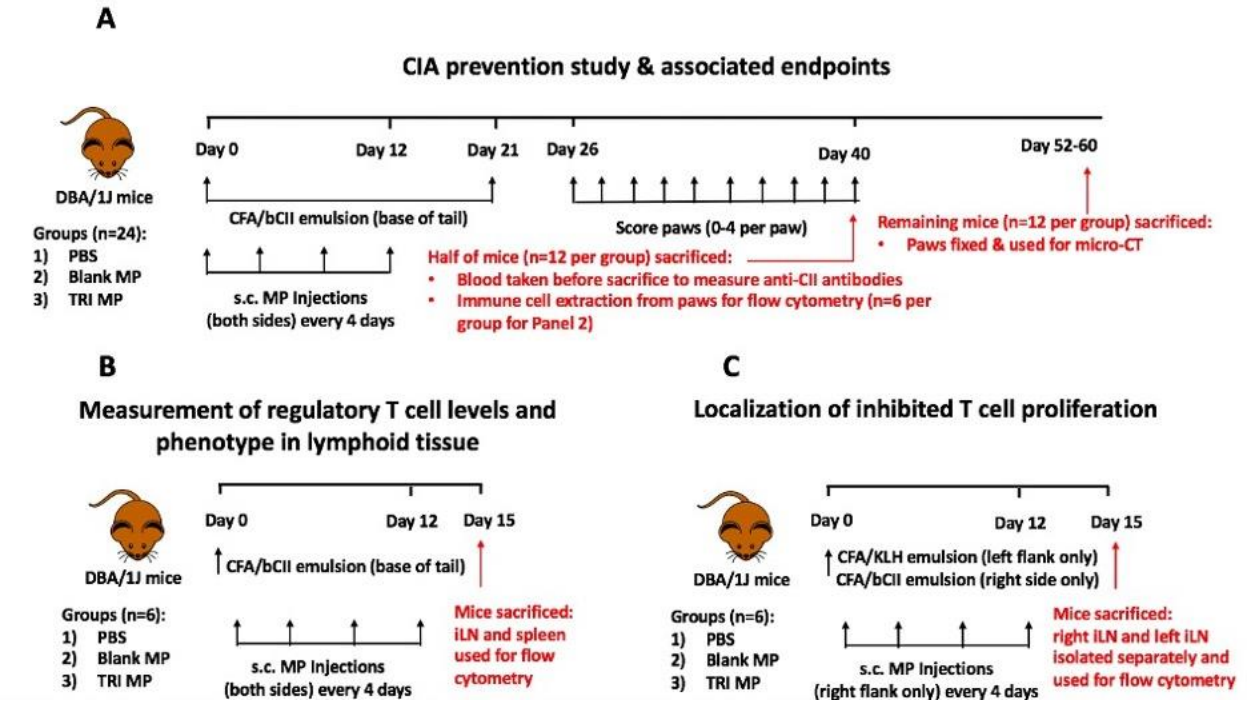

Figure 3.3 Experimental timelines for microparticle treated animal studies. **A) Timeline for CIA prevention and associated endpoints. Mice (n=24 per group) were immunized with an emulsion of complete Freund's adjuvant (CFA) and bovine collagen II (bCII) at the base of the tail on Day 0 and subcutaneously (s.c.) injected with PBS or microparticles (MP) by both hind limbs every 4 days between Day 0 and Day 12. Mice were scored by a blinded individual for signs of arthritis between Day 26 and Day 40 (Figure 3.4), at which point half of mice were sacrificed and used to measure serum auto-antibodies (Figures 3.6 and 3.7) and to extract immune cells from the paws (Figures 3.12 and 3.13). The other half of mice were left until Day 52-60 to allow sufficient time for inflammation to result in bone erosion, and then sacrificed and used for micro-computed tomography (CT) (Figure 3.5). B) Timeline for measurement of regulatory T cell levels and phenotype in lymphoid tissue. Mice (n=6 per group) treated as in A), but sacrificed at Day 15 to assess T cells at a time point close to MP administration to assess regulatory T cell levels and phenotype in the draining inguinal lymph nodes (iLN) and spleen (Figures 3.8 – 3.10). C) Timeline for localization of inhibited T cell proliferation. Mice (n=6 per group) were immunized with bCII by the base of the tail on the right side only, and on the left flank an emulsion of CFA and Keyhole limpet hemocyanin (KLH) was given. Mice were treated with PBS or MP as described above, but only on the right flank. T cell responses were assessed for both the draining iLN (right side) and contralateral iLN (left side) relative to MP localization (Figure 3.11).**

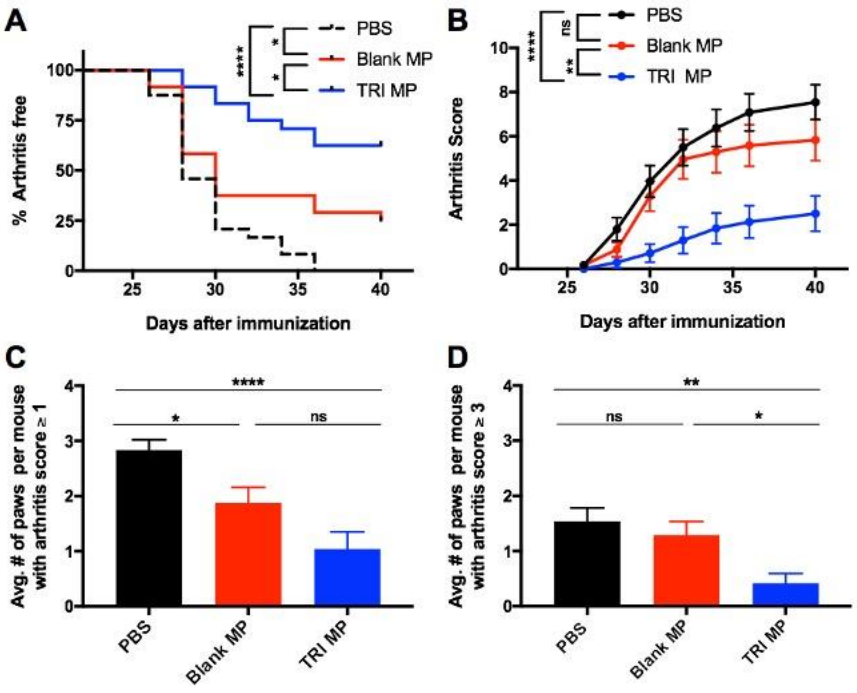

Figure 3.4 TRI MP administration reduces incidence and severity of CIA onset. **A) Survival curve indicating percentage of mice that remained arthritis free (score of 0 for all paws). B) Arthritis scores over time. A two-way ANOVA was performed, followed by Tukey post-hoc analysis to compare the mean of every group with the mean of every other group at each time point. Significance labels apply to all time points from Day 32 on. C-D) Average number of paws per mouse with arthritis score greater than or equal to specified threshold of 1 (C) or 3 (D) at Day 40. n = 24 mice per group, data presented as mean ± SEM, and the following cutoffs were used for significance: * p < 0.05, ** p < 0.01, **** p < 0.0001.**

3.3.2 Correlation demonstrated between arthritis clinical score and bone erosion

To determine if the reduction in paw inflammation observed with TRI MP administration was associated with less bone erosion, micro-computed tomography (CT) scans were performed on fixed hind paws from mice sacrificed between Day 52 and Day 60. Visible full-thickness bone erosions could be detected at the MTP joints in some paws with high clinical arthritis scores (Figure 3.5 A). Quantification of the relationship between MTP joint bone volume and arthritis score for an individual paw demonstrated a negative, moderate strength (Spearman r = -0.573), and significant ($p < 0.0001$) correlation (Figure 3.5 B). Notably there is some variability in the

joint bone volume among paws that had arthritis scores of zero at Day 40. While some of this may be natural variation present in healthy paws (i.e. bone volumes of ~ $4mm^3$ – $5mm^3$), some of the lower bone volume measurements in this group may reflect the delayed emergence of arthritis between Day 40 and Day 60 in corresponding mice. Despite this variability, the moderate strength and significant correlation observed suggest that on average the arthritis score is still a good predictor of bone erosion. When the data is presented by treatment group, the TRI MP group has significantly (one-way ANOVA, Tukey post-hoc, $p < 0.001$) more joint bone volume than the PBS group (Figure 3.5 C). Depending on the severity, an arthritic bone erosion should theoretically result in a loss of joint bone volume (V) and/or an increase in joint bone surface area (SA) due to the irregular nature of bone erosions. Together this would result in an increased surface area to volume ratio (SA/V). As expected, there was a positive, moderate strength (Spearman $r = 0.699$), and significant ($p < 0.0001$) correlation between MTP joint bone surface area to volume ratio and arthritis score (Figure 3.5 D). Likewise, relative to PBS treatment, TRI MP treatment significantly (one-way ANOVA, Tukey post-hoc, $p < 0.001$) prevented the increased joint bone surface area to volume ratio associated with arthritis (Figure 3.5 E). Together these findings demonstrate that a reduced arthritis score was associated with protection from bone erosion, and on average TRI MP treated mice exhibited less bone erosion.

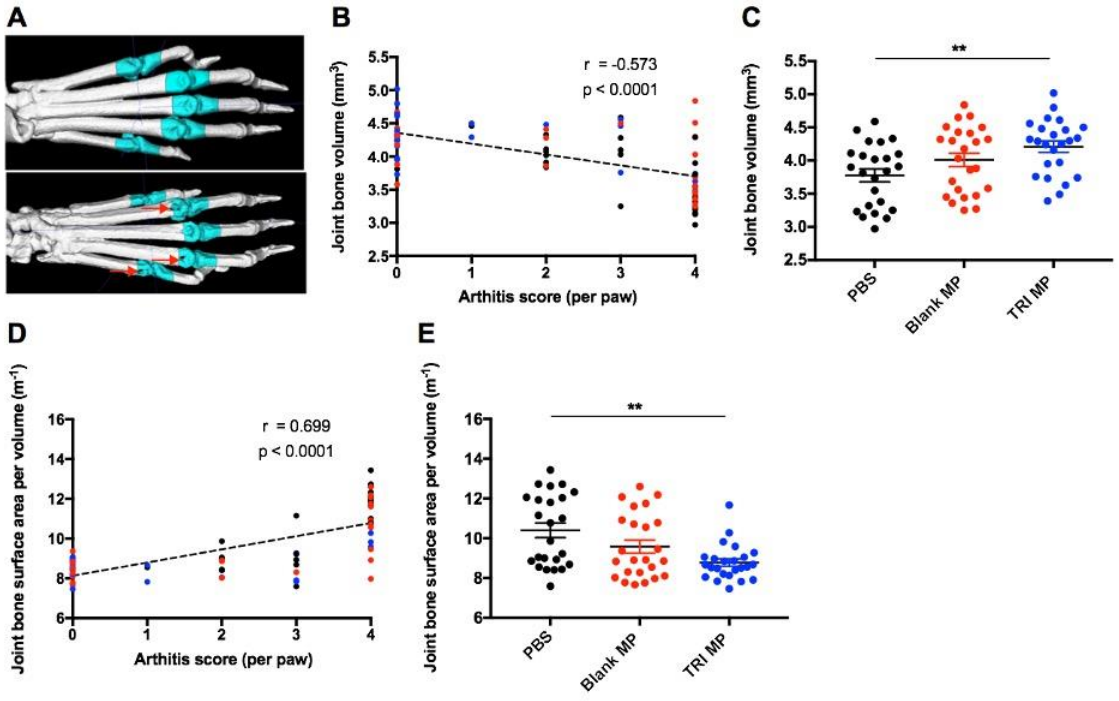

Figure 3.5 Lower arthritis score correlates with less bone erosion. **A) Representative 3D reconstructions of micro-CT scans showing a paw without bone erosion (top) and a paw with severe bone erosion (bottom). Joint masks indicated in cyan were the regions used to calculate joint bone volume. B) Joint bone volume versus arthritis score at Day 40 for individual hind paws. Color coded based on treatment group: black – PBS, red – Blank MP, blue – TRI MP. Spearman correlation coefficient and p value for correlation are indicated. C) Average joint bone volume by treatment group. D) Joint bone surface area to volume ratio versus arthritis score at Day 40 for individual hind paws. Color coded based on treatment group: black – PBS, red – Blank MP, blue – TRI MP. Spearman correlation coefficient and p value for correlation are indicated. E) Average joint bone surface area to volume ratio by treatment group. n = 24 paws (12 mice) per group, data presented as mean ± SEM, and the following cutoffs were used for significance: ** p < 0.01.**

3.3.3 Auto-antibodies are reduced in mice that are administered TRI MP

To begin to understand the mechanism by which TRI MP is acting, serum taken on Day 40 was used in indirect ELISAs with bCII as the antigen to measure levels of anti-CII IgG antibodies (Ab). Representative serial dilution curves (Figure 3.6 A) show one TRI MP mouse (blue) with a particularly left-shifted curve, and thus reduced anti-CII IgG Ab titer. Ab titer was normalized to the titer of a monoclonal CII Ab included on each plate to account for plate-to-plate variability. A

plot of normalized anti-CII IgG Ab titer vs. arthritis score had a weak (Spearman r = 0.303) and non-significant (p = 0.0817) correlation (Figure 3.6 B). However, TRI MP treatment significantly (one-way ANOVA, Tukey post-hoc, $p < 0.05$) lowered the average anti-CII IgG Ab titer by approximately 40% relative to PBS treatment (Figure 3.6 C). These results demonstrate that TRI MP significantly reduced the level of an arthritis causing auto-antibody but did not completely block auto-antibody generation even in mice that had no signs of arthritis. The lack of correlation between anti-CII IgG Ab titer and arthritis clinical score suggests that the mechanism of TRI MP action is not a reduction of the concentration or affinity of total anti-CII IgG Ab. While the anti-CII IgG level has been associated with CIA disease severity in a few studies[497,506], there is also evidence that the percentage of anti-CII IgG that is of the Th1 associated IgG2a isotype[530] and not the overall IgG level predicts susceptibility to CIA since IgG2a is associated with complement system activity[457,471]. Therefore, anti-CII IgG2a Ab titers were also assessed. There was no correlation between anti-CII IgG2a Ab titers and arthritis score and no differences in anti-CII IgG2a Ab titers between treatment groups (Figure 3.7).

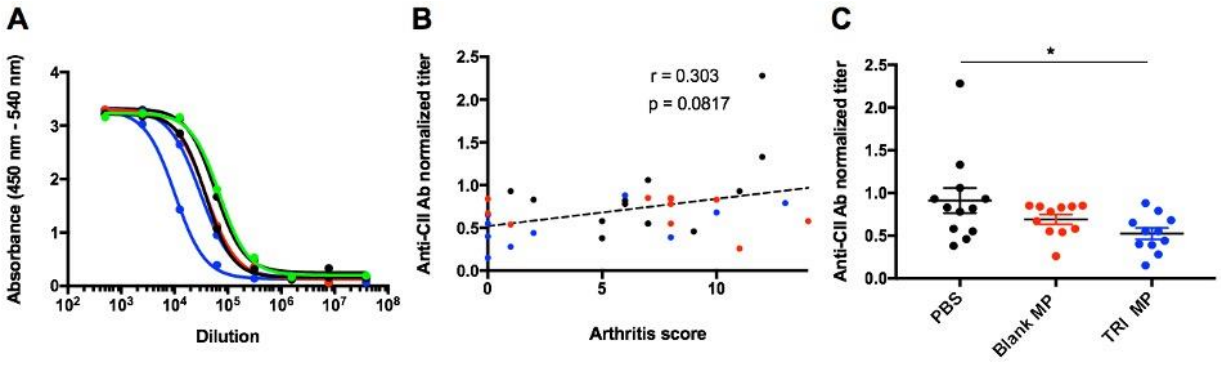

Figure 3.6 TRI MP administration lowers level of anti-collagen II IgG antibodies. **A) Representative serial dilution curves with each curve corresponding to a single mouse, black – PBS treated mouse, red – Blank MP treated mouse, blue – TRI MP treated mouse, green – monoclonal anti-collagen II (CII) antibody (Ab) (Clone 2B1.5) used as standard. B) Normalized anti-CII IgG Ab titer versus arthritis score. Ab titer was defined as the dilution corresponding to the half-maximal absorbance in the linear section of the dilution curve, or the IC50 value using a non-linear four parameter regression. Normalized titer was calculated by dividing the titer by that of the 2B1.5 Ab standard for a given plate. Color coded based on treatment group: black – PBS, red – Blank MP, blue – TRI MP. Spearman correlation coefficient and p value for correlation are indicated. C) Average normalized anti-CII IgG Ab titer by treatment group. n = 12 mice per group, data presented as mean ± SEM, and the following cutoffs were used for significance: * $p < 0.05$.**

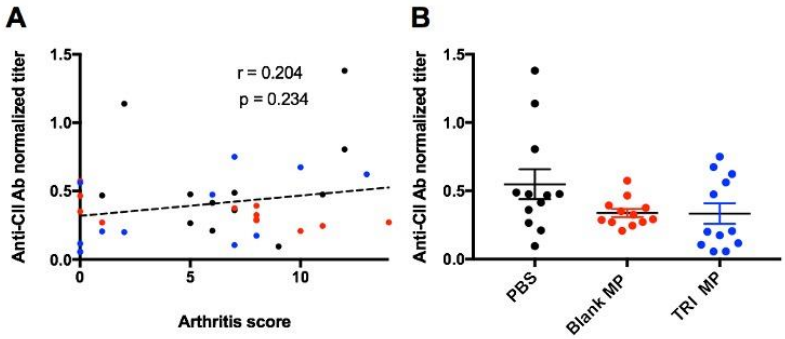

Figure 3.7 Assessment of anti-collagen II IgG 2a antibodies. **A) Normalized anti-CII IgG2a Ab titer versus arthritis score. Ab titer was defined as the dilution corresponding to the half-maximal absorbance in the linear section of the dilution curve, or the IC50 value using a non-linear four parameter regression. Normalized titer was calculated by dividing the titer by that of the 2B1.5 clone Ab standard for a given plate. Color coded based on treatment group: black – PBS, red – Blank MP, blue – TRI MP. Spearman correlation coefficient and p value for correlation are indicated. B) Average normalized anti-CII IgG 2a Ab titer by treatment group. n = 12 mice per group, data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group. All comparisons were non-significant.**

3.3.4 TRI MP treatment increases a $CD4^+$ T cell population with elevated regulatory markers in the draining lymph node and spleen

To investigate whether regulatory T cells could be playing a role in TRI MP prevention of CIA, mice were immunized with bCII, injected with PBS, Blank MP, or TRI MP every 4 days, and sacrificed on Day 15. While there was not a significant increase in the levels of $FoxP3^+CD25^+$ Tregs in the draining lymph node (inguinal, iLN) (Figure 3.8 A and B) or spleen (Figure 3.8 A and D) of TRI MP treated mice relative to controls, there was a significant increase (one-way ANOVA, Tukey post-hoc, $p < 0.01$) in $FoxP3^- CD25^+$ T cells relative to PBS treated mice in the iLN (Figure 3.8 C) and spleen (Figure 3.8 E). Likewise, no significant increase in $FoxP3^+$ Tregs was observed at a later time point (Day 35) in the pilot study with daily injections of un-encapsulated TRI factors (Figure 3.2). Several markers associated with regulatory T cell function were also assessed to evaluate how their expression on the $FoxP3^- CD25^+$ population compared to that of conventional $CD4^+$ T cells ($FoxP3^- CD25^-$) and Tregs ($FoxP3^+ CD25^+$), as well as whether TRI MP led to evaluated expression of these markers relative to control treatments on either the $FoxP3^- CD25^+$ or Treg populations. The analyzed markers included: latency-associated peptide (LAP), part of the latent TGF-beta complex; CTLA-4, a checkpoint molecule that blocks CD80/86 co-stimulation; and CD73, an enzyme which degrades AMP to immunosuppressive adenosine. When mice from all treatment groups were pooled together in the analysis, the $FoxP3^- CD25^+$ population had significantly (One-way ANOVA, Tukey post-hoc for T cell population, $p < 0.01$ or $p < 0.0001$) higher expression of LAP, CTLA-4, and CD73 than the conventional $CD4^+$ T cells ($FoxP3^- CD25^-$) population in both the iLN and spleen (Figure 3.8 F-L). However, while TRI MP treatment resulted in significantly elevated expression of CD73 for the iLN $FoxP3^- CD25^+$ population, TRI MP led to trends toward reduction (and one significant example) of LAP and CTLA-4 expression

for the FoxP3$^-$ CD25$^+$ and/or Treg (FoxP3$^+$ CD25$^+$) populations in the iLN and spleen (Figure 3.9). It is possible that reduced inflammation in TRI MP treated mice prevented the upregulation of these suppressive markers. Tbet expression was also assessed, but an appreciable Tbet$^+$ population was not detected (Figure 3.10). While TRI MP treatment did not increase the levels of conventional FoxP3$^+$ Tregs or increase expression of suppressive markers on these cells, it did increase a population of activated CD4$^+$ T cells (FoxP3$^-$CD25$^+$) that had elevated levels of suppressive markers.

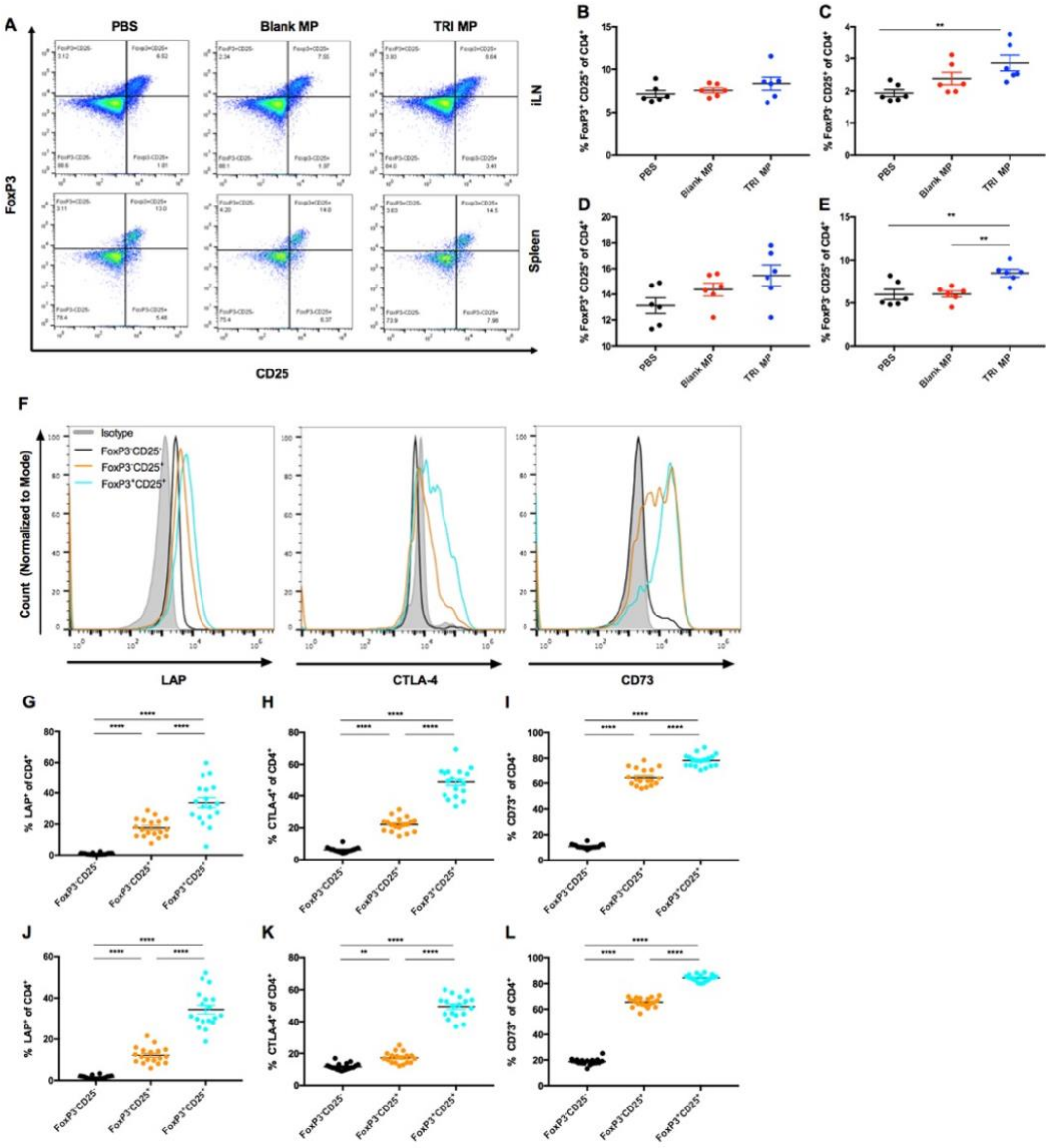

Figure 3.8 TRI MP leads to more CD25$^+$FoxP3$^-$ T cells, which express elevated levels of LAP, CTLA-4, and CD73. **A) Representative pseudocolor plots of CD25 expression versus FoxP3 expression for CD4$^+$ cells from the iLN (top row) or spleen (bottom row) of mice treated with PBS (left column), Blank MP (center column), or TRI MP (right column). B-E) Quantification of plots from A, showing percentage of CD4$^+$ cells that are FoxP3$^+$CD25$^+$ (B, E) or FoxP3$^-$CD25$^+$ (C, D) by treatment group for the iLN (B, C) and spleen (D, E). F) Representative histogram plots of LAP (left), CTLA-4 (middle), and CD73 (right) expression for the isotype control (shaded gray), the FoxP3$^-$CD25$^-$ population (black), the FoxP3$^-$CD25$^+$ population (orange), and the FoxP3$^+$CD25$^+$ (cyan) population (from a PBS treated mouse). G-L) Quantification of the percentage of CD4$^+$ T cell populations that are LAP$^+$ (G,J), CTLA-4$^+$ (H, K), or CD73$^+$ (I, L) relative to isotype control. Presented by CD4$^+$ T cell population (FoxP3$^-$CD25$^-$, FoxP3$^-$CD25$^+$, and FoxP3$^+$CD25$^+$) for the iLN (G-I) and spleen (J-L). n = 6 mice per treatment group and n=18 mice per CD4$^+$ T cell population group, data presented as mean ± SEM, and the following cutoffs were used for significance: ** p < 0.01, *** p < 0.001, **** p < 0.0001.**

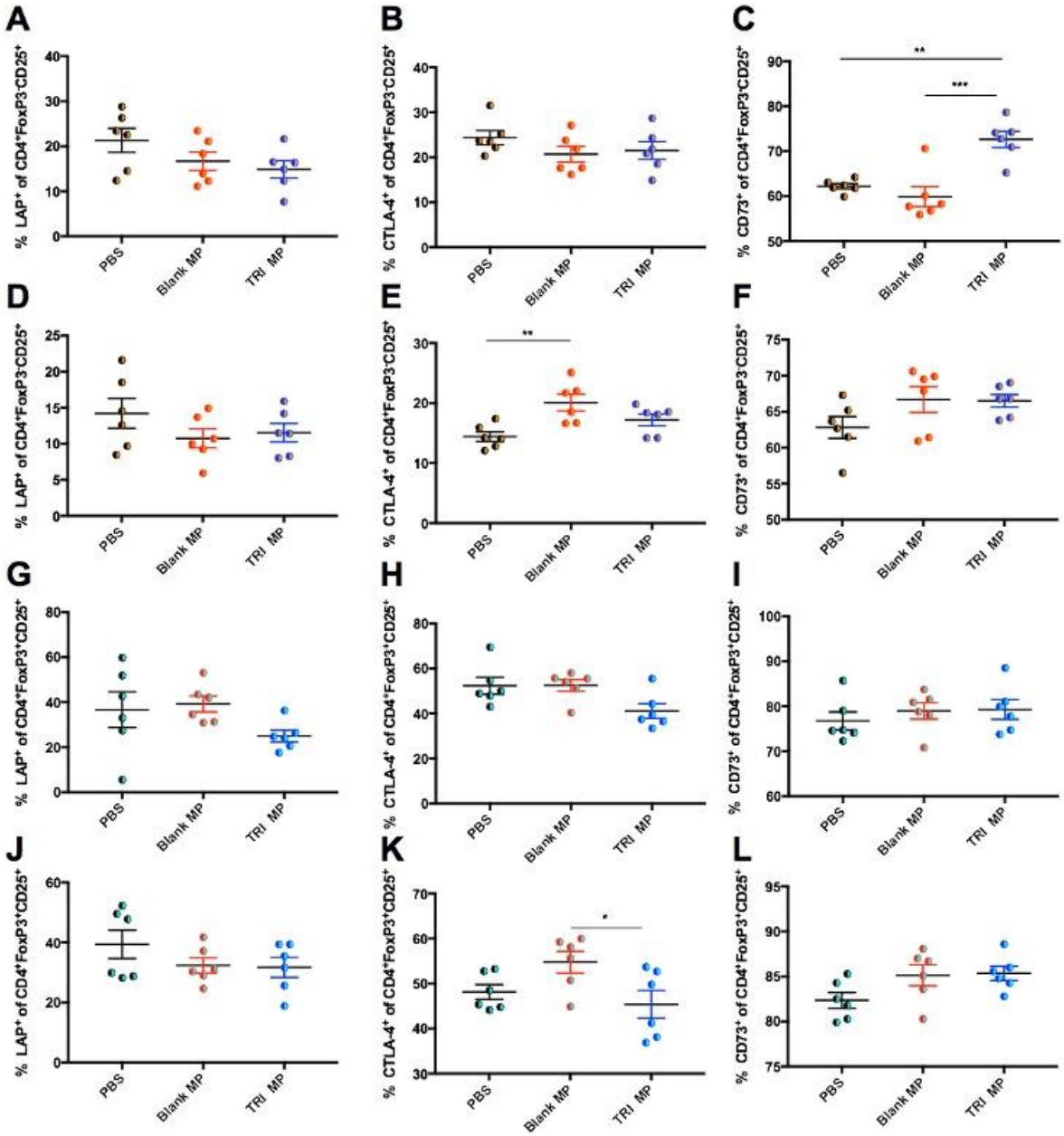

Figure 3.9 Analysis of LAP, CTLA-4, and CD73 expression by treatment group. **Quantification of the percentage of the indicated CD4$^+$ T cell population that are LAP$^+$, CTLA-4$^+$,or CD73$^+$ relative to isotype control. Complimentary analysis to Figure 3.8, but presented by treatment group (PBS, Blank MP, or TRI MP). Graphs are for the FoxP3$^-$CD25$^+$ population in the iLN (A-C), the FoxP3$^-$CD25$^+$ population in the spleen (D-F), the FoxP3$^+$CD25$^+$ population in the iLN (G-I), or the FoxP3$^+$CD25$^+$ population in the spleen (J-L).). n = 6 mice per group, data presented as mean ± SEM, and the following cutoffs were used for significance: ** p < 0.01, *** p < 0.001, **** p < 0.0001. All other comparisons were non-significant**

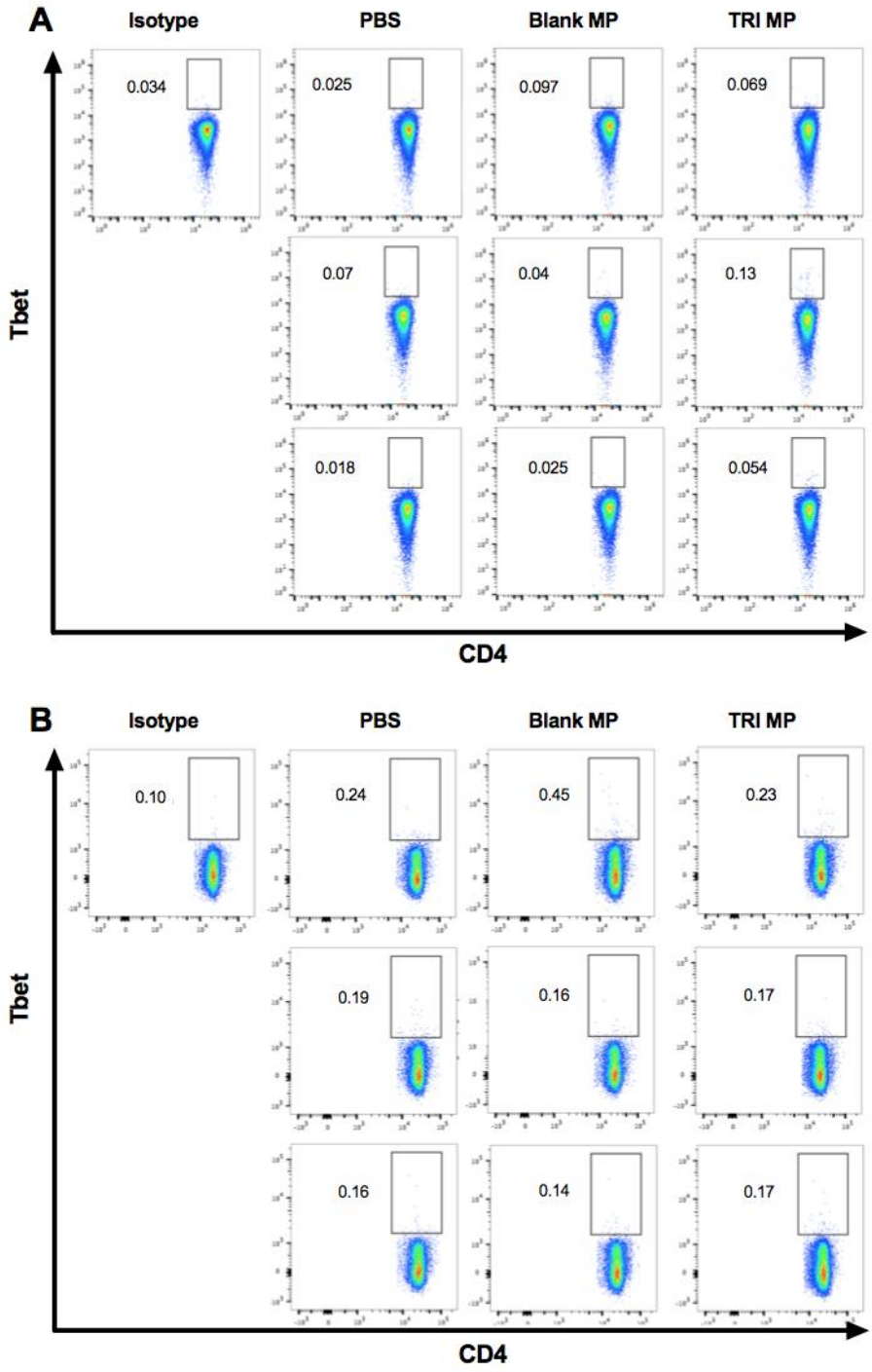

Figure 3.10 Lack of appreciable Tbet^{+} population in the iLN at Day 15 staining with two different antibody clones. **Representative flow plots showing CD4 expression versus Tbet expression showing isotype control or 3 different samples each for PBS, Blank MP, and TRI MP treatments (grouped by column). A) Samples stained with Tbet antibody clone 4B10. B) Samples stained with Tbet antibody clone O4-06.**

3.3.5 The effects of the dose of TRI MP administered are not localized to the draining lymph node

In order to evaluate the role of TRI MP suppression of T cell proliferation in arthritis protection as well as the localization of this immunosuppression, mice were immunized with KLH on one flank and immunized with bCII along with PBS, Blank MP, or TRI MP on the other flank. The iLN of the TRI MP treated flank had a trend towards reduced cell numbers, and significantly (One-way ANOVA, Tukey post-hoc, $p < 0.01$) reduced proliferation of $CD4^+$ T cells (Figure 3.11 A and B). There also was an increase in the $FoxP3^-CD25^+$ population (Figure 3.11 C) consistent with Figure 3.8 C. However, the contralateral limb in TRI MP treated mice also had the response to immunization suppressed to a similar degree. The contralateral iLN of the TRI MP group had a trend towards reduced cell numbers and reduced proliferation, with significant differences (One-way ANOVA, Tukey post-hoc, $p < 0.01$) observed relative to the Blank MP group (Figure 3.11 D and E). There was also a significant increase (One-way ANOVA, Tukey post-hoc, $p < 0.01$) in the $FoxP3^-CD25^+$ population in the contralateral iLN (Figure 3.11 F). These results suggest that the actions of TRI MP were not localized to the draining LN, as similar levels of reduced cellular proliferation and an increased regulatory population were observed in both the draining and contralateral iLN.

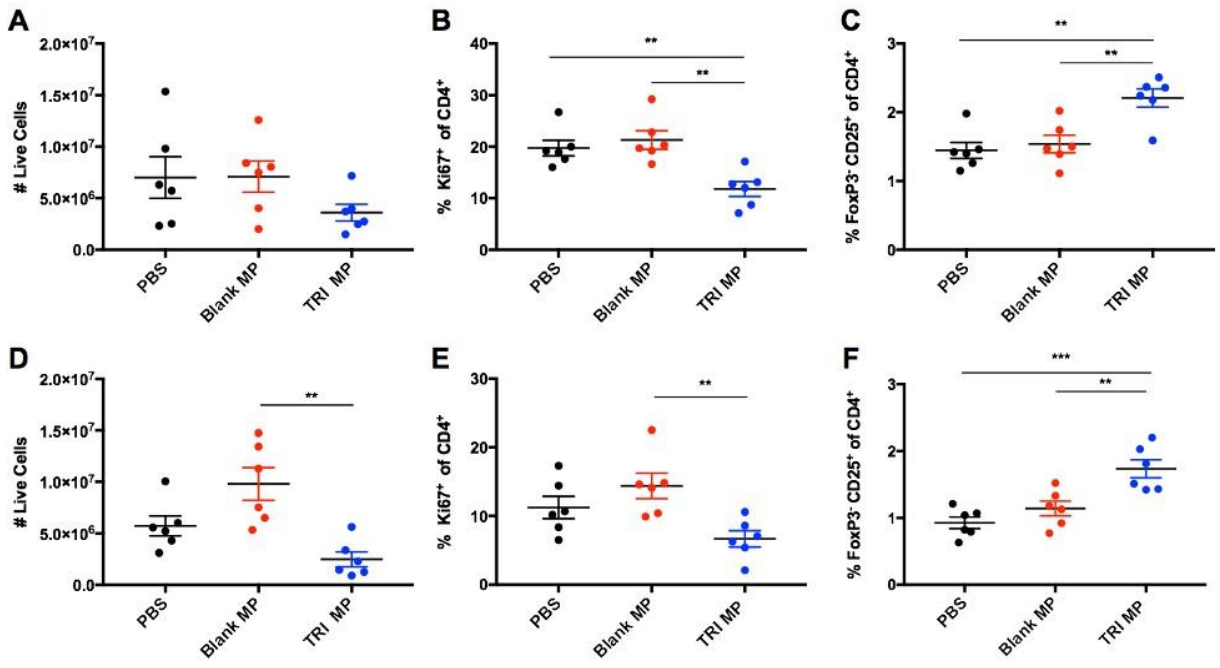

Figure 3.11 TRI MP reduces conventional T cell proliferation and expands a regulatory population not only for the draining lymph node, but also in the contralateral lymph node for a non-arthritic immunization.

A) Number of live cells in draining iLN as determined using counting beads. B) Percentage of CD4+ T cells expressing the proliferation marker Ki67 in the the draining LN. C) Percentage of CD4+ T cells that are FoxP3-CD25+ in the draining LN. D-F) Same as A-C, but for the contralateral LN instead of the draining LN. n = 6 mice per group, data presented as mean ± SEM, and the following cutoffs were used for significance: ** $p < 0.01$, *** $p < 0.001$.

3.3.6 Lower arthritis score associated with less immune infiltrate and inflammatory cytokine in the paws

To assess how TRI MP treatment altered the amount and characteristics of immune infiltrate in the inflamed paws themselves, immune cells were extracted from paws between Day 40 - 42. Since TRI MP was shown to reduce $CD4^+$ T cell proliferation and expand a $FoxP3^-CD25^+$ population expressing suppressive markers in the draining LN (Figures 3.8 and 3.11), the accumulation of $CD4^+$ T cells in the paws and fraction of them that were $FoxP3^-CD25^+$ was assessed. A moderate (Spearman r = 0.634) and significant ($p < 0.0001$) positive correlation was

observed between the number of $CD4^+$ T cells in the paws and the arthritis score (Figure 3.12 A). While mice with lower arthritis scores had fewer number of $CD4^+$ T cells in the paws, a larger percentage of these $CD4^+$ T cells were $FoxP3^-CD25^+$ (Figure 3.12 B). Although TRI MP treated mice did not have a significantly different $FoxP3^-CD25^+$ cell population relative to PBS and Blank MP controls (Figure 3.12 C), the average for TRI MP was slightly larger driven by three TRI MP treated mice with arthritis scores of zero and greater than 20% of CD4 T cells expressing the $FoxP3^-CD25^+$ phenotype (Figure 3.12 C). Notably, the percentage of $CD4^+$ T cells expressing FoxP3 was not significantly correlated with arthritis score or significantly increased with TRI MP treatment (Figure 3.13). Given the paradigm of auto-antibodies and $CD4^+$ T cells promoting myeloid cell recruitment and expansion in CIA, the size of the overall immune infiltrate in the paws and levels of monocytes/macrophages and neutrophils were assessed. There was a significant ($p < 0.0001$) positive correlation between the amount of immune infiltrate in the paws, as defined by CD45 expression, and the arthritis score (Figure 3.12 D). Not only did the number of immune cells present in the paws increase with higher arthritis scores, but the composition of the $CD45^+$ immune population changed as well. The percentages of monocytes/macrophages ($CD11b^+Ly\text{-}6G^-Ly\text{-}6C^+$)[531] and neutrophils ($CD11b^+Ly\text{-}6G^+$) among $CD45^+$ cells were significantly ($p = 0.0001$ and $p = 0.002$ respectively) and positively correlated with arthritis score (Figure 3.12 E and F). The percentage of monocytes/macrophages and neutrophils in the paws of bCII-immunized mice was also noticeably higher than that of un-immunized mice (Figure 3.13). Due to the role of TNF-α in causing paw redness and swelling, myeloid cell expression of TNF-α was also measured. Both the number of TNF-α^+ monocytes/macrophages and the number of TNF-α^+ neutrophils were significantly ($p = 0.001$ and $p = 0.019$) and positively correlated with arthritis score (Figure 3.12 G and H). Taken together, these findings are consistent with a scenario in which mice with low

arthritis scores have less $CD4^+$ T cell infiltrate and/or a higher proportion of regulatory $FoxP3^-$ $CD25^+$ cells, resulting in reduced infiltrate of myeloid cells and less production of an inflammatory cytokine responsible for paw redness and swelling.

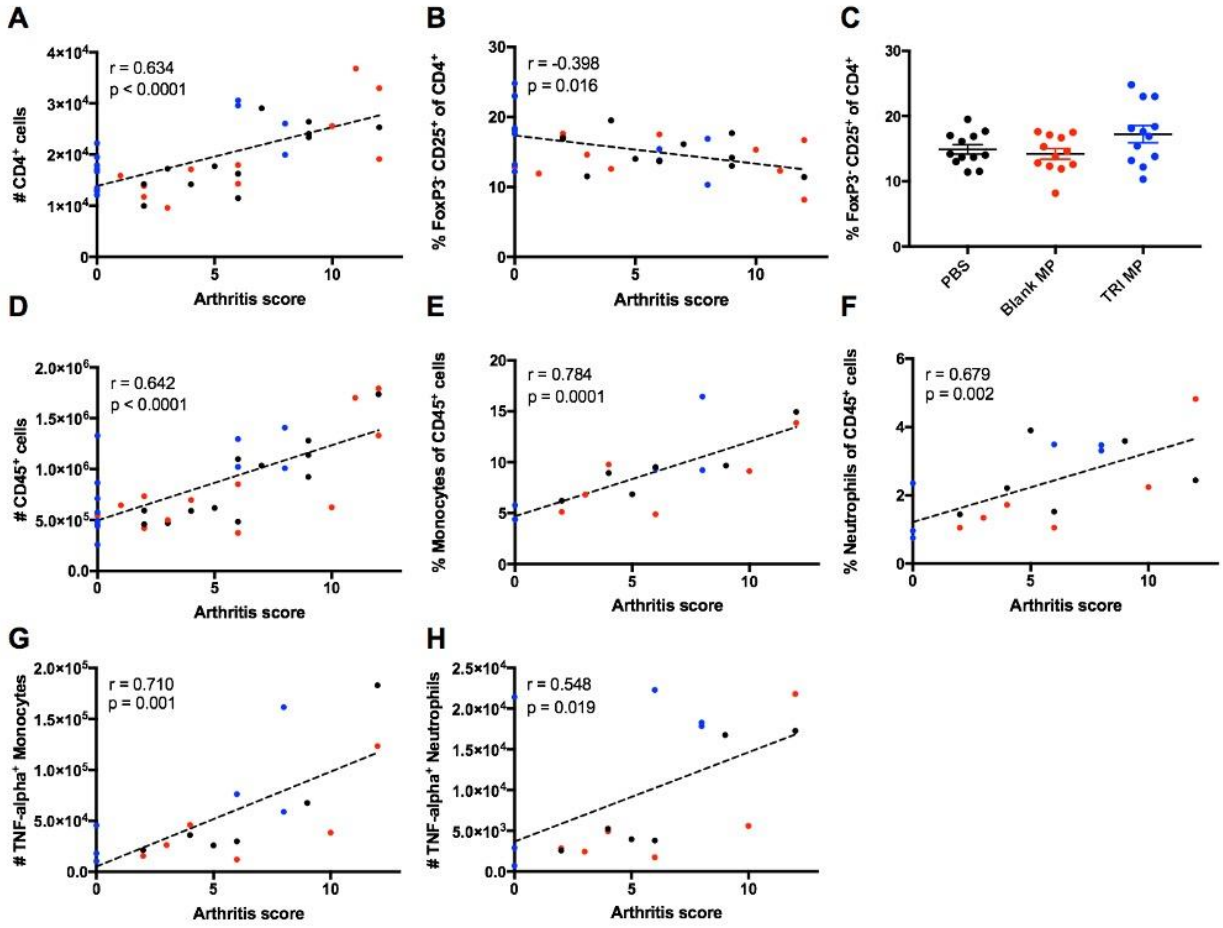

Figure 3.12 Lower arthritis score correlates with less $CD4^+$ T cells, a higher proportion of regulatory cells, and less inflammatory innate immune cells in the paws. **A,B,D-H) Indicated parameter of the paw immune infiltrate versus arthritis score (Day 40-42). Spearman correlation coefficient and p value for correlation are indicated. n = 6-12 mice per group. These include the number of $CD4^+$ T cells (A), the percentage of $CD4^+$ T cells that are $FoxP3^-$ and $CD25^+$ (B), the number of $CD45^+$ immune cells (D), the percentage of $CD45^+$ cells that are monocytes/macrophages ($CD11b^+Ly\text{-}6G^-Ly\text{-}6C^+$) (E), the percentage of $CD45^+$ cells that are neutrophils ($CD11b^+Ly\text{-}6G^+$) (F), the number of TNF-α expressing monocytes/macrophages (G), and the number of TNF-α expressing neutrophils (H). C) Percentage of $CD4^+$ T cells that are $FoxP3^-CD25^+$ by treatment group. n = 12 mice per group, data presented as mean ± SEM.**

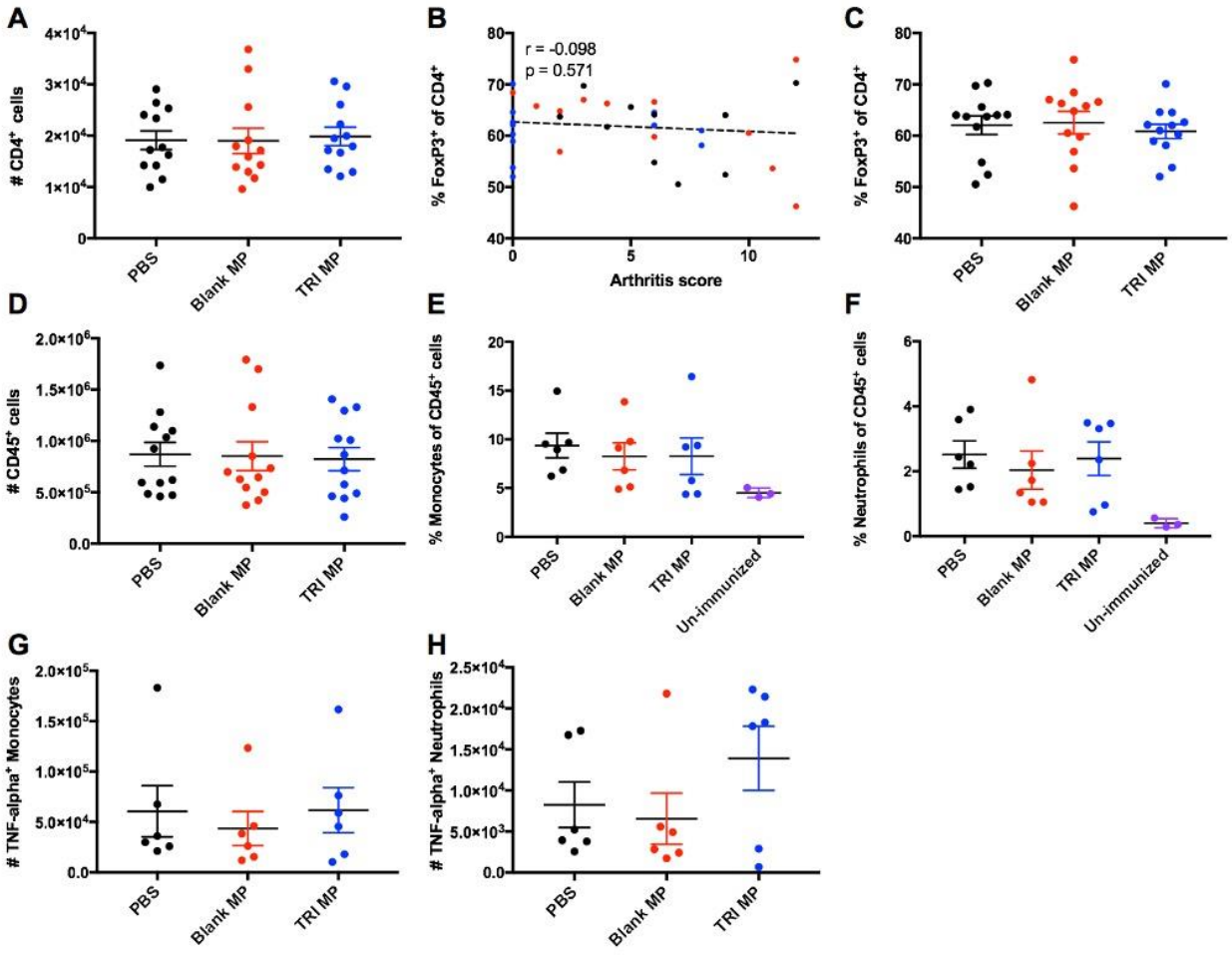

Figure 3.13 Additional analysis of paw immune infiltrate. **A,C,D-H) Indicated parameter of the paw immune infiltrate by treatment group (Day 40-42). In two of these (E and F), mice that were not immunized with bCII or treated in any other way are included as an additional control. n = 6-12 mice per group (n= 3 un-immunized), data presented as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey post-hoc analysis in order to compare the mean of every group with the mean of every other group. All comparisons were non-signficant. These include the number of $CD4^+$ T cells (A), the percentage of $CD4^+$ T cells that are $FoxP3^+$ (C), the number of $CD45^+$ immune cells (D), the percentage of $CD45^+$ cells that are monocytes/macrophages ($CD11b^+Ly\text{-}6G^-Ly\text{-}6C^+$) (E), the percentage of $CD45^+$ cells that are neutrophils ($CD11b^+Ly\text{-}6G^+$) (F), the number of TNF-α expressing monocytes/macrophages (G), and the number of TNF-α expressing neutrophils (H). B) Percentage of $CD4^+$ T cells that are $FoxP3^+$ versus arthritis score. Spearman correlation coefficient and p value for correlation are indicated. n = 12 mice per group.**

3.4 Discussion

New therapeutic approaches to RA are necessary as a large number of patients do not respond sufficiently to existing treatments. In particular, approaches aiming to maintain or restore Treg – Teff balance are of considerable interest because of the role this balance has in influencing disease progression, both in RA and the murine model of CIA. We have previously explored microparticle formulations that expand Tregs and limit Teff levels, resulting in disease prevention or therapeutic treatment in several preclinical models. Here we evaluated the ability of TRI MP to prevent the development of arthritis in the CIA model, and explored the mechanism behind disease prevention. Mice given s.c. TRI MP injections every four days between Day 0-12 following bCII immunization had significantly reduced incidence of arthritis and severity of arthritis relative to both PBS and Blank MP treated control groups (Figure 3.4). The protection provided by TRI MP not only served to block tissue swelling, but also prevented bone erosion in the digits relative to PBS but not relative to Blank MP (Figure 3.5). While this may in part reflect some protective effect of Blank MP as discussed below, the lack of significant difference between Blank MP and TRI MP in bone erosion also likely reflects some limitations of the bone erosion assessment given the robust differences observed between Blank MP and TRI MP in Figure 3.4. First although all PBS treated mice developed arthritis, only a small fraction had paws with substantial bone erosions. While this is likely a natural reflection of the fact that sufficient severity and duration of inflammation must occur to result in bone erosion, it means that differences between treatment groups will be accordingly harder to detect. Secondly, there was a relatively large degree of variability in the bone volume measurements of mice without arthritis relative to the magnitude of bone volume reduction in mice with bone erosions (Figure 3.5 B). The assessment of bone surface area to volume ratio as opposed to bone volume resulted in a stronger correlation with arthritis

score and a larger trend towards a difference between Blank MP and TRI MP (Figure 3.5 D and E). This may have been due an ability of the surface area to volume ratio to partially mitigate these limitations. For example, minor bone erosions would be expected to be disproportionally detected by surface area and the surface area to volume ratio may help reduce natural variability in the size of healthy (arthritis score of zero) joints. Taken together these factors of a high bar for detection and high variability may have contributed to the lack of significant difference observed for bone erosions between Blank MP and TRI MP based on the sample size studied. However, a substantial and significant difference was still demonstrated with TRI MP treatment compared to both PBS and Blank MP for the primary endpoints of the CIA model, arthritis incidence and arthritis score (Figure 3.4 A and B).

The first step in examining the mechanism by which TRI MP achieved these preventative effects was assessing levels of anti-CII auto-antibodies. While TRI MP significantly lowered titers of anti-CII IgG Ab relative to the PBS control, there were still similar titers in mice who were arthritis free and those who developed severe arthritis (Figure 3.6). Likewise, no correlation between titers of anti-CII IgG2a and clinical arthritis score was observed (Figure 3.7). This suggests that TRI MP was either affecting other aspects of the Ab response, such as epitope spreading, and/or affecting immune cell recruitment/expansion in the paws. The impact of TRI MP administration on the $CD4^+$ T cell population was assessed next since TRI MP has been proven to influence $CD4^+$ Treg and Teff levels in other disease models[408–410], and $CD4^+$ T cells contribute to CIA disease severity independently of helping with Ab production[456]. While TRI MP treatment did not result in increased levels of canonical $FoxP3^+$ Tregs in the draining LN or spleen at the Day 15 time point, it did result in increased levels of a $FoxP3^-CD25^+$ T cell population with elevated expression of several suppressive molecules utilized by Tregs including LAP, CTLA-4,

and CD73 (Figure 3.8). TRI MP also had an anti-proliferative effect, reducing immunization-induced expansion of LN cell numbers and the proliferation of $CD4^+$ T cells in the LN at Day 15 (Figure 3.11). To understand how these early TRI MP induced changes in the periphery protected against the development of arthritis between Days 26-40, paw immune infiltrate was analyzed at the experimental endpoint. Mice with lower arthritis scores had less $CD4^+$ T cells in the paws, but a larger percentage of cells were $FoxP3^-CD25^+$ (Figure 3.12). Additional correlations showed that lower arthritis scores were associated with reduced overall immune infiltration, reduced myeloid cell representation among the infiltrate, and less TNF-α producing myeloid cells (Figure 3.12). These findings are consistent with a mechanism in which TRI MP decreases arthritis score by limiting immunization-induced expansion of $CD4^+$ T cells directly and/or through an increased regulatory $FoxP3^-CD25^+$ population in the periphery, resulting in less $CD4^+$ T cell recruitment and/or the migration of $FoxP3^-CD25^+$ T cells to the paws, and in turn less recruitment and activation of myeloid cells to produce arthritis causing inflammatory cytokines. Although significant correlations in agreement with this mechanism were observed for Figure 3.12, when the data was presented by treatment group significant differences were not observed between TRI MP and other treatment groups (Figures 3.12 and 3.13). This may be because of a lower sample size in this experiment than the prevention study, less separation between the TRI MP and control group arthritis scores in the cohort used for this experiment, and/or because the phases of paw inflammation are dynamic with the timing of disease onset varied among mice. While significant correlations with arthritis score cannot definitely prove the order or causality of the proposed mechanism of action for TRI MP, literature on the immunological processes of CIA development supports this sequence of events[454]. While it is possible that TRI MP administration does not directly cause all of the arthritis score associated changes in paw immune infiltrate observed, if

TNF-α is directly responsible for the redness and swelling measured in arthritis scores[454,463], then at the very least TRI MP must reduce TNF-α production in order to lower arthritis scores.

Previous studies investigating TRI MP have demonstrated that the combination of all three types of MP in TRI MP was more effective than any single MP factor or any dual combination of factors[408–410], so only Blank MP and PBS alone controls were evaluated here. The Blank MP control exhibits a trend in the same direction as TRI MP in several figures, including figures where Blank MP is significantly different from PBS (Figure 3.4 A and C) or Blank MP is not significantly different from TRI MP (Figures 3.5 C, 3.6 C, and 3.8 C). These findings may be due to the immunomodulatory properties of PLGA microparticles themselves. Notably, lactic acid from PLGA MP degradation was previously shown to inhibit dendritic cell maturation, possibly by interfering with NF-κB activation[532]. Furthermore, i.v. injected PLGA NP prevented auto-immunity by causing monocytes/neutrophils that phagocytosed them to traffic to the liver and spleen instead of the site of inflammation[395]. While the average diameter of TRI MP was approximately 15-20 μm (Figure 3.1), a size likely too large to be phagocytosed by APCs, there is a relatively broad distribution of MP size with some small enough to be phagocytosed (albeit with those smaller microparticles in the distribution representing a much smaller quantity of overall % encapsulated active ingredients). These effects could be more pronounced in this model relative to past models TRI MP have been used in due to a higher dose and frequency of microparticle administration. Despite any protective effects observed with the Blank MP group, the drugs delivered by TRI MP still have a substantial and significant role in reducing arthritis incidence and severity (Figure 3.4 A and B).

While TRI MP was hypothesized to increase levels of FoxP3$^+$ Tregs in the CIA model based on experience with TRI MP in most other disease models, a previous TRI MP study also

observed increases in a population of FoxP3^{-}CD25^{+} cells similar to the one observed here and the regulatory function of this regulatory population was demonstrated through a T cell suppression assay. Specifically, in an OVA protein-specific contact hypersensitivity model, TRI MP administration led to a significant increase in the percentage of OVA-specific CD4^{+} T cells that were FoxP3^{-}CD25^{+} Tbet^{neg} but not a significant increase in the percentage of CD4^{+} T cells that were FoxP3^{+}[408]. Because this was an adoptive transfer model involving use of congenic (CD45.2) OT-II T cell clone, the percentage of transferred CD4^{+} T cells expressing FoxP3^{+} was negligible and over 90% of the (CD45.2^{+}CD4^{+}) CD25^{+} population in the draining LN of TRI MP treated mice was made up of FoxP3^{-}CD25^{+} Tbet^{neg} cells as opposed to FoxP3^{+}CD25^{+} cells[408]. Thus, when CD45.2^{+}CD4^{+}CD25^{+} T cells were sorted and shown to inhibit conventional (CD4^{+}CD25^{-}) T cell proliferation in a suppression assay at ratios as low as 1 CD25^{+} T cell : 8 conventional T cells[408], it was clear that the FoxP3^{-}CD25^{+} Tbet^{neg} population had suppressive function. Here we observed increases in a similar population with likely regulatory function that was characterized as FoxP3^{-} CD25^{+} and had elevated expression of LAP, CTLA-4, and CD73 (Figure 3.8). However, because of the much higher level of FoxP3^{+}CD25^{+} Tregs in the CIA model, the FoxP3^{-}CD25^{+} population accounts for only ~25% of the CD25^{+} population in the draining LN of TRI MP treated mice (Figure 3.8). Therefore, a suppression assay using CD25^{+} regulatory cells in the CIA model would be unlikely to be informative due to an inability to distinguish the suppressive contribution of the FoxP3^{-} in the presence of a much larger population of suppressive FoxP3^{+} cells. Although it cannot definitively be claimed that the FoxP3^{-}CD25^{+} population observed here is not an activated effector population, the increased levels of suppressive markers expressed by this population, similarity to a verified suppressive population observed using TRI MP in a different disease model, and the observations that TRI MP provided strong CIA protection while inhibiting CD4^{+} T cell

proliferation together provide strong evidence for the regulatory nature of the $FoxP3^{-}CD25^{+}$ population increased by TRI MP. The reason that an increase in $FoxP3^{+}$ expression was not observed in this model is unclear, but may have to do with the MP dose used, MP injection location, use of CFA as the priming agent, and/or the single initiating antigen in this model as opposed to previous models eliciting more polyclonal responses.

Further developing TRI MP towards clinical use for arthritis will require dose optimization to minimize any non-specific immunosuppression. The use of subcutaneous MP delivery, depending on the drug delivered and it's dose, may be able to keep delivery relatively localized to the injection site[306]. This is of particular interest when delivering immunomodulatory or immunosuppressive agents, so that the ability of the immune system to fight pathogens in other tissues is not impaired. A previous study evaluating hind limb allotransplantation found that TRI MP injected in the contralateral limb was not effective in prolonging graft survival relative to TRI MP injected in the transplanted limb, indicating that the immunomodulatory effects of TRI MP were restricted to the local area/antigens[410]. Here, we found that TRI MP administered on one limb reduced proliferation and expanded a regulatory population in the contralateral limb (Figure 3.11). The reason for this discrepancy may be the larger dose of TRI MP used in this study, and in particular, the dose of rapamycin. Unlike active TGF-β and IL-2, which have serum half-lives of only 2-4 minutes when i.v. injected[520,521], rapamycin has a serum half-life of 6 hours when i.v. injected[533] which may permit greater systemic distribution than the other TRI MP components. Although TRI MP dosing used in this study was based on a pilot using daily injections of un-encapsulated drugs and a lower TRI dose provided limited arthritis protection (Figure 3.2), it is possible that further optimization of dose and delivery kinetics to use a rapamycin dose in between that of high and low tested doses and/or lowering the rapamycin dose while increasing doses of

TGF-β and IL-2 yields a formulation capable of preventing CIA development without causing systemic immunosuppression. When TRI MP was previously shown to be more effective than a comparable dose of un-encapsulated TRI factors in a different model, both were given at the same frequency (one administration for a shorter timeline)[408]. While the pilot experiment using the higher dose of un-encapsulated TRI factors for TRI MP dose estimation led to an arthritis score of similar magnitude to TRI MP, the sample size of this group was substantially smaller (n=6 vs. n=24) and the daily delivery of un-encapsulated TRI factors partially mimicked the sustained delivery role of microparticles which were given less frequently in the later prevention study. A direct comparison of TRI MP to un-encapsulated TRI factors administered with the same frequency should be evaluated in the CIA model after future optimization of TRI factor dose. Pharmacokinetic studies will ultimately be necessary to support TRI MP translation for arthritis or other indications, however radiolabeled agents may be required given the extremely small amount of cytokines released.

In summary, this study found that TRI MP was able to significantly reduce the incidence, severity, and associated bone erosion of arthritis induced by collagen II immunization. The mechanism of this protective effect involved reduced $CD4^+$ T cell proliferation and an increased regulatory population in the periphery following TRI MP administration, and these changes were also reflected in the paws during arthritis onset and associated with reduced recruitment/expansion of TNF-α producing myeloid cells. The next steps in the development of TRI MP as a therapy for arthritis include identifying optimal dosing to prevent CIA without causing systemic immunosuppression and evaluating the ability of therapeutic TRI MP administration to reverse established arthritis for clinical relevance.

4.0 Conclusions and future work

4.1 Summary of results

In this thesis the ability of TRI MP to prevent autoimmune disease development was evaluated in models of type 1 diabetes (T1D) and collagen-induced arthritis (CIA), and although there was technically significant disease prevention in both models relative to PBS controls, TRI MP did not function as intended in either model in terms of providing substantial local expansion of $FoxP3^+$ Tregs. Although various alterations to both TRI MP and insulin peptide delivery were explored in the T1D model, the increase in insulin-specific Tregs observed in the draining lymph node following injection of TRI MP with insulin peptide was not substantial enough to affect overall Tregs or insulin-specific Tregs in the pancreatic lymph node. Furthermore, this increase in insulin-specific Tregs was not functionally meaningful as TRI MP + insulin peptide failed to provide additional T1D protection relative to that provided by insulin peptide alone in the Blank MP + insulin peptide group. While TRI MP led to a substantial reduction in both arthritis incidence and arthritis scores in the CIA model, this was associated with an increase of $FoxP3^-CD25^+$ population with likely regulatory function instead of an increase in $FoxP3^+$ Tregs and the immunosuppressive effects of TRI MP were not localized to the draining lymph node. Possible reasons for the lack of substantial $FoxP3^+$ Treg expansion can be broadly categorized as timing/location of assessment issues, delivery issues, or model issues and are discussed in detail below.

4.2 Possible reasons for the lack of substantial FoxP3$^+$ Treg expansion

4.2.1 Possible timing/location issues

It is possible that TRI MP did lead to more substantial increases in FoxP3$^+$ Tregs, however, that flow cytometry analysis was not conducted at the appropriate time point and/or tissue location to observe that change. In the T1D study it is highly improbable that TRI MP + insulin peptide (relative to Blank MP + insulin peptide) led to a substantial increase in FoxP3$^+$ Tregs outside of the locations (iLN, pLN, spleen and islets in some mice) and time points assessed (Day 10, Day 24 in one group, Week 35 in pilot prevention study), because of its inability to provide additional T1D protection beyond that of insulin peptide emulsion in either spontaneous or adoptive transfer prevention models.

In the CIA study it is more plausible that a substantial FoxP3$^+$ Treg increase occurred at a time point and/or in a tissue that was not assessed. The time points assessed included Day 15 after the first TRI MP administration (iLN and spleen), Day 35 (the iLN in pilot study), and Day 40 (paws). While the Day 15 time point was selected as an "early" time point soon after all the TRI MP injections were given, it is possible that a larger difference in Tregs between groups would be observed if a much earlier time point was chosen such as 4 days after a single TRI MP administration[408], either because of a transient peak in inflammation or because of the dispersal/trafficking of Tregs out of the draining lymph at a later time point. Immune infiltrate of the paws was assessed at Day 40 because arthritis scores were found to be relatively stable by this time point. However, it is likewise possible that Treg levels in the paws may have already peaked and subsided by this time point, especially in mice that were largely protected from paw inflammation. Detection of a Teff population (Th1 or Th17) and calculation of a Treg/Teff ratio,

or assessment of paw infiltrate at a consistent time after arthritis onset in each mouse may improve the likelihood of seeing significant differences between treatment groups. Lastly, it is worth mentioning that for some locations/time points it is possible that a significant increase in FoxP3$^+$ Tregs could be observed if the n was larger (Figures 3.2 and 3.8), but the magnitude of such changes would still be very small unlike the 1.5-2 fold increases observed in other models[408–410].

4.2.2 Possible Delivery Issues – Drug/MP Dosing

Aspects of TRI MP delivery that differed between the autoimmune models explored here and past models for which TRI MP has been evaluated that could explain discrepancies include the MP doses/drug doses/drug release kinetics and the distance between the site of inflammation and TRI MP injection location. Although there were some differences in MP formulation (polymers/fabrication parameters), MP dose administered, and reported cumulative release (particularly for TGF-β) among prior TRI MP studies models[408–410], initial experiments using TRI MP in the T1D model were within these ranges and two different MP formulations were tested. Although the final dose of TRI MP used in this model (9.6 mg TGF-β MP, 7.5 mg of IL-2 MP, and 4 mg Rapa MP per injection per side) exceeded that used in past studies, this dose was found to be slightly more effective than the original dose in terms of insulin-specific Treg expansion in the draining lymph node (especially when combined with insulin peptide delivery changes).

The use of tetramer staining gives some additional insight into how both the level of insulin-specific T cells and the percentage of insulin-specific T cells that are Tregs affect the overall Treg level. Notably, the final formulation of TRI MP + insulin peptide did achieve nearly a 2x fold increase in the percentage of insulin-specific T cells that were Tregs in the iLN (Figure 2.11). This is similar to changes in Treg percentages observed at the polyclonal level in other

models evaluating TRI MP models[408–410]. However, due to the facts that insulin-specific T cells were still less than 0.3% of $CD4^+$ T cells in the iLN after expansion, the baseline level of insulin-specific T cells in the pLN was higher than the iLN, and the baseline frequency of Tregs among insulin-specific T cells in the pLN was greater than the iLN, significant changes to insulin-specific and polyclonal Tregs levels in the pLN were not observed (Figure 2.11). Although the IL-2 component of TRI MP could have a role in promoting Treg expansion in models in which TRI MP has been effective, presumably antigen/inflammation is primarily responsible for T cell expansion and TRI MP is primarily responsible for converting activated T cells to Tregs (importantly TRI MP did not reduce insulin-specific T cell expansion in the T1D model). This suggests that a lack of sufficient Treg levels was more due to issues with antigen delivery and the expansion of insulin-specific T cells than it was the conversion of those cells to Tregs by TRI MP. All of this information on TRI MP dosing and insulin-specific Treg levels taken together suggests that it is unlikely that the TRI MP formulation/dosing differences could account for the inadequate Treg induction observed in the T1D model.

In the CIA model the MP dose used was even higher and although disease protection was provided, the dose of drug(s) and/or MP used may not have been optimal for localized Treg induction. The MP dose used in the CIA model (15 TGF-β MP, 5 mg IL-2 MP, and 15 mg Rapa MP per injection per side with more injection administration times– two for Rapa and four for cytokines) was based on a pilot study using un-encapsulated TRI factors where a corresponding dose showed potential for reducing arthritis score but a lower dose did not (Figure 3.2). Thus, the MP dosing used seemed necessary for CIA prevention at the time, and the impact on arthritis development was comparable to that of daily administration of un-encapsulated TRI factors (refer to Section 3.4 for more nuanced assessment) suggesting that the TRI drugs were releasing from

MP appropriately. Additionally, using MP dosing comparable to that of the T1D study was ineffective in the CIA model, although those batches of MP were found to have uncharacteristically low drug loading and release (data not shown). However, the finding that TRI MP was having systemic immunosuppressive effects in the CIA model (Figure 3.11) in contrast to the local effects observed in past models[408,410] suggests that TRI MP dosing was not optimal. Specifically, the dose of rapamycin was likely responsible given that it was administered at a thousand-fold higher dose and has a longer half-lifer than the cytokines (Section 3.4).

In addition to causing systemic immunosuppression, it is also possible that the relatively large amount of rapamycin in the TRI MP dose administered could be limiting the degree of Treg expansion. Although Tregs are less dependent on mTOR than Teff, they still require weak mTOR signaling[517,534]. This is consistent with the finding that while higher concentrations of rapamycin during in vitro Treg expansion helped to maintain a greater Treg purity, they also reduced the amount of Treg expansion[535]. The impact of rapamycin dose on Treg expansion in vivo has not directly been studied and higher doses of rapamycin than those used by TRI MP in the CIA model have been found to be conducive to Treg expansion in vivo [354,361,517]. However, it is possible that the ideal rapamycin dose for Treg expansion varies from model to model since the degree to which rapamycin enhances Treg levels is thought to be influenced by the strength of TCR stimulation and co-stimulation[517].

4.2.3 Possible Delivery Issues – MP injection site

In previous models that TRI MP has been evaluated in, MP were injected into the tissue that was being protected from inflammation and/or proximal to that tissue such that released TRI factors would affect the dLN for that tissue models[408–410]. Since such an injection was of

questionable feasibility in a model of T1D, TRI MP was instead injected at an irrelevant site (hind limb) with auto-antigen in order to induce/expand antigen-specific Tregs. However, this approach meant TRI MP was not near the disease-associated source of inflammation which may contribute to APC recruitment, increased antigen presentation, and/or IL-2 production needed for higher levels of Treg induction/expansion[518,536]. Additionally, an injection site away from the target tissue could reduce some of the protective effects TRI MP may have in addition to Treg induction/expansion, such as the direct suppressive effects of TGF-β and rapamycin on APCs and Teffs at the in site of inflammation and corresponding draining LN (in this case islets and pLN)[362,363,517]. While this fails to account for why there was not a higher level of FoxP3$^+$ Tregs at the dLN for the TRI MP injection site (the iLN), it may have contributed to the ineffectiveness of TRI MP in preventing disease development in the T1D model.

TRI MP was injected at a site that shared a dLN with the source of disease inducing inflammation in the CIA model, so injection site should not have been a contributing factor in terms of FoxP3$^+$ Treg levels or disease protection.

4.2.4 Possible Model Issues – Disease type and animal strain

In addition to delivery system differences, differences between diseases models in which TRI MP had been previously tested and the T1D and CIA models explored here could explain the lack of robust expansion of FoxP3$^+$ Tregs. Notable model differences include the disease type, animal strain, inflammation type, and antigen format. Unlike previous disease models that TRI MP had been tested in, both NOD T1D and CIA are models of autoimmunity. This means that T cells recognizing relevant antigens may not have as strong of a pMHC-TCR interaction on average[89], and they may be more likely to have an activated phenotype[64] (particularly for T1D

since it is a spontaneous model) both of which could theoretically impair the degree of Treg induction/expansion achieved. The success of other auto-antigen based approaches in increasing Treg levels and preventing disease development in both the spontaneous T1D and CIA models (Sections 1.2.3 and 1.4.2) suggests that this is not an insurmountable obstacle. Although it is possible that the success of Treg-based treatment in models of autoimmunity could vary depending on the degree to which their mechanism relies on Treg expansion vs. Treg induction, an experiment comparing TRI MP + auto-antigen and TRI MP + irrelevant antigen in the NOD model suggested that the use of auto-antigen was not substantially altering the ability of TRI MP to increase Treg levels (Section 2.3.4).

Similarly, the T1D and CIA models use specific mouse strains (NOD and DBA/1) that are different from mouse and rat strains previously used in models that TRI MP had been evaluated in. The NOD mouse strain in particular is known to have a variety of genetic polymorphisms that affect both APC and Treg function (Sections 1.1.3.2 and 1.1.4.2), and the ability of common DC subsets to induce and expand Tregs has been suggested to be impaired in this strain[146,147]. However, again the success of other auto-antigen based approaches in NOD mice suggests that these challenges can be overcome, particularly if an approach's mechanism of action depends on other APC subsets and/or regulatory populations[289,295]. Comparison of the effectiveness of TRI MP for Treg induction in NOD mice to B6g7 suggested that defects associated with the NOD strain were also not impairing the effectiveness of TRI MP in the T1D model (Section 2.3.4). However, the fact that immunization with a CFA/auto-antigen emulsion led to significant increase in FoxP3$^+$ Tregs in the iLN for NOD mice but not DBA/1 mice suggests that there may be differences in how amenable these strains are to Treg induction (i.e. a relatively greater predisposition towards Th17

or Tfh induction, either in general or in response to CFA) or that bCII induces a weaker response than insulin peptide).

4.2.5 Possible Model Issues – Inflammation type and antigen format

Other differences between the models that TRI MP had been previously evaluated in and the models tested here include the type of inflammation used and the antigen format. Previous models evaluating TRI MP used a hapten, Concanavalin A (Con A), or transplantation to induce inflammation[408–410]. In the CIA model CFA was used for this purpose and while NOD mice spontaneously develop T1D, CFA was also used to deliver antigen in some experiments with the aim of inducing local inflammation to elicit a more robust Treg response. Theoretically, a failure for CFA to enable TRI MP to induce/expand Tregs could either be due to CFA inducing too strong of an inflammatory response or too weak of an inflammatory response. CFA could be inducing too strong of an inflammatory response if the signal it provided for Th1 (or Th17) Teff induction was too strong for TRI MP to overcome and skew naïve T cell differentiation towards a Treg phenotype. However, the lack of significantly different Treg levels when administering insulin peptide in CFA compared to IFA or PBS (Figure 2.9) as well as a lack of a substantial Tbet^{+} population observed in the CIA model (Figure 3.10) or in a T1D-related experiment in B6g7 mice (data not shown) suggest that too strong inflammation from CFA was not a problem. Since greater DC levels enables greater Treg levels and Tregs depend on IL-2 produced by Teff[536,537], it is also possible that CFA did not elicit a strong enough initial inflammatory response with enough APC recruitment and/or IL-2 to support robust Treg induction/expansion greater than that induced by antigen alone. This is particularly true of the T1D model where CFA is protective against disease development and IFA was used in the final TRI MP formulation, but for the CIA model CFA

would be expected to induce a sufficient inflammatory response compatible with Treg expansion since it is capable of inducing disease.

The antigen format refers to the reliance on endogenous vs. delivered antigen, whether an immune response was induced to a single epitope or a multiple epitopes/antigens, how an antigen was delivered (route/delivery system), and what form it was delivered in (protein, peptide, modified, etc.) Past models using TRI MP have not given antigen in addition to the source of inflammation and have used models presumably involving T cells of many antigen specificities [408–410]. Although a single auto-antigen was exogenously administered in the T1D model, Tregs of a single antigen specificity and regulatory cell-based approaches using a single antigen (in some cases insulin or insulin peptide) have had success in this model (Table 1-1). However, many of these approaches using MP or NP either administered auto-antigen (with or without immunomodulatory agents) through routes with greater systemic exposure (ex: i.v. or i.p.) or used GM-CSF to enhance DC recruitment if using s.c. administration (Table 1-1). Therefore, the combination of administering TRI MP with a single exogenous antigen and a lack of sufficient interaction between DCs presenting that antigen and antigen-specific T cells due to the site and route of administration could plausibly explain the ineffectiveness of TRI MP in T1D in light of both its success in other models and the success of similar approaches in the NOD model of T1D.

In the CIA model, the auto-antigen used in attempt to provide auto-antigen specificity was the CII used for disease induction. While approaches administering CII have been effective at preventing arthritis in the CIA model (Section 1.4.2), in these instances CII was given under tolerogenic conditions (orally) separately from the CII given with CFA for disease induction. Although such an approach could also be explored with TRI MP by giving CII with TRI MP at a

distant site from the CII/CFA emulsion, it would be analogous to that used in the T1D model for which TRI MP had no added benefit.

4.3 Conclusions

In a model of T1D TRI MP increased levels of insulin-specific Tregs in the draining lymph node when co-administered with insulin peptide, however these changes were not substantial enough to effect Treg levels in the pLN or to have added benefit beyond that of insulin-peptide alone in providing T1D protection. The most plausible explanation for these findings is the combination of administering TRI MP at a distant site from inflammation (thus potentially losing some of the direct suppressive effects of TRI MP) and the lack of sufficient exposure of delivered agents to insulin-specific T cells due to the site and route of administration limiting the expansion of insulin-specific Tregs. In the CIA model of arthritis TRI MP significantly reduced the incidence of arthritis and arthritis severity. While this disease protection was associated with reduced T cell proliferation and increased levels of a likely regulatory population, an increase in $FoxP3^+$ Tregs was not observed and evidence of systemic immunosuppression was found. The most likely explanation for these findings is that the dose of the TRI MP factors used in this model was not optimal with too high a dose of rapamycin. Collectively this data suggests that TRI MP will have the greatest utility for models in which auto-antigen alone is not protective and that TRI MP dosing (including the relative ratios of each of the TRI factors) may need to be fine-tuned in each model to ensure increases in Tregs (or the Treg/Teff ratio) without systemic immunosuppression.

4.4 Future work

4.4.1 Changes to the delivery system or drug cargo of TRI MP

4.4.1.1 MP or NP delivery system changes

TRI MP was sufficiently effective at providing sustained release of encapsulated drug as discussed in Section 4.2.2. However, an ideal drug delivery for the TRI factors would have greater drug loading, a more linear release profile for drug release, and sustained release of large drug levels to the avoid the need for frequent administration. One method of improving TRI MP towards these goals would be to fabricate TRI MP using microfluidic chips instead of a sonication and homogenization based approach[538,539]. Microfluidic fabrication is capable of achieving a more homogenous MP size distribution, which in turn is thought to lead to greater drug loading and more linear drug release[538]. However, even with such an approach, maximum cytokine drug loading would likely remain on the order of ~100 ng per mg MP (i.e. 10% by mass). A recent approach of creating protein nanogels out of IL-2/Fc fusion protein and reversible cross-linkers has the potentially for substantially higher drug loading[540]. However, this approach would also need to be adapted for TGF-β, and because these nanogels were designed to be coupled to i.v. administered cells, the persistence and release kinetics after s.c. administration would need to be studied. Alterations to the nanogel size and cross-linker type may be able to influence these properties if needed. An osmotic minipump could also be used for the delivery of TRI factors. Advantages of this approach are its proven track record, the ability to provide sustained linear release of relatively high (compared to MP) doses of TRI factors, and the ability to use a catheter to direct drug delivery to the organ/region of interest. A notable disadvantage is that an osmotic

minipump is a device which must be implanted as well as removed unlike biodegradable MP or NP systems.

4.4.1.2 TRI factor drug changes

The effect of TRI MP on Treg levels is thought to be due primarily to the induction and expansion of pTregs as opposed to through the expansion of tTregs. It is possible that the reliance of TRI MP in pTregs is a limitation given that these cells are relatively slow to develop demethylation at the FoxP3 conserved non-coding sequence 2 (CNS2) locus and other loci that are important for Treg stability[345]. The inflammatory cytokine milieu found at tissues under autoimmune attack is particularly challenging for Treg stability[236,240,541], and may be difficult for newly generated pTreg to overcome. Therefore, modification to the drugs delivered by TRI MP in order to promote tTreg expansion instead of pTreg induction may result in greater effectiveness especially in the context of autoimmunity.

An important first step would be to verify that TRI MP is primarily acting through pTregs instead of tTregs. This would require using a model (as well as also ideally having a FoxP3-EGFP mouse strain) in which TRI MP had a substantial impact on the Treg population such that a sufficient population of Tregs could be isolated and the majority of these Tregs would be generated/derived as a result of TRI MP administration (ex: the hapten model of contact dermatitis[408]). Tregs from TRI MP and Blank MP treated mice (as well as additional controls such as *in vitro* generated iTregs) would have FoxP3 CNS2 demethylation assessed by bisulfite sequencing in order to give a relative measure of tTreg vs. pTreg frequency and Treg stability.

If TRI MP was associated with pTreg induction and reduced Treg stability, then several approaches could be taken to address this limitation. First, the time point used for assessing CNS2 demethylation could be re-evaluated as a later time point (ex: 2-3 weeks as opposed to 4 days)

would likely result in greater FoxP3 demethylation for any pTregs. It is also possible that the presence of the tolerogenic environment provided by TRI MP gives sufficient time for pTregs to become stable even in the face of inflammatory cytokines. This could be explored by treating mice with TRI MP (in an inflammatory model) for different lengths of time, using FACS to sort the resulting Treg population, and then assessing Treg stability (maintenance of FoxP3 expression) when re-stimulated *in vitro* in the presence and absence of inflammatory cytokines.

One way to harness tTreg instead of pTregs would be through Treg recruitment instead of Treg induction. Our group has also previously developed MP which release the chemokine CCL22 MP for Treg recruitment and shown the ability of these CCL22 MP to increase Treg levels and prevent disease development in several preclinical models[542–545]. This approach is presumably dependent on MP injection at the site of inflammation, and so while it would be a consideration in the CIA model (especially since TRI MP was unable to increase $FoxP3^+$ Treg levels), it would not be a viable option in the T1D model. It is also important to consider that Tregs recruited by CCL22 would not be exclusively antigen-specific and thus presumably not as potent as those generated by TRI MP. However, it is possible that a non-specific Treg population recruited by CCL22 could result in a relevant (auto-antigen) antigen-specific population via infectious tolerance.

Instead of replacing the TRI MP system, individual drugs could be replaced to favor tTreg expansion over pTreg induction. Both IL-2 and rapamycin would be expected to be conducive to tTreg expansion, so the exclusion of TGF-β would theoretically have more of a tTreg expansion effect than a pTreg induction effect. However, past models evaluating TRI MP have shown it to be more effective than any of the dual combinations of factors including IL-2 and rapamycin[408–410]. This could either be due to the role of TGF-β in Treg induction and/or other immunosuppressive effects on APCs and Teffs. However, it remains possible that replacing TGF-

β (or an alternative TRI MP factor) with a different immunomodulatory agent could result in improved results and more of a tTreg response. One option would be to replace TGF-β with an histone deacetylase inhibitor (HDACi). These HDACi have been shown to increase Treg levels and function through a variety of mechanisms. For example, HDACi can enhance Treg suppressive function by promoting FoxP3 acetylation which improves it DNA binding ability and by reducing the expression of inflammatory cytokines and co-stimulation by APCs via increased STAT3 acetylation[546–549]. Our group has also shown that the MP delivery of the HDACi SAHA (N-hydroxy-N0-phenyl-octanediamide, suberoylanilide hydroxamic acid) was able to increase FoxP3 expression in a model of dry eye disease[550].

4.4.2 T1D model

4.4.2.1 Delivery of TRI factors to the pancreas with an osmotic minipump

A promising approach to continue the T1D project would be using a delivery system that localizes the TRI factors by the pancreas for localized immunomodulation. Such an approach would avoid issues with the delivery of auto-antigen and degree of corresponding T cell expansion by relying on endogenous antigen presentation, avoid having to parse the protective effects of TRI MP from those of auto-antigen alone, and deliver TRI factors in a manner more similar to how TRI MP has been used in past models with a greater likelihood of benefiting from presumed direct effects of TRI factors on Teff and APCs in the pLN/pancreas. An osmotic minipump and catheter have been previously used for local delivery to the pancreas[551], so this approach could be used to deliver a TRI solution (2-20 ng/day TGF-β, 2-20 ng/day IL-2, and 0.5-2.5 μg/day rapamycin). An important first step would be to do a dosing study to ensure that local TRI delivery is not causing islet toxicity or having systemic immunosuppressive effects. This would involve implanting mice

with osmotic minipumps delivering PBS only, TRI dose 1 (ex: 20 ng/day TGF-β and IL-2, 2.5 μg/day rapamycin), or TRI dose 2 (ex: 20 ng/day TGF-β and IL-2, 0.5 μg/day rapamycin) for 2 weeks and immunizing all mice s.c. by the hind limb with HEL protein in CFA at 1 week after pump implantation with mice sacrificed at 3 weeks. Islet toxicity would be assessed by checking blood glucose of all mice twice a week, by performing a glucose tolerance test at the end of 3 weeks, and by performing histology with TUNEL staining on pancreatic tissue from sacrificed mice[525,552,553]. A daily i.p. injection of 50 μg/day (~ 2.5 mg/kg) rapamycin could also evaluated as a positive control. The macroscopic appearance of the liver would also be checked in all animals due to the observation of liver damage with i.p. TRI MP administration, and histological assessment by a pathologist would be explored if needed. Systemic immunosuppression would be assessed by comparing the levels of HEL tetramer$^+$ and Ki67$^+$ CD4 T cells in immunized mice treated with PBS versus those treated with TRI factors. A HEL re-stimulation assay to assess IFN-γ production through ELISA could also be performed.

Due to the pancreas-gut axis[162], TRI factors delivered locally to the pancreas could also have immunosuppressive effects on the gut without causing systemic immunosuppression. It is unclear if such an effect would be harmful, neutral, or beneficial. The gut already is a relatively tolerogenic site at baseline[366], so it is possible that TRI factor delivery to the gut would either have no effect or help to support this baseline phenotype. However, it is also theoretically possible that the presence of TRI factors in the gut could inhibit or alter the timing of microbial antigen encounter that is important for proper regulatory cell development[24]. A first step towards assessing the impact of osmotic pump – TRI treatment on the gut would be to compare Treg levels in the mesenteric LN and lamina propria in treated mice to control (osmotic pump – PBS treated) mice. If Treg levels in the gut are abnormal following osmotic pump – TRI treatment, then the functional

relevance of these effects could be explored with a dextran sulfate sodium (DSS) induced colitis model. Specifically, DSS induced colitis severity (weight loss, histology score, and colon length) in mice previously given osmotic pump – TRI treatment could be compared to colitis severity in mice previously given osmotic pump – PBS treatment.

Once a TRI dose is selected, the level of Tregs and the ratio of $FoxP3^+$ Tregs to $Tbet^+$ Teff in the pLN and pancreas 3 weeks after treatment would be assessed in osmotic pump – PBS and osmotic pump – TRI treated mice. T1D prevention and reversal studies could then be performed. For the reversal study mice would be considered diabetic upon 2 consecutive blood glucose readings greater than 300 mg/dL and after temporary restoration of blood glucose to 300 mg/dL with daily insulin administration[294], mice would be implanted with osmotic pumps for PBS or TRI treatment. Blood glucose would be monitored twice-weekly for 2 months and reversal assessed as stable blood glucose below 200 mg/dL. In all prevention and reversal studies, osmotic pumps would need to be explanted after 3 weeks and additional controls (single and/or dual TRI factors) could be tested if the osmotic pump – TRI treatment was successful.

If osmotic pump delivery of TRI factors is successful in the T1D prevention model but not in the T1D reversal model, then temporary systemic immunosuppression could be used for induction therapy, analogous to how a calcineurin inhibitor (FK506) and anti-lymphocyte serum (ALS) were used in conjunction with TRI MP in a transplantation model[410]. Nearly all approaches that have successfully induced remission (or prolonged survival) in diabetic NOD mice were initiated within 2 weeks of the diagnosis of T1D onset[379], suggesting that there may not be sufficient β cell mass remaining after this point. It is expected that the temporary local immunosuppressive effects of the TRI factors are sufficient to prevent further β cell destruction even before the treatment can induce/expand the Tregs needed for longer term protection.

However, induction systemic immunosuppression could be beneficial in this regard by further protecting remaining β cell mass from inflammatory immune cells until the Treg-based protective effects of TRI factor delivery have time to develop.

4.4.2.2 Insulin peptide dose modifications and alternative/additional auto-antigens

Other approaches could also be explored with the goal increasing the interaction between auto-antigen and the corresponding auto-reactive T cells. As discussed in Section 2.4, adding GM-CSF to the TRI MP or trying to re-formulate TRI MP as i.v. or i.p. administered particles would not be ideal from a publication or translation perspective. Alternatively, the use of a greater dose of insulin peptide or alternative/additional auto-antigens could be evaluated. The 100 μg of insulin peptide (or insulin peptide mimotope) was chosen because other groups delivering insulin peptide or mimotopes for T1D prevention (including in IFA, osmotic mini-pumps, or in MP) have consistently used this dose or less for T1D prevention [354,371,374,396]. It is possible that a larger dose of insulin peptide would enable the induction/expansion of more insulin-specific Tregs (such that there was a greater increase in polyclonal Treg levels even if the percentage of Tregs among insulin-specific T cells remained unchanged from the current data). However it is also possible that the level of insulin peptide used is already in excess for the number of insulin-specific T cells circulating through the iLN or that a higher dose of insulin peptide without TRI MP would further increase the percentage of mice that remain T1D-free, thereby further shrinking the opportunity to see additional benefit with TRI MP.

Alternative/additional auto-antigens such as BDC2.5 mimotope, preproinsulin, or β cell lysate could also be considered in an effort to promote a greater expansion of auto-reactive T cells that could be converted to Tregs by TRI MP. Specifically, the ability of these auto-antigens

(administered in PBS or IFA) co-administered with TRI MP to increase Treg levels in the iLN and pLN as well as suppress spontaneous and adoptive transfer models of T1D could be assessed. The use of a larger number of auto-antigen epitopes would theoretically be a great way to overcome the observed limited expansion of insulin-specific T cells. Islet-specific T cells of multiple antigen-specificities could be converted to Tregs, reducing the dependence on the magnitude of expansion for any one antigen specificity. In addition to being able to generate a larger number of islet-specific Tregs, the use of multiple antigens would also generate a more diverse Treg population that may be more effective in suppressing diabetogenic T cells. β cell lysate would be particularly promising in this regard due to its ability to replicate the diversity of auto-antigens found in β cells as well as the format (i.e. PTMs) and the relative doses of different auto-antigens. However there would also be several potential drawbacks to using alternative/additional auto-antigens. First, using multiple auto-antigens would compromise the ability to use tetramer staining to track antigen-specific populations and potentially the ability to distinguish and characterize Tregs generated as a result of treatment from pre-existing Tregs. This would be a relatively minor sacrifice if it led to improved disease outcomes and it could also be overcome by using combining insulin peptide with a select few additional epitopes, such as BDC2.5. Second, the quality and dose of auto-antigen epitopes used matters in addition to the quantity of epitopes used. Ins B:9-23 was selected as the auto-antigen for the studies performed in Chapter 2 because it is thought to be the key initiating antigen in the NOD model[104], and although the expansion of insulin-specific cells was insufficient to achieve the expected outcomes when combined with TRI MP, Ins B:9-23 elicits a relatively strong proliferative response compared to an equimolar dose of full length insulin protein despite the latter containing more auto-reactive epitopes [73]. Thus, not all epitopes are equal and the size of the responding population and the average affinity for cognate antigen will likely

be better for some antigens than others based on central and peripheral tolerance differences. Using a source of auto-antigen with greater epitopes/diversity (i.e. lysate) will providing more different types of antigens will presumably restrict the dose and presentation of the most potent epitopes. Ultimately experimentation would be needed to determine if the potential advantages to using a diverse antigen source such as β cell lysate, the larger pool of antigen-specific precursors and more diverse Treg population, outweigh these drawbacks.

4.4.2.3 Mechanistic exploration of the regulatory cell based protective effects of insulin peptide

Additional experiments could be performed to better understand the regulatory cell based protective effects of Blank MP + insulin peptide/IFA. Specifically, the adoptive transfer experiment using iLN lymphocytes from Blank MP + insulin peptide/IFA treated mice (Section 2.2.7) could be repeated in which 5 million total lymphocytes, the number of FoxP3-GFP$^+$ Tregs corresponding to 5 million lymphocytes, or an equal dose of Tregs from untreated mice could all be adoptively transferred into NOD.scid recipients. Comparison of these first two groups would indicate if the protective effects were occurring through FoxP3$^+$ Tregs. If the effect was not through Tregs, then other populations such Th2 cells, Tr1 cells, or CD25$^+$ cells could be explored. If the effect was through Tregs, then a comparison between the second and third groups would indicate whether the protective effects were due to a boost in Treg numbers or an increased function (or increased frequency of insulin-specific Tregs among total Tregs). In the latter case a flow panel to assess functional and activation markers (ex: CD44, CD25, LAP, CD39, CD73, KLRG1, ICOS, PD-1, NRP1, CTLA-4, Tbet, Helios, and IL-10) for insulin tetramer$^+$ and polyclonal Tregs could be evaluated in Blank MP + IFA/insulin peptide treated and untreated mice.

4.4.3 CIA model

4.4.3.1 TRI MP dose-finding

The next step for the CIA project would be to do a dose-finding study to attempt to identify a dose of TRI MP that is capable in reducing the incidence and severity of CIA in a preventative model (and also ideally expanding FoxP3$^+$ Tregs) without causing systemic immunosuppression. Initial experiments would focus on the systemic immunosuppression aspect first (conducted as described in Section 3.2.7) since this is a shorter experimental timeline and has less variability than the prevention study arthritis scores, thus requiring fewer mice per group. The first new dose combinations to be tested would reduce the amount of Rapa MP (i.e. from 15 mg to 5 mg or 1.5 mg per injection) since the hypothesis is that rapamycin more than the cytokines is responsible for systemic immunosuppression due to its higher dose and longer half-life. Once one or more candidate TRI MP doses that do not cause systemic immunosuppression are identified, they would be tested for their ability to prevent CIA induction (as described in Section 3.2.3). If one of these new TRI MP doses is successful, additional controls including the single factor MP controls (i.e. TGF-β MP only, Rapa MP only, or IL-2 MP only) as well as equivalent doses of un-encapsulated TRI administered at the same injection frequency as TRI MP would also be tested. Experiments assessing the level of Tregs and Teff in the iLN and the paws would also be re-assessed (as described in Sections 3.2.6 and 3.2.8) under this new dosing regimen with staining for RORγT to assess Th17 Teff.

4.4.3.2 CIA adoptive transfer model to assess regulatory population contribution

If the above studies are successful, then the next steps would be to assess the relative contribution of localized inhibition of T cell proliferation and regulatory cell expansion to the

protective effects of TRI MP and to evaluate therapeutic TRI MP administration in established CIA. Although reductions in T cell proliferation and an increase in a $FoxP3^{-}CD25^{+}$ were observed with TRI MP treatment (Sections 3.3.4 and 3.3.5) the relative contributions of each of these to the observed protective effects remains unclear. The role of regulatory cells in the protective effects of TRI MP would be assessed by adoptive transfer similar to what was performed in the T1D model (as described in Section 2.2.7). Specifically, mice immunized with CFA/bCII and treated with either Blank MP or TRI MP would be sacrificed at Day 15 to be used as cell donors. The iLN would be removed and then 3 million cells from these MP treated mice (or unimmunized control mice) would be i.v. transferred into recipient mice on Day 0 at the same time as bCII immunization. These recipient mice would then receive a second bCII immunization and be monitored for arthritis development as previously described (Section 3.2.3). If the incidence and/or severity of CIA was significantly reduced in mice receiving cells from TRI MP treated mice, then it would suggest that at least part of the protective effects of TRI MP were through effects on the size or potency of a regulatory population. If DBA/1J FoxP3-EGFP mice could be obtained, then FACS could be used to isolate the $FoxP3^{-}CD25^{+}$ population (from both TRI MP treated mice and controls) to assess its suppressive function *in vitro* and *in vivo.* Treg suppression assays could be used to compare the potency of $FoxP3^{-}CD25^{+}$ cells to $FoxP3^{+}CD25^{+}$ cells as described in Section 2.2.5. Additionally, a sorted $FoxP3^{-}CD25^{+}$ population or a $FoxP3^{-}CD25^{+}$ depleted population from TRI MP or Blank MP treated mice could be used in the adoptive transfer CIA model described above.

4.4.3.3 Therapeutic administration of TRI MP in model of established CIA

Although TRI MP had a substantial impact on arthritis incidence and severity in a preventative model (Section 3.3.1), a more clinically relevant model would be a therapeutic model in which TRI MP is administered after the onset of arthritis. This could be performed by waiting

until Day 30 after the initial immunization to treat mice with MP, at which point stratified randomization based on arthritis clinical score would be used to sort mice into treatment groups. Mice would then be monitored out to Day 60 for changes to arthritis score and bone erosion would be assessed as previously described (Section 3.2.4) at the study end point. In addition to MP controls, proven treatment approaches such as anti-TNF-α or anti- IL-1β could be used as a benchmark to which TRI MP could be compared[554,555].

4.4.4 Testing TRI MP with human cells and humanized mice

If above approaches using TRI MP (or an alternative delivery system for TRI factors) discussed above are successful, then next steps towards the translation of TRI MP for the treatment of T1D and RA would be to evaluate the ability of TRI MP to expand relevant antigen-specific human regulatory cell populations. TRI MP could be made as described for pre-clinical models, but human IL-2 would be used instead of murine IL-2 (human TGF-β is already used for TRI MP tested in mice). A first step towards this would be stimulating PBMCs from patients with auto-antigen in the presence or absence of TRI MP (and controls). For T1D, PBMCs from HLA-DQ8+ would be stimulated with insulin peptide for 14 days and then stained with insulin tetramer (DQ8/B:10-23)[110,112] as well as CD3, CD4, CD25, CD127, and FoxP3 to assess insulin-specific Treg levels. Additional staining for Teff transcription factors (Tbet and RORγT and inflammatory cytokines (ex: IFN-γ, IL-17, IL-6, and TNF-α) could also be performed. Since TRI MP would be expected to be given as an intra-articular injection without exogenous antigen for the treatment of RA, an *in vitro* experiment using PBMCs from SE allele+ (HLA-DR4) patients would stimulate T cells with a mixture of citrullinated proteins (i.e. collagen II, aggrecan, vimentin, and fibrinogen[556].

The same markers described would be assessed for Treg and Teff levels, but at a polyclonal level instead of via tetramer staining. Notably, if work in NOD mice suggested that either exogenous antigen with T1D was not necessary (i.e. osmotic minipump delivery of TRI factors) or that multiple auto-antigens were necessary (i.e. TRI MP with β cell lysate), then a mixed auto-antigen stimulation and polyclonal staining approach without tetramer could be used for T1D patient PBMCs as well.

If TRI MP was able to increase auto-antigen-specific Treg levels and/or reduce Teff and inflammatory cytokine levels in PBMCs from T1D and/or RA patients *in vitro*, then the next step would be to evaluate the ability of TRI MP to replicate this effect *in vivo* using humanized mouse models. Specifically, NOD.scid Il2rg$^{-/-}$ (NSG) mice transgenic for human HLA (DQ8 for T1D or DR4 for RA) would be re-constituted using peripheral blood from HLA matched patients (and patient synovial tissue in the case of RA)[295,385,557]. The ability of TRI MP to increase the levels of Tregs in the pLN (T1D) or the dLN of the implanted synovial tissue and TRI MP injection site (RA) would then be assessed.

Bibliography

[1] Eisenbarth, G. S. Type I diabetes mellitus. A chronic autoimmune disease. *New England Journal of Medicine* vol. 314 1360–1368 (1986).

[2] Katsarou, A. *et al.* Type 1 diabetes mellitus. *Nat. Rev. Dis. Prim.* **3**, 1–17 (2017).

[3] CDC. *Centers for Disease Control and Prevention. National Diabetes Statistics Report, 2020. Atlanta, GA: Centers for Disease Control and Prevention, U.S. Dept of Health and Human Services*. (2020).

[4] Imperatore, G. *et al.* Projections of type 1 and type 2 diabetes burden in the U.S. population aged <20 years through 2050: Dynamic modeling of incidence, mortality, and population growth. *Diabetes Care* **35**, 2515–2520 (2012).

[5] Gerstein, H. C. Diabetes: Dysglycaemia as a cause of cardiovascular outcomes. *Nature Reviews Endocrinology* vol. 11 508–510 (2015).

[6] Hsieh, A. & Twigg, S. M. The enigma of the dead-in-bed syndrome: Challenges in predicting and preventing this devastating complication of type 1 diabetes. *Journal of Diabetes and its Complications* vol. 28 585–587 (2014).

[7] Steffes, M. W., Sibley, S., Jackson, M. & Thomas, W. β-cell function and the development of diabetes-related complications in the diabetes control and complications trial. *Diabetes Care* **26**, 832–836 (2003).

[8] Weinstock, R. S. *et al.* Severe hypoglycemia and diabetic ketoacidosis in adults with type 1 diabetes: Results from the T1D exchange clinic registry. *J. Clin. Endocrinol. Metab.* **98**, 3411–3419 (2013).

[9] Anderson, M. S. & Bluestone, J. A. THE NOD MOUSE: A Model of Immune Dysregulation. *Annu. Rev. Immunol* **23**, 447–85 (2005).

[10] Hinman, R. M. & Cambier, J. C. Role of B Lymphocytes in the Pathogenesis of Type 1 Diabetes. *Current Diabetes Reports* vol. 14 1–7 (2014).

[11] Wong, F. S. *et al.* Investigation of the role of B-cells in type 1 diabetes in the NOD mouse. *Diabetes* **53**, 2581–2587 (2004).

[12] Pugliese, A. Autoreactive T cells in type 1 diabetes. *Journal of Clinical Investigation* vol. 127 2881–2891 (2017).

[13] Marré, M. L. & Piganelli, J. D. Environmental factors contribute to β cell endoplasmic reticulum stress and neo-antigen formation in type 1 diabetes. *Front. Endocrinol. (Lausanne).* **8**, 262 (2017).

[14] Thayer, T. C., Wilson, S. B. & Mathews, C. E. Use of nonobese diabetic mice to understand human type 1 diabetes. *Endocrinology and Metabolism Clinics of North America* vol. 39 541–561 (2010).

[15] Mathis, D., Vence, L. & Benoist, C. β-cell death during progression to diabetes. *Nature* vol. 414 792–798 (2001).

[16] Padgett, L. E., Broniowska, K. A., Hansen, P. A., Corbett, J. A. & Tse, H. M. The role of reactive oxygen species and proinflammatory cytokines in type 1 diabetes pathogenesis. *Ann. N. Y. Acad. Sci.* **1281**, 16–35 (2013).

[17] Concannon P, Rich SS, N. G. Genetics of type 1A diabetes. *New England Journal of Medicine* vol. 360 1646–54 (2009).

[18] Pociot, F. & Lernmark, Å. Genetic risk factors for type 1 diabetes. *The Lancet* vol. 387 2331–2339 (2016).

[19] Noble, J. A. & Valdes, A. M. Genetics of the HLA region in the prediction of type 1 diabetes. *Curr. Diab. Rep.* **11**, 533–542 (2011).

[20] Herold, K. C., Vignali, D. A. A., Cooke, A. & Bluestone, J. A. Type 1 diabetes: Translating mechanistic observations into effective clinical outcomes. *Nature Reviews Immunology* vol. 13 243–256 (2013).

[21] Fujinami, R. S., Von Herrath, M. G., Christen, U. & Whitton, J. L. Molecular mimicry, bystander activation, or viral persistence: Infections and autoimmune disease. *Clinical Microbiology Reviews* vol. 19 80–94 (2006).

[22] Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* **81**, 1–5 (2007).

[23] Bach, J. F. & Chatenoud, L. The hygiene hypothesis: An explanation for the increased frequency of insulin-dependent diabetes. *Cold Spring Harb. Perspect. Med.* **2**, (2012).

[24] Knoop, K. A. *et al.* Microbial antigen encounter during a preweaning interval is critical for tolerance to gut bacteria. *Sci. Immunol.* **2**, (2017).

[25] Zhou, H. *et al.* Evaluating the Causal Role of Gut Microbiota in Type 1 Diabetes and Its Possible Pathogenic Mechanisms. *Frontiers in Endocrinology* vol. 11 125 (2020).

[26] Kikutani, H. & Makino, S. The murine autoimmune diabetes model: NOD and related strains. *Adv. Immunol.* **51**, 285–322 (1992).

[27] Atkinson, M. A. Evaluating preclinical efficacy. *Science Translational Medicine* vol. 3 96cm22-96cm22 (2011).

[28] Motta, V. N. *et al.* Identification of the Inflammasome Nlrp1b as the Candidate Gene Conferring Diabetes Risk at the Idd4.1 Locus in the Nonobese Diabetic Mouse . *J. Immunol.*

194, 5663–5673 (2015).

[29] Shultz, L. D. *et al.* Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180–91 (1995).

[30] Dobbs, C. M. & Haskins, K. Comparison of a T Cell Clone and of T Cells from a TCR Transgenic Mouse: TCR Transgenic T Cells Specific for Self-Antigen Are Atypical. *J. Immunol.* **166**, 2495–2504 (2001).

[31] Delong, T. *et al.* Diabetogenic T-cell clones recognize an altered peptide of chromogranin A. *Diabetes* **61**, 3239–3246 (2012).

[32] Chan, A. Y. & Anderson, M. S. Central tolerance to self revealed by the autoimmune regulator. *Ann. N. Y. Acad. Sci.* **1356**, 80–89 (2015).

[33] Josefowicz, S. Z., Lu, L.-F. & Rudensky, A. Y. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu. Rev. Immunol.* **30**, 531–564 (2012).

[34] Mueller, D. L. Mechanisms maintaining peripheral tolerance. *Nature Immunology* vol. 11 21–27 (2010).

[35] Hamilton-Williams, E. E., Bergot, A. S., Reeves, P. L. S. & Steptoe, R. J. Maintenance of peripheral tolerance to islet antigens. *Journal of Autoimmunity* vol. 72 118–125 (2016).

[36] Martinov, T. & Fife, B. T. Type 1 diabetes pathogenesis and the role of inhibitory receptors in islet tolerance. *Annals of the New York Academy of Sciences* 73–103 (2019) doi:10.1111/nyas.14106.

[37] Santegoets, S. J. A. M. *et al.* Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunol. Immunother.* **64**, 1271–1286 (2015).

[38] Roncarolo, M. G., Gregori, S., Bacchetta, R., Battaglia, M. & Gagliani, N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. *Immunity* vol. 49 1004–1019 (2018).

[39] Collison, L. W. *et al.* IL-35-mediated induction of a potent regulatory T cell population. *Nat. Immunol.* **11**, 1093–1101 (2010).

[40] Sarantopoulos, S., Lu, L. & Cantor, H. Qa-1 restriction of CD8+ suppressor T cells. *Journal of Clinical Investigation* vol. 114 1218–1221 (2004).

[41] Singh, A. K., Tripathi, P. & Cardell, S. L. Type II NKT Cells: An Elusive Population With Immunoregulatory Properties. *Frontiers in immunology* vol. 9 1969 (2018).

[42] Rosser, E. C. & Mauri, C. Regulatory B Cells: Origin, Phenotype, and Function. *Immunity* vol. 42 607–612 (2015).

[43] Schmidt, A., Oberle, N. & Krammer, P. H. Molecular mechanisms of Treg-mediated T cell suppression. *Front. Immunol.* **3**, 1–20 (2012).

[44] Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nature Reviews Immunology* vol. 8 523–532 (2008).

[45] Prochazka, M., Serreze, D. V., Worthen, S. M. & Leiter, E. H. Genetic control of diabetogenesis in NOD/Lt mice. Development and analysis of congenic stocks. *Diabetes* **38**, 1446–1455 (1989).

[46] Fernando, M. M. A. *et al.* Defining the Role of the MHC in Autoimmunity: A Review and Pooled Analysis. *PLoS Genet.* **4**, e1000024 (2008).

[47] Tisch, R. & McDevitt, H. Insulin-dependent diabetes mellitus. *Cell* vol. 85 291–297 (1996).

[48] Tsai, S. & Santamaria, P. MHC class II polymorphisms, autoreactive T-cells, and autoimmunity. *Frontiers in Immunology* vol. 4 (2013).

[49] Tsai, S., Serra, P., Clemente-Casares, X., Slattery, R. M. & Santamaria, P. Dendritic Cell–Dependent In Vivo Generation of Autoregulatory T Cells by Antidiabetogenic MHC Class II. *J. Immunol.* **191**, 70–82 (2013).

[50] Tsai, S. *et al.* Antidiabetogenic MHC class II promotes the differentiation of MHC-promiscuous autoreactive T cells into FOXP3+ regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3471–3476 (2013).

[51] Reizis, B., Altmann, D. M. & Cohen, I. R. Biochemical characterization of the human diabetes-associated HLA-DQ8 allelic product: Similarity to the major histocompatibility complex class II I-Ag7 protein of non-obese diabetic mice. *Eur. J. Immunol.* **27**, 2478–2483 (1997).

[52] Hu, X. *et al.* Additive and interaction effects at three amino acid positions in HLA-DQ and HLA-DR molecules drive type 1 diabetes risk. *Nat. Genet.* **47**, 898–905 (2015).

[53] Stratmann, T. *et al.* The I-A g7 MHC Class II Molecule Linked to Murine Diabetes Is a Promiscuous Peptide Binder . *J. Immunol.* **165**, 3214–3225 (2000).

[54] Carrasco-Marin, E., Shimizu, J., Kanagawa, O. & Unanue, E. R. The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. *J. Immunol.* **156**, (1996).

[55] Sato, A. K., Sturniolo, T., Sinigaglia, F. & Stern, L. J. Substitution of aspartic acid at β57 with alanine alters MHC class II peptide binding activity but not protein stability: HLA-DQ (α1*0201, β1*0302) and (α1*0201, β1*0303). *Hum. Immunol.* **60**, 1227–1236 (1999).

[56] Johnson, M. B., Cerosaletti, K., Flanagan, S. E. & Buckner, J. H. Genetic Mechanisms Highlight Shared Pathways for the Pathogenesis of Polygenic Type 1 Diabetes and Monogenic Autoimmune Diabetes. *Current Diabetes Reports* vol. 19 (2019).

[57] Clark, M., Kroger, C. J. & Tisch, R. M. Type 1 diabetes: A chronic anti-self-inflammatory response. *Frontiers in Immunology* vol. 8 1898 (2017).

[58] Atlan-Gepner, C. *et al.* Disorganization of thymic medulla precedes evolution towards diabetes in female NOD mice. *Autoimmunity* **31**, 249–260 (1999).

[59] Kishimoto, H. & Sprent, J. A defect in central tolerance in NOD mice. *Nat. Immunol.* **2**, 1025–1031 (2001).

[60] Zucchelli, S. *et al.* Defective central tolerance induction in NOD mice: Genomics and genetics. *Immunity* **22**, 385–396 (2005).

[61] Hu, Y., Nakagawa, Y., Purushotham, K. R. & Humphreys-Beher, M. G. Functional changes in salivary glands of autoimmune disease-prone NOD mice. *Am. J. Physiol. - Endocrinol. Metab.* **263**, (1992).

[62] Many, M. C., Maniratunga, S. & Denef, J. F. The non-obese diabetic (NOD) mouse: An animal model for autoimmune thyroiditis. in *Experimental and Clinical Endocrinology and Diabetes* vol. 104 17–20 (Exp Clin Endocrinol Diabetes, 1996).

[63] Stratmann, T. *et al.* Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity. *J. Clin. Invest.* **112**, 902–914 (2003).

[64] Pauken, K. E. *et al.* Cutting Edge: Type 1 Diabetes Occurs despite Robust Anergy among Endogenous Insulin-Specific CD4 T Cells in NOD Mice. *J. Immunol.* **191**, 4913–4917 (2013).

[65] Mingueneau, M., Jiang, W., Feuerer, M., Mathis, D. & Benoist, C. Thymic negative selection is functional in NOD mice. *J. Exp. Med.* **209**, 623–637 (2012).

[66] Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 1032–1039 (2001) doi:10.1038/ni723.

[67] Pugliese, A. *et al.* Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J. Clin. Invest.* **107**, 555–564 (2001).

[68] Chentoufi, A. A. & Polychronakos, C. Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: The mechanism by which the IDDM2 locus may predispose to diabetes. *Diabetes* **51**, 1383–1390 (2002).

[69] Brimnes, M. K. *et al.* Low expression of insulin in the thymus of non-obese diabetic mice. *J. Autoimmun.* **19**, 203–213 (2002).

[70] Jaeckel, E., Lipes, M. A. & Von Boehmer, H. Recessive tolerance to preproinsulin 2 reduces but does not abolish type 1 diabetes. *Nat. Immunol.* **5**, 1028–1035 (2004).

[71] Prabakar, K. R. & Pugliese, A. Peripheral antigen-expressing cells in type 1 diabetes.

Current Diabetes Reports vol. 4 101–107 (2004).

[72] Hurtenbach, U. & Maurer, C. Type I diabetes in NOD mice is not associated with insulin-specific, autoreactive T cells. *J. Autoimmun.* **2**, 151–161 (1989).

[73] Mohan, J. F. *et al.* Unique autoreactive T cells recognize insulin peptides generated within the islets of Langerhans in autoimmune diabetes. *Nat. Immunol.* **11**, 350–354 (2010).

[74] Thébault-Baumont, K. *et al.* Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. *J. Clin. Invest.* **111**, 851–857 (2003).

[75] Moriyama, H. *et al.* Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 10376–10381 (2003).

[76] French, M. B. *et al.* Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice. *Diabetes* **46**, 34–39 (1997).

[77] Vafiadis, P. *et al.* Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat. Genet.* **15**, 289–292 (1997).

[78] Cai, C. Q., Zhang, T., Breslin, M. B., Giraud, M. & Lan, M. S. Both polymorphic variable number of tandem repeats and autoimmune regulator modulate differential expression of insulin in human thymic epithelial cells. *Diabetes* **60**, 336–344 (2011).

[79] Durinovic-Bellò, I., Hummel, M. & Ziegler, A. G. Cellular immune response to diverse islet cell antigens in IDDM. *Diabetes* **45**, 795–800 (1996).

[80] Sarugeri, E. *et al.* Autoimmune responses to the β cell autoantigen, insulin, and the INS VNTR-IDDM2 locus. *Clin. Exp. Immunol.* **114**, 370–376 (1998).

[81] Ellis, T. *et al.* Cellular immune responses against proinsulin: No evidence for enhanced reactivity in individuals with IDDM. *Diabetes* **48**, 299–303 (1999).

[82] Semana, G., Gausling, R., Jackson, R. A. & Hafler, D. A. T cell autoreactivity to proinsulin epitopes in diabetic patients and healthy subjects. *J. Autoimmun.* **12**, 259–267 (1999).

[83] Durinovic-Belló, I. *et al.* Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin. *Genes Immun.* **11**, 188–193 (2010).

[84] Durinovic-Belló, I., Gersuk, V. H., Ni, C., Wu, R. & Thorpe, J. Avidity-Dependent Programming of Autoreactive T Cells in T1D. *PLoS One* **9**, (2014).

[85] Motzo, C. *et al.* Heterogeneity in the magnitude of the insulin gene effect on HLA risk in type 1 diabetes. *Diabetes* **53**, 3286–3291 (2004).

[86] Laine, A. P. *et al.* The human leukocyte antigen genotype has a modest effect on the insulin gene polymorphism-associated susceptibility to type 1 diabetes in the Finnish population.

Tissue Antigens **63**, 72–74 (2004).

[87] Gardner, J. M. *et al.* Deletional tolerance mediated by extrathymic aire-expressing cells. *Science (80-.).* **321**, 843–847 (2008).

[88] Han, B. *et al.* Developmental control of CD8+ T cell-avidity maturation in autoimmune diabetes. *J. Clin. Invest.* **115**, 1879–1887 (2005).

[89] Harbige, J., Eichmann, M. & Peakman, M. New insights into non-conventional epitopes as T cell targets: The missing link for breaking immune tolerance in autoimmune disease? *Journal of Autoimmunity* vol. 84 12–20 (2017).

[90] Delong, T. *et al.* Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science (80-.).* **351**, 711–714 (2016).

[91] Marré, M. L. *et al.* Inherent ER stress in pancreatic islet β cells causes self-recognition by autoreactive T cells in type 1 diabetes. *J. Autoimmun.* **72**, 33–46 (2016).

[92] McLaughlin, R. J., Spindler, M. P., van Lummel, M. & Roep, B. O. Where, How, and When: Positioning Posttranslational Modification Within Type 1 Diabetes Pathogenesis. *Current Diabetes Reports* vol. 16 (2016).

[93] Koning, F., Thomas, R., Rossjohn, J. & Toes, R. E. Coeliac disease and rheumatoid arthritis: Similar mechanisms, different antigens. *Nature Reviews Rheumatology* vol. 11 450–461 (2015).

[94] Rondas, D. *et al.* Citrullinated Glucose-Regulated Protein 78 Is an Autoantigen in Type 1 Diabetes. *Diabetes* **64**, 573–586 (2015).

[95] Marre, M. L. *et al.* Modifying enzymes are elicited by ER stress, generating epitopes that are selectively recognized by CD4 + T cells in patients with type 1 diabetes. *Diabetes* **67**, 1356–1368 (2018).

[96] Mcginty, J. W. *et al.* Recognition of Posttranslationally Modified GAD65 Epitopes in Subjects With Type 1 Diabetes. *Diabetes* **63**, 3033–3040 (2014).

[97] Van Lummel, M. *et al.* Posttranslational modification of HLA-DQ binding islet autoantigens in type 1 diabetes. *Diabetes* **63**, 237–247 (2014).

[98] Gottlieb, P. A. *et al.* Chromogranin A is a T cell antigen in human type 1 diabetes. *J. Autoimmun.* **50**, 38–41 (2014).

[99] Mannering, S. I. *et al.* The insulin A-chain epitope recognized by human T cells is posttranslationally modified. *J. Exp. Med.* **202**, 1191–1197 (2005).

[100] Wiles, T. A. *et al.* An insulin-IAPP hybrid peptide is an endogenous antigen for CD4 T cells in the non-obese diabetic mouse. *J. Autoimmun.* **78**, 11–18 (2017).

[101] Babon, J. A. B. *et al.* Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. *Nat. Med.* **22**, 1482–1487 (2016).

[102] Liepe, J. *et al.* A large fraction of HLA class I ligands are proteasome-generated spliced peptides. *Science (80-.).* **354**, 354–358 (2016).

[103] Ebstein, F. *et al.* Proteasomes generate spliced epitopes by two different mechanisms and as efficiently as non-spliced epitopes. *Sci. Rep.* **6**, (2016).

[104] Nakayama, M. *et al.* Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* **435**, 220–223 (2005).

[105] Wegmann, D. R., Norbury-Glaseru, M. & Danielf, D. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol.* **24**, 1853–1857 (1994).

[106] Mohan, J. F., Petzold, S. J. & Unanue, E. R. Register shifting of an insulin peptide-MHC complex allows diabetogenic T cells to escape thymic deletion. *J. Exp. Med.* **208**, 2375–2383 (2011).

[107] Crawford, F. *et al.* Specificity and detection of insulin-reactive CD4 +T cells in type 1 diabetes in the nonobese diabetic (NOD) mouse. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 16729–16734 (2011).

[108] Wan, X. *et al.* Pancreatic islets communicate with lymphoid tissues via exocytosis of insulin peptides. *Nature* **560**, 107–111 (2018).

[109] Alleva, D. G. *et al.* A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. *J. Clin. Invest.* **107**, 173–180 (2001).

[110] Yang, J. *et al.* Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 14840–14845 (2014).

[111] Zhang, L., Nakayama, M. & Eisenbarth, G. S. Insulin as an autoantigen in NOD/human diabetes. *Current Opinion in Immunology* vol. 20 111–118 (2008).

[112] Spanier, J. A. *et al.* Increased effector memory insulin-specific CD4+ T cells correlate with insulin autoantibodies in patients with recent-onset type 1 diabetes. *Diabetes* **66**, 3051–3060 (2017).

[113] Arif, S. *et al.* Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J. Clin. Invest.* **113**, 451–463 (2004).

[114] Danke, N. A., Yang, J., Greenbaum, C. & Kwok, W. W. Comparative study of GAD65-specific CD4+ T cells in healthy and type 1 diabetic subjects. *J. Autoimmun.* **25**, 303–311 (2005).

[115] Malhotra, D. *et al.* Tolerance is established in polyclonal CD4 + T cells by distinct mechanisms, according to self-peptide expression patterns. *Nat. Immunol.* **17**, 187–195 (2016).

[116] Legoux, F. P. *et al.* CD4+ T Cell Tolerance to Tissue-Restricted Self Antigens Is Mediated by Antigen-Specific Regulatory T Cells Rather Than Deletion. *Immunity* **43**, 896–908 (2015).

[117] Trudeau, J. D. *et al. Perspectives in Diabetes Neonatal-Cell Apoptosis A Trigger for Autoimmune Diabetes? DIABETES* vol. 49 (2000).

[118] Turley, S., Poirot, L., Hattori, M., Benoist, C. & Mathis, D. Physiological β Cell Death Triggers Priming of Self-reactive T Cells by Dendritic Cells in a Type-1 Diabetes Model. *J. Exp. Med.* **198**, 1527–1537 (2003).

[119] Gagnerault, M. C., Luan, J. J., Lotton, C. & Lepault, F. Pancreatic lymph nodes are required for priming of β cell reactive T cells in NOD mice. *J. Exp. Med.* **196**, 369–377 (2002).

[120] Kassem, S. A., Ariel, I., Thornton, P. S., Scheimberg, I. & Glaser, B. β-Cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. *Diabetes* **49**, 1325–1333 (2000).

[121] Nagata, S., Hanayama, R. & Kawane, K. Autoimmunity and the Clearance of Dead Cells. *Cell* vol. 140 619–630 (2010).

[122] O'Brien, B. A. *et al.* A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse. *J. Autoimmun.* **26**, 104–115 (2006).

[123] Diana, J. *et al.* Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. *Nat. Med.* **19**, 65–73 (2013).

[124] Berry, G. J. *et al.* Genome-Wide Transcriptional Analyses of Islet-Specific CD4 + T Cells Identify Idd9 Genes Controlling Diabetogenic T Cell Function . *J. Immunol.* **194**, 2654–2663 (2015).

[125] Lyons, P. A. *et al.* The NOD Idd9 genetic interval influences the pathogenicity of insulitis and contains molecular variants of Cd30, Tnfr2, and Cd137. *Immunity* **13**, 107–115 (2000).

[126] Martinez, X. *et al.* CD8 + T Cell Tolerance in Nonobese Diabetic Mice Is Restored by Insulin-Dependent Diabetes Resistance Alleles . *J. Immunol.* **175**, 1677–1685 (2005).

[127] HAMILTON-WILLIAMS, E. E. *et al.* The Use of Idd Congenic Mice to Identify Checkpoints of Peripheral Tolerance to Islet Antigen. *Ann. N. Y. Acad. Sci.* **1103**, 118–127 (2007).

[128] Hamilton-Williams, E. E. *et al.* Cellular mechanisms of restored β-cell tolerance mediated by protective alleles of Idd3 and Idd5. *Diabetes* **61**, 166–174 (2012).

[129] Kochupurakkal, N. M., Kruger, A. J., Tripathi, S., Zhu, B. & Adams, L. T. Blockade of the Programmed Death-1 (PD1) Pathway Undermines Potent Genetic Protection from Type 1 Diabetes. *PLoS One* **9**, 89561 (2014).

[130] Van Belle, T. L., Nierkens, S., Arens, R. & von Herrath, M. G. Interleukin-21 Receptor-Mediated Signals Control Autoreactive T Cell Infiltration in Pancreatic Islets. *Immunity* **36**, 1060–1072 (2012).

[131] King, C., Ilic, A., Koelsch, K. & Sarvetnick, N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* **117**, 265–277 (2004).

[132] Dai, Y. D. *et al.* Slc11a1 enhances the autoimmune diabetogenic T-cell response by altering processing and presentation of pancreatic islet antigens. *Diabetes* **58**, 156–164 (2009).

[133] Stober, C. B., Brode, S., White, J. K., Popoff, J. F. & Blackwell, J. M. Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function. *Infect. Immun.* **75**, 5059–5067 (2007).

[134] Price, J. D. & Tarbell, K. V. The role of dendritic cell subsets and innate immunity in the pathogenesis of type 1 diabetes and other autoimmune diseases. *Front. Immunol.* **6**, (2015).

[135] Marleau, A. M., Summers, K. L. & Singh, B. Differential Contributions of APC Subsets to T Cell Activation in Nonobese Diabetic Mice. *J. Immunol.* **180**, 5235–5249 (2008).

[136] Vasquez, A. C., Feili-Hariri, M., Tan, R. J. & Morel, P. A. Qualitative and quantitative abnormalities in splenic dendritic cell populations in NOD mice. *Clin. Exp. Immunol.* **135**, 209–218 (2004).

[137] Lau-Kilby, A. W. *et al.* Interleukin-2 inhibits FMS-like tyrosine kinase 3 receptor ligand (flt3L)-dependent development and function of conventional and plasmacytoid dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2408–2413 (2011).

[138] Yamanouchi, J. *et al.* Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat. Genet.* **39**, 329–337 (2007).

[139] Pelletier, A. N. & Lesage, S. The Idd13 congenic interval defines the number of merocytic dendritic cells, anovel trait associated with autoimmune diabetes susceptibility. *J. Autoimmun.* **43**, 70–77 (2013).

[140] Reboulet, R. A., Hennies, C. M., Garcia, Z., Nierkens, S. & Janssen, E. M. Prolonged Antigen Storage Endows Merocytic Dendritic Cells with Enhanced Capacity To Prime Anti-Tumor Responses in Tumor-Bearing Mice. *J. Immunol.* **185**, 3337–3347 (2010).

[141] Katz, J. D., Ondr, J. K., Opoka, R. J., Garcia, Z. & Janssen, E. M. Cutting Edge: Merocytic Dendritic Cells Break T Cell Tolerance to β Cell Antigens in Nonobese Diabetic Mouse Diabetes. *J. Immunol.* **185**, 1999–2003 (2010).

[142] Alleva, D. G., Pavlovich, R. P., Grant, C., Kaser, S. B. & Beller, D. I. Aberrant macrophage cytokine production is a conserved feature among autoimmune-prone mouse strains: Elevated interleukin (IL)-12 and an imbalance in tumor necrosis factor-α and IL-10 define a unique cytokine profile in macrophages from young nonobese diabetic mice. *Diabetes* **49**, 1106–1115 (2000).

[143] Baldwin, A. S., Tisch Donald Weaver, R. J., Poligone, B. & Bui, T. B Kinase κ a Hyperactive I B Regulation Due to κ Exhibit a Defect in NF-Dendritic Cells from Nonobese Diabetic Mice. *J Immunol Ref.* **167**, 1461–1468 (2001).

[144] Balasa, B. *et al.* CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. *J. Immunol.* **159**, 4620–7 (1997).

[145] Amrani, A. *et al.* CD154-dependent priming of diabetogenic CD4+ T cells dissociated from activation of antigen-presenting cells. *Immunity* **16**, 719–732 (2002).

[146] Price, J. D. *et al.* CD8 + dendritic cell-mediated tolerance of autoreactive CD4 + T cells is deficient in NOD mice and can be corrected by blocking CD40L . *J. Leukoc. Biol.* **95**, 325–336 (2014).

[147] Price, J. D., Hotta-Iwamura, C., Zhao, Y., Beauchamp, N. M. & Tarbell, K. V. DCIR2+ cDC2 DCs and Zbtb32 restore CD4+ T-cell tolerance and inhibit diabetes. *Diabetes* **64**, 3521–3531 (2015).

[148] Bouma, G. *et al.* NOD mice have a severly impaired ability to recruit leukocytes into sites of inflammation. *Eur. J. Immunol.* **35**, 225–235 (2005).

[149] Kriegel, M. A., Rathinam, C. & Flavell, R. A. Pancreatic islet expression of chemokine CCL2 suppresses autoimmune diabetes via tolerogenic CD11c + CD11b + dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 3457–3462 (2012).

[150] Hotta-Iwamura, C. & Tarbell, K. V. Type 1 diabetes genetic susceptibility and dendritic cell function: potential targets for treatment. *J. Leukoc. Biol.* **100**, 65–80 (2016).

[151] Zhang, J. *et al.* The autoimmune diseaseg-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. *Nat. Genet.* **43**, 902–907 (2011).

[152] Rawlings, D. J., Dai, X. & Buckner, J. H. The Role of PTPN22 Risk Variant in the Development of Autoimmunity: Finding Common Ground between Mouse and Human. *J. Immunol.* **194**, 2977–2984 (2015).

[153] Lavrikova, E. Y. *et al.* The carriage of the type 1 diabetes-associated R262W variant of human LNK correlates with increased proliferation of peripheral blood monocytes in diabetic patients. *Pediatr. Diabetes* **12**, 127–132 (2011).

[154] Zervou, M. I., Mamoulakis, D., Panierakis, C., Boumpas, D. T. & Goulielmos, G. N. STAT4: A risk factor for type 1 diabetes? *Hum. Immunol.* **69**, 647–650 (2008).

[155] Fernando Shahijanian, Grant P. Parnell, Fiona C. McKay, Prudence N. Gatt, Maryam Shojoei, Kate S. O'Connor, Stephen D. Schibeci, Fabienne Brilot, Christopher Liddle, Marcel Batten, ANZgene Multiple Sclerosis Genetics Consortium, Graeme J. Stewart, D. R. B. The CYP27B1 variant associated with an increased risk of autoimmune disease is underexpressed in tolerizing dendritic cells. *Hum. Mol. Genet.* **23**, 1425–1434 (2013).

[156] Mollah, Z. U. A. *et al. Abnormal NF-B Function Characterizes Human Type 1 Diabetes Dendritic Cells and Monocytes 1*. *The Journal of Immunology* vol. 180 www.jimmunol.org (2008).

[157] Chen, Y. G. *et al.* Molecular signatures differentiate immune states in type 1 diabetic families. *Diabetes* **63**, 3960–3973 (2014).

[158] Wen, L. *et al.* Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* **455**, 1109–1113 (2008).

[159] Sorini, C. *et al.* Loss of gut barrier integrity triggers activation of islet-reactive T cells and autoimmune diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 15140–15149 (2019).

[160] Sofi, M. H. *et al.* Polysaccharide a-dependent opposing effects of mucosal and systemic exposures to human gut commensal bacteroides fragilis in type 1 diabetes. *Diabetes* **68**, 1975–1989 (2019).

[161] Costa, F. R. C. *et al.* Gut microbiota translocation to the pancreatic lymph nodes triggers NOD2 activation and contributes to T1D onset. *J. Exp. Med.* **213**, 1223–1239 (2016).

[162] Turley, S. J., Lee, J. W., Dutton-Swain, N., Mathis, D. & Benoist, C. Endocrine self and gut non-self intersect in the pancreatic lymph nodes. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17729–17733 (2005).

[163] Meddings, J. B., Jarand, J., Urbanski, S. J., Hardin, J. & Gall, D. G. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **276**, (1999).

[164] Neu, J. *et al.* Changes in intestinal morphology and permeability in the Biobreeding rat before the onset of type 1 diabetes. *J. Pediatr. Gastroenterol. Nutr.* **40**, 589–595 (2005).

[165] Tai, N. *et al.* Microbial antigen mimics activate diabetogenic CD8 T cells in NOD mice. *J. Exp. Med.* **213**, 2129–2146 (2016).

[166] Hu, Y. *et al.* Different immunological responses to early-life antibiotic exposure affecting autoimmune diabetes development in NOD mice. *J. Autoimmun.* **72**, 47–56 (2016).

[167] Kriegel, M. A. *et al.* Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11548–11553 (2011).

[168] Mariño, E. *et al.* Gut microbial metabolites limit the frequency of autoimmune T cells and

protect against type 1 diabetes. *Nat. Immunol.* **18**, 552–562 (2017).

[169] Fukuda, S. *et al.* Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* **469**, 543–549 (2011).

[170] Plöger, S. *et al.* Microbial butyrate and its role for barrier function in the gastrointestinal tract. *Ann. N. Y. Acad. Sci.* **1258**, 52–59 (2012).

[171] Peng, L., Li, Z.-R., Green, R. S., Holzman, I. R. & Lin, J. Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers. *J. Nutr.* **139**, 1619–1625 (2009).

[172] Bosi, E. *et al.* Increased intestinal permeability precedes clinical onset of type 1 diabetes. *Diabetologia* **49**, 2824–2827 (2006).

[173] Sapone, A. *et al.* Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* **55**, 1443–1449 (2006).

[174] Harbison, J. E. *et al.* Gut microbiome dysbiosis and increased intestinal permeability in children with islet autoimmunity and type 1 diabetes: A prospective cohort study. *Pediatr. Diabetes* **20**, 574–583 (2019).

[175] Knip, M. & Siljander, H. The role of the intestinal microbiota in type 1 diabetes mellitus. *Nature Reviews Endocrinology* vol. 12 154–167 (2016).

[176] Vatanen, T. *et al.* The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. *Nature* **562**, 589–594 (2018).

[177] Drescher, K. M., von Herrath, M. & Tracy, S. Enteroviruses, hygiene and type 1 diabetes: Toward a preventive vaccine. *Reviews in Medical Virology* vol. 25 19–32 (2015).

[178] Tracy, S. *et al.* Toward Testing the Hypothesis that Group B Coxsackieviruses (CVB) Trigger Insulin-Dependent Diabetes: Inoculating Nonobese Diabetic Mice with CVB Markedly Lowers Diabetes Incidence. *J. Virol.* **76**, 12097–12111 (2002).

[179] Horwitz, M. S. *et al.* Diabetes induced by Coxsackie virus: Initiation by bystander damage and not molecular mimicry. *Nat. Med.* **4**, 781–785 (1998).

[180] Tian, J., Lehmann, P. V. & Kaufman, D. L. T Cell Cross-reactivity between Coxsackievirus and Glutamate Decarboxylase Is Associated with a Murine Diabetes Susceptibility Allele. *J. Exp. Med.* **180**, 1979–1984 (1994).

[181] Christen, U. *et al.* A viral epitope that mimics a self antigen can accelerate but not initiate autoimmune diabetes. *J. Clin. Invest.* **114**, 1290–1298 (2004).

[182] Ehl, S., Hombach, J., Aichele, P., Hengartner, H. & Zinkernagel, R. M. Bystander activation of cytotoxic T cells: Studies on the mechanism and evaluation of in vivo significance in a transgenic mouse model. *J. Exp. Med.* **185**, 1241–1251 (1997).

[183] Serreze, D. V, Ottendorfer, E. W., Elliss, T. M., Gauntt, C. J. & Atkinson, M. A. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. *Diabetes* **49**, 708–711 (2000).

[184] Drescher, K. M., Kono, K., Bopegamage, S., Carson, S. D. & Tracy, S. Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: Insulitis determines susceptibility of pancreatic islets to virus infection. *Virology* **329**, 381–394 (2004).

[185] Kanno, T. *et al.* Group B Coxsackievirus Diabetogenic Phenotype Correlates with Replication Efficiency. *J. Virol.* **80**, 5637–5643 (2006).

[186] Serreze, D. V *et al.* Diabetes Acceleration or Prevention by a Coxsackievirus B4 Infection: Critical Requirements for both Interleukin-4 and Gamma Interferon. *J. Virol.* **79**, 1045–1052 (2005).

[187] Horwitz, M. S., Ilic, A., Fine, C., Balasa, B. & Sarvetnick, N. Coxsackieviral-mediated diabetes: Induction requires antigen-presenting cells and is accompanied by phagocytosis of beta cells. *Clin. Immunol.* **110**, 134–144 (2004).

[188] Sioofy-Khojine, A. B. *et al.* Coxsackievirus B1 infections are associated with the initiation of insulin-driven autoimmunity that progresses to type 1 diabetes. *Diabetologia* **61**, 1193–1202 (2018).

[189] Laitinen, O. H. *et al.* Coxsackievirus b1 is associated with induction of β-cells autoimmunity that portends type 1 diabetes. *Diabetes* **63**, 446–455 (2014).

[190] Atkinson, M. A. *et al.* Cellular immunity to a determinant common to glutamate decarboxylase and Coxsackie virus in insulin-dependent diabetes. *J. Clin. Invest.* **94**, 2125–2129 (1994).

[191] Honeyman, M. C., Stone, N. L. & Harrison, L. C. *Molecular Medicine T-Cell Epitopes in Type 1 Diabetes Autoantigen Tyrosine Phosphatase IA-2: Potential for Mimicry with Rotavirus and Other Environmental Agents*. *Molecular Medicine* vol. 4 (1998).

[192] Hiemstra, H. S. *et al.* Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3988–3991 (2001).

[193] De Beeck, A. O. & Eizirik, D. L. Viral infections in type 1 diabetes mellitus-why the β cells? *Nature Reviews Endocrinology* vol. 12 263–273 (2016).

[194] Schloot, N. C. *et al.* Molecular mimicry in type 1 diabetes mellitus revisited: T-cell clones to GAD65 peptides with sequence homology to coxsackie or proinsulin peptides do not crossreact with homologous counterpart. *Hum. Immunol.* **62**, 299–309 (2001).

[195] Schneider, D. A. & von Herrath, M. G. Potential viral pathogenic mechanism in human type 1 diabetes. *Diabetologia* **57**, 2009–2018 (2014).

[196] Dunne, J. L. *et al.* Rationale for enteroviral vaccination and antiviral therapies in human type 1 diabetes. *Diabetologia* **62**, 744–753 (2019).

[197] Campanella, M. *et al.* The Coxsackievirus 2B Protein Suppresses Apoptotic Host Cell Responses by Manipulating Intracellular Ca2+ Homeostasis. *J. Biol. Chem.* **279**, 18440–18450 (2004).

[198] Hanafusa, T. & Imagawa, A. Fulminant type 1 diabetes: a novel clinical entity requiring special attention by all medical practitioners. *Nat. Clin. Pr. Endocrinol. Metab.* **3**, 36–45 (2007).

[199] Akatsuka, H. *et al.* A case of fulminant type 1 diabetes with coxsackie B4 virus infection diagnosed by elevated serum levels of neutralizing antibody. *Diabetes Res. Clin. Pract.* **84**, e50–e52 (2009).

[200] Richardson, S. J. *et al.* Islet cell hyperexpression of HLA class I antigens: a defining feature in type 1 diabetes. *Diabetologia* **59**, 2448–2458 (2016).

[201] Colli, M. L. *et al.* Exposure to the viral by-product dsrna or coxsackievirus b5 triggers pancreatic beta cell apoptosis via a bim/ mcl-1 imbalance. *PLoS Pathog.* **7**, (2011).

[202] Chen, Z., Herman, A. E., Matos, M., Mathis, D. & Benoist, C. Where CD4+CD25+ T reg cells impinge on autoimmune diabetes. *J. Exp. Med.* **202**, 1387–1397 (2005).

[203] Wang, C.-J. *et al.* Protective Role of Programmed Death 1 Ligand 1 (PD-L1) in Nonobese Diabetic Mice The Paradox in Transgenic Models. *Diabetes* **57**, 1861–1869 (2008).

[204] Zhang, Q. & Vignali, D. A. A. Co-stimulatory and Co-inhibitory Pathways in Autoimmunity. *Immunity* vol. 44 1034–1051 (2016).

[205] Ansari, M. J. I. *et al.* The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med.* **198**, 63–69 (2003).

[206] Lühder, F., Höglund, P., Allison, J. P., Benoist, C. & Mathis, D. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *J. Exp. Med.* **187**, 427–432 (1998).

[207] Ueda, H. *et al.* Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* **423**, 506–511 (2003).

[208] Araki, M. *et al.* Genetic Evidence That the Differential Expression of the Ligand-Independent Isoform of CTLA-4 Is the Molecular Basis of the Idd5.1 Type 1 Diabetes Region in Nonobese Diabetic Mice. *J. Immunol.* **183**, 5146–5157 (2009).

[209] Stumpf, M., Zhou, X. & Bluestone, J. A. The B7-Independent Isoform of CTLA-4 Functions To Regulate Autoimmune Diabetes. *J. Immunol.* **190**, 961–969 (2013).

[210] Dahlén, E., Hedlund, G. & Dawe, K. Low CD86 Expression in the Nonobese Diabetic

Mouse Results in the Impairment of Both T Cell Activation and CTLA-4 Up-Regulation. *J. Immunol.* **164**, 2444–2456 (2000).

[211] Cannons, J. L. *et al.* Genetic and functional association of the immune signaling molecule 4-1BB (CD137/TNFRSF9) with type 1 diabetes. *J. Autoimmun.* **25**, 13–20 (2005).

[212] Kachapati, K. *et al.* The B10 Idd9.3 Locus Mediates Accumulation of Functionally Superior CD137 + Regulatory T Cells in the Nonobese Diabetic Type 1 Diabetes Model . *J. Immunol.* **189**, 5001–5015 (2012).

[213] Nielsen, C., Hansen, D., Husby, S., Jacobsen, B. B. & Lillevang, S. T. Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. *Tissue Antigens* **62**, 492–497 (2003).

[214] Quandt, Z., Young, A. & Anderson, M. Immune checkpoint inhibitor diabetes mellitus: a novel form of autoimmune diabetes. *Clin. Exp. Immunol.* **200**, 131–140 (2020).

[215] Stamatouli, A. M. *et al.* Collateral damage: Insulin-dependent diabetes induced with checkpoint inhibitors. in *Diabetes* vol. 67 1471–1480 (American Diabetes Association Inc., 2018).

[216] Buckner, J. H. Mechanisms of impaired regulation by CD4+ CD25+ FOXP3+ regulatory T cells in human autoimmune diseases. *Nature Reviews Immunology* vol. 10 849–859 (2010).

[217] Feuerer, M. *et al.* Enhanced thymic selection of FoxP3+ regulatory T cells in the NOD mouse model of autoimmune diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 18181–18186 (2007).

[218] Alard, P. *et al.* Deficiency in NOD antigen-presenting cell function may be responsible for suboptimal CD4+CD25+ T-cell-mediated regulation and type 1 diabetes development in NOD mice. *Diabetes* **55**, 2098–2105 (2006).

[219] Wu, A. J., Hua, H., Munson, S. H. & McDevitt, H. O. Tumor necrosis factor-α regulation of CD4+C25+ T cell levels in NOD mice. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12287–12292 (2002).

[220] D'Alise, A. M. *et al.* The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 19857–19862 (2008).

[221] Mellanby, R. J., Thomas, D., Phillips, J. M. & Cooke, A. Diabetes in non-obese diabetic mice is not associated with quantitative changes in CD4+ CD25+ Foxp3+ regulatory T cells. *Immunology* **121**, 15–28 (2007).

[222] Malek, T. R. & Bayer, A. L. Tolerance, not immunity, crucially depends on IL-2. *Nature Reviews Immunology* vol. 4 665–674 (2004).

[223] Tang, Q. *et al.* Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction. *Immunity* **28**, 687–697 (2008).

[224] Grinberg-Bleyer, Y. *et al.* IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J. Exp. Med.* **207**, 1871–1878 (2010).

[225] Furusawa, Y. *et al.* Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* **504**, 446–450 (2013).

[226] Arpaia, N. *et al.* Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451–455 (2013).

[227] Luo, X. *et al.* Dendritic cells with TGF-β1 differentiate naïve CD4 +CD25- T cells into islet-protective Foxp3+ regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2821–2826 (2007).

[228] Zheng, Y. *et al.* Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* **463**, 808–812 (2010).

[229] Schuster, C., Jonas, F., Zhao, F. & Kissler, S. Peripherally induced regulatory T cells contribute to the control of autoimmune diabetes in the NOD mouse model. *Eur. J. Immunol.* **48**, 1211–1216 (2018).

[230] Holohan, D. R., Van Gool, F. & Bluestone, J. A. Thymically-derived Foxp3+ regulatory T cells are the primary regulators of type 1 diabetes in the non-obese diabetic mouse model. *PLoS One* **14**, (2019).

[231] Sgouroudis, E., Albanese, A. & Piccirillo, C. A. Impact of Protective IL-2 Allelic Variants on CD4 + Foxp3 + Regulatory T Cell Function In Situ and Resistance to Autoimmune Diabetes in NOD Mice . *J. Immunol.* **181**, 6283–6292 (2008).

[232] Yamanouchi, J. *et al.* Idd9.1 locus controls the suppressive activity of FoxP3+CD4 +CD25+ regulatory T-cells. *Diabetes* **59**, 272–281 (2010).

[233] Gregori, S., Giarratana, N., Smiroldo, S. & Adorini, L. Dynamics of Pathogenic and Suppressor T Cells in Autoimmune Diabetes Development. *J. Immunol.* **171**, 4040–4047 (2003).

[234] You, S. *et al.* Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* **54**, 1415–1422 (2005).

[235] Clough, L. E. *et al.* Release from Regulatory T Cell-Mediated Suppression during the Onset of Tissue-Specific Autoimmunity Is Associated with Elevated IL-21. *J. Immunol.* **180**, 5393–5401 (2008).

[236] Zhou, X. *et al.* Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* **10**, 1000–1007 (2009).

[237] Rubtsov, Y. P. *et al.* Stability of the regulatory T cell lineage in vivo. *Science (80-.).* **329**, 1667–1671 (2010).

[238] Sakaguchi, S., Vignali, D. A. A., Rudensky, A. Y., Niec, R. E. & Waldmann, H. The plasticity and stability of regulatory T cells. *Nature Reviews Immunology* vol. 13 461–467 (2013).

[239] Miyao, T. *et al.* Plasticity of Foxp3 + T Cells Reflects Promiscuous Foxp3 Expression in Conventional T Cells but Not Reprogramming of Regulatory T Cells. *Immunity* **36**, 262–275 (2012).

[240] Bailey-Bucktrout, S. L. *et al.* Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity* **39**, 949–962 (2013).

[241] Kornete, M., Sgouroudis, E. & Piccirillo, C. A. ICOS-Dependent Homeostasis and Function of Foxp3 + Regulatory T Cells in Islets of Nonobese Diabetic Mice . *J. Immunol.* **188**, 1064–1074 (2012).

[242] Brusko, T. *et al.* No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. *Diabetes* **56**, 604–612 (2007).

[243] Jeker, L. T., Bour-Jordan, H. & Bluestone, J. A. Breakdown in peripheral tolerance in type 1 diabetes in mice and humans. *Cold Spring Harb. Perspect. Med.* **2**, (2012).

[244] Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* **203**, 1701–1711 (2006).

[245] Long, S. A. *et al.* Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4+CD25+ regulatory T-cells of type 1 diabetic subjects. *Diabetes* **59**, 407–415 (2010).

[246] Johnson, M. B., Cerosaletti, K., Flanagan, S. E. & Buckner, J. H. Genetic Mechanisms Highlight Shared Pathways for the Pathogenesis of Polygenic Type 1 Diabetes and Monogenic Autoimmune Diabetes. *Current Diabetes Reports* vol. 19 (2019).

[247] Hull, C. M., Peakman, M. & Tree, T. I. M. Regulatory T cell dysfunction in type 1 diabetes: what's broken and how can we fix it? *Diabetologia* vol. 60 1839–1850 (2017).

[248] Long, S. A. *et al.* An autoimmune-associated variant in PTPN2 reveals an impairment of IL-2R signaling in CD4+ T cells. *Genes Immun.* **12**, 116–125 (2011).

[249] Garg, G. *et al.* Type 1 Diabetes-Associated IL2RA Variation Lowers IL-2 Signaling and Contributes to Diminished CD4 + CD25 + Regulatory T Cell Function . *J. Immunol.* **188**, 4644–4653 (2012).

[250] Yang, J. H. M. *et al.* Natural variation in interleukin-2 sensitivity influences regulatory T-cell frequency and function in individuals with long-standing type 1 diabetes. *Diabetes* **64**, 3891–3902 (2015).

[251] Schneider, A. *et al.* The Effector T Cells of Diabetic Subjects Are Resistant to Regulation via CD4 + FOXP3 + Regulatory T Cells . *J. Immunol.* **181**, 7350–7355 (2008).

[252] Lawson, J. M. *et al.* Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. *Clin. Exp. Immunol.* **154**, 353–359 (2008).

[253] Glisic-Milosavljevic, S. *et al.* At-risk and recent-onset type 1 diabetic subjects have increased apoptosis in the CD4+CD25+high T-cell fraction. *PLoS One* **2**, 146 (2007).

[254] Jana, S. *et al.* The type of responder T-cell has a significant impact in a human in vitro suppression assay. *PLoS One* **5**, (2010).

[255] Bettini, M. *et al.* TCR Affinity and Tolerance Mechanisms Converge To Shape T Cell Diabetogenic Potential. *J. Immunol.* **193**, 571–579 (2014).

[256] Kieback, E. *et al.* Thymus-Derived Regulatory T Cells Are Positively Selected on Natural Self-Antigen through Cognate Interactions of High Functional Avidity. *Immunity* **44**, 1114–1126 (2016).

[257] Petersen, J. *et al.* T-cell receptor recognition of HLA-DQ2-gliadin complexes associated with celiac disease. *Nat. Struct. Mol. Biol.* **21**, 480–488 (2014).

[258] Christen, U., Hintermann, E., Holdener, M. & von Herrath, M. G. Viral triggers for autoimmunity: Is the 'glass of molecular mimicry' half full or half empty? *Journal of Autoimmunity* vol. 34 38–44 (2010).

[259] Baker, R. L. *et al.* CD4 T cells reactive to hybrid insulin peptides are indicators of disease activity in the NOD mouse. in *Diabetes* vol. 67 1836–1846 (2018).

[260] Hapil, F. Z. & Wingender, G. The interaction between invariant Natural Killer T cells and the mucosal microbiota. *Immunology* **155**, 164–175 (2018).

[261] Sadelain, M. W. J., Qin, H. Y., Lauzon, J. & Singh, B. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* **39**, 583–589 (1990).

[262] Lee, I.-F., van den Elzen, P., Tan, R. & Priatel, J. J. NKT Cells Are Required for Complete Freund's Adjuvant-Mediated Protection from Autoimmune Diabetes. *J. Immunol.* **187**, 2898–2904 (2011).

[263] Tian, B. *et al.* Upregulating CD4+CD25+FOXP3+ regulatory T cells in pancreatic lymph nodes in diabetic NOD mice by adjuvant immunotherapy. *Transplantation* **87**, 198–206 (2009).

[264] Aumeunier, A. *et al.* Systemic toll-like receptor stimulation suppresses experimental allergic asthma and autoimmune diabetes in NOD mice. *PLoS One* **5**, (2010).

[265] Bach, J. F. The hygiene hypothesis in autoimmunity: The role of pathogens and commensals. *Nature Reviews Immunology* vol. 18 105–120 (2018).

[266] Filippi, C. M., Estes, E. A., Oldham, J. E. & Von Herrath, M. G. Immunoregulatory mechanisms triggered by viral infections protect from type 1 diabetes in mice. *J. Clin.*

Invest. **119**, 1515–1523 (2009).

[267] Patterson, C. C. *et al.* Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet* **373**, 2027–2033 (2009).

[268] Patterson, C. C. *et al.* Trends in childhood type 1 diabetes incidence in Europe during 1989-2008: Evidence of non-uniformity over time in rates of increase. *Diabetologia* vol. 55 2142–2147 (2012).

[269] Bach, J.-F. The Effect of Infections on Susceptibility to Autoimmune and Allergic Diseases. *N. Engl. J. Med.* **347**, 911–920 (2002).

[270] Viskari, H. R. *et al.* Can enterovirus infections explain the increasing incidence of type 1 diabetes? *Diabetes Care* vol. 23 414–416 (2000).

[271] Pang, H. *et al.* Advances in Knowledge of Candidate Genes Acting at the Beta-Cell Level in the Pathogenesis of T1DM. *Frontiers in Endocrinology* vol. 11 119 (2020).

[272] Dooley, J. *et al.* Genetic predisposition for beta cell fragility underlies type 1 and type 2 diabetes. *Nat. Genet.* **48**, 519–527 (2016).

[273] Thompson, P. J. *et al.* Targeted Elimination of Senescent Beta Cells Prevents Type 1 Diabetes. *Cell Metab.* **29**, 1045-1060.e10 (2019).

[274] Richards, D. M., Kyewski, B. & Feuerer, M. Re-examining the Nature and Function of Self-Reactive T cells. *Trends in Immunology* vol. 37 114–125 (2016).

[275] Richards, D. M. *et al.* The Contained Self-Reactive Peripheral T Cell Repertoire: Size, Diversity, and Cellular Composition. *J. Immunol.* **195**, 2067–2079 (2015).

[276] Yu, W. *et al.* Clonal Deletion Prunes but Does Not Eliminate Self-Specific αβ CD8+ T Lymphocytes. *Immunity* **42**, 929–941 (2015).

[277] Ziegler, A. G. *et al.* Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA - J. Am. Med. Assoc.* **309**, 2473–2479 (2013).

[278] Insel, R. A. *et al.* Staging presymptomatic type 1 diabetes: A scientific statement of jdrf, the endocrine society, and the American diabetes association. *Diabetes Care* **38**, 1964–1974 (2015).

[279] Gepts, W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* **14**, 619–633 (1965).

[280] Klinke, D. J. Extent of Beta Cell Destruction Is Important but Insufficient to Predict the Onset of Type 1 Diabetes Mellitus. *PLoS One* **3**, e1374 (2008).

[281] Atkinson, M. A., Roep, B. O., Posgai, A., Wheeler, D. C. S. & Peakman, M. The challenge

of modulating β-cell autoimmunity in type 1 diabetes. *The Lancet Diabetes and Endocrinology* vol. 7 52–64 (2019).

[282] Steele, C. *et al.* Insulin Secretion in Type 1 Diabetes. *Diabetes* **53**, 426–433 (2004).

[283] Unger, R. H. & Grundy, S. Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes. *Diabetologia* vol. 28 119–121 (1985).

[284] Eizirik, D. L., Colli, M. L. & Ortis, F. The role of inflammation in insulitis and B-cell loss in type 1 diabetes. *Nature Reviews Endocrinology* vol. 5 219–226 (2009).

[285] Abdul-Rasoul, M., Habib, H. & Al-Khouly, M. 'The honeymoon phase' in children with type 1 diabetes mellitus: Frequency, duration, and influential factors. *Pediatr. Diabetes* **7**, 101–107 (2006).

[286] Palmer, J. P. *et al.* C-Peptide Is the Appropriate Outcome Measure for Type 1 Diabetes Clinical Trials to Preserve β-Cell Function: Report of an ADA Workshop, 21-22 October 2001. in *Diabetes* vol. 53 250–264 (American Diabetes Association, 2004).

[287] Tarbell, K. V., Yamazaki, S., Olson, K., Toy, P. & Steinman, R. M. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J. Exp. Med.* **199**, 1467–1477 (2004).

[288] Tarbell, K. V. *et al.* Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J. Exp. Med.* **204**, 191–201 (2007).

[289] Prasad, S. *et al.* Tolerogenic Ag-PLG nanoparticles induce tregs to suppress activated diabetogenic CD4 and CD8 T cells. *J. Autoimmun.* **89**, 112–124 (2018).

[290] Wilson, D. S. *et al.* Synthetically glycosylated antigens induce antigen-specific tolerance and prevent the onset of diabetes. *Nat. Biomed. Eng.* **3**, 817–829 (2019).

[291] Tang, Q. *et al.* In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* **199**, 1455–1465 (2004).

[292] Lewis, J. S. *et al.* Dual-Sized Microparticle System for Generating Suppressive Dendritic Cells Prevents and Reverses Type 1 Diabetes in the Nonobese Diabetic Mouse Model. *ACS Biomater. Sci. Eng.* **5**, 2631–2646 (2019).

[293] Elias, D. & Cohen, I. R. Peptide therapy for diabetes in NOD mice. *Lancet* **343**, 704–706 (1994).

[294] Phillips, B. *et al.* A microsphere-based vaccine prevents and reverses new-onset autoimmune diabetes. *Diabetes* **57**, 1544–1555 (2008).

[295] Clemente-Casares, X. *et al.* Expanding antigen-specific regulatory networks to treat

autoimmunity. *Nature* **530**, 434–440 (2016).

[296] Akbarpour, M. *et al.* Insulin B chain 9-23 gene transfer to hepatocytes protects from type 1 diabetes by inducing Ag-specific FoxP3+ Tregs. *Sci. Transl. Med.* **7**, 289ra81-289ra81 (2015).

[297] Mbongue, J. C. *et al.* Reversal of New Onset Type 1 Diabetes by Oral Salmonella-Based Combination Therapy and Mediated by Regulatory T-Cells in NOD Mice. *Front. Immunol.* **10**, 320 (2019).

[298] Agulnick, A. D. *et al.* Insulin-Producing Endocrine Cells Differentiated In Vitro From Human Embryonic Stem Cells Function in Macroencapsulation Devices In Vivo. *Stem Cells Transl. Med.* **4**, 1214–1222 (2015).

[299] Vegas, A. J. *et al.* Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. *Nat. Med.* **22**, 306–311 (2016).

[300] Chang, R. *et al.* Nanoporous Immunoprotective Device for Stem-Cell-Derived β-Cell Replacement Therapy. *ACS Nano* **11**, 7747–7757 (2017).

[301] Xiao, X. *et al.* Endogenous Reprogramming of Alpha Cells into Beta Cells, Induced by Viral Gene Therapy, Reverses Autoimmune Diabetes. *Cell Stem Cell* **22**, 78-90.e4 (2018).

[302] Salisbury, E. M., Game, D. S. & Lechler, R. I. Transplantation tolerance. *Pediatric Nephrology* vol. 29 2263–2272 (2014).

[303] Zhang, L. *et al.* Monoclonal antibody blocking the recognition of an insulin peptide-MHC complex modulates type 1 diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 2656–2661 (2014).

[304] Ostrov, D. A. *et al.* Methyldopa blocks MHC class II binding to disease-specific antigens in autoimmune diabetes. *J. Clin. Invest.* **128**, 1888–1902 (2018).

[305] Serra, P. & Santamaria, P. Antigen-specific therapeutic approaches for autoimmunity. *Nature Biotechnology* vol. 37 238–251 (2019).

[306] Kohane, D. S. Microparticles and nanoparticles for drug delivery. *Biotechnology and Bioengineering* vol. 96 203–209 (2007).

[307] Feili-Hariri, M. *et al.* Immunotherapy of NOD mice with bone marrow-derived dendritic cells. *Diabetes* **48**, 2300–2308 (1999).

[308] Feili-Hariri, M., Falkner, D. H. & Morel, P. A. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *Eur. J. Immunol.* **32**, 2021–2030 (2002).

[309] Machen, J. *et al.* Antisense Oligonucleotides Down-Regulating Costimulation Confer Diabetes-Preventive Properties to Nonobese Diabetic Mouse Dendritic Cells. *J. Immunol.* **173**, 4331–4341 (2004).

[310] Funda, D. P. *et al.* Antigen loading (e.g., Glutamic Acid Decarboxylase 65) of Tolerogenic DCs (tolDCs) reduces their capacity to prevent diabetes in the non-obese diabetes (NOD)-severe combined immunodeficiency model of adoptive cotransfer of diabetes as well as in NOD mice. *Front. Immunol.* **9**, 290 (2018).

[311] Tai, N. *et al.* IL-10-conditioned dendritic cells prevent autoimmune diabetes in NOD and humanized HLA-DQ8/RIP-B7.1 mice. *Clin. Immunol.* **139**, 336–349 (2011).

[312] Van Duivenvoorde, L. M. *et al.* Antigen-specific immunomodulation of collagen-induced arthritis with tumor necrosis factor-stimulated dendritic cells. *Arthritis Rheum.* **50**, 3354–3364 (2004).

[313] Martin, E. *et al.* Antigen-specific suppression of established arthritis in mice by dendritic cells deficient in NF-κB. *Arthritis Rheum.* **56**, 2255–2266 (2007).

[314] Stoop, J. N. *et al.* Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses. *Arthritis Rheum.* **62**, 3656–3665 (2010).

[315] Menges, M. *et al.* Repetitive injections of dendritic cells matured with tumor necrosis factor α induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* **195**, 15–21 (2002).

[316] Haase, C., Yu, L., Eisenbarth, G. & Markholst, H. Antigen-dependent immunotherapy of non-obese diabetic mice with immature dendritic cells. *Clin. Exp. Immunol.* **160**, 331–339 (2010).

[317] Lo, J., Xia, C.-Q., Peng, R. & Clare-Salzler, M. J. Immature Dendritic Cell Therapy Confers Durable Immune Modulation in an Antigen-Dependent and Antigen-Independent Manner in Nonobese Diabetic Mice. *J. Immunol. Res.* **2018**, 5463879 (2018).

[318] Di Caro, V. *et al.* Involvement of suppressive B-lymphocytes in the mechanism of tolerogenic dendritic cell reversal of type 1 diabetes in NOD mice. *PLoS One* **9**, e83575–e83575 (2014).

[319] Giannoukakis, N., Phillips, B., Finegold, D., Harnaha, J. & Trucco, M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* **34**, 2026–2032 (2011).

[320] Qin, S. *et al.* 'Infectious' transplantation tolerance. *Science (80-.).* **259**, 974–977 (1993).

[321] Lennon, G. P. *et al.* T Cell Islet Accumulation in Type 1 Diabetes Is a Tightly Regulated, Cell-Autonomous Event. *Immunity* **31**, 643–653 (2009).

[322] Spence, A. *et al.* Revealing the specificity of regulatory T cells in murine autoimmune diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 5265–5270 (2018).

[323] Tan, T. G., Mathis, D. & Benoist, C. Singular role for T-BET+CXCR3+ regulatory T cells

in protection from autoimmune diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 14103–14108 (2016).

[324] Sprouse, M. L. *et al.* High self-reactivity drives T-bet and potentiates Treg function in tissue-specific autoimmunity. *JCI insight* **3**, (2018).

[325] Van Der Net, J. B., Bushell, A., Wood, K. J. & Harden, P. N. Regulatory T cells: First steps of clinical application in solid organ transplantation. *Transplant International* vol. 29 3–11 (2016).

[326] Romano, M., Fanelli, G., Albany, C. J., Giganti, G. & Lombardi, G. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. *Frontiers in Immunology* vol. 10 43 (2019).

[327] Bluestone, J. A. *et al.* Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci. Transl. Med.* **7**, 315ra189-315ra189 (2015).

[328] Marek-Trzonkowska, N. *et al.* Therapy of type 1 diabetes with CD4+CD25highCD127-regulatory T cells prolongs survival of pancreatic islets - Results of one year follow-up. *Clin. Immunol.* **153**, 23–30 (2014).

[329] Gitelman, S. E. & Bluestone, J. A. Regulatory T cell therapy for type 1 diabetes: May the force be with you. *Journal of Autoimmunity* vol. 71 78–87 (2016).

[330] Brunstein, C. G. *et al.* Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation. *Biology of Blood and Marrow Transplantation* vol. 19 1271–1273 (2013).

[331] Mohanty, R. *et al.* CAR T cell therapy: A new era for cancer treatment (Review). *Oncology Reports* vol. 42 2183–2195 (2019).

[332] Maldini, C. R., Ellis, G. I. & Riley, J. L. CAR T cells for infection, autoimmunity and allotransplantation. *Nature Reviews Immunology* vol. 18 605–616 (2018).

[333] Elinav, E., Adam, N., Waks, T. & Eshhar, Z. Amelioration of Colitis by Genetically Engineered Murine Regulatory T Cells Redirected by Antigen-Specific Chimeric Receptor. *Gastroenterology* **136**, 1721–1731 (2009).

[334] Fransson, M. *et al.* CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J. Neuroinflammation* **9**, 576 (2012).

[335] MacDonald, K. G. *et al.* Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J. Clin. Invest.* **126**, 1413–1424 (2016).

[336] Boardman, D. A. *et al.* Expression of a Chimeric Antigen Receptor Specific for Donor HLA Class I Enhances the Potency of Human Regulatory T Cells in Preventing Human Skin Transplant Rejection. *Am. J. Transplant.* **17**, 931–943 (2017).

[337] Raffin, C., Vo, L. T. & Bluestone, J. A. Treg cell-based therapies: challenges and perspectives. *Nature Reviews Immunology* vol. 20 158–172 (2020).

[338] Walker, A. J. *et al.* Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase. *Mol. Ther.* **25**, 2189–2201 (2017).

[339] Tenspolde, M. *et al.* Regulatory T cells engineered with a novel insulin-specific chimeric antigen receptor as a candidate immunotherapy for type 1 diabetes. *J. Autoimmun.* **103**, 102289 (2019).

[340] Boroughs, A. C. *et al.* Chimeric antigen receptor costimulation domains modulate human regulatory T cell function. *JCI Insight* **4**, (2019).

[341] Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science (80-.).* **299**, 1057–1061 (2003).

[342] Wright, G. P. *et al.* Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 19078–19083 (2009).

[343] Beavis, P. A. *et al.* Resistance to regulatory T cell-mediated suppression in rheumatoid arthritis can be bypassed by ectopic foxp3 expression in pathogenic synovial T cells. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 16717–16722 (2011).

[344] Honaker, Y. *et al. Gene editing to induce FOXP3 expression in human CD4 + T cells leads to a stable regulatory phenotype and function*. *Sci. Transl. Med* vol. 12 http://stm.sciencemag.org/ (2020).

[345] Ohkura, N. *et al.* T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression Are Independent and Complementary Events Required for Treg Cell Development. *Immunity* **37**, 785–799 (2012).

[346] Morikawa, H. *et al.* Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5289–5294 (2014).

[347] Arvey, A. *et al.* Genetic and epigenetic variation in the lineage specification of regulatory T cells. *Elife* **4**, (2015).

[348] Riley, J. L., June, C. H. & Blazar, B. R. Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning. *Immunity* **30**, 656–665 (2009).

[349] Phillips, B. E., Garciafigueroa, Y., Engman, C., Trucco, M. & Giannoukakis, N. Tolerogenic dendritic cells and T-regulatory cells at the clinical trials crossroad for the treatment of autoimmune disease; emphasis on type 1 diabetes therapy. *Frontiers in Immunology* vol. 10 148 (2019).

[350] Turner, M. S., Kane, L. P. & Morel, P. A. Dominant Role of Antigen Dose in CD4 + Foxp3 + Regulatory T Cell Induction and Expansion . *J. Immunol.* **183**, 4895–4903 (2009).

[351] Gabryšová, L. *et al.* Integrated T-cell receptor and costimulatory signals determine TGF-β-dependent differentiation and maintenance of Foxp3+ regulatory T cells. *Eur. J. Immunol.* **41**, 1242–1248 (2011).

[352] Fan, M. Y. & Turka, L. A. Immunometabolism and PI(3)K signaling as a link between IL-2, Foxp3 expression, and suppressor function in regulatory T cells. *Frontiers in Immunology* vol. 9 1 (2018).

[353] Long, S. A. *et al.* Low-Dose Antigen Promotes Induction of FOXP3 in Human CD4 + T Cells. *J. Immunol.* **187**, 3511–3520 (2011).

[354] Daniel, C., Weigmann, B., Bronson, R. & von Boehmer, H. Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimetope. *J. Exp. Med.* **208**, 1501–1510 (2011).

[355] Coppieters, K. T., Harrison, L. C. & Von Herrath, M. G. Trials in type 1 diabetes: Antigen-specific therapies. *Clinical Immunology* vol. 149 345–355 (2013).

[356] Zhang, Z. J., Davidsont, L., Eisenbartht, G. & Weiner, H. L. *Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin (diabetes/tolerance/autolmmunity/lmmunotherapy/insulin)*. *Proc. Natl. Acad. Sci. USA* vol. 88 (1991).

[357] Petersen, J. S. *et al.* Coupling of oral human or porcine insulin to the B subunit of cholera toxin (CTB) overcomes critical antigenic differences for prevention of type I diabetes. *Clin. Exp. Immunol.* **134**, 38–45 (2003).

[358] Oliveira, V. G., Caridade, M., Paiva, R. S., Demengeot, J. & Graca, L. Sub-optimal CD4+ T-cell activation triggers autonomous TGF-β-dependent conversion to Foxp3+ regulatory T cells. *Eur. J. Immunol.* **41**, 1249–1255 (2011).

[359] Battaglia, M. *et al.* Rapamycin Promotes Expansion of Functional CD4 + CD25 + FOXP3 + Regulatory T Cells of Both Healthy Subjects and Type 1 Diabetic Patients. *J. Immunol.* **177**, 8338–8347 (2006).

[360] Chen, W. J. *et al.* Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. *J. Exp. Med.* **198**, 1875–1886 (2003).

[361] Daniel, C., Wennhold, K., Kim, H. J. & Von Boehmer, H. Enhancement of antigen-specific Treg vaccination in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 16246–16251 (2010).

[362] Travis, M. A. & Sheppard, D. TGF-β Activation and Function in Immunity. *Annu. Rev. Immunol.* **32**, 51–82 (2014).

[363] Stallone, G. *et al.* mTOR inhibitors effects on regulatory T cells and on dendritic cells. *Journal of Translational Medicine* vol. 14 (2016).

[364] Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).

[365] Fujihashi, K. *et al.* Peyer's patches are required for oral tolerance to proteins. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3310–3315 (2001).

[366] Mowat, A. M. I. Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews Immunology* vol. 3 331–341 (2003).

[367] Wang, X. *et al.* Mechanism of oral tolerance induction to therapeutic proteins. *Advanced Drug Delivery Reviews* vol. 65 759–773 (2013).

[368] Gottschalk, R. A., Corse, E. & Allison, J. P. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J. Exp. Med.* **207**, 1701–1711 (2010).

[369] Gottschalk, R. A. *et al.* Distinct influences of peptide-MHC quality and quantity on in vivo T-cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 881–886 (2012).

[370] Atkinson, M. A., Maclaren, N. K. & Luchetta, R. Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. *Diabetes* **39**, 933–937 (1990).

[371] Daniel, D. & Wegmann, D. R. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc. Natl. Acad. Sci. U. S. A.* **93**, 956–960 (1996).

[372] Liu, E., Abiru, N., Moriyama, H., Miao, D. & Eisenbarth, G. S. Induction of insulin autoantibodies and protection from diabetes with subcutaneous insulin B:9-23 peptide without adjuvant. in *Annals of the New York Academy of Sciences* vol. 958 224–227 (New York Academy of Sciences, 2002).

[373] Muir, A. *et al.* Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-γ transcription. *J. Clin. Invest.* **95**, 628–634 (1995).

[374] Alleva, D. G. *et al.* Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9-23) peptide. *Diabetes* **51**, 2126–2134 (2002).

[375] Tian, J. *et al.* Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J. Exp. Med.* **183**, 1561–1567 (1996).

[376] Petersen, J. S. *et al.* Neonatal tolerization with glutamic acid decarboxylase but not with bovine serum albumin delays the onset of diabetes in NOD mice. *Diabetes* **43**, 1478–1484 (1994).

[377] Elias, D. *et al.* Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3088–3091 (1991).

[378] Elias, D. *et al.* Hsp60 Peptide Therapy of NOD Mouse Diabetes Induces a Th2 Cytokine Burst and Downregulates Autoimmunity to Various -Cell Antigens. *Diabetes* **46**, 758–765 (1997).

[379] Shoda, L. K. M. *et al.* A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity* vol. 23 115–126 (2005).

[380] Lee, H. C., Kim, S. J., Kim, K. S., Shin, H. C. & Yoon, J. W. Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue. *Nature* **408**, 483–488 (2000).

[381] Lee, H. C., Kim, S. J., Kim, K. S., Shin, H. C. & Yoon, J. W. Erratum: Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue (Nature (2000) 408 (483-488)). *Nature* vol. 458 660 (2009).

[382] Bowman, M. & Atkinson, M. Heat shock protein therapy fails to prevent diabetes in NOD mice. *Diabetologia* vol. 45 1350–1351 (2002).

[383] Zanin-Zhorov, A. *et al.* Heat shock protein 60 enhances CD4+CD25+ regulatory T cell function via innate TLR2 signaling. *Journal of Clinical Investigation* vol. 116 2022–2032 (2006).

[384] Jun, J. C. *et al.* T cell-intrinsic TLR2 stimulation promotes IL-10 expression and suppressive activity by CD45RbHi T cells. *PLoS One* **12**, e0180688 (2017).

[385] Serr, I. *et al.* Type 1 diabetes vaccine candidates promote human Foxp3+ Treg induction in humanized mice. *Nat. Commun.* **7**, (2016).

[386] Bergman, M. L. *et al.* Tolerogenic insulin peptide therapy precipitates type 1 diabetes. *Journal of Experimental Medicine* vol. 214 2153–2156 (2017).

[387] Daniel, C., Weigmann, B. & von Boehmer, H. Reply to 'Tolerogenic insulin peptide therapy precipitates type 1 diabetes'. *Journal of Experimental Medicine* vol. 214 2157–2159 (2017).

[388] Crispe, I. N. The liver as a lymphoid organ. *Annual Review of Immunology* vol. 27 147–163 (2009).

[389] Chen, Y. *et al.* Targeted delivery of antigen to intestinal dendritic cells induces oral tolerance and prevents autoimmune diabetes in NOD mice. *Diabetologia* **61**, 1384–1396 (2018).

[390] Yamazaki, S. *et al.* CD8 + CD205 + Splenic Dendritic Cells Are Specialized to Induce Foxp3 + Regulatory T Cells . *J. Immunol.* **181**, 6923–6933 (2008).

[391] Hotta-Iwamura, C. *et al.* Low CD25 on autoreactive Tregs impairs tolerance via low dose

IL-2 and antigen delivery. *J. Autoimmun.* **90**, 39–48 (2018).

[392] Getts, D. R. *et al.* Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. *Nat. Biotechnol.* **30**, 1217–1224 (2012).

[393] Getts, D. R. *et al.* Therapeutic inflammatory monocyte modulation using immune-modifying microparticles. *Sci. Transl. Med.* **6**, 219ra7-219ra7 (2014).

[394] Jamison, B. L. *et al.* Nanoparticles Containing an Insulin–ChgA Hybrid Peptide Protect from Transfer of Autoimmune Diabetes by Shifting the Balance between Effector T Cells and Regulatory T Cells. *J. Immunol.* **203**, 48–57 (2019).

[395] Saito, E. *et al.* Designing drug-free biodegradable nanoparticles to modulate inflammatory monocytes and neutrophils for ameliorating inflammation. *J. Control. Release* **300**, 185–196 (2019).

[396] Lewis, J. S. *et al.* A combination dual-sized microparticle system modulates dendritic cells and prevents type 1 diabetes in prediabetic NOD mice. *Clin. Immunol.* **160**, 90–102 (2015).

[397] Sugarman, J., Tsai, S., Santamaria, P. & Khadra, A. Quantifying the importance of pMHC valency, total pMHC dose and frequency on nanoparticle therapeutic efficacy. *Immunol. Cell Biol.* **91**, 350–359 (2013).

[398] Han, B. *et al.* Prevention of diabetes by manipulation of anti-IGRP autoimmunity: High efficiency of a low-affinity peptide. *Nat. Med.* **11**, 645–652 (2005).

[399] Tsai, S. *et al.* Reversal of Autoimmunity by Boosting Memory-like Autoregulatory T Cells. *Immunity* **32**, 568–580 (2010).

[400] Fadok, V. A., Bratton, D. L., Guthrie, L. & Henson, P. M. Differential Effects of Apoptotic Versus Lysed Cells on Macrophage Production of Cytokines: Role of Proteases. *J. Immunol.* **166**, 6847–6854 (2001).

[401] A-Gonzalez, N. *et al.* Apoptotic Cells Promote Their Own Clearance and Immune Tolerance through Activation of the Nuclear Receptor LXR. *Immunity* **31**, 245–258 (2009).

[402] Xia, C. Q. *et al.* Transfusion of apoptotic β-cells induces immune tolerance to β-cell antigens and prevents type 1 diabetes in NOD mice. *Diabetes* **56**, 2116–2123 (2007).

[403] Prasad, S., Xu, D. & Miller, S. D. Tolerance strategies employing antigen-coupled apoptotic cells and carboxylated PLG nanoparticles for the treatment of type 1 diabetes. *Review of Diabetic Studies* vol. 9 319–327 (2012).

[404] Getts, D. R. *et al.* Tolerance Induced by Apoptotic Antigen-Coupled Leukocytes Is Induced by PD-L1 + and IL-10–Producing Splenic Macrophages and Maintained by T Regulatory Cells . *J. Immunol.* **187**, 2405–2417 (2011).

[405] Prasad, S., Kohm, A. P., McMahon, J. S., Luo, X. & Miller, S. D. Pathogenesis of NOD diabetes is initiated by reactivity to the insulin B chain 9-23 epitope and involves functional epitope spreading. *J. Autoimmun.* **39**, 347–353 (2012).

[406] Pishesha, N. *et al.* Engineered erythrocytes covalently linked to antigenic peptides can protect against autoimmune disease. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 3157–3162 (2017).

[407] Chen, Y. G. *et al.* Molecular signatures differentiate immune states in type 1 diabetic families. *Diabetes* **63**, 3960–3973 (2014).

[408] Balmert, S. C. *et al.* In vivo induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis. *J. Control. Release* **261**, 223–233 (2017).

[409] Ratay, M. L. *et al.* TRI Microspheres prevent key signs of dry eye disease in a murine, inflammatory model. *Sci. Rep.* **7**, 1–9 (2017).

[410] Fisher, J. D. *et al.* Treg-inducing microparticles promote donor-specific tolerance in experimental vascularized composite allotransplantation. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 25784–25789 (2019).

[411] Engman, C. *et al.* Generation of antigen-specific Foxp3+ regulatory T-cells in vivo following administration of diabetes-reversing tolerogenic microspheres does not require provision of antigen in the formulation. *Clin. Immunol.* **160**, 103–123 (2015).

[412] Quintana, F. J. *et al.* An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 20768–20773 (2010).

[413] Yeste, A., Nadeau, M., Burns, E. J., Weiner, H. L. & Quintana, F. J. Nanoparticle-mediated codelivery of myelin antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 11270–11275 (2012).

[414] Yeste, A. *et al.* Tolerogenic nanoparticles inhibit T cell-mediated autoimmunity through SOCS2. *Sci. Signal.* **9**, (2016).

[415] Ali, O. A., Huebsch, N., Cao, L., Dranoff, G. & Mooney, D. J. Infection-mimicking materials to program dendritic cells in situ. *Nat. Mater.* **8**, 151–158 (2009).

[416] Verbeke, C. S. *et al.* Multicomponent Injectable Hydrogels for Antigen-Specific Tolerogenic Immune Modulation. *Adv. Healthc. Mater.* **6**, (2017).

[417] Husseiny, M. I. *et al.* Factors affecting Salmonella-based combination immunotherapy for prevention of type 1 diabetes in non-obese diabetic mice. *Vaccine* **36**, 8008–8018 (2018).

[418] Roep, B. O., Wheeler, D. C. S. & Peakman, M. Antigen-based immune modulation therapy for type 1 diabetes: the era of precision medicine. *The Lancet Diabetes and Endocrinology* vol. 7 65–74 (2019).

[419] Pozzilli, P. *et al.* No effect of oral insulin on residual beta-cell function in recent-onset Type I diabetes (the IMDIAB VII). *Diabetologia* **43**, 1000–1004 (2000).

[420] Skyler, J. S. Effects of oral insulin in relatives of patients with type 1 diabetes: The diabetes prevention trial-type 1. *Diabetes Care* **28**, 1068–1076 (2005).

[421] Krischer, J. *et al.* Effect of oral insulin on prevention of diabetes in relatives of patients with type 1 diabetes: A randomized clinical trial. *JAMA - J. Am. Med. Assoc.* **318**, 1891–1902 (2017).

[422] Fourlanos, S. *et al.* Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes. *Diabetes* **60**, 1237–1245 (2011).

[423] Walter, M., Philotheou, A., Bonnici, F., Ziegler, A. G. & Jimenez, R. No effect of the altered peptide ligand NBI-6024 on β-cell residual function and insulin needs in new-onset type 1 diabetes. *Diabetes Care* **32**, 2036–2040 (2009).

[424] Ludvigsson, J. *et al.* GAD65 Antigen Therapy in Recently Diagnosed Type 1 Diabetes Mellitus. *N. Engl. J. Med.* **366**, 433–442 (2012).

[425] Wherrett, D. K. *et al.* Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: A randomised double-blind trial. *Lancet* **378**, 319–327 (2011).

[426] Lazar, L. *et al.* Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: A randomised, double-blind phase II study. *Diabetes. Metab. Res. Rev.* **23**, 286–291 (2007).

[427] Schloot, N. C. *et al.* Effect of heat shock protein peptide DiaPep277 on β-cell function in paediatric and adult patients with recent-onset diabetes mellitus type 1: Two prospective, randomized, double-blind phase II trials. *Diabetes. Metab. Res. Rev.* **23**, 276–285 (2007).

[428] Bonifacio, E. *et al.* Effects of high-dose oral insulin on immune responses in children at high risk for type 1 diabetes: The Pre-POINT randomized clinical trial. *JAMA - J. Am. Med. Assoc.* **313**, 1541–1549 (2015).

[429] Harrison, L. C. *et al.* Pancreatic β-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes. *Diabetes Care* **27**, 2348–2355 (2004).

[430] Alleva, D. G. *et al.* Immunomodulation in type 1 diabetes by NBI-6024, an altered peptide ligand of the insulin B(9-23) epitope. *Scand. J. Immunol.* **63**, 59–69 (2006).

[431] Axelsson, S., Hjorth, M., Ludvigsson, J. & Casas, R. Decreased GAD65-specific Th1/Tc1 phenotype in children with Type1 diabetes treated with GAD-alum. *Diabet. Med.* **29**, 1272–1278 (2012).

[432] Hjorth, M. *et al.* GAD-alum treatment induces GAD 65 -specific CD4 + CD25 high FOXP3

+ cells in type 1 diabetic patients. *Clin. Immunol.* **138**, 117–126 (2011).

[433] Ali, M. A. *et al.* Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes. *Sci. Transl. Med.* **9**, (2017).

[434] Arif, S. *et al.* GAD-alum immunotherapy in type 1 diabetes expands bifunctional Th1/Th2 autoreactive CD4 T cells. *Diabetologia* **63**, 1186–1198 (2020).

[435] Carballido, J. M. & Santamaria, P. Taming autoimmunity: Translating antigen-specific approaches to induce immune tolerance. *J. Exp. Med.* **216**, 247–250 (2019).

[436] Kappos, L. *et al.* Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. *Nat. Med.* **6**, 1176–1182 (2000).

[437] Bielekova, B. *et al.* Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: Results of a phase II clinical trial with an altered peptide ligand. *Nat. Med.* **6**, 1167–1175 (2000).

[438] Mirmira, R. G., Sims, E. K., Syed, F. & Evans-Molina, C. Biomarkers of β-Cell Stress and Death in Type 1 Diabetes. *Current Diabetes Reports* vol. 16 95 (2016).

[439] Tobón, G. J., Youinou, P. & Saraux, A. The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. *J. Autoimmun.* **35**, 10–14 (2010).

[440] Myasoedova, E., Crowson, C. S., Kremers, H. M., Therneau, T. M. & Gabriel, S. E. Is the incidence of rheumatoid arthritis rising? Results from Olmsted County, Minnesota, 1955-2007. *Arthritis Rheum.* **62**, 1576–1582 (2010).

[441] Lundkvist, J., Kastäng, F. & Kobelt, G. The burden of rheumatoid arthritis and access to treatment: Health burden and costs. *Eur. J. Heal. Econ.* **8**, S49-60 (2008).

[442] Smolen, J. S. & Aletaha, D. Rheumatoid arthritis therapy reappraisal: Strategies, opportunities and challenges. *Nature Reviews Rheumatology* vol. 11 276–289 (2015).

[443] Taylor, P. C. Clinical efficacy of launched JAK inhibitors in rheumatoid arthritis. *Rheumatology (Oxford).* **58**, i17–i26 (2019).

[444] Smolen, J. S. *et al.* Rheumatoid arthritis. *Nat. Rev. Dis. Prim.* **4**, 1–23 (2018).

[445] Malmström, V., Catrina, A. I. & Klareskog, L. The immunopathogenesis of seropositive rheumatoid arthritis: From triggering to targeting. *Nature Reviews Immunology* vol. 17 60–75 (2017).

[446] Schellekens, G. A., De Jong, B. A. W., Van Den Hoogen, F. H. J., Van De Putte, L. B. A. & Van Venrooij, W. J. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest.* **101**, 273–281 (1998).

[447] Girbal-Neuhauser, E. *et al.* The Epitopes Targeted by the Rheumatoid Arthritis-Associated Antifilaggrin Autoantibodies are Posttranslationally Generated on Various Sites of (Pro)Filaggrin by Deimination of Arginine Residues. *J. Immunol.* **162**, 585 LP – 594 (1999).

[448] Schellekens, G. A. *et al.* The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* **43**, 155–163 (2000).

[449] Rantapää-Dahlqvist, S. *et al.* Antibodies Against Cyclic Citrullinated Peptide and IgA Rheumatoid Factor Predict the Development of Rheumatoid Arthritis. *Arthritis Rheum.* **48**, 2741–2749 (2003).

[450] Nielen, M. M. J. *et al.* Specific Autoantibodies Precede the Symptoms of Rheumatoid Arthritis: A Study of Serial Measurements in Blood Donors. *Arthritis Rheum.* **50**, 380–386 (2004).

[451] Trentham, D. E., Townes, A. S. & Kang, A. H. Autoimmunity to type II collagen: An experimental model of arthritis*. *J. Exp. Med.* **146**, 857–868 (1977).

[452] Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A. & Mosedale, B. Immunisation against heterologous type II collagen induces arthritis in mice [13]. *Nature* vol. 283 666–668 (1980).

[453] Barck, K. H. *et al.* Quantification of cortical bone loss and repair for therapeutic evaluation in collagen-induced arthritis, by micro-computed tomography and automated image analysis. *Arthritis Rheum.* **50**, 3377–3386 (2004).

[454] Luross, J. A. & Williams, N. A. The genetic and immunopathological processes underlying collagen-induced arthritis. *Immunology* **103**, 407–416 (2001).

[455] Kuhn, K. A. *et al.* Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *J. Clin. Invest.* **116**, 961–973 (2006).

[456] Nandakumar, K. S., Bäcklund, J., Vestberg, M. & Holmdahl, R. Collagen type II (CII)-specific antibodies induce arthritis in the absence of T or B cells but the arthritis progression is enhanced by CII-reactive T cells. *Arthritis Res. Ther.* **6**, (2004).

[457] Watson, W. C. & Townes, A. S. Genetic susceptibility to murine collagen II autoimmune arthritis proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J. Exp. Med.* **162**, 1878–1891 (1985).

[458] Ehinger, M. *et al.* Influence of CD4 or CD8 deficiency on collagen-induced arthritis. *Immunology* **103**, 291–300 (2001).

[459] Ranges, G. E., Sriram, S. & Cooper, S. M. Prevention of type II collagen-induced arthritis by in vivo treatment with anti-L3T4. *J. Exp. Med.* **162**, 1105–1110 (1985).

[460] Yoshino, S., Cleland, L. G. & Mayrhofer, G. Treatment of collagen-induced arthritis in rats

with a monoclonal antibody against the α/β T cell antigen receptor. *Arthritis Rheum.* **34**, 1039–1047 (1991).

[461] Cordova, K. N., Willis, V. C., Haskins, K. & Holers, V. M. A Citrullinated Fibrinogen-Specific T Cell Line Enhances Autoimmune Arthritis in a Mouse Model of Rheumatoid Arthritis. *J. Immunol.* **190**, 1457–1465 (2013).

[462] Marinova-Mutafchieva, L. *et al.* Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA). *Clin. Exp. Immunol.* **107**, 507–512 (1997).

[463] Williams, R. O., Feldmann, M. & Maini, R. N. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9784–9788 (1992).

[464] Padyukov, L. *et al.* A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis. *Ann. Rheum. Dis.* **70**, 259–265 (2011).

[465] Källberg, H. *et al.* Gene-gene and gene-environment interactions involving HLA-BRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis. *Am. J. Hum. Genet.* **80**, 867–875 (2007).

[466] Raychaudhuri, S. *et al.* Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat. Genet.* **44**, 291–296 (2012).

[467] Scally, S. W. *et al.* A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis. *J. Exp. Med.* **210**, 2569–2582 (2013).

[468] James, E. A. *et al.* Citrulline-specific Th1 cells are increased in rheumatoid arthritis and their frequency is influenced by disease duration and therapy. *Arthritis Rheumatol.* **66**, 1712–1722 (2014).

[469] Chemin, K. *et al.* A Novel HLA-DRB1∗10:01-Restricted T Cell Epitope from Citrullinated Type II Collagen Relevant to Rheumatoid Arthritis. *Arthritis Rheumatol.* **68**, 1124–1135 (2016).

[470] Gerstner, C. *et al.* Functional and Structural Characterization of a Novel HLA-DRB1*04:01-Restricted α-Enolase T Cell Epitope in Rheumatoid Arthritis. *Front. Immunol.* **7**, 14 (2016).

[471] Wooley, P. H., Luthra, H. S., Stuart, J. M. & David, C. S. Type II collagen-induced arthritis in mice: I. Major histocompatibility complex (I Region) linkage and antibody correlates. *J. Exp. Med.* **154**, 688–700 (1981).

[472] Rosloniec, E. F. *et al.* Induction of Autoimmune Arthritis in HLA-DR4 (DRB1*0401) Transgenic Mice by Immunization with Human and Bovine Type II Collagen. *J. Immunol.*

160, (1998).

[473] Holmdahl, R. *et al.* Type II Collagen Autoimmunity in Animals and Provocations Leading to Arthritis. *Immunol. Rev.* **118**, 193–232 (1990).

[474] Malmstrom, V. *et al.* Systemic versus cartilage-specific expression of a type II collagen-specific T-cell epitope determines the level of tolerance and susceptibility to arthritis. *Immunology* **93**, 4480–4485 (1996).

[475] Klareskog, L. *et al.* A new model for an etiology of rheumatoid arthritis: Smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum.* **54**, 38–46 (2006).

[476] Stolt, P. *et al.* Silica exposure among male current smokers is associated with a high risk of developing ACPA-positive rheumatoid arthritis. *Ann. Rheum. Dis.* **69**, 1072–1076 (2010).

[477] Mikuls, T. R. *et al.* Antibody responses to Porphyromonas gingivalis (P. gingivalis) in subjects with rheumatoid arthritis and periodontitis. *Int. Immunopharmacol.* **9**, 38–42 (2009).

[478] Wegner, N. *et al.* Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunological Reviews* vol. 233 34–54 (2010).

[479] Kharlamova, N. *et al.* Antibodies to Porphyromonas gingivalis Indicate Interaction between Oral Infection, Smoking, and Risk Genes in Rheumatoid Arthritis Etiology. *Arthritis Rheumatol.* **68**, 604–613 (2016).

[480] McGraw, W. T., Potempa, J., Farley, D. & Travis, J. Purification, characterization, and sequence analysis of a potential virulence factor from Porphyromonas gingivalis, peptidylarginine deiminase. *Infect. Immun.* **67**, 3248–3256 (1999).

[481] Scher, J. U. *et al.* Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife* **2013**, (2013).

[482] Wu, H. J. *et al.* Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* **32**, 815–827 (2010).

[483] Abdollahi-Roodsaz, S. *et al.* Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. *J. Clin. Invest.* **118**, 205–216 (2008).

[484] Okada, Y. *et al.* Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature* **506**, 376–381 (2014).

[485] Raychaudhuri, S. Recent advances in the genetics of rheumatoid arthritis. *Current Opinion in Rheumatology* vol. 22 109–118 (2010).

[486] Billiau, A. & Matthys, P. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J. Leukoc. Biol.* **70**, 849–60 (2001).

[487] Cao, D. Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. *Eur. J. Immunol.* **33**, 215–223 (2003).

[488] Mottonen, M. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* **140**, 360–367 (2005).

[489] Lawson, C. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. *Rheumatology* **45**, 1210–1217 (2006).

[490] Han, G. CD4+CD25high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. *Cell. Immunol.* **253**, 92–101 (2008).

[491] Ehrenstein, M. R. *et al.* Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. *J. Exp. Med.* **200**, 277–285 (2004).

[492] Nadkarni, S., Mauri, C. & Ehrenstein, M. R. Anti-TNF-α therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-β. *J. Exp. Med.* **204**, 33–39 (2007).

[493] Flores-Borja, F., Jury, E. C., Mauri, C. & Ehrenstein, M. R. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 19396–19401 (2008).

[494] Han, S., Li, Y., Mao, Y. & Xie, Y. Meta-analysis of the association of CTLA-4 exon-1 +49A/G polymorphism with rheumatoid arthritis. *Hum. Genet.* **118**, 123–132 (2005).

[495] Seidl, C. *et al.* CTLA4 codon 17 dimorphism in patients with rheumatoid arthritis. *Tissue Antigens* **51**, 62–66 (1998).

[496] van Amelsfort, J. M. R. *et al.* Proinflammatory mediator–induced reversal of CD4+,CD25+ regulatory T cell–mediated suppression in rheumatoid arthritis. *Arthritis Rheum.* **56**, 732–742 (2007).

[497] Morgan, M. E. *et al.* CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. *Arthritis Rheum.* **48**, 1452–1460 (2003).

[498] Benham, H. *et al.* Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci. Transl. Med.* **7**, (2015).

[499] Kong, N. *et al.* Antigen-specific transforming growth factor β-induced Treg cells, but not natural Treg cells, ameliorate autoimmune arthritis in mice by shifting the Th17/Treg cell balance from Th17 predominance to Treg cell predominance. *Arthritis Rheum.* **64**, 2548–2558 (2012).

[500] Sun, G. *et al.* Adoptive Induced Antigen-Specific Treg Cells Reverse Inflammation in Collagen-Induced Arthritis Mouse Model. *Inflammation* **41**, 485–495 (2018).

[501] Lim, D. S., Kang, M. S., Jeong, J. A. & Bae, Y. S. Semi-mature DC are immunogenic and not tolerogenic when inoculated at a high dose in collagen-induced arthritis mice. *Eur. J. Immunol.* **39**, 1334–1343 (2009).

[502] Voigtländer, C. *et al.* Dendritic cells matured with TNF can be further activated in vitro and after subcutaneous injection in vivo which converts their tolerogenicity into immunogenicity. *J. Immunother.* **29**, 407–415 (2006).

[503] Thompson, H. S. G., Harper, N., Bevan, D. J. & Staines, N. A. Suppression of collagen induced arthritis by oral administration of type II collagen: Changes in immune and arthritic responses mediated by active peripheral suppression. *Autoimmunity* **16**, 189–199 (1993).

[504] Min, S. Y. *et al.* Induction of IL-10-producing CD4+CD25+ T cells in animal model of collagen-induced arthritis by oral administration of type II collagen. *Arthritis Res. Ther.* **6**, R213 (2004).

[505] Gertel, S., Serre, G., Shoenfeld, Y. & Amital, H. Immune Tolerance Induction with Multiepitope Peptide Derived from Citrullinated Autoantigens Attenuates Arthritis Manifestations in Adjuvant Arthritis Rats. *J. Immunol.* **194**, 5674–5680 (2015).

[506] Dzhambazov, B. *et al.* Therapeutic Vaccination of Active Arthritis with a Glycosylated Collagen Type II Peptide in Complex with MHC Class II Molecules. *J. Immunol.* **176**, 1525–1533 (2006).

[507] Trentham, D. E. *et al.* Effects of oral administration of type II collagen on rheumatoid arthritis. *Science (80-.).* **261**, 1727–1730 (1993).

[508] Sieper, J. *et al.* Oral type II collagen treatment in early rheumatoid arthritis. *Arthritis Rheum.* **39**, 41–51 (1996).

[509] Choy, E. H. S. *et al.* Control of Rheumatoid Arthritis by Oral Tolerance. *Arthritis Rheum.* **44**, 1993–1997 (2001).

[510] Kavanaugh, A. *et al. Allele and Antigen-Specific Treatment of Rheumatoid Arthritis: A Double Blind, Placebo Controlled Phase 1 Trial*. www.jrheum.org (2003).

[511] Koffeman, E. C. *et al.* Epitope-specific immunotherapy of rheumatoid arthritis: Clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum.* **60**, 3207–3216 (2009).

[512] Albani, S., Koffeman, E. C. & Prakken, B. Induction of immune tolerance in the treatment of rheumatoid arthritis. *Nature Reviews Rheumatology* vol. 7 272–281 (2011).

[513] Thomas, R. Dendritic cells and the promise of antigen-specific therapy in rheumatoid arthritis. *Arthritis Research and Therapy* vol. 15 204 (2013).

[514] Capini, C. *et al.* Antigen-Specific Suppression of Inflammatory Arthritis Using Liposomes.

J. Immunol. **182**, 3556–3565 (2009).

[515] Paik, J., Duggan, S. T. & Keam, S. J. Triamcinolone Acetonide Extended-Release: A Review in Osteoarthritis Pain of the Knee. *Drugs* vol. 79 455–462 (2019).

[516] Jhunjhunwala, S. *et al.* Controlled release formulations of IL-2, TGF-β1 and rapamycin for the induction of regulatory T cells. *J. Control. Release* **159**, 78–84 (2012).

[517] Shan, J. *et al.* The effects of rapamycin on regulatory T cells: Its potential time-dependent role in inducing transplant tolerance. *Immunology Letters* vol. 162 74–86 (2015).

[518] Klatzmann, D. & Abbas, A. K. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nature Reviews Immunology* vol. 15 283–294 (2015).

[519] Saez, V. *et al.* Extraction of PLGA-microencapsulated proteins using a two-immiscible liquid phases system containing surfactants. *Pharm. Res.* **30**, 606–615 (2013).

[520] Donohue, J. H. & Rosenberg, S. A. The fate of interleukin-2 after in vivo administration. *J. Immunol.* **130**, 2203–8 (1983).

[521] Coffey, R. J., Kost, L. J., Lyons, R. M., Moses, H. L. & LaRusso, N. F. Hepatic processing of transforming growth factor β in the rat: Uptake, metabolism, and biliary excretion. *J. Clin. Invest.* **80**, 750–757 (1987).

[522] Dooley, S. & Ten Dijke, P. TGF-β in progression of liver disease. *Cell and Tissue Research* vol. 347 245–256 (2012).

[523] Chow, Z., Banerjee, A. & Hickey, M. J. Controlling the fire-tissue-specific mechanisms of effector regulatory T-cell homing. *Immunology and Cell Biology* vol. 93 355–363 (2015).

[524] Tang, Q. *et al.* Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat. Immunol.* **7**, 83–92 (2006).

[525] Barlow, A. D., Nicholson, M. L. & Herbert, T. P. Evidence for rapamycin toxicity in pancreatic β-Cells and a review of the underlying molecular mechanisms. *Diabetes* vol. 62 2674–2682 (2013).

[526] Williams, R. O. Collagen-induced arthritis in mice: A major role for tumor necrosis factor-α. *Methods Mol. Biol.* **361**, 265–284 (2007).

[527] Ganesan, K. *et al.* Gender differences and protective effects of testosterone in collagen induced arthritis in rats. *Rheumatol. Int.* **28**, 345–353 (2008).

[528] Yushkevich, P. A. *et al.* User-guided 3D active contour segmentation of anatomical structures: Significantly improved efficiency and reliability. *Neuroimage* **31**, 1116–1128 (2006).

[529] Akitsu, A. & Iwakura, Y. Isolation of Joint-infiltrating Cells. *BIO-PROTOCOL* **6**, (2016).

[530] Deenick, E. K., Hasbold, J. & Hodgkin, P. D. Decision criteria for resolving isotype switching conflicts by B cells. *Eur. J. Immunol.* **35**, 2949–2955 (2005).

[531] Rose, S., Misharin, A. & Perlman, H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytom. Part A* **81 A**, 343–350 (2012).

[532] Allen, R. P., Bolandparvaz, A., Ma, J. A., Manickam, V. A. & Lewis, J. S. Latent, Immunosuppressive Nature of Poly(lactic- co -glycolic acid) Microparticles. *ACS Biomater. Sci. Eng.* **4**, 900–918 (2018).

[533] Comas, M. *et al.* New nanoformulation of rapamycin rapatar extends lifespan in homozygous p53-/- mice by delaying carcinogenesis. *Aging (Albany. NY).* **4**, 715–722 (2012).

[534] Zeng, H. *et al.* MTORC1 couples immune signals and metabolic programming to establish T reg-cell function. *Nature* **499**, 485–490 (2013).

[535] Strauss, L., Czystowska, M., Szajnik, M., Mandapathil, M. & Whiteside, T. L. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. *PLoS One* **4**, (2009).

[536] Darrasse-Jèze, G. *et al.* Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J. Exp. Med.* **206**, 1853–1862 (2009).

[537] Askenasy, N., Kaminitz, A. & Yarkoni, S. Mechanisms of T regulatory cell function. *Autoimmunity Reviews* vol. 7 370–375 (2008).

[538] Xu, Q. *et al.* Preparation of monodisperse biodegradable polymer microparticles using a microfluidic flow-focusing device for controlled drug delivery. *Small* **5**, 1575–1581 (2009).

[539] Duncanson, W. J. *et al.* Microfluidic synthesis of advanced microparticles for encapsulation and controlled release. *Lab on a Chip* vol. 12 2135–2145 (2012).

[540] Eskandari, S. K. *et al.* Regulatory T cells engineered with TCR signaling-responsive IL-2 nanogels suppress alloimmunity in sites of antigen encounter. *Sci. Transl. Med.* **12**, (2020).

[541] Komatsu, N. *et al.* Pathogenic conversion of Foxp3 + T cells into TH17 cells in autoimmune arthritis. *Nat. Med.* **20**, 62–68 (2014).

[542] Jhunjhunwala, S. *et al.* Bioinspired controlled release of CCL22 recruits regulatory T cells in vivo. *Adv. Mater.* **24**, 4735–4738 (2012).

[543] Glowacki, A. J. *et al.* Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 18525–18530 (2013).

[544] Ratay, M. L. *et al.* Treg-recruiting microspheres prevent inflammation in a murine model of dry eye disease. *J. Control. Release* **258**, 208–217 (2017).

[545] Fisher, J. D. *et al.* In situ recruitment of regulatory T cells promotes donor-specific tolerance in vascularized composite allotransplantation. *Sci. Adv.* **6**, (2020).

[546] Tao, R. *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat. Med.* **13**, 1299–1307 (2007).

[547] Li, B. *et al.* FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 4571–4576 (2007).

[548] Frikeche, J. *et al.* Impact of HDAC inhibitors on dendritic cell functions. *Experimental Hematology* vol. 40 783–791 (2012).

[549] Sun, Y. *et al.* Cutting Edge: Negative Regulation of Dendritic Cells through Acetylation of the Nonhistone Protein STAT-3. *J. Immunol.* **182**, 5899–5903 (2009).

[550] Ratay, M. L., Balmert, S. C., Bassin, E. J. & Little, S. R. Controlled release of an HDAC inhibitor for reduction of inflammation in dry eye disease. *Acta Biomater.* **71**, 261–270 (2018).

[551] Casazza, A. *et al.* Tumour growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform. *EMBO Mol. Med.* **4**, 234–250 (2012).

[552] Zhang, N. *et al.* Sirolimus is associated with reduced islet engraftment and impaired β-cell function. *Diabetes* **55**, 2429–2436 (2006).

[553] Fraenkel, M. *et al.* mTOR inhibition by rapamycin prevents β-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. *Diabetes* **57**, 945–957 (2008).

[554] Joosten, L. A. B. *et al.* Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. *Arthritis Res.* **1**, 81–91 (1999).

[555] Williams, R. O., Marinova-Mutafchieva, L., Feldmann, M. & Maini, R. N. Evaluation of TNF-α and IL-1 Blockade in Collagen-Induced Arthritis and Comparison with Combined Anti-TNF-α/Anti-CD4 Therapy. *J. Immunol.* **165**, 7240–7245 (2000).

[556] Law, S. C. *et al.* T-cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles. *Arthritis Res. Ther.* **14**, R118 (2012).

[557] Schinnerling, K., Rosas, C., Soto, L., Thomas, R. & Aguillón, J. C. Humanized mouse models of rheumatoid arthritis for studies on immunopathogenesis and preclinical testing of cell-based therapies. *Frontiers in Immunology* vol. 10 203 (2019).