The Role of T cell-associated Polarizing Transcription Factors in Dendritic Cell Priming of T cells towards Immunity or Tolerance; Role of T-bet or Foxp3 Ectopic Expression in Dendritic Cells

By

Michael Wheeler Lipscomb

B.S., University of California, Los Angeles (UCLA), 2003

M.S., California State University, Los Angeles (CSULA), 2005

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This dissertation was presented

by

Michael Wheeler Lipscomb

It was defended on

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and approved by

Jon D Piganelli, PhD

Adrian Morelli, MD, PhD

Per H Basse, MD, PhD

Pawel Kalinski, MD, PhD

Walter J. Storkus, PhD
Dissertation Director

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Abstract

Dendritic cells (DC) are professional antigen presenting cells that can prime naïve T cells to elicit immunity or tolerance. The ability to regulate immunity or tolerance is governed by the "type" of polarization state of activated T cells. T-bet has been identified as the master regulator of Type 1 polarization in T cells, and its expression is essential for immunity. Interestingly, T-bet is also expressed in DC and its abolishment has been shown to impair Type-1 T cell responses in limited studies. Conversely, Foxp3 expression in T regulatory cells engenders a tolerogenic phenotype that can suppress T cell responses (as well as DC induction of immunity). Foxp3 expression in non-T cell subsets, such as adenocarinoma, has also shown immunosuppressive characteristics in the tumor microenvironment and draining lymph nodes. Therefore, I examined the role of T-bet or Foxp3 expression in the DC in modulating T cell responses. T cells were primed with T-bet expressing DC (DC.T-bet) or Foxp3 expressing DC (DC.Foxp3) and responses were thoroughly investigated. DC.T-bet potently primed naïve T cells towards Type 1 immunity, inducing 2-3 fold increased levels of T-bet, IFNγ, CXCR3, and Granzyme-B. Little-to-no changes were found in DC costimulatory molecule expression, however, DC were completely impaired in production of pro-inflammatory and Type-1-inducing cytokines. We confirmed cytokine-independent Type 1 polarization of T cells via neutralization studies. In analogous studies, Foxp3 ectopic expression in DC was found to restrain Type 1 and 17 polarized T cell responses while concomitantly generate CD4+Foxp3+CD25+ T regulatory cell subsets that co-expressed high levels of CTLA-4, CD25, NRP-1 and GITR. These *in vitro* generated T regulatory cells (by DC.Foxp3 and not control DC) suppressed both naïve and

memory CD8+ T cell proliferation and IFNγ production. Neutralizing agents confirmed that the tryptophan catabolizing enzyme-IDO and the immunosuppressive cytokine TGFβ were partially dependent for both suppressing Type 1 T cell responses and generating functionally suppressive Tregs. In summation, this work shows that T-bet and Foxp3 expression in DC play similar roles to expression in T cells by governing immunity or tolerance, respectively.

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PREFACE

This body of work is dedicated to the honor of Vickie Anne Wheeler Lipscomb, as a testament for her intelligence, drive, courage, audacity, tenacity and discipline; to which she humbly bestowed upon me. Additionally, with great honor goes to my father, Wayne Harding Lipscomb, who continues to support, sacrifice, and reinforce the foundation of my growth as an individual. Last, but not least, I thank my brother, Alex-Andre, who has, and continues to, guide me in all planes of existence as a soldier bound by loyalty, integrity, commitment, and honor.

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INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells that sit at the interface of orchestrating immunity or tolerance (1-3). Investigations targeting DC immunobiology have delineated that the combination of antigen presentation quantity and quality, coupled with co-stimulatory or co-inhibitory molecules, and the specific cytokine and/or soluble factor profile can generate a variable array of T cell responses (4-8). These T cell responses, whether they be cell-mediated (Type-1, Th17 or Treg) or humoral (Type-2) can effectively and efficiently remove pathogens, or conversely, induce tolerance (i.e. T regulatory subsets) to mitigate effector T cell responses (9-14). In essence, it was identification of transcription factors linked to the commitment, or *polarization*, of these T cell cohorts that helped solidify the functional differences and underlying mechanism(s) that discriminate these specific T cell subsets (9; 14-16). Interestingly, these transcription factors can directly, or indirectly, suppress the expression of other T cell subset transcription factors to enforce functional polarity. For example, T-bet, which is associated with Type-1 polarized T cell responses, was found to antagonize GATA-3, the Type-2 polarizing transcription factor, to drive T cells towards a strict IFNγ^{+}IL-4^{-} cytokine production profile associated with host protection against viral pathogens and cancer cells (17-20). In the absence of T-bet, GATA-3 function is unopposed, yielding T cells obliged towards T_H2 commitment (IL-4^{+}IFNγ^{-} cytokine profile), to yield host protection against bacteria and multi-cellular pathogens (14; 20). Similarly, it was found that Foxp3, the principle transcription factor for T regulatory cells, and RORγt, the master regulator of T_H17 responses, functioned in a mutually exclusive manner. These factors directly antagonize each other's expression and\or ability to operate as transcription factors (21-23). While the functional correlates of these transactivator proteins has become well-established in T cells, their roles and the roles of similar proteins in alternate immune cells that are capable of exhibiting differential states of functional polarization (such as DC) remains poorly understood. A report by the Glimcher group has

shown that deletion of T-bet expression in DC lead to impairment of Type-1 T cell responses, as measured by reduced IFNγ production from wild-type responder T cells (24). Additionally, Foxp3 expression in pancreatic carcinoma cells has recently been associated with immunosuppression (25).

Given the importance of Type-1 immunity to cancer immunotherapy and anergy/Treg-type T cell responses in the setting of allograft transplant retention, these reports led me to question two things. First, could overexpression of T-bet in DC engender protective Type-1 T cell responses (i.e. engineering of Type-1-polarized DC)? Secondly, could DC engineered to express Foxp3 become regulatory DC that neutralize Type-1 immunity and\or expand T regulatory cell subsets, plausibly benefitting the autoimmunity and tissue transplantation fields? Ultimately, I believe that the investigation of (these T cell-associated) transcription factors in DC may help to elucidate variable and\or additional signals involved in corollary T cell polarization. Furthermore, if successful, these *ex vivo* modified DC may provide valuable immunotherapeutic tools to generate antigen-specific and\or global immunity in the fields of cancer and infectious disease or tolerance in the fields of transplantation and autoimmunity.

1.1 T cells

T cells are integral components of the adaptive immune system, serving as principle eradicators of cellular pathogens. Such protection is afforded by both direct, *cytolytic* effector functions mediated by T cells, and by the indirect action of T cell produced-cytokines that exert anti-proliferative or pro-apoptotic pressure on invading pathogens. In most cases, T cell recognition of pathogens occurs via the interaction of the T cell receptor (TCR; along with the CD4 or CD8 co-receptors) with MHC (or HLA) complexes on host cells that contain pathogen-derived peptide sequences (3; 9; 26; 27). A diverse T cell repertoire can be mobilized against MHC-presented, pathogen-derived peptides due to the variable combinations of alpha and beta TCR chains that function as a unit in binding to the MHC/peptide complex (28; 29). Thymic (central) tolerance mechanisms removes most high-avidity, auto(self)-reactive T cell clones (negative selection), while sustaining only those T cell clones capable of recognizing self-MHC complexes to a limited, non-pathogenic level (i.e. positive selection) (30; 31). Once T cells enter the peripheral circulation and encounter a "cognate" antigen via their TCR complex, rapid clonotypic proliferation can occur, after which the expanded T cell population undergoes contraction and establishes a small cohort of "memory" T cells (4; 27; 32). Such memory T cells may then rapidly expand and acquire effector function in response to antigen restimulation, serving to protect the host against secondary infections or transformed cells (33; 34).

A further level of refinement associated with T cell responses is reflected in the ability of these T cells to become functionally committed to one (of at least) four distinct polarization states: Type-1 ($CD4^+$ T helper (T_H) 1 cells; $CD8^+$ cytotoxic T lymphocytes (CTL)) (35; 36), Type-2 ($CD4^+$ T_H2) (36; 37), Type-17 ($CD4^+$ T_H17) (12), and T regulatory (Tregs) cell subsets (13). Although cytokine profile is predominately used to identify the differing T cell functional subsets (**Table 1**), these functions have more recently been affiliated with the "up-stream" restricted use of transcription factors T-bet (Type-1) (38; 39), GATA-3 (Type-2) (12), RORγt (Type-17) (16),

and Foxp3 (Tregs) (13). Memory T cells developed under polarizing conditions tend to retain their specific polarized states, and have long been considered unable to be reprogrammed (or *repolarized*) to an alternate polarization state (33). Most recently, however, this concept has been called into question (40; 41).

Type	Transcription Factor	Cytokine Profile	Additional Associated Markers
Type 1	T-bet	IFNγ	CXCR3, IL-12Rβ2
Type 2	GATA-3	IL-4, IL-5, IL-13	IL-4R
Type 17	RORγt	IL-17	CCR6, IL-23R
Tregs	Foxp3	IL-35	CXCR4, CD25, CTLA-4, Neuropilin-1, GITR, OX40

Table 1. Cytokine, master transcriptional regulator, and additional-associated markers associated with polarization states of $CD4^+$ T cells

Regardless, antigen-specific T cells respond appropriately to rechallenge by invading pathogens and altered host somatic cells (infected or mutated). For example, viral or bacterial infection of host cells allows for presentation of foreign peptides via MHC class I or II molecules to which both Type-1 polarized $CD8^+$ effector and $CD4^+$ T cells can be primed against. Alternatively, the presence of free circulating bacteria can be eradicated by the effective reciprocal cross-talk of Type-2 polarized T_H cells (T_H2), antigen presenting cells (APC) and B cells to produce antibodies, and cytokines or Type-17 polarized T_H cells (T_H17) that can effectively mobilize/recruit neutrophils and macrophages that mediate the eradication of invading pathogenic organisms. Although confounding at first, T regulatory cells have been

shown to play important roles by prevention of exacerbated immune responses that can lead to pathological conditions if left unregulated (in a state of chronic antigenic stimulation).

1.1.1 T-bet and Immunity

T-bet is the hallmark transcription factor for Type 1 immunity, expressed in both $CD8^+$ effector and $CD4^+$ helper T cell subsets (38; 42; 43). T-bet expression in T cells induces IFNγ expression, among other Type 1-associated functions, as well as is induced\regulated by IFNγ, which acts through the IFNγ receptor-STAT1 signaling cascade to further promote T-bet expression (17; 44). Also of note, IL-12p70, a potent Type-1 polarizing cytokine which acts through the IL-12 receptor-STAT4 signaling cascade, does not induce T-bet expression directly (17). Further evidence suggests that T_H1 cell fate is determined prior to IL-12 signaling, and still occurs in STAT4-defecient mice (17; 45-48), whereas STAT1 knockout model systems are deficient in T_H1 programming (49). Murine T cells deficient in T-bet expression exhibit poor T_H1 functionality, and are characterized by impaired IFNγ production, impaired production of Granzyme-B and perforin, and poor mobilization to sites of infection due to loss of the chemokine receptor CXCR3 (19; 46; 50-52). Furthermore, ectopic (transgene) expression of T-bet in naïve $CD4^+$ T cells or T_H2 polarized cells convey unto these T cells the phenotypic characteristics of Type-1 immune cells (i.e. expression of high levels of IFNγ and a concomitant abolishment of IL-4 production by acting on GATA-3 functioning(18; 19; 53). These works have established T-bet as the principle transcription factor for driving T cells towards Type-1 immunity. However, additional work has shown that T-bet can regulate Type-1 immunity in non-T cell lineages, including natural killer (NK)(54), natural killer T (NKT) (55), and dendritic cells (DC) (24; 56), questioning whether T-bet serves a pleiotropic Type 1 immunity-governor in immune cells.

1.1.2 Foxp3 and Tolerance

Foxp3 transcription is essential to the generation of regulatory T cells (57). Although Foxp3 expression is ubiquitous, its absence in T cells leads to exacerbated T cell responses that mediate autoimmunity and other pathological disorders (58; 59). Foxp3 expression in T cells engenders potent suppression of both naïve and memory T cell proliferation and effector functions (60). Overexpression of Foxp3 in naïve $CD4^+$ T cells or pre-committed T_H1 cells converts these subsets into T regulatory cells that expressed high levels of IL-2 receptor alpha chain (CD25), cytotoxic T lymphocyte antigen-4 (CTLA-4) and Neuropilin-1 (NRP1), among other proteins, and these genetically-engineered T cells suppress T effector cell function and proliferation (61; 62). However, Treg subsets have also been identified that can exert immunosuppressive effects even though they fail to express Foxp3; these subsets remain comparatively poorly-defined (63). Alternatively, the existence of the $CD8^+Foxp3^+$ Treg subset has been recently revitalized (64-67), suggesting that both $CD4^+$ and $CD8^+$ T cell subsets can contribute to tolerance using a range of mechanisms associated with immunosuppression.

Of further interest, cancer cells have been recently shown to overexpress Foxp3 in the tumor microenvironment. These Foxp3 expressing cancer cells elicit potent suppression of infiltrating T effector cell functions and proliferation (25). Thus it remains formally possible that the immunosuppressive functions linked to Foxp3 may be the result of its expression in a myriad of cell types, including immune privileged tissues, fetal tissues, and myeloid cell subsets, such as macrophages or DC.

1.2 $CD8^+$ and $CD4^+$ Effector T cells

$CD8^+$ T effector (cytotoxic T lymphocytes; CTL) cells are MHC class I-restricted T cell subsets that are capable of mediating potent cell-cell contact-dependent destruction of target cells and secreting Type-1-associated, inflammatory cytokines/chemokines. The CD8 co-receptor molecule, on the surface of these T cell subsets, interacts directly with the alpha-3 domain of the MHC class I heavy chain expressed by APC or target cells, fortifying the signal strength of TCR-MHC interactions and lowering the activation threshold for responder T cells in response to cognate antigen (68; 69). In the absence of CD8, the ability of CTL to interact with the MHC class I/peptide complexes on the surface of target cells is critically hindered (68). MHC class I is ubiquitously present on all cells of a given individual, with the exception of certain tissues found in immune privileged sites and low levels observed on red blood cells (70). All somatic cells appear competent to process and present antigenic-peptide, whether self or foreign, in the context of MHC class I molecules; thus allowing recognition and *sampling engagement* of $CD8^+$ effector T cells with the vast majority of self cells (69). Upon specific recognition, CD8+ T cell clones may proliferate, and acquire effector functions/molecules (i.e. perforin, granzyme, IFNγ, TNF-a, Fas (CD95), TRAIL, among others; (68)**)**. Indeed, abolishment of one or more of these factors impairs $CD8^+$ T effector cell-mediated clearance of pathogen-infected or transformed cells.

$CD4^+$ T cells are termed *helper* cells because they assist in modulating corollary CD8+ T cell response or B-cell production of antibodies (71). T helper (T_H) cell subsets have also been shown to license DC to promote, or suppress, various T cell polarizing states (72). For example, T_H1 cells can license DC to polarize $CD8^+$ T effector cells into CTL through upregulation of CD40, a surface bound-molecule that interacts with CD40 ligand present on activated T cells (73-75). Alternatively, T_H2 cells have been shown to form a bridge between B cells and DC to augment effective activation and effector function of B cells, including antibody

isotype switching and the release of pro-inflammatory cytokines (76). Finally, newly-described T_H17 cells, seem to bridge the innate and adaptive arms of the immune system by effectively activating and mobilizing innate immune cells (i.e. predominately neutrophils) to mediate clearance of extracellular pathogens (12).

Similar to the importance of memory $CD8^+$ T cells, memory $CD4^+$ T cells are also important for rapidly mounting immunity upon subsequent exposures of a given antigen (77). Memory T cells are not as reliant on survival or homeostatic factors (vs. naïve T cells) and can rapidly expand (vs. naïve T cells) to protect the host against subsequent infection(s) (78).

1.3 T regulatory cell subsets

T regulatory cell subsets (Tregs) were originally identified as a small subset of T cells normally present at approximately 5-10% of total T cells and constitutively expressing CD25, the IL-2 receptor alpha chain (79; 80). Such Tregs are naturally selected during their thymic development, and thus termed *natural* Tregs. Alternatively, naïve T cells induced in the periphery upon antigen exposure towards T regulatory fate are preferentially termed *peripheral* Tregs.

Subsequent works identified that Foxp3 was the master regulator of the T regulatory cell phenotype, regulating high level expression of CD25, CTLA-4, and neuropilin-1 (NRP1), as well as complexing with various histone deacetylases (HDAC) and transcription factors to control the immunosuppressive functions of T cells (61; 81). $Foxp3^+CD25^+$ Tregs are the predominant form of regulatory T cell found in the body, with more recent references made to a minority population of CD8+ Treg cells (64). Treg generation is rigidly dependent on the presence of TGFβ during naïve T cell priming (82). As TGFβ is not generally expressed by DC, it is believed the lymph node tissues and activated T cells contribute the major levels of TGFβ; thereby playing a part in

the regulation of T cell polarization in tandem with DC (83). The presence of IL-6 or IL-21 have been shown to counteract the role of Treg onset, enforcing conversion of naïve T cells instead into T_H17 subsets (84; 85). This further confirms the mutual exclusivity of the Treg vs. Th17 polarization states, and allows for finely-tuned/-balanced immunity based on microenvironmental cues.

T regulatory cells are potent suppressors of both T effector proliferation and effector functions, such as expression of IFNγ, in a predominantly cell-contact-dependent manner (86). A more limited literature suggests that T regulatory cells secrete soluble suppressive factors (87). Host absence of T regulatory cells, as modeled in mice deficient in Foxp3 expression, leads to extensive (auto)immune pathology in association with uncontrolled T cell proliferation and inflammatory T cell effector functions (58; 88). Conversely, adoptive transfer of *ex vivo* developed Treg cells in the context of autoimmunity and transplant models leads to amelioration of immune pathology (89).

1.4 Dendritic Cells

Dendritic cells (DC) are specialized antigen presenting cells that are responsible for the *priming* (activation and polarization) of specific T cells. This role is dependent upon DC presentation of peptides within their cell-surface MHC class I or II molecules (to CD8+ or CD4+ T cells, respectively), levels of expressed co-stimulatory vs. co-inhibitory molecules, and the array of cytokines an chemokines elaborated by DC at the time of cognate DC-T cell interaction (2; 3; 90; 91). Absence or functional incompetence of DC leads to severely impaired T cell responses, as well as, to perturbed B cell, NK, and alternate innate immune cell functions; highlighting the importance of DC in orchestrating immune responses (91; 92). DC are localized

in tissues and act as sentinel scavengers that continuously uptake, process, and present *self* and *foreign* antigens to T cells (90; 93). Tissue DC are specialized to traffic from peripheral sites to draining lymph nodes, through both the lymphatics and the blood, with intent to present antigens to nodal (largely naïve) T cells (4). DC themselves do not discriminate between foreign vs. self peptides, but rather leave this distinction up to the thymically-trained T cells, that then respond "appropriately" to a given antigenic peptide epitope.

DC are equipped to provide support for the activation, proliferation, and polarization of responder T cells based on cues contributed within the tissue environment in which antigen has been acquired. DC express Toll like-receptors (TLR), as well as other "danger" recognizing sensors, that can readily engage foreign particles and antigens, such as peptidoglycan present on the surface of bacteria, double stranded RNA present in viruses, and foreign DNA sequences (differentially methylated CpG strands) (90; 94). Other "danger" signals to DC include pro-inflammatory molecules elaborated by damaged tissues, such as IFNα, IL-1β or TNFα, or elevated levels of free ATP, among others (90). The triggering of these TLRs or other danger sensors on DC promotes their *maturation,* leading to DC upregulation of pMHC complexes on their cell surface (i.e. signal 1), elevation in levels of costimulatory molecules, such as B7.1 (CD80) and B7.2 (CD86) (i.e. signal 2), and typically robust production of polarizing cytokines, such as IL-12, IL-6, and\or IL-23, to name a few (i.e. signal 3 (2; 4)). Receipt of danger signals also coordinately alters the expression of chemokine receptors on DC, allowing them to exit peripheral tissue sites and home to the tissue-draining lymph nodes, where a concentrated pool of naïve T cell responders may be found (2; 36). The DC phenotypic transformation from peripheral *(resting, immature*) to transport-competent *(mature)*, ensures the acquisition of a balanced level of signals 1-3 to prompt an appropriately polarized and "addressed" cohort of responder T cells that are then competent to return to tissue sites of antigenic "insult" and to mediate requisite effector functions within that site to resolve any condition of "danger" (1).

1.4.1 Type 1 polarized DC subsets

Type 1 polarized T cell responses appear optimally developed by stimulation with DC subsets that exhibit elevated levels of signals 1-3, specifically increased expression of MHC class I/II molecules, B7 family member costimulatory molecules and expression of IL-12 family member cytokines (36; 95-97). Absence of one or more of these signals has been shown to limit Type 1 polarized T cell responses. pMHC complexes, along with high levels of costimulatory and adhesion molecules, increases the contact duration of T cells, specifically naïve T cells, with DC to facilitate immunity (32; 98; 99). Therefore, it is of no surprise that DC that have not been activated to become mature (i.e. immature, resting) are poor stimulators of T cell responses. These immature DC, instead, induce states of T cell anergy or apoptosis, or selectively promote T regulatory cells (100). Type 1 polarized DC are optimized in their capacity to produce high levels of the hallmark Type-1 inducible factor IL-12p70 (36; 95). Exogenous addition of IL-12p70 with anti-CD3, which acts as a mitogenic agonist of the TCR receptor, mimicking TCR:pMHC engagement, plus anti-CD28, which acts as an agonist of the costimulatory receptor on T cells, mimicking B7.1 or B7.2 engagement with CD28, potently primes T cells towards Type 1 polarization (101). These T cells express elevated levels of T-bet, granzyme-B (in $CD8^+$ T effectors), CXCR3, and the IL-12 receptor (IL-12R) high affinity β2 chain. DC-produced IL-12p70 then further reinforces Type 1 responses by signaling through the IL-12Rβ1β2 heterodimeric receptor complex via STAT4 activation and reinforcement of IFNγ expression. Such committed Type 1 polarized T cells express little-to-no IL-4, GATA-3, IL-10, or Foxp3, and exhibit potent anti-tumor and anti-viral effector function.

Although IL-12p70, and related IL-12 family members IL-23 and IL-27, have been shown to augment Type 1 polarized T cell states, additional studies demonstrate the clear importance of signal 1 and signal 2 in polarizing T cells. Indeed, high levels of costimulatory molecules on DC, such as B7.1, or cell adhesion molecules, such as ICAM-1, have been shown to gear early activated T cells towards Type 1 immunity, independent of IL-12 family member cytokine provision (102-104). Several studies have also shown that the strength of TCR:pMHC interactions, along with increased engagement of T cell and DC through adhesion molecule upregulation, can directly dictate the magnitude of Type 1 polarized T cell responses (105). Therefore, the plasticity of DC, with regard to the provision of signals 1-3 to T cells, dictates not only responder T cell quantity, but also, T cell quality (based on polarization state, resistance to apoptosis, etc,).

1.4.2 Tolerogenic DC subsets

DC can induce tolerance, as opposed to immunity, in responder T cells (106). These DC subsets, so-called tolerogenic DC, are more prevalent in the tumor-bearing host and may aid in the progression of cancer by dampening immune surveillance. Studies have revealed that the very plastic nature of DC allows for cues within the tissue microenvironment to condition DC in a manner that dictates the quality of corollary T responses evoked in tissue-draining lymph nodes. For instance, the demand of the tumorogenic program to repair, regenerate, and grow mandates pro-inflammatory responses be restrained suggests the need to modulate DC to skew towards tolerance to aid in suppression of immune responses. In particular, “tolerogenic” DC express low levels of pMHC complexes, elevated co-inhibitory molecule expression (such as B7H1 (programmed death ligand-1 (PDL1)) or B7-DC), and\or reduced levels of Type 1 polarizing cytokines (such as IL-12 family members) and increased production of

immunosuppressive cytokines (such as IL-10, VEGF, and\or TGFβ (8)). Tolerogenic DC may also express high levels of the tryptophan catabolizing enzyme, indoleamine-2,3-dioxygenase (IDO), which degrades free tryptophan and "starves" responder T cells of an essential amino acid (107; 108). This commonly results in increased Fas/Fas-L-dependent T cell apoptosis (8; 108). The use of tolerogenic DC *in vivo* could ultimately provide a means to promote specific immune tolerance in order to treat clinical conditions associated with states of exacerbated, or uncontrolled inflammatory-type immune responses, such as autoimmunity and allograft rejection. Further characterization and study of the interplay between the microenvironment and DC may lead to a better understanding of how tolerogenic DC are "born" and how such conditions may be manipulated in vivo or in vitro to better define treatment regimens for patients where tolerogenic DC are desired or not.

1.5 Dendritic Cell based-Vaccines

Dendritic cell (DC) based-vaccines are believed to hold translational promise as "biologic adjuvants" for eliciting Ag-specific immunity or tolerance (109). Several factors make DC attractive immunotherapeutic agents, including (but not limited to): DC are the principle APC that induce T cell responses, they are abundant (as monocytes precursors isolated from blood) and easily differentiated *ex vivo*, and they can be *matured* into a Type 1- or tolerogenic-DC through modulation with TLR agonists, cytokines, or genetic reprogramming (110). More appropriately, *ex vivo*-modified DC can be used to expand Type 1 polarized antigen-specific T cell responses towards immunity or tolerance *in vivo* (110; 111).

1.5.1 Cancer Immunotherapy

Transformed and virally-infected human cells presented a unique cohort of antigenic peptides on their cell surfaces in the context of MHC (called Human Leukocyte Antigens (HLA) in humans) class I and II molecules. In the case of cancer cells, a tumor-associated antigen may reflect a normal "self" protein that is grossly over-expressed when compared with normal tissue, or it could reflect a neo-antigen that is generated via genetic mutation or the awakening of a normally silent gene product due to hypomethylation of tumor cell DNA (as in the case of cancer-testis antigens, etc.) (112). While drawing significant enthusiasm for their performance, clinical trials using vaccines based on such antigens have, thus far, proven rather disappointing in yielding objective clinical responses (OCR). Current limitations may include the type of DC accessed by the vaccine in vivo, the low avidity of the responder T cell pools that cannot directly recognize tumor cells, the inappropriate polarization of the responder T cell pool, the inability of vaccine-induced T cells to traffic into tumor sites in vivo, and the poor survival of DC and/or vaccine-induced T cells within the tumor-bearing host, among others.

DC-based vaccines provide an alternative means by which to effectively prime therapeutic anti-tumor antigen-specific T cells in vivo. In such a paradigm, DC can be developed *ex vivo* to attain a strict, committed Type-1 polarization state, before being loaded with tumor antigens or being injected into tumor lesions, where these APC may capture and process tumor antigens into MHC–presented peptides. Consequent trafficking of ex vivo or in vivo loaded DC to the draining lymph nodes (DLN) would allow for the activation and development of Type-1, anti-tumor T cells that could mediate superior therapeutic effects (113). While current DC-based cancer vaccines have yielded only modest successes (114), the likelihood of beneficial outcome might be further enhanced if the conditioned DC used in the vaccine promoted Type-1 T cell responses that were refractory to tumor-associated immune deviation and which were enhanced in their tropism into the tumor afflicted tissues.

It is also important to note that cancer cells are not homogenous in their expression of either target antigens or MHC class I/II molecules, making it somewhat unrealistic for T cell responses to be curative in many cases. However, since Type-1 DC can also be potent stimulators of innate effector cells, such as NK cells, that may actually recognize MHC-loss tumors better than their MHC+ counterparts, such vaccine approaches may still have a fighting chance in patients harboring MHC-loss tumors (113). The *trick*, of course, then becomes how to generate *ex vivo* DC that are committed to induce strong, long-lived Type-1 polarization in responder innate and adaptive immune cells.

1.5.2 Quest for Tolerance in Autoimmunity and Transplantation

Autoimmunity and transplant rejection are undesirable pathological conditions orchestrated primarily by DC, T cells and B cells (115; 116). In the case of autoimmunity, self reactive-T cells or B cells can attack *normal* cells of the body in an antigen-specific manner. These events are mishaps by the body to which *self* is recognized mistakenly as *non-self*. In the case of transplantation, reactive T cells to non-foreign tissue are a natural, but *undesirable* outcome. In such case, the allogenic MHC alleles represent strong immunogens for both CD4+ and CD8+ host T cell recognition of grafted tissues. Even in the face of extensive tissue type matching, shared HLA alleles may present so-called “minor histocompatibility” antigenic peptides that prompt host immunity against grafts (116). In cases of both autoimmunity and transplantation, T or B cells elicit undesirable immune responses. *In vitro* (mouse and human) and *in vivo* (human) experiments using tolerogenic DC have shown that vaccine-suppressed T and B cell responses yield reduced tissue-specific damage (autoimmune models) and increased graft survival (transplant models) (8; 89; 117-120).

1.6. Basis for this Project

The molecular cloning of transactivator molecules, such as T-bet and Foxp3, has allowed for the performance of mechanistic studies designed to dissect their roles in T cell functional programming. Given the increasing sense that such factors dictate polarized states in non-T cells, including members of the innate system (DC and NK cells), as well as, cancer cells, etc., I chose to investigate whether the enforced expression of T-bet or Foxp3 cDNA in human DC conferred Type-1 vs. regulatory capacity on these APC, respectively. This was a logical endeavor in my mind as both T-bet and Foxp3 are potent transcription factors that can complex with histone acetylases and deactylases, bind numerous promoter and repressor gene segments, and regulate several downstream cascade of proteins that would be presumed to impact DC phenotype and function (15; 121-123). The engineering of DC with T-bet cDNA serves both a basic and translational immunology project, since T-bet can be expressed naturally by at least a minor subset of DC (in association with Type-1 immunity) and expression of T-bet in engineered DC might yield a therapeutic modality to enforce protective Type-1 immunity that would not be easily silenced in the tumor microenvironment (due to T-bet transcription being driven-off a non-genomic promoter). While natural Foxp3 expression has yet to be reported in DC, we have anecdotal data that CD11c+ DC may express Foxp3 within progressively-growing murine tumor lesions, suggesting the association of Foxp3+ DC with immunosuppression. This supports the potential basic and translational aspects for studying DC engineered with Foxp3 cDNA to understand mechanisms of action (that might be antagonized in cancer therapies) or for application in the settings of autoimmune disease and allograft rejection, where antigen-specific immune tolerance is a major goal.

1.7 Summary

Expanding on the work performed by the Glimcher group, I evaluated the role of T-bet expression in DC (DC.T-bet); characterizing the DC.T-bet phenotype, engendered T cell responses, and mechanisms of Type 1 immunity induced in responder T cells upon priming by DC.T-bet. To achieve this, DC were transduced with an adenoviral vector encoding human T-bet and subsequently evaluated for phenotypic alterations and ability to promote T cell responses, using superantigen and antigen-specific model systems. Both systems showed DC.T-bet induction of enhanced Type 1 immunity from responding naïve, but not memory, T cells. Strikingly, the ability of DC.T-bet to induce Type 1 immunity in T cells was not dependent on IL-12 or IFNγ production. While the specific mechanism of action remains unresolved, further studies can be built upon this foundation, including molecular analyses of Type 1 polarization states imparted in DC.T-bet, analyses of the *in vivo* efficacy of DC.T-bet-based vaccines in promoting protective/therapeutic anti-tumor immunity, and proteomic/genomic and signal transduction analyses to identify downstream molecules linked to the mechanisms of action for DC.T-bet in preferentially promoting Type-1 T cell responses.

Analogous to the T-bet studies, I investigated the role of Foxp3 expression in DC. In a manner complementary to that for the field of tumor immunology, the autoimmunity and transplant fields have shifted their approaches towards the harnessing of tolerogenic DC as an immunotherapy modality. I investigated the role of Foxp3 expressing DC (DC.Foxp3) to yield a tolerogenic DC capable of silencing Type-1 immunity while concomitantly fostering Treg responses *in vitro*. Unlike the DC.T-bet studies, I was able to implicate both IDO and TGFβ as partial mediators of immunosuppression associated with DC.Foxp3-based stimulation of naïve T cells. My data provide a solid foundation for future *in vivo* studies designed to assess the ability

of *ex vivo* DC.Foxp3 to prevent or ameliorate autoimmune pathology and/or prolong allograft tissue survival. Furthermore, this work opens the door for further investigation of the impact of natural, endogenous expression of Foxp3 or other FOX -related family members (i.e. Foxo3) on DC functional polarity and corollary immune responses to these APC.

Preface Chapter 2

Using an adenoviral vector to transduce human T-bet into monocyte-derived DC, I characterized the phenotype of these DC and investigated the functional polarization and mechanisms of Type 1 induced immunity in responding T cells *in vitro*. The predominant work of these studies used the non-specific superantigen model system, which bridges MHC class II molecules to the TCR of subsets of CD8 or CD4 T cells to mimic signal 1, in order to evaluate T cell responses. Here, I showed that DC.T-bet induces potent Type 1 immunity in T cells as characterized by both Type-1 associated surface molecule expression and elevated production of the hallmark Type 1 cytokine IFN-γ in T cells. We used multiple assays to ensure the Type 1 induction was induced by DC.T-bet into T cells, which included cell sorting, single cell-analysis by flow cytometry, and immunohistochemical analyses. Furthermore, to investigate the mechanisms of Type 1 immunity, we utilized neutralization studies of costimulatory and cytokines, and further identified that Type 1 polarization was induced via an IL-12- and IFNγ-independent mechanism. Finally, to show that this was not an artifact of the superantigen model, and to show the potential utility of DC.T-bet in anti-tumor vaccines, we tested the ability peptide-pulsed DC.T-bet to prime anti-melanoma CD8 T cells isolated from normal, healthy donors *in vitro*. The results of the antigen-specific studies parallel the superantigen model system, showing that DC.T-bet induces superior (tumor) antigen-specific Type 1 immunity, supporting the translation of DC.T-bet as a potential cancer vaccine component.

2. Enforced T-bet expression in DC induces IL-12- and IFNγ- independent Type-1 T cell responses and concomitantly suppresses T_H2 and T regulatory subsets

Lipscomb MW[1,2], Chen Lu,[1,2] Goldbach CJ[3], Watkins SC[3], Taylor JL[2], Wesa AK[1,2], Storkus WJ[1,2]

Departments of [1]Immunology, [2]Dermatology, [3]Center for Biologic Imaging at the University of Pittsburgh, Pittsburgh, PA

All results reported in this data were obtained by Michael W. Lipscomb. Christina Goldbach performed immunohistochemical and imaging analysis of T-bet expression in DC. JLT cloned hT-bet and generated the Ad.hT-bet vector used to infect human DC.

2.1. Abstract

T-bet has been well-characterized as an important transcription factor required for Type-1-polarized responses in T_H1, $CD8^+$ T, and natural killer cells. Additionally, T-bet expression in myeloid cells, specifically dendritic cells (DC), appears important in the ability of these antigen-presenting cells (APC) to promote such Type-1 immune responses. In this study, we investigated the regulation of Type-1 polarized responses in T cells by DC ectopically (over)expressing T-bet (DC.T-bet) after transduction with a recombinant adenovirus encoding full-length human T-bet. Naïve T cells primed by DC.T-bet (vs. control DC) produced more IFNγ and less IL-4 in both the $CD4^+$ and $CD8^+$ T cell responder subsets, while simultaneously restraining frequencies of $CD4^+CD25^+Foxp3^+$ T regulatory cells. Somewhat counter-intuitively, DC.T-bet production of IL-12 family member cytokines (i.e. IL-12p70, IL-23 and IL-27) and TNFα were completely abolished, suggesting that promotion of Type-1 immunity functions independently of these inflammatory cytokines. IL-12 independence in DC.T-bet function was confirmed using neutralization antibodies to IL-12p70, which had no effect on the Type-1 polarization of T cell responders. Our preliminary data also suggests that DC.T-bet suppresses Type-2 T cells and $Foxp3^+$ Treg differentiation from naive T cells, making these cells attractive adjuvants for clinic translation in the setting of cancer or chronic infectious disease

2.2 Introduction

DC are antigen-presenting cells with a unique and indispensable ability to induce primary T, B, and NK cell immune responses. However, the complexity and plasticity of DC are governed by different microenvironmental conditions that can induce contrasting states of immunity and tolerance (3; 91; 124). T cell priming involves the interaction of peptide-loaded major histocompatibility complexes (MHC) expressed by DC with the Ag-specific T cell receptor (TCR) on the T cell surface. Costimulatory molecule and cytokine signaling govern polarization and maintenance of T cell proliferation and differentiation, induction of effector and/or helper functions, and generation of memory T cell pools (27; 125; 126). The functional polarization of specific T cells is dictated, in large part, by the profile of cytokines produced by DC upon their engagement with T cells; although limited data also argues for the involvement of co-stimulatory molecules in polarizing responses (91).

Type-1 DC (DC1) activate and polarize αβ-T, macrophages, NK, NKT, and γδ-T cells into Type-1 effector cells that are competent to mediate the clearance of viral infected- and transformed cells, as well as some extracellular parasites (36; 127). Activation of Type-1 T cells is marked by the distinct upregulation of functional IL-12 receptor (IL-12R), accumulation of cytolytic granules (Granzyme-B and Perforin), increased IFNγ and decreased IL-10 and IL-4 cytokine production, and increased expression of the CXCR3 and CCR5 chemokine receptors; all of which are important for effective T cell-mediated clearance of pathogens (127). Additionally, bi-directional crosstalk between $CD8^+$ T cells or NK cells may reinforce Type-1 responses by effectively allowing DC to further promote Type-1 polarized T cell responses (128).

T-bet has been identified as the master regulator of T_H1 commitment in $CD4^+$ T cells and, most recently, a major transcriptional regulator of NK and $CD8^+$ T cell cytokine, cytolytic and migratory functions (35; 38; 39; 54; 55). T-bet acts as a transcription activator for inducing

secretion of IFNγ, suppressing IL-4, promoting CXCR3 expression, and upregulation of the IL-12 receptor β2 chain (IL-12Rβ2) in T cells, NK, NKT and γδ-T cells (38; 52). The ability of any of these cell types to assume functional Type-1 polarization is impaired in mice deficient in T-bet (39; 123; 129). Interestingly, T-bet has been shown to bind the same subset of promoter regions in B, NK, and T cells, albeit with reported differential gene regulation (39; 123; 129). Studies assessing T-bet expression in DC have demonstrated that DC production of IFNγ correlated directly with levels of T-bet expressed in these cells (24). Lack of T-bet expression did not impair the maturation of bone marrow-derived or splenic DC, but this deficiency did impair the ability of DC to activate T_H1 responses *in vivo* (24). Additionally, Wang et al. found that T-bet expression in DC regulated inflammatory cytokines and chemokines production, including IL-1 and MIP-1α (130). Notably, the Glimcher group showed that T-bet expression in DC directly correlated with TNFα production and the development of ulcerative colitis in murine models (131). These findings support a hypothesis that T-bet expression in DC is crucial for the development of Type-1 cell-mediated immunity, although its mechanism of action remains poorly illuminated.

The unique ability of DC to induce and sustain primary immune responses makes them optimal candidates for vaccination protocols (91; 111). DC vaccinations continue to appear safe and although limited clinical responses have been achieved, the optimal type of DC for vaccination still remains to be determined. Challenges include making DC noncompliant to the negative influences of the tumor microenvironment; i.e. retaining Ag-presenting function and Type-1 polarization (3). Therefore, the generation of a “rigid” DC that mediates sustained immunostimulatory functions by expressing high Type-1 differentiating costimulatory molecules and cytokines appears critical to the optimal priming and maintenance of effector T cells exhibiting potent anti-tumor reactivity (109). Furthermore, the reduction and/or absence of regulatory costimulatory molecules and cytokines would also be preferred in order to avoid the development of immune tolerance and/or the induction of Treg cells. The successful completion

of these studies may define a novel format of functionally “locked”’ DC for use in vaccines designed to promote therapeutically meaningful Type 1 immunity.

2.3 Materials and Methods

2.3.1 Adenovirus and Construct

Human T-bet (hT-bet) was PCR cloned from peripheral blood lymphocytes using the following primers: hT-bet: Fwd 5'-GTCGACGACGGCTACGGGAAGGTG-3', Rev 5'-GGATCCTTAGTCGGTGTCCTCCAACC-3'. The product was then digested with the restriction enzymes SalI and BamHI and the 1.7Kb fragment containing full-length hT-bet was ligated into the adenoviral-Cre-Lox (Adlox) vector. After sequence validation, recombinant adenoviruses were generated by co-transfection of Adlox.hT-bet and helper virus DNA into the adenoviral packaging cell line CRE8. The harvested recombinant adenoviral hT-bet (AdhT-bet) was purified by cesium chloride density-gradient centrifugation and subsequent dialysis before storage in 3% threoalose at -80°C. Titers of viral particles were estimated by optical density. The mock (empty) adenoviral vector Adψ5 was used as a negative control. Immature DC were transduced on day 5 of culture at an MOI of 300. Briefly, DC were transduced for 2h at RT, washed twice, and resuspended in AIM-V media (Life Technologies, Inc., Grand Island, NY) supplemented with rhIL-4 (20 ng/ml, Peprotech, Rocky Hill, NJ) and rhGM-CSF (1000 U/ml; Leukine; Amgen Inc., Thousand Oaks, CA) before being incubated for an additional 48h at 37°C under 5% CO_2 tension. Transduction efficiency was 63 ± 18% for all experiments (n = 13) as determined by intracellular staining for T-bet monitored by flow cytometry.

2.3.2. Isolation of donor Dendritic and T cells

DC (> 95% CD11c^{+}CD14^{-}) were generated from plastic-adherent monocytes isolated from the peripheral blood of normal donors and patients with melanoma, with donor consent under IRB-approved protocols, as previously described (132). Non-adherent cells, enriched in T cells, were collected and stored at -80°C for 5-7 days during the DC culture period. After thawing, naïve or memory T cells were negatively-isolated using CD45RO or CD45RA MACS^{TM} microbeads

(Miltenyi Biotec, Auburn, CA), respectively, per the manufacturer's protocols, yielding cell populations of > 98% purity. $CD4^{pos}$ or $CD8^{pos}$ naïve or memory T cell subsets were further isolated by positive-selection using specific MACS™ microbeads.

2.3.3 Dendritic and T cell coculture studies; superantigen (SEB) model

Naïve or memory T cells were plated with SEB pulsed-DC.T-bet or control DC at an E:T ratio of 1:10 in TcMEM. Supernatant of DC-T cell cocultures were collected on day 3 and analyzed for IFN-γ production by ELISA. Additionally, on day 3, $CD4^{pos}$ T cells were separated from DC by MACS™. Total RNA was isolated for RT-PCR analysis or T cells were co-stained with mAbs to CD4, CD212 and T-bet for flow cytometric analysis. In additional studies, naïve or memory T cells cultured with SEB pulsed-DC.T-bet or control DC were restimulated on day 5 with DC.T-bet or control DC supplemented with 20 U/ml of rhIL-2 (Peprotech) and 5 ng/ml of rhIL-7 (Sigma-Aldrich). T cells were replenished with TcMEM supplemented with IL-2 and IL-7 every other day. On days 12 or 14, cells were collected and assayed for cytokine (IFN-γ, IL-4, and IL-10), cell surface (CXCR3), and intracellular (Foxp3 and Granzyme-B) protein expression by flow cytometry. To evaluate intracellular cytokine expression, cells were stimulated with PMA (1 μg/ml; Sigma-Aldrich) and Ionomycin (10 ng/ml; Sigma-Aldrich) for 4h, with 2 μM monensin (Sigma-Aldrich) added over the final 2h of culture. For CFSE proliferation assays, Sorted $CD45RO^{neg}$ (naïve) or $CD45RA^{neg}$ (memory) T cells were labeled with 1.5 μM of CFSE (Sigma-Aldrich) in PBS for 15 minutes at 37°C. T cells were then washed twice and resuspended in TcMEM for 30 min at 37°C. 1×10^5 T cells were added to DC in a final concentration of 100 U/ml of rhIL-2 (Peprotech) in TcMEM. Responder T cells were evaluated on day 3 for CFSE dilution by three-color flow cytometry performed on CSFE-labeled cell using IFNγ and CD3 mAbs.

2.3.4. Antigen Specific T cell studies

Following monocyte separation, non-adherent cells were isolated from PBMC and cryopreserved until needed. DC.T-bet or control DC were prepared as afore mentioned. Prior to coculture with T cells, DC were pulsed with tumor antigenic class I A2-restricted peptides: gp100 (209–217), Tyrosinase (368–376D), or EphA2 (288-296) for 3 h at 37°C in AIM-V medium (GIBCO-Invitrogen, Carlsbad, CA). Naïve CD8 T cells were isolated by first depleting the memory subsets using CD45RO microbeads (Miltenyi; MACS^{TM}) and further positively selected for CD8+ T cell subsets using the respected CD8 microbeads (Miltenyi). DC.T-bet or control DC were plated with autologous CD8+ T cells at a 1:10 in T cell medium containing 5ng/ml of IL-7. After 7 days, T cells were restimulated with either the respected DC.T-bet or control DC, immature DC, or irradiated (at 5000 rads) PBMC supplemented with 20 U/ml of IL-2 and 5ng/ml of IL-7 for an additional 7 days prior to analysis of antigen-specific T cell responses.

2.3.5. Readout Assays

2.3.5.1. ELISPOT

On day 14 of antigen specific-T cell assays, the frequencies of peptide-specific CD8+ T cell responders were measured using anti-human IFN-γ ELISPOT assays, as previously described. CD8^{+} T cells were added to ELISPOT wells at a 5:1 ratio of T cell to antigen presenting cell, to which we used T2 immortalized cell line, which expresses class I A2 and class II DR4 MHC molecules. EphA2, gp100, or Tyrosinase peptides, as afore mentioned, were added at a final concentration of 10 µg/ml. ELISPOT plates were incubated at 37°C for 24h prior to development and evaluation using an ImmunoSpot automatic plate reader (Cellular Technology Ltd., Cleveland, OH), as previously reported. The number of peptide-specific CD8^{+} T cell responders were statistically compared to the background number of IFNγ spots produced by T cells in

response to APCs pulsed with HIV peptides. Positive control wells contained T cells and 10 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich).

2.3.5.2. Flow Cytometry and Immunohistochemical Analysis

For cell surface staining using flow cytometry, cells were collected and re-suspended at 2 x 10^5 cells/100 µl in 96-well V-bottom plates in PBS with 2% BSA and 0.2% NaN_3 (FACS buffer), then washed twice before blocking in 3% human serum. Antibody staining was performed for 30 minutes at 4°C at a dilution of 1:20, after which, cells were either analyzed by flow cytometry or resuspended in Fix/Perm buffer (eBioscience) for subsequent intracellular staining. Briefly, for intracellular staining, cells were washed in 1X permeabilization buffer (eBioscience) prior to blocking with 10% human serum for 10 minutes at 4°C. Antibody staining was performed at 4°C for 45-60 minutes. Cells were then washed twice in 1X permeabilization buffer, resuspended in FACS buffer, and analyzed by flow cytometry. For immunofluorescence microscopy, 1 x 10^5 DC were cytospun, fixed onto slides and co-stained with anti-T-bet (Santa Cruz Biotechnologies), nuclear dye (DAPI), and rhodamine phalloidin. Fluorescence images were then captured using an Olympus BX51 microscope (Olympus America, Melville, NY).

2.3.5.3. Reverse Transcriptase-PCR

For mRNA analysis, DC were harvested on day 2 (48h post-transduction) and MACS^{TM} isolated naïve or memory CD4^{pos} T cells were harvested on day 3 after initial priming by DC. RNA was isolated with Trizol (Inivitrogen, Carlsbad, CA). Reverse transcription was performed using MuLV reverse transcriptase (Applied Biosystems, Carlsbad, CA) and Random Hexamers (Applied Biosystems). Semi-quantitative PCR was used to amplify cDNA for expression of gene-specific products. Specific primers were used for IL-12p35, IL-12/23p40, IL-23p19, IL-27p28, IL-27pEBI-3, IL-15, IL-18, IL-10, TGFβ, IFN-α, and IFN-γ as previously described(132). Additional primer sequences included: T-bet: Fwd 5'-CCACCAGCCACTACAGGATG-3' and Rev 5'-

GGACGCCCCCTTGTTGTTT-3'; GATA-3: Fwd 5'-GTGCTTTTTAACATCGACGGTC-3' and Rev 5'- AGGGGCTGAGATTCCAGGG-3'; Foxp3: Fwd 5'-GCACCTTCCCAAATCCCAGT-3' and Rev 5'-TAGGGTTGGAACACCTGCTG-3'; and RORγt: Fwd 5'-AAATCTGTGGGGACAAGTCG-3' and Rev 5'-TGAGGGTATCTGCTCCTTGG-3'. β-actin primers were used as an internal positive control (132).

2.3.5.4. Western Blotting

DC were harvested 24h after adenoviral transduction. Total cellular protein was collected at 1 x 10^5 DC per 50µl as lysate prior to subjection onto SDS-PAGE. The gel was next transferred onto a nitrocellulose blot, prior to staining with primary antibodies to T-bet, Bcl-xL, Bcl-2, Mcl-1 or Survivin (Santa Cruz Biotechnologies) overnight. After washing in 5% PBS\Tween, blots were stained with secondary antibodies for 1 hour conjugated to horse radish-peroxidase (HRP) and subsequently assessed. β-actin levels were analyzed by treating the blots with 2% NaN_3 (Sodium Azide) for 1 h in the presence of primary Abs to β-actin generated from different species as the primary Abs of the protein of interest (i.e. T-bet, Bcl-xL, Bcl-2, Mcl-1 or Survivn) analyzed. After overnight incubation, blots were washed and stained with secondary Abs conjugated to HRP and analyzed.

2.3.5.5. Neutralization Studies

Neutralizing anti-hIL-12p70 polyclonal Ab (pAb; R&D Systems), anti-hIL-12Rβ2 pAb (R&D Systems) anti-hIFNγ pAb (R&D Systems), anti-hIFNγR1 pAb (R&D Systems), anti-hIL-23 pAb (R&D Systems), anti-IL-27R pAb (TCCR/WSX-1; R&D Systems), and anti-CD27 pAb (R&D Systems) were used at a final concentration of 10 μg/ml. Additionally, Notch-Fc was used at a final concentration of 10μg/ml. Briefly, DC.T-bet or control DC were plated with naïve or memory T cells at a DC:T cell ratio of 1:10 in triplicate in 96-flat bottom plates in the presence or

absence of blocking Abs. On day 3, cell-free supernatants were collected and evaluated using IFN-γ ELISA.

2.4. Results

2.4.1. Phenotype of T-bet expressing DC (DC.T-bet)

Human DC were generated from monocytes via culture in rhIL-4 and rhGM-CSF for 5 days prior to being transduced with recombinant adenovirus encoding human T-bet (DC.T-bet) or control Ad.ψ5 (DC.ψ5) for a subsequent 48h period. DC generated under known Type-1 polarizing conditions were also assessed for endogenous T-bet expression. Harvested DC was analyzed for T-bet mRNA (via reverse transcriptase-PCR; **Fig. 1A**) and protein expression (via western blot and flow cytometry; **Fig. 1B and 1C**). As shown in **Fig. 1A**, T-bet expression in untreated immature DC (DC.null) and DC.ψ5 was very low (at both the transcript and protein levels), with modest expression levels augmented by culture with inflammatory cytokines (i.e. IFN-α, IFN-γ, TNF-α for DCα1; (95)); IL-12 for DC-IL12 (133); IFN-γ for DC-LPS/IFN-γ, TLR ligands (poly I:C for DC1α; LPS for DC-LPS/IFN-γ), a macrocyclic lactone known to mature DC (bryostatin-1 for DC-BS1; (134)), or infection with AdhT-bet, which yielded a DC population that was approximately 70% T-bet positive (**Fig. 1C**). Notably, confocal immunofluorescence microscopy analyses revealed that T-bet was expressed predominantly in the nucleus of DC.T-bet cells (**Fig. 1D**).

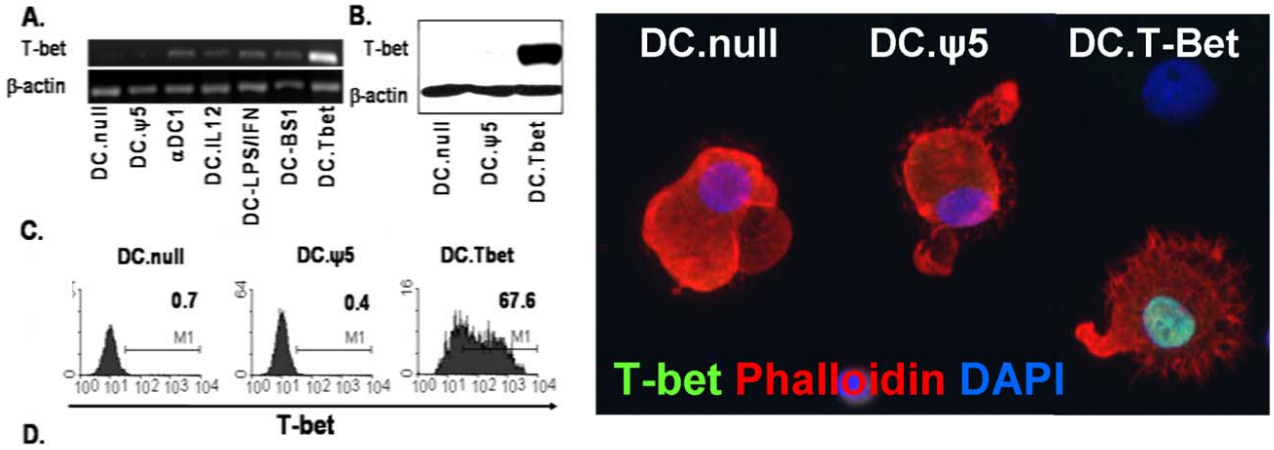

Figure 1.T-bet expression in DC. Adenoviral construct encoding human T-bet was transduced into monocyte-derived DC. (A) T-bet expression was evaluated by reverse transcriptase PCR in control DC (DC.null and DCψ5), alpha DC1 (Wesa et al), DC adenovirally transduced with human IL-12, DC stimulated with LPS and IFNγ, DC stimulated with 1ng/ml of bryostatin-1 for 4h, and T-bet transduced DC. Protein expression was confirmed in DC.T-bet by (B) western blotting and (C) flow cytometry. (D) T-bet localized to the nucleaus in T-bet transduced DC.

Confirming the protein presence and localization of T-bet to the nucleus of DC, we next evaluated changes in co-stimulatory molecule and cytokine profiles as a consequence of downstream signaling by T-bet expression in DC. Flow cytometric analysis, via co-staining of T-bet with variable co-stimulatory\inhibitory molecules, revealed little-to-no changes in mean fluorescent intensity (MFI) levels compared to backbone transduced DC (DC.ψ5) or non-transduced DC (**Fig. 2A**). Although we found T-bet positive DC to express elevated levels of CD11c, CD80, CD86, and ICAM-1, within the transduced cohort, subsequent analysis with adenoviral GFP revealed no marked differences (**data not shown**), suggesting that the actual adenoviral vector was selective in infection of DC within a cohort. Analysis of mRNA transcriptional profile revealed little to no changes in the IL-12 family of cytokines, including the shared IL-12\23p40, IL-12p35, IL-23p19, and IL-27 EBI3 subunits (**Fig. 2B)**. However, we found IL-27p28 subunits to be restrained in expression, suggesting that IL-27 protein expression would be abolished. We did not find DC.T-bet to express IFNγ as investigated at the mRNA

(**Fig. 2B**) or protein level (**data not shown**). Additionally, evaluation of IL-10, TGFβ, IL-18, and IL-15 revealed no differences between the control DC and DC.T-bet (**Fi.g 2B**). However, evaluation of protein expression varied greatly from observed mRNA transcription. DC.T-bet had completely abolished expression of IL-12p70, IL-23p19, and TNFα, which are Type 1 and Type 17-differentiating and supporting cytokines for T cells polarization (**Fig. 2C**). Additionally, we found IL-10 and IL-6 to also be completely abolished in DC.T-bet (**data not shown**). It has been reported that cytokines, most notably IL-2, IFNγ, and TNFα in T cells, are regulated post-transcriptionally through various mechanisms, including mRNA degradation, protein recruitment for silencing, and destabilization (135), suggesting a potential mechanism of cytokine regulation in DC.T-bet at the transcription level. Finally, evaluation of survival and anti-apoptotic proteins associated with DC by western blot revealed no significant changes in DC.T-bet compared to control DC (**Fig. 2D**).

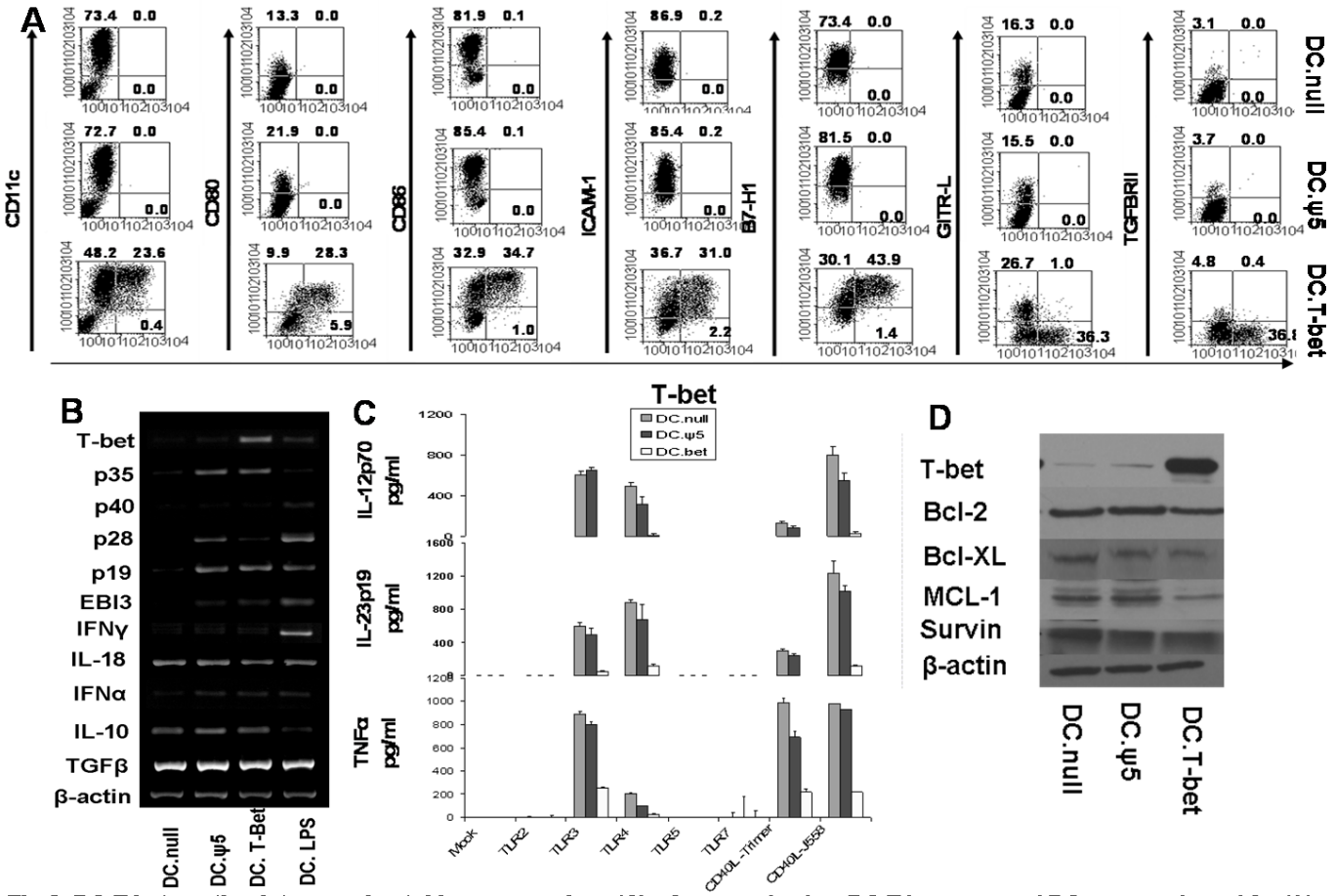

Fig 2. DC.T-bet costimulatory and cytokine expression. 48h after transduction, DC.T-bet or control DC were evaluated for (A) costimulatory\ coinhibitory molecule expression. (B) mRNA was isolated from DC and evaluated for changes in transcription levels of various cytokines. LPS stimulated DC (DC.LPS) was additionally included as a control group. (C) DC were stimulated with various TLR or CD40 agonists and measured for IL-12p70, IL-23p19, or TNFα production by ELISA assays. (D) Western Blot was performed on pro- and anti-apoptotic molecules in DC.T-bet or control DC

2.4.2. Early Type 1 T cell responses induced by DC.T-bet

Differential DC.T-bet expression of cytokines did suggest that the shape of the T cell response may be modulated. To investigate T cell responses, I chose to use the superantigen model, employing staphylococcus enterotoxin B fragment, to bridge the MHC of DC.T-bet or control DC and the TCR of responding T cells. This bypassed the necessity for antigen specificity, allowing the differential expression of costimulatory, adhesion, and accessory molecules, along with cytokine profile expression, to dictate T cell responses. Furthermore, work by Kalinski et al and Kapsenberg, among others, have shown that the SEB-superantigen

model, under low doses, is unable to skew T cell responses; thereby acting solely as a bypass bridge to initial signal 1 of MHC:TCR engagement (136; 137). Briefly, DC.T-bet or control DC was pulsed with 1 ng/ml of SEB in AIM-V medium in 37°C for 3 h. Autologous T cells were isolated from non-adherent PBMC using MACS^{TM} isolation. Naïve T cells were attained by depleting memory cell subsets using CD45RO microbeads, whereas memory T cells were isolated by depleting naïve cell subsets with the CD45RA microbeads. The choice for depletion was to allow further positive isolation of naïve or memory CD4^{+} or CD8^{+} T cell populations. DC.T-bet or control DC and naïve or memory T cells were plated in a 96-Flat bottom plate at a ratio of 1:10, respectively, in T cell medium. Harvest of supernatant on day 3 revealed a 4 fold increase in IFNγ in the naïve (CD45RO depleted) T cell subsets, but no change in the memory subsets (**Fig. 3A**). It is important to note again that DC.T-bet showed no differential expression of IFNγ mRNA transcription as assessed by RT-PCR (**Fig. 2B)**, nor presence of IFNγ protein expression by ELISA, even after treatment with TLR or CD40 agonists (**data not shown**). I next wanted to evaluate additional early T cell polarization by assessing "master" T helper subset transcriptional factors. DC.T-bet or control DC were cultured with naïve CD4^{+} (CD45RO^{neg}CD4^{+}; MACS^{TM} isolation of CD4 positive cells from depleted CD45RO^{neg} group) T cell subsets for 3 days. Total cells were collected and further purified by using CD3 microbead isolation via MACS^{TM}. Greater than 98% of isolated cells were CD3^{+}CD4^{+} T cells (**data not shown**). RNA was extracted from these purified CD3^{+}CD4^{+} T helper cell subsets and analyzed for expression of the polarizing transcription factors: T-bet (T_H1), GATA-3 (T_H2), RORγt (T_H17), and Foxp3 (T regulatory). DC.T-bet expanded T cells had much higher levels of T-bet with a concomitant decrease in GATA-3 and RORγt compared to control DC expanded T cells (**Fig. 3B**). I confirmed greater T-bet expression on the protein level in DC.T-bet primed naïve T cells by performing flow cytometry analysis, gating on CD4^{+} T cell subsets co-stained with T-bet and IL-12Rβ2 expression (**data not shown**). Also of interest, we found an increase in cluster size of DC.T-bet primed T cells compared to control DC primed T cells (**Fig. 3C**). Recent literature

has shown that T cell clusters are important for paracrine secretion and signaling of IL-2 to promote Type 1 polarization and T cell responses (138).

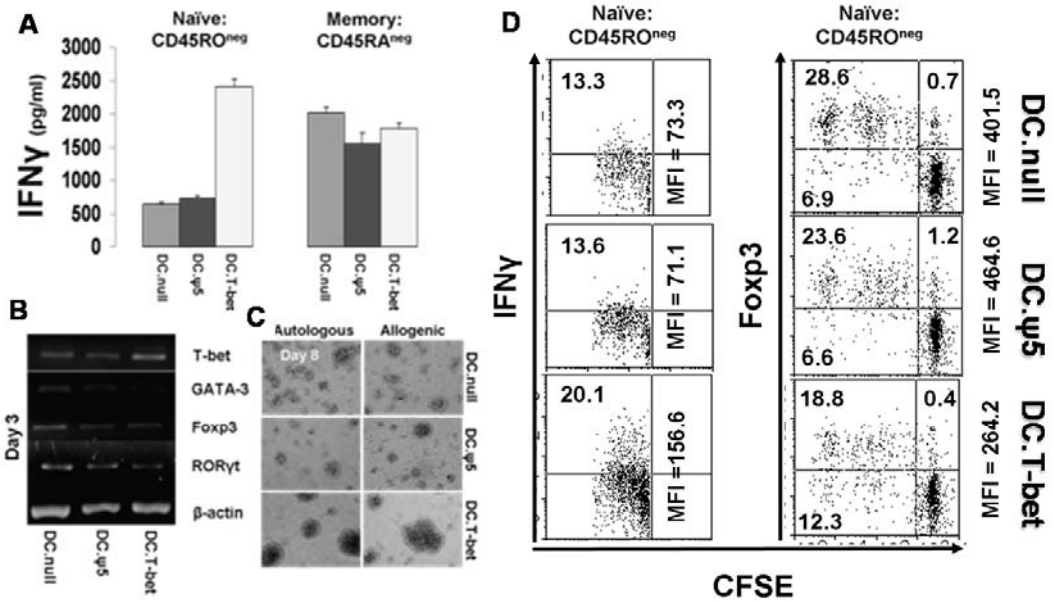

Figure 3. Early Type 1 polarization in naïve T cells primed by DC.T-bet. DC.T-bet and control DC were cocultured at a 1:10 ratio with naïve or memory T cells and assessed on day 3 for T cell responses. (A) DC.T-bet primed naïve, but not memory, T cells expressed elevated levels of IFNγ production compared to control DC primed T cells. (B) Isolation of CD4+ T cells after 3 day culture with DC.T-bet revealed a skew towards Type 1 polarization by enhancing upregulation of T-bet and downregulation of GATA-3 and RORγt as assessed by RT-PCR and normalized to beta-actin levels. (C) DC.T-bet expanded T cells had fewer, but larger cluster aggregates of responding T cells in both autologous and allogenic settings. (D) CFSE proliferation co-stained with anti-IFNγ (on day 3) and anti-Foxp3 (on day 5) revealed that proliferation rates were equal to control DC primed T cells, but that DC.T-bet primed naïve T cells expressed greater frequency and MFI level of IFNγ and reduced levels of Foxp3 co-expression with CFSE.

Furthermore, it was to be delineated whether DC.T-bet induced higher levels of IFNγ production or, alternatively, expanded a greater pool of IFNγ producing cells by enhancing proliferation status. CFSE-labeled naïve or memory T cells were cultured with DC.T-bet or control DC for 3 days, prior to co-staining cells with IFNγ to directly assess proliferation of responding T cells co-expressing IFNγ. Initial priming of naïve T cells with DC.T-bet did not yield a greater proliferation advantage compared to control DC expanded T cells (**Fig. 3D)**. However, it was clear that DC.T-bet expanding T cells had both higher frequency and MFI levels of IFNγ production, suggesting that a greater proportion of T cells in the initial culture were enforced to express *higher* levels of IFNγ (**Fig. 3D and Fig. 4**). As to be expected, memory T cells were unable to

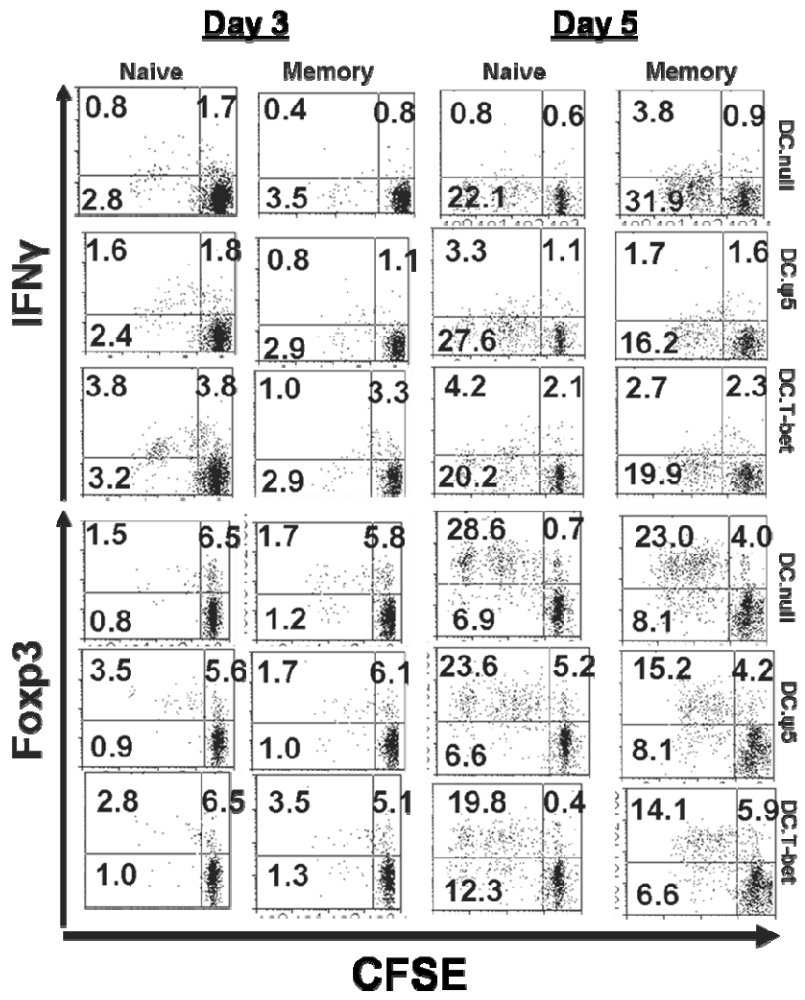

Figure 4. DC.T-bet primed T cells begin to express lower levels of Foxp3 at day 5. DC.T-bet or control DC were cultured with CFSE labeled autologus naïve or memory T cells in 96-round bottom plates in the presence of 100U/ml of IL-2. Cells were collected on days 3-5 and assessed for (A) IFNγ production or (B) Foxp3 expression against CFSE dilution

be repolarized to a greater proportion of IFNγ producing cells. Not surprising, Foxp3 level of expression didn't change at the day 3 time point (**Fig. 3D and Fig 4)**, corroborating recent findings that Foxp3 expression is an early T cell activation marker (139). However, at day 5, Foxp3 expression in DC.T-bet primed naïve T cells began to *diverge* from control DC primed and proliferating T cells as assessed by co-staining Foxp3 versus CFSE dilution **(Fig. 4)**. These data sets led us to question the effect that DC.T-bet has on T cell polarization, and whether there was a concomitant increase in Type 1 and reduction in IL-4$^+$ T_H2 and Foxp3$^+$ Treg subsets.

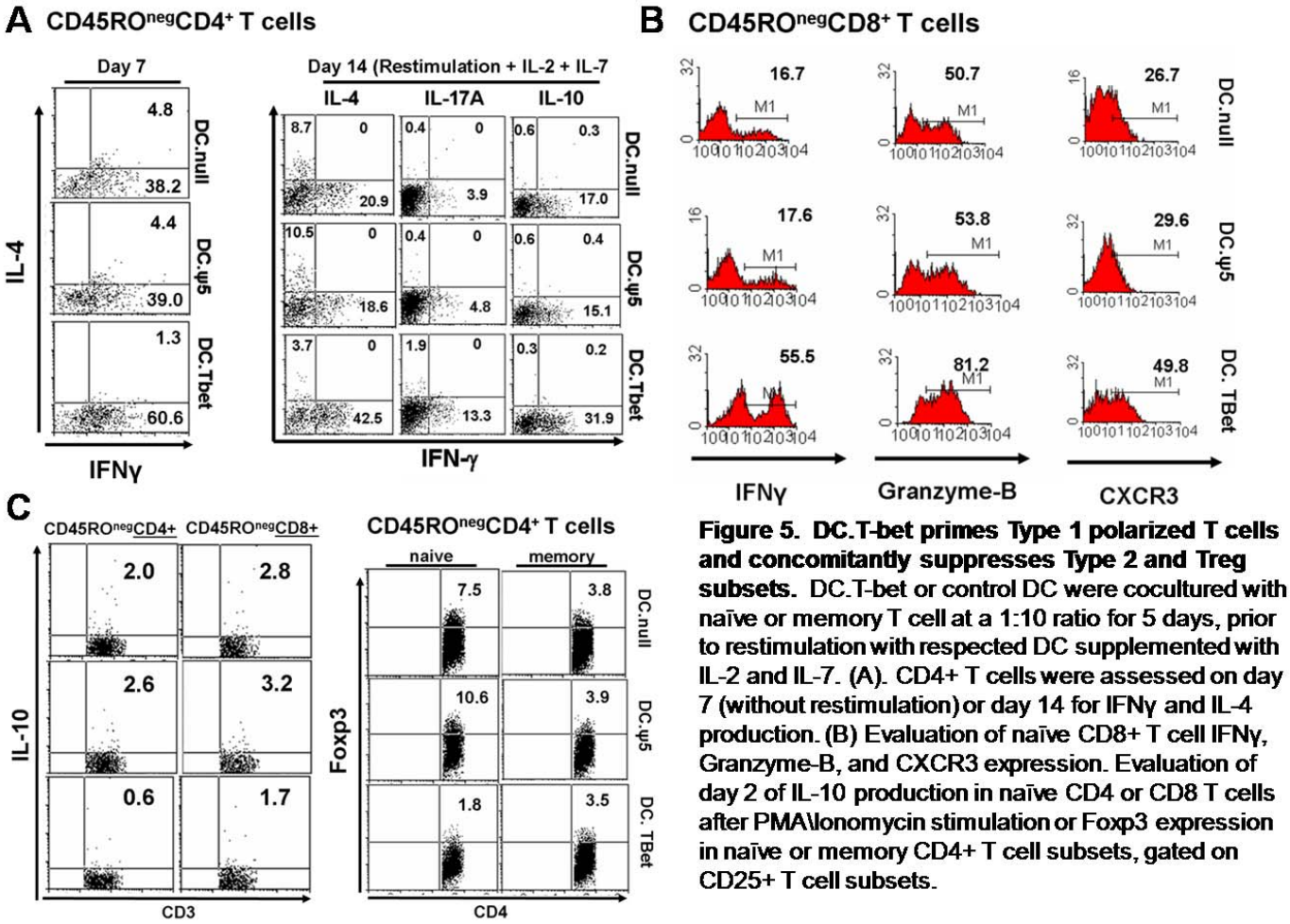

Figure 5. DC.T-bet primes Type 1 polarized T cells and concomitantly suppresses Type 2 and Treg subsets. DC.T-bet or control DC were cocultured with naïve or memory T cell at a 1:10 ratio for 5 days, prior to restimulation with respected DC supplemented with IL-2 and IL-7. (A). CD4+ T cells were assessed on day 7 (without restimulation) or day 14 for IFNγ and IL-4 production. (B) Evaluation of naïve CD8+ T cell IFNγ, Granzyme-B, and CXCR3 expression. Evaluation of day 2 of IL-10 production in naïve CD4 or CD8 T cells after PMA\Ionomycin stimulation or Foxp3 expression in naïve or memory CD4+ T cell subsets, gated on CD25+ T cell subsets.

2.4.3. Concomitant induction of Type 1 and suppression of Type 2 and T regulatory cells

Early T cell responses showed that DC.T-bet primed naïve T cells elicited potent induction of IFNγ production, with associated increases in T-bet expression with concomitant decreases in GATA-3 (mRNA) and Foxp3 (protein; day 5 analysis). However, functional differentiation, or polarization, of T cells is generally evaluated after additional restimulation and evaluation post-14 days after primary stimulation. Furthermore, rigid cytokine profile and additional Type 1-associated markers and effector functions, including chemokine receptors, such as CXCR3 (Type 1-associated) or CCR4 (Type 2-associated), late antigen receptors, such

as VLA-4 (Type 1-associated), and effector molecules, such as granzyme-B, Fas-ligand and perforin (Type 1-associated) can arise at later time points. In these series of studies, DC.T-bet or control DC were co-cultured with naïve or memory T cells for 5 days, prior to restimulation with respected SEB pulsed-DC supplemented with IL-2 and IL-7. T cell cytokine profiles were evaluated by ELISA and flow cytometry on days 7 and 14 upon PMA and Ionomycin restimulation in the presence of Monensin, an inhibitor of cellular secretion. For non-cytokine profiles (such as surface-bound proteins or intracellular granules), T cells were analyzed by flow cytometry on days 14 or 21. Flow cytometry was performed by co-staining and gating on CD8 or CD4 T cell subsets from bulk naïve ($CD45RO^{neg}$) or memory ($CD45RA^{neg}$) and staining for cytokines or cell surface molecules. In analogous studies, naïve or memory T cells were further isolated for $CD4^+$ or $CD8^+$ T cell subsets to assess the direct effect of DC.T-bet on each cell subset. DC.T-bet increased the frequency of IFNγ producing $CD45RO^{neg}CD4^+$ T cells as early as day 7 and on day 14 (**Fig. 5A**), while concomitantly decreasing IL-4, IL-17A and IL-10 production (**Fig. 5A).** DC.T-bet also augmented $CD8^+$ T cell IFNγ, CXCR3 and Granzyme-B production from naïve precursors (**Fig. 5B)**. Finally, DC.T-bet skewed T cells away from T regulatory phenotype, as indicative of the restrained IL-10 production (indicative of Tr1-like cells) and $CD4^+CD25^+Foxp3^+$ T regulatory cell subsets (**Fig. 5C**). Cumulatively, these data sets show that DC.T-bet enforced a Type 1 polarization that overrides induction of Type-2 or T regulatory phenotypes.

2.4.4 Antigen specific-Type 1 polarization

My studies with DC.T-bet up to this point have used an artificial system to engage TCR:MHC for priming responder T cells. Although the superantigen system has been shown to be unbiased, and I have included DC.null and DC.ψ5 expanded T cells as internal controls in all experiments, it was left to be seen if DC.T-bet can impart Type 1 polarization using cognate

MHC:peptide engagement of responding naïve T cells via TCR recognition and activation. Briefly, DC.T-bet or control DC were pulsed for 3h in 37°C with gp100, tyrosinase, and EphA2 HLA-A2 class (MHC Class I) restricted peptides. DC were then washed twice and plated with autologous naïve $CD8^+$ T cell (CD45RO depleted CD8 positive isolated) at a ratio of 1:10 in T cell medium supplemented with IL-7. 7 days later, T cells were restimulated with either peptide pulsed (respected)-DC.T-bet or -control DC, peptide pulsed-immature DC, or peptide pulsed-irradiated bulk PBMC supplemented with IL-2 and IL-7. On day 14, T cells were collected and plated in triplicate with HLA-$A2^+DR4^+$ restricted-T2 cell (a cell line abolished in ability of TAP1, and thus unable to process and present antigen) in presence of individual peptides for gp100, EphA2, or HIV (negative control) peptides onto an anti-IFNγ stained nitrocellulose 96-flat bottom plate (Millipore) in AIM-V for ELISPOT analysis or in a 96-Flat bottom plate (non-coated, non-stained) for collection of supernatant for ELISA. 24h later, the nitrocellulose plate was developed for ELISPOT analysis and the frequency of spots was detected using Immunospot detection analysis. DC.T-bet induced both a higher frequency and antigen-specific T cell subsets primed against EphA2 and gp100 upon restimulation with DC.T-bet, iDC, or irradiated PBMC, compared to control DC expanded T cells (**Fig. 6A**). After 3 days of culture in the non-coated, non-stained plate, supernatant was collected and analyzed for IFNγ expression by ELISA. DC.T-bet antigen-specific responding T cells showed higher levels of total IFNγ production upon stimulation with specific peptide, paralleling ELISPOT data sets (**Fig 6B**). Analogously, DC.T-bet or control DC primed T cells, on day 14, were restimulated with T2 cells pulsed with gp100, Tyrosinase, MART-1, or EphA2 for 24 h, with the final 6 h of culture supplemented with 2 μM of monensin. T cells were collected and co-stained with respected tetramer and ant-IFNγ, prior to flow cytometric analysis. DC.T-bet produced a two fold increase in the frequency and MFI of IFNγ in both MART-1 and EphA2 antigen-specific $CD8^+$ T cell responders (**Fig. 6C**). Thus DC.T-bet primes a greater proportion of naïve CD8 T cells to

become IFNγ producers in an antigen specific manner, analogous to the data achieved using the superantigen model system.

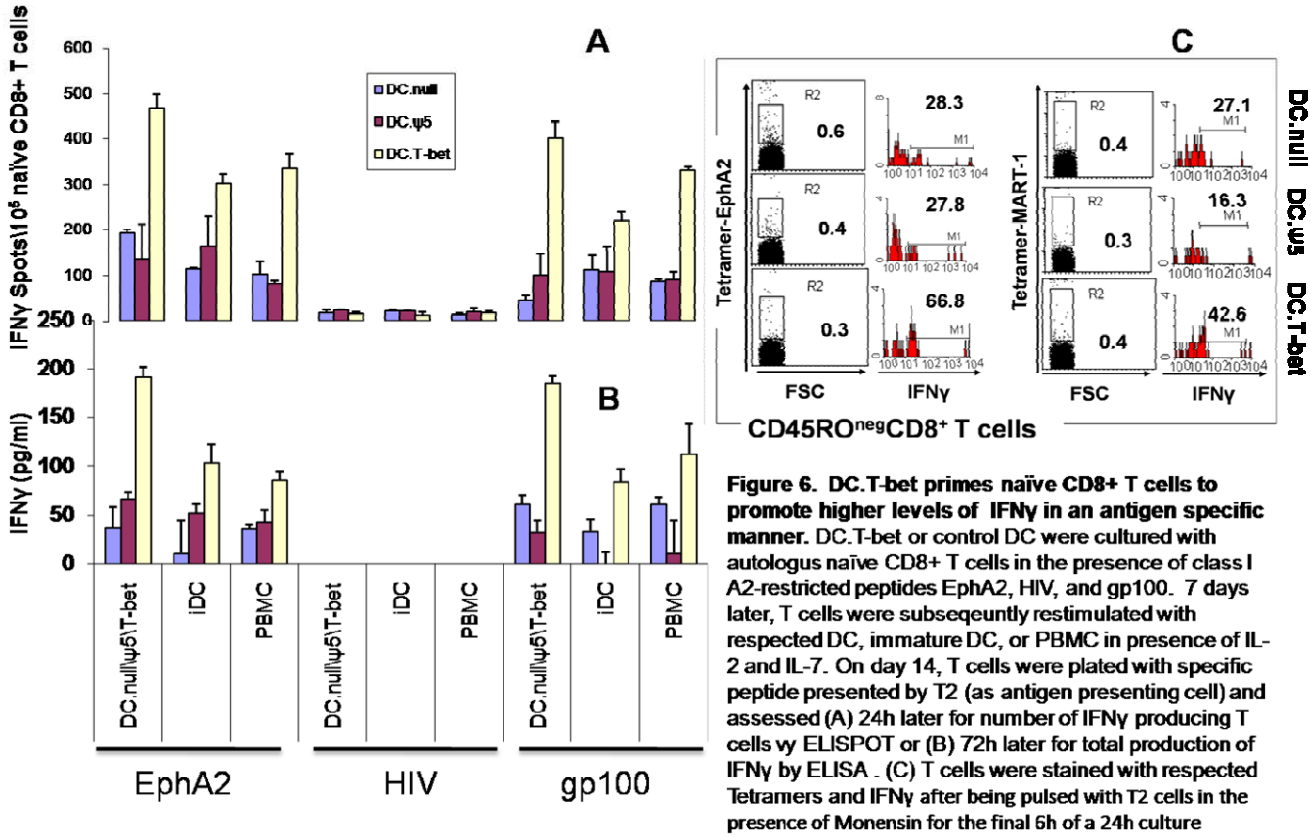

Figure 6. DC.T-bet primes naïve CD8+ T cells to promote higher levels of IFNγ in an antigen specific manner. DC.T-bet or control DC were cultured with autologus naïve CD8+ T cells in the presence of class I A2-restricted peptides EphA2, HIV, and gp100. 7 days later, T cells were subseqeuntly restimulated with respected DC, immature DC, or PBMC in presence of IL-2 and IL-7. On day 14, T cells were plated with specific peptide presented by T2 (as antigen presenting cell) and assessed (A) 24h later for number of IFNγ producing T cells vy ELISPOT or (B) 72h later for total production of IFNγ by ELISA . (C) T cells were stained with respected Tetramers and IFNγ after being pulsed with T2 cells in the presence of Monensin for the final 6h of a 24h culture

2.4.5. IL-12-and IFNγ-Independent Type 1 Polarization of T cells

After confirming DC.T-bet induction of Type 1 polarization, both in the superantigen and antigen-specific model systems, I next set to identify the mechanisms that impart Type 1 polarization to responding T cells. Initial TCR:MHC engagement along with costimulatory molecule signaling, such as CD80 or CD86 engagement with cognate CD28 (or CTLA-4) receptor, is critical for full T cell activation (9). However, the presence (or absence) of *polarizing*

cytokines, such as IL-12, IFNγ, IL-27, IL-6, IL-10 and\or TGFβ dictate the polarization state (of specific T helper differentiation) in responding naïve T cells (9). Confounding, the primary candidates such as IL-12p70, IL-27, and IFNγ (**Fig 2B and 2C)** expression were absent in DC.T-bet. This led me to question whether DC.T-bet produces a novel cytokine that functions as a potent Type 1 inducing trans factor. To test this, I performed transwell studies. Briefly, 5 x 10^5 DC.T-bet or control DC were co-cultured in the bottom chamber of a transwell plate for 24h with 1 x 10^6 naïve T cells in the upper chamber. 1 x 10^5 non-transduced SEB pulsed-DC (or bystander DC) or 1 x 10^5 anti-CD3\CD28 microbeads (dynabeads; Invitrogen) were added to the top chamber as stimulating agents. Even with five times the number of DC.T-bet to bystander DC, DC.T-bet was unable to induce T cells towards Type 1 immunity when separated by a transwell (**Fig 7A**). These results suggested that DC.T-bet does not impart Type 1 induction via distal, or trans, mechanisms.

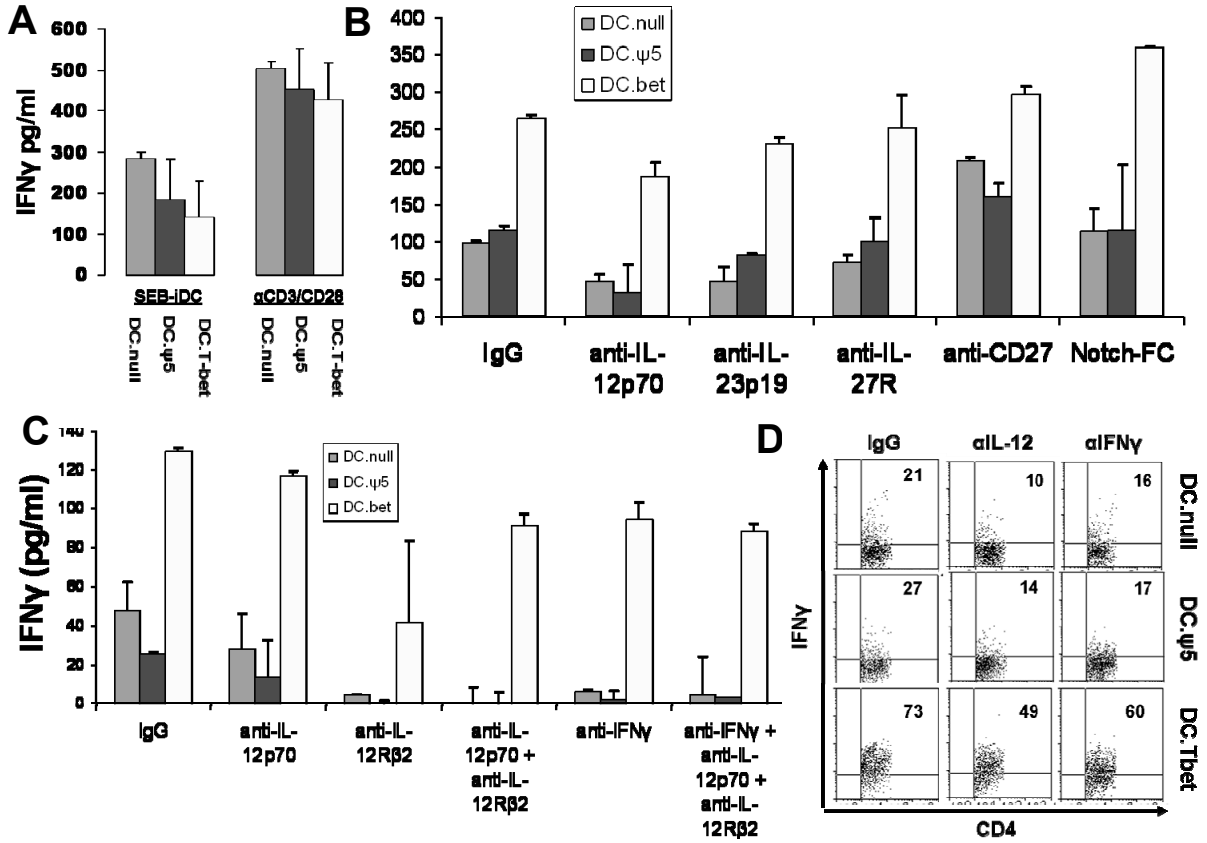

Figure 7. DC.T-bet primes Type 1 polarized T cells via IL-12- and IFN- independent mechanisms. (A) DC.T-bet or control DC were plated in the bottom chamber of a transwell plate for 24 h prior to addition of (non-transduced immature DC pulsed with SEB and naïve T cells. After 48h, supernatant was collected and analyzed for IFNγ production. **(**B-C**)** DC.T-bet or control DC were cocultured with naïve or memory T cell at a 1:10 ratio for 3 days in the presence or absence of indicated neutralizing Abs. (D) DC.T-bet or control DC primed T cells in presence of IgG controls, anti-IL-12p70 + anti-IL-12Rβ2 (αIL-12) or anti-IFNγ and anti-IFNγR1 were evaluated for intracelular IFNγ expression on day 14, after restimulation on day 5 with respected DC supplemented with IL-2 and IL-7

Neutralizing Abs were used to confirm that the polarizing cytokines, IL-12, IL-23, IL-27 were not involved in DC.T-bet induction of Type-1 immunity. Other novel *cis* acting mechanisms of Type 1 induction in T cells that act independent of IL-12 have been shown, including CD27-CD70 engagement (140) or delta like-4 (DLL4) engagement with cognate notch ligand (141). Neutralization of the CD27 receptor and addition of the notch-Fc ligand to block CD27-CD70 and DLL4-Notch engagement, respectively, was unable to alleviate Type 1 polarization induced by DC.T-bet in responding naïve T cells (**Fig 7B**). Although T-bet expression in T cells is IL-12

independent, its expression is STAT1 dependent upon engagement of IFNγ-IFNγR\STAT1 and\or TCR:MHC signaling. Early data sets show that DC.T-bet induced-higher levels of T-bet and IL-12Rβ2 expression in responding naïve T cells compared to controls (**Fig 3C**), however we were unsure if this upregulation and production of IFNγ was dependent on initial IFNγ signaling via STAT1. Therefore, I neutralized interactions of IFNγ-IFNγ receptor by addition of anti-IFNγR1, which did not disrupt the ability of DC.T-bet to promote Type 1 polarization (**Fig. 7C and 7D**). Interestingly, this also suggest that the autocrine feedback signaling of IFNγ receptor by activated T cells may not be necessary to promote Type 1 polarization, but that DC.T-bet guidance directed T cells towards Type 1 (including upregulation of T-bet in T cells) polarization in an IFNγ- and IL-12-independent mechanism. Because of the redundancies and complexities of IFNγ-IFNγR signaling and T-bet, additional studies will need to be performed to highlight the role of IFNγ in DC.T-bet priming of naïve T cell responses.

2.5. Discussion

Previous studies using T-bet knockout DC to prime wild type T cells showed an impaired Type 1 immune response, principally measured by IFNγ, in an *in vivo* mouse model (24). In another study, TLR stimulation, primarily by CpG, to induce full activation and induction of Type 1 immunity was impaired in T-bet knockout-DC (56). This suggested that natural, endogenous expression of T-bet in dendritic cells played a significant role in modulating Type 1 polarized T cell responses. In these studies, I investigated T-bet's role in human DC by ectopically overexpressing T-bet via an expression vector and assessed T cell response *in vitro*.

T-bet transduction into DC via adenoviral vectors yielded an efficiency of 66-80% T-bet^{+} DC within the transduced cohort. In assessing the DC phenotype, little-to-no observable changes in costimulatory molecules were identified; however the cytokine profile expressed by DC.T-bet would have suggested impaired Type 1 immunity, as the IL-12 family related cytokines (IL-12p70, IL-23, and IL-27) were all abolished. Utilizing multiple TLR agonists and CD40 agonists, which have shown *in vitro* the ability to rapidly produce inflammatory cytokines in DC, achieved little-to-no expression of IL-12 family-related cytokines. However, more confounding was that the mRNA expression levels didn't correlate directly to protein secretion levels, suggesting that T-bet expression in DC may promote post-transcriptional or translational modification of cytokines, as has been shown in previous studies (135). Notably, post-transcriptional regulation of cytokines has been well documented within T cells and shown that activation under different polarizing cues can induce various mechanisms of post-transcriptional regulation. Additional, albeit less examined, roles of post-transcriptional regulation have also been highlighted in DC (142). Alternatively, the inconsistency (predominately in analysis of mRNA expression) could be a result of the heterogeneous makeup of T-bet expressing and non-Tbet expressing DC within the evaluated transduced DC.T-bet cohort (i.e. approximately 60% of DC expressed T-bet). Therefore, future studies isolating for a pure population of T-bet positive DC should be utilized to improve the consistency in DC phenotype studies. It also

should be noted that mRNA expression profile represents a snapshot in time of the dynamic kinetic events of cytokine transcription, translation, and secretion in DC. ELISA analysis represents the culmination of captured cytokine over time. mRNA analysis of p35 expression in DC.LPS represents 24h post stimulation, and its lower levels most likely represent the exhaustion and\or induction of negative feedback mechanisms to turn off p35 mRNA expression at the time point evaluated. Other indexes, such as IL-27p28, EBI3, IL-p12p40, and IL-10 serves as additional internal controls to which we could compare DC.T-bet to TLR-stimulated DC (i.e. DC.LPS). DC.LPS consistently produced high levels of IL-12p70 when assayed by ELISA over a 24h stimulation period, similar to control DC (**data not shown**).

Although cytokines presented by APC and in the local microenvironment have been shown to be responsible for T cell differentiation, non-cytokine mediated factors have also been identified. Most notably, DC and T cells have been shown to modulate the balance between immunity and regulation (or tolerance) by the length of these interactions in the lymph nodes (98; 143). Notably, studies have come to show that DC dictate the polarization state of T cells prior to cytokine secretion (i.e prior to IL-12-STAT4 signaling; (45)). Not surprising, as T-bet absence in DC was shown to impair Type 1 polarized T cell responses in the mouse model, I found that DC.T-bet induced a significant, up to four-fold, increase in IFNγ production from responding naïve, but not memory, T cells. However, I was unsure whether this was due to a greater conversion of naïve T cells towards the Type 1 polarized state or whether DC.T-bet increased the level of proliferation of Type 1 T cells. DC.T-bet converted a greater proportion of T cells expanded from the naïve pool to IFNγ^{+} T cells, as assessed by proliferation versus IFNγ production, paralleling published reports that T-bet deficient DC did not affect\alter the proliferation profile\potential of responding T cells (24). Flow cytometric analysis of CFSE vs. IFNγ represents a snapshot in time, whereas ELISA data represents the culmination of changes over time. Memory T cells, as defined, responded much early to DC stimulation for secretion of IFNγ. Naïve cells, however, required differentiation and polarization prior to secretory release of

cytokines. The data presented is a snapshot of the optimal time for IFNγ against CFSE dilution for naïve responding T cells, whereas we consistently found memory T cell to have an optimal snapshot at day 1-2 (**data not shown**) and an appearance of less production of IFNγ at day 3 (**Figures 3 and 4).** Concomitantly, DC.T-bet also restrained Foxp3 expression in responding T cells, which was observable beginning at the day 5 time point, suggesting that there was a reciprocal impairment in selection for T regulatory subsets. Although Foxp3 is indeed co-expressed, as a marker for activation, in T cells primed by DC.T-bet and control DC, we continually found that Fox3 expression began to drop more pronounced in DC.T-bet primed compared to control DC primed T cells. This may reflect the speculation in recent literatures that different Foxp3 isoforms can contribute different activation and suppressive cues. DC.T-bet may then be able to more profoundly skew against the suppressive isoforms of Foxp3 to which our Ab (PCH101; eBioscience) identifies (22; 144). Continued investigation of early T cell responses after priming by DC.T-bet or control DC further revealed that the transcription factor T-bet was upregulated in naïve primed T cells by DC.T-bet (DC.T-bet was removed from responding T cells by MACS isolation of $CD3^+$ T cells; yielding 99% purity for $CD3^+CD4^+$ T cells. This was further evaluated by flow cytometry, gating on the $CD3^+CD4^+$ T cell population and co-staining with anti-T-bet and anti-IL-12Rβ2; yielding a 4 fold increase in T-bet and IL-12Rβ2 over control DC expanded naïve T cells. All experiments were performed with at least three different donors in at least three independent experiments to which the trends were always similar in the ability of DC.T-bet to induce Type 1 polarization in early responding T cells.

The next series of studies established the commitment profile of DC.T-bet primed naïve or memory T cells. As T cells require strong activation signals to fully commit towards differentiation, or polarization, I assessed T cell responses after 14 days, with restimulation of DC.T-bet or control DC on day 5 supplemented with IL-2 and IL-7. Evaluating both the $CD4^+$ and $CD8^+$ naïve and memory pools, DC.T-bet was found to induce potent Type 1 polarization in *only* responding naïve T cells by additionally upregulating Type 1-associated effector molecules,

including IL-12Rβ1β2, CXCR3 (chemokine receptor important for trafficking into sites of inflammation) and Granzyme-B (in $CD8^+$ T cells) in addition to IFNγ, as assessed on the single cell level by flow. Furthermore, this response came with a concomitant mutual skew away from the IL-4^+IFNγ^- T helper cell subsets (T_H2 cells), were there was a consistent reduction in the frequency of IL-4 producing cells in responding naïve T cells. Because CFSE proliferation assays revealed no significant changes in expansion between DC.T-bet or control DC primed T cells, we concluded that DC.T-bet was indeed biasing a greater proportion of naïve T cells towards the Type 1 fate upon initial priming and away from $CD25^+Foxp3^+$ T regulatory cells. Additionally, IL-10 production (potentially from $CD8^+$ T effector cells, T_H1 or Tr1-like cells) was restrained. Thus T-bet expression in DC conferred a bias towards Type 1 immunity against Type 2 or T regulatory T cell subsets in responding naïve, but not memory, $CD4^+$ and $CD8^+$ T cells. For polarization studies, all experiments were performed with at least three different donors in at least three independent experiments of $CD45RO^{neg}$ and $CD45RO^{neg}CD4^+$ or $CD45RO^{neg}CD8^+$ isolated cell subsets, to which the trends were always similar in the ability of DC.T-bet to induce Type 1 polarization. Differences in total expression of cytokines and surface markers varied by donor, but DC.T-bet was always found to significantly increase the production of Type 1 and restrain Type 2 and Treg phenotypes compared to control DC expanded T cells.

DC.T-bet priming of antigen specific T cell responses led to analogous results to the superantigen model system, yielding a three to four fold increase in IFNγ producing $CD8^+$ effector T cells primed from an autologous naïve pool as assessed by both ELISPOT (and ELISA) and Flow cytometric analysis. Furthermore, the induction of Type 1 immunity was not induced in a “bystander” fashion, as restimulation with non-specific peptides (HIV) yielded no measurable IFNγ producing spots or detectable levels of IFNγ by ELISA in DC.T-bet or control DC expanded $CD8^+$ T cells. Thus DC.T-bet induction of Type 1 polarized T cells, similar to the evaluation of OVA-specific responding T cells in the mouse model performed by the Glimcher

group, functioned in an antigen-specific fashion that increased the level of IFNγ producing $CD8^+$ T cells (albeit their system assessed $CD4^+$ T_H subsets).

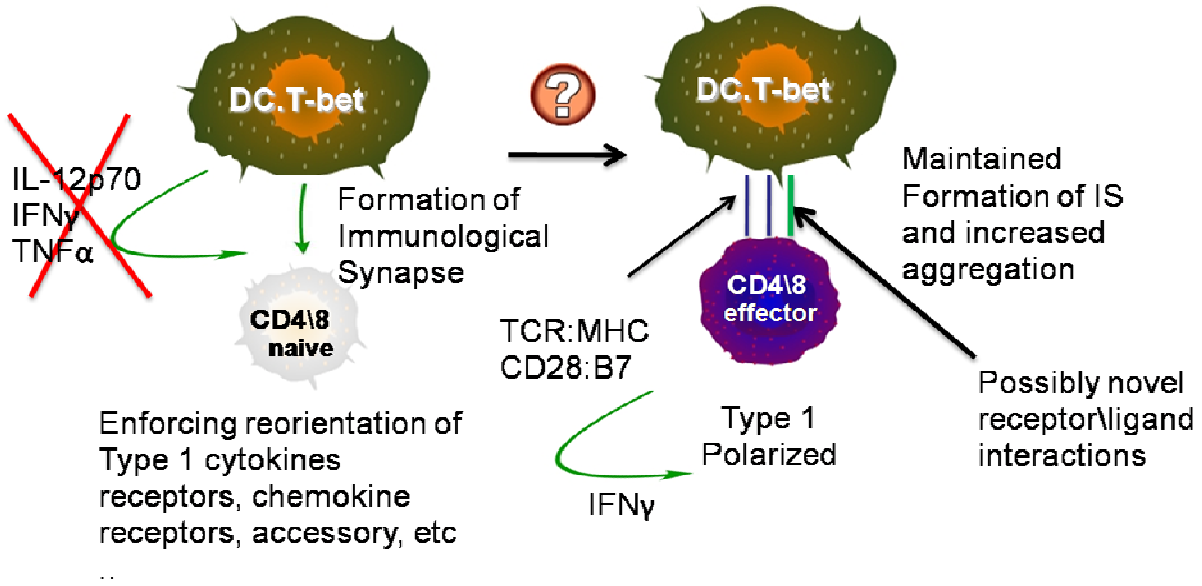

Figure 8. Propsed DC.T-bet mechanisms of polarization.

Confirming that DC.T-bet induction of Type 1 polarization is not an artifact of the superantigen model system but is applicably seen in an antigen-specific model system, it remained to be uncovered the novel mechanisms that induced Type 1 polarization in DC.T-bet. Likely candidates weren't readily obvious for DC.T-bet induction of Type 1 immunity, as early studies showed abolished IL-12 family member cytokines, no IFNγ production, nor inflammatory cytokines, such as TNFα or IL-6. This compounded with little-to-no changes in survival (as assessed by pro- anti-apopotic protein expression and Annexin-V staining) or costimulatory molecules, made the investigation of the mechanisms of this Type 1 polarization novel. Using the transwell system, we confirmed that DC.T-bet exerted its Type 1 induction in a contact, or possibly proximity, dependent fashion, as there were no changes between responding T cells primed by DC.T-bet or control DC when separated. Further confirmation of IL-12 family-independency was achieved by using neutralizing Abs to IL-12p70, IL-12Rβ2, IL-23p19, or IL-27

receptor. In additional studies, DC.T-bet compared to αDC1 (132) in ability to prime IFNγ from responder naïve. However, in the presence of neutralizing Abs to IL-12p70, αDC1 induction of IFNγ from responder T cells was reduced by > 50%, whereas DC.T-bet was reduced by < 5% (**data not shown**). This confirmed that DC.T-bet functioned in an IL-12 independent method and that the neutralizing Ab was functional. I also evaluated other literature noted IL-12 independent mechanisms of Type 1 polarization, in blocking CD27-CD70 interactions and notch-delta like-4 (DLL4) engagement to which DC.T-bet induction of Type 1 polarization was also independent of these mechanisms. Neutralization of IFNγ and the IFNγ receptor (IFNγR1), which has been shown to induce endogenous T-bet expression in T cells, via STAT1-dependent signaling, did not alleviate Type 1 polarization. Thus blocking IL-12p70 and IL-12Rβ2 or IFNγ and IFNγ receptor were unable to dampen the ability of DC.T-bet to induce Type 1 polarization in responding naïve T cells. Whether STAT4 or STAT1 are activated and translocated remains to be addressed, but it should be noted that studies have shown STAT1-independent pathways as important for regulation of antiviral immunity (145; 146). This corroborates studies by the Glimcher group showing the important of T-bet for viral responses via CpG signaling in DC (56). This is consistent with literature reports, that have shown that T-bet in T cells is induced prior to IL-12 (i.e. in an IL-12 independent fashion) via interaction with cognate DC (45). Thus, as DC are not principle producers of IFNγ, it can be reasoned that DC, which may be polarized, can regulate Type 1 polarization in responding cognate T cells through TCR:MHC engagement coupled with costimulatory and\or adhesion molecules (independent of cytokine). These may include enhanced LFA-1:ICAM-1 engagement to maintain long-term DC contacts via adhesion for costimulatory signaling and effectively stabilizing the supramolcular activation cluster (SMAC) (143; 147), constitutive expression of B7 costimulatory molecules (i.e. unable to be downregulated due to constitutive signaling by T-bet in DC) that could enhance the ability of DC to reorient cytokine receptor inclusion and\or actin rearragement to deliver Type 1 signaling

cues (148), and\or expression of novel cell-cell (or proximal) molecular interactions to enforce Type 1 polarization all within the context of the immune synapse (**Figure 8**).

Because of the heterogeneous population of DC.T-bet, the necessity to move into the mouse model *and* develop a bicistronic GFP reporter (human and mouse) T-bet adenoviral vector is best suited for further mechanistic evaluation of DC.T-bet. Murine models would allow use of distinct knockout systems to critically evaluate mechanisms of DC.T-bet mediated polarization of T cells, beyond the use of neutralizing antibodies, siRNA, or other limited knock-down studies that can be achieved (and be recapitulated) in human model systems. GFP reporter T-bet vector will provide a way to sort for positive T-bet DC within the transduced cohort, allowing for pure populations for proteomic or microarray analysis. Investigation of the mechanisms may yield new novel approaches to induction of Type 1 immunity, possibly at the level of DC-T cell interface during priming, whether this may be contributed to adhesion, accessory, or, costimulatory molecules remains to be seen. However, the therapeutic potential of DC.T-bet is potent; as it requires limited *ex vivo* manipulation to promote IL-12- and IFNγ-independent potent Type 1 polarized immune responses.

Preface Chapter 3

The quest for tolerance in both the autoimmunity and transplant settings is dependent on the specific suppression of unwarranted or autoreactive T cell immunopathology. With the identification of Foxp3^{+} T regulatory (Tregs) cell subsets and tolerogenic DC, which can either induce Tregs or create a hyporesponsive atmosphere, the field has shifted to harness immunotherapeutic agents to alleviate pathologic inflammatory disease states. Interestingly, DC, the principle APC which have been associated with T cell immunity, are once again at the forefront as prime APC capable of generating a tolerogenic or suppressive environment based on conditioning that allows them to directly suppress T effector cells and indirectly foster the development of Tregs that serve as immune suppressors.

The goal of this project was to investigate the potential of Foxp3, the master regulator of the suppressive functions associated with Treg, to promote the establishment of tolerogenic DC after delivery of Foxp3 (via adenoviral vector encoding hFoxp3) into human monocyte-derived DC.

3. Enforced expression of Foxp3 in DC generates tolerance by both expanding functional T regulatory cell subsets and directly inducing hyporesponsiveness in T effector cells.

Lipscomb MW[1,2], Goldbach CJ[3], Watkins SC[3], Taylor JL[1], Wesa AK[1,2], Storkus WJ[1,2].

From the Departments of [1]Dermatology, [2]Immunology, [3]Center for Biological Imaging
University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

All data and results reported in this study were obtained by Michael W. Lipscomb. Christina Goldbach performed immunohistochemical analysis for identification of Foxp3 expression in DC. JLT cloned hFoxp3 and generated the rAd.hFoxp3 vector used to infect human DC.

3.1. Abstract

Recently, Foxp3 expression and associated suppressive mechanisms have been identified outside the previous localized T lymphocyte population and into endothelial and epithelial cells. This, coupled with the quest for a potent tolerogenic dendritic cell for immunotherapy, led to the investigation of Foxp3 expression in DC, as a potential modulator of DC induced-T cell responses. Enforced expression of Foxp3 into monocyte-derived DC engendered a potent tolerogenic DC (DC.Foxp3) that markedly suppressed proliferation of both autologous and allogenic naïve T cells. Furthermore, DC.Foxp3 restrained Type 1 polarization (as measured by IFNγ, CXCR3, T-bet, and Granzyme-B) and effector functions in both $CD8^+$ and $CD4^+$ responding naïve T cells. Reciprocally, IL-4 producing-T_H2 cell were preferentially selected for, yielding a 4-fold increase in IL-4^+IFNγ^- T cells compared to control DC primed naïve T cells. Concomitantly, we saw as much as a five-fold increase in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs that co-expressed CTLA-4, GITR, and Neuropilin-1. DC.Foxp3 expanded Tregs were potent suppressors of responding T cell proliferation and production of IFNγ. Furthermore, we found IL-17A and RORγt expression restrained. Investigation of the mechanisms revealed that both IDO and TGFβ partly played roles in governing suppression, whereas DC.Foxp3 mediated suppression was not dependent on PD-L1, IL-10, or Fas. Interestingly, the phenotype of these Foxp3 expressing DC resulted in complete abolishment of the cytokines IL-12p70, IL-23, IL-6, and IL-10 as assessed on the protein level. These results identify a potent tolerogenic-engineered DC that restrains Type 1 and 17 T effector functions and in tandem increases the frequency of functional suppressive $CD25^+Foxp3^+$ Tregs and IL-4 producing-T_H2 cells.

3.2. Introduction

Autoimmunity and transplantation rejection are pathological disorders characterized by undesirable or unwarranted cellular and/or antibody-mediated tissue destruction and/or disorder (149). Briefly, autoimmune responses are characterized by the adaptive arms autoreactivity against self antigens leading to inflammatory injury or disruption of physiological processes, with chronic activity leading to exacerbated tissue destruction and systemic disorders (150). Autologous antigen-specific T cells (autoAg) that are Type 1 and/or Type 17 polarized $CD4^+$ T cells, T_H1 and T_H17, respectively, are the primary mediators of immunopathology (151; 152). They mediate immunity by upregulation of IFNγ and/or IL-17 and activation and mobilization of $CD8^+$ cytotoxic T lymphocytes (CTL) and innate immune cells, such as neutrophils and macrophages (151; 153-155). Transplant graft rejection, although a normal process of non-self recognition, is an unwanted T cell alloreactive response primarily orchestrated by $CD4^+$ T helper cell subsets leading to cellular-mediated tissue destruction of the graft. A larger scale model of transplant disease is seen in graft versus host disease (GVHD), which is characterized by alloreactive response of donor transferred T cells to recipient tissues via recognition of foreign MHC molecules and/or minor histocompatibility alloantigens (MinHC; (156)). Additionally, T helper cells are required for initiation/augmentation of B cell auto- and alloantibody responses that contribute to autoimmune responses, hyper acute graft rejection, and chronic transplant diseases (157). In both settings, experimental evidence has shown that T cell responses, specifically $CD4^+$ T helper responses, are the principle mediators causing pathological disease or disorders (158-160)

Effective immunotherapeutic treatments for transplantation and autoimmunity are dependent on the ability to inhibit and/or ablate antigen-specific donor and host tissue destruction by immune cells (161-163). Antigen-specific tolerance would effectively allow virally infected and transformed cells and extracellular pathogen clearance, while maintaining tolerance to

endogenous or transplanted normal tissues (162). Both transplant and autoimmunity fields have begun to realize the potential of dendritic cells (DC) as clinical adjuvants or vaccines. Originally herald as inducers of antigen-specific T cell responses, DC are also potent suppressors of immunity by inducing T cell tolerance (3; 90; 100; 158; 164). Tolerogenic DC can directly restrain Type 1 and Type 17 T cell polarized responses and/or indirectly differentiate naïve T cells into Foxp3^{+} T regulatory (Tregs) or IL-10^{+}Foxp3^{-} T regulatory-1 cells (Tr1; (100; 119; 120; 165-169)). Furthermore, Tregs were able to in turn re-condition "normal" DC to tolerogenic status to induce Tr1-like cells from T effector cells (170; 171). It is still unclear exactly what cis and/or trans molecular factors define tolerogenic DC subsets that can confer inhibition of effector T cell responses and\or expand IL-10^{+}Tr1-like and Foxp3^{+}Treg subsets. However, shifting polarizing stimuli towards negative costimulatory molecules and immunosuppressive cytokines have been shown to be important in peripheral tolerance (8; 36; 106; 126; 172).

Directly restraining Type 1 and Type 17 responses is largely dependent on DC signaling, as well as microenvironmental cues, during DC-T cell interaction. IL-12 family members have been shown to be indispensable in augmenting both auto- and alloreactive T cell responses (1; 27; 172). Specifically, IL-12p70 and IL-27 induce Type 1 polarization, whereas IL-23 production modulates T_H17 cell survival and effector functions (173; 174).. Clear evidence has additionally shown that DC can also expand Foxp3^{+} and IL-10^{+} regulatory T cell subsets from naïve T cells (165; 168). The role of Tregs as suppressors of immunity has been well characterized and shown to be indispensable for peripheral tolerance, effectively controlling immunopathological events in both autoimmunity and allograft rejection (143; 175; 176).

In vivo, Foxp3^{+}Tregs act directly to restrain T effector functions, by mechanisms that are still being delineated, and indirectly through re-conditioning dendritic cells to limit the priming of autoreactive T cells, ultimately restraining and/or skewing T cell differentiation, acquisition, and/or elicitation of effector functions (143; 176; 177). Additionally, IL-10^{+}Tr1 cells also have been shown to directly and indirectly, through DC modulation, repress T cell expansion and

functions by inhibiting DC antigen presentation, maturation, and ability to prime Type 1 polarized T cell responses (118; 120; 165; 172). Furthermore, high levels of IL-10 expression by DC, often engendered by maturation in presence of IL-10, has been shown to generate both Foxp3^{+}Tregs and IL-10^{+}Tr1 cells (100; 118). The reciprocal interplay between Tregs/Tr1-like cells and DC have been shown to be important in induction and maintenance of tolerance both *in vivo* and *in vitro* (167; 178). Thus, the generation of tolerogenic DC that can skew T cells away from Type 1 and Type 17 differentiation towards Foxp3^{+}Tregs and/or Tr1-like cells are valuable therapeutic tools that can perturb autoimmune- and transplant graft rejection-associated pathologies.

Since its discovery, the role of Foxp3 in T cells has been explored and acknowledged to play potent suppressive roles in restraining immunopathogenic events in both autoimmunity and transplantation (58-61; 151; 179). Mice absent of Foxp3 develop severe pathogenic autoimmune diseases (180). Recently, expression of Foxp3 in adenocarinoma epithelial cells conferred immunosuppressive roles that effectively ablated T cell responses (25). Finally, the role of FOX-related family of proteins has also been shown to modulate immunity in the myeloid lineage, specifically DC. Foxo3 deficiency in DC was found to confer proliferation advantage in responding T cells, suggesting that Foxo3 acted to perturb the magnitude of T cell immune responses (181). Currently, there is no literature governing the potential role of Foxp3 in DC, however, we hypothesized that Foxp3 may act as a universal suppressor of immune functions, and may confer potent tolerogenic properties in dendritic cells.

3.3. Materials and Methods

3.3.1. Adenovirus Construct

Human Foxp3 (hFoxp3) was cloned from human peripheral blood lymphocytes using PCR amplification. The product was then digested with restriction enzymes and ligated into an adenoviral-Cre-Lox (Adlox) plasmid vector. Recombinant adenoviruses were generated by co-transfection of the hFoxp3 Adlox with helper virus DNA into the adenoviral packaging cell line CRE8 that expresses Cre recombinase. Recombinant adenoviral vectors were propagated on CRE8 cells, purified by cesium chloride density-gradient centrifugation and subsequent dialysis, before storage in 3% threalose at -80C. Titers of viral particles were determined by optical densitometry. The mock adenoviral vector Adψ5 was used as control. All experiments used a multiplicity of infection (MOI) at 600.

3.3.2. Isolation of normal donor DC and T cells

Human peripheral blood monocytes (PBMC) were isolated from heparinized human peripheral blood by Ficoll density gradient centrifugation, allowed to adhere for 1 hour, washed extensively, and supplemented with 20ng/ml of IL-4 (Peprotech) and 10^3 units/ml of GM-CSF (Leukotriene) in AIM-V for 5 or 6 days. Greater than 95% of cells were $CD11c^+CD14^-$ (immature DC). Naïve or memory T cells were isolated by depletion of $CD45RO^+$ or $CD45RA^+$ cells from PBLs, respectively, using MACS™ microbeads (Miltenyi). Subsequent positive selection for $CD4^+$ or $CD8^+$ T cells was performed on depleted naïve (CD45RO-) or memory (CD45RA-) T cells. Flow cytometric analysis for purity of naïve or memory CD4+ or CD8+ T cells was: $CD4^+CD45RA^+ \geq 98\%$, $CD8^+CD45RA^+ \geq 98\%$, $CD4^+CD45RO^+ \geq 97\%$ and $CD8^+CD45RO^+ \geq 98\%$.

3.3.3. DC and T cell coculture; autologous vs. allogenic responses

3.3.3.1 CFSE Proliferation Assays

Autologous (pulsed with staphylococcus enterotoxin B (SEB; Sigma) at 1ng/ml for 3hrs in 37C to bridge the TCR:MHC engagement of responding T cells) or allogenic DC.Foxp3 or control DC were cultured with naïve or memory T cells at a ratio of 1:10, respectively. 1 x 10^5 T cells, labeled with 0.5μM of CFSE, were cocultured with DC in a final concentration of 100U/ml of rhIL-2 (Peprotech) in TcMEM for 3 days, prior to flow cytometric analysis.

3.3.3.2. T cell Stimulation Assays

T cells were plated with SEB pulsed-autologous or allogenic-DC.Foxp3 or control DC at a DC:T cell ratio of 1:10. T cells were restimulated with respected DC on day 5 and supplemented with 20u/ml of rhIL-2 (Peprotech) and 5ng/ml of rhIL-7 (Sigma). T cells were supplemented with IL-2 and IL-7 every other day for an additional 7-9 days (for cytokine analysis) or 15 days (for Foxp3, CTLA-4, GITR, NRP-1 and CD25 expression). Cells were collected and evaluated for cytokine, surface, and intracellular protein expression by flow cytometry. To evaluate cytokine expression, cells were stimulated with PMA (1μg/ml) and Ionomycin (10ng/ml) for 4 hours with 2nM of monensin (Sigma) added for the final 2 hours of culture.

3.3.3.3. Treg suppression of T cell proliferation and IFNγ production

$CD25^+$ DC.Foxp3 or control DC expanded autologous or allogenic $CD4^+$ T cells, isolated by MACS™ on day 21, were co-cultured with CFSE labeled naïve or memory $CD8^+$ responder T cells (MACSTM isolated) at a ratio of 1:10, respectively in presence of 100 U/ml of IL-2 onto plates pre-coated overnight with 10μg/ml of anti-CD3/CD28 or ant-CD3. Cells were assessed for CFSE dilution on day 4 by flow cytometry. For suppression of effector functions, $CD8^+$ naïve or memory T cells were activated with anti-CD3/CD28 dynabeads (Invitrogen) in presence of 10^3

U/ml of and 50ng/ml of IL-12 for 8 days. $CD25^+$ DC.Foxp3 expanded or control DC expanded $CD4^+$ T cells were then cocultured with these $CD8^+$ T cells in the presence of PMA/Ionomycin and 2μM of Monensin for 4hrs, prior to harvesting cells and co-staining with anti-CD3 and IFNγ.

3.3.4. Readout Assays

3.3.4.1. Flow cytometry and Immunohistochemical Analysis

For cell surface staining using flow cytometry, cells were collected and re-suspended at 2 x 10^5 cells/100 μl in 96-well V-bottom plates in PBS with 2% BSA and 0.2% NaN_3 (FACS buffer), then washed twice before blocking in 3% human serum. Antibody staining was performed for 30 minutes at 4^oC at a dilution of 1:20, after which, cells were either analyzed by flow cytometry or resuspended in Fix/Perm buffer (eBioscience) for subsequent intracellular staining. Briefly, for intracellular staining, cells were washed twice in 1X permeabilization buffer (eBioscience) prior to blocking with 10% human serum for 10 minutes at 4^oC. Antibody staining was performed at 4^oC for 45-60 minutes. Cells were then washed twice in 1X permeabilization buffer, resuspended in FACS buffer, and analyzed by flow cytometry. For immunofluorescence microscopy, 1 x 10^5 DC were cytospun, fixed onto slides and co-stained with anti-T-bet (Santa Cruz), nuclear dye (DAPI), and rhodamine phalloidin. Fluorescence images were then captured using an Olympus BX51 microscope (Olympus America, Melville, NY).

3.3.4.2. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For mRNA analysis, DC were harvested on day 2 (48h post-transduction) and MACS™ isolated naïve or memory $CD4^{pos}$ T cells were harvested on day 3 after initial priming by DC. RNA was isolated with Trizol (Inivitrogen, Carlsbad, CA). Reverse transcription was performed using MuLV reverse transcriptase (Applied Biosystems, Carlsbad, CA) and Random Hexamers (Applied Biosystems). Semi-quantitative PCR was used to amplify cDNA for expression of gene-

specific products. Specific primers were used for IL-12p35, IL-12/23p40, IL-23p19, IL-27p28, IL-27pEBI-3, IL-15, IL-18, IL-10, TGFβ, IFN-α, and IFN-γ as previously described(132). Additional primer sequences included: T-bet: Fwd 5'-CCACCAGCCACTACAGGATG-3' and Rev 5'-GGACGCCCCCTTGTTGTTT-3'; GATA-3: Fwd 5'-GTGCTTTTTAACATCGACGGTC-3' and Rev 5'- AGGGGCTGAGATTCCAGGG-3'; Foxp3: Fwd 5'-GCACCTTCCCAAATCCCAGT-3' and Rev 5'-TAGGGTTGGAACACCTGCTG-3'; and RORγt: Fwd 5'-AAATCTGTGGGGACAAGTCG-3' and Rev 5'-TGAGGGTATCTGCTCCTTGG-3'. β-actin primers were used as an internal positive control (132).

3.3.5 Neutralization Studies

Neutralizing anti-CD95 polyclonal Ab (pAb; Raybiotech), anti-TGFβ pAb (R&D Systems) anti-hIL-10 pAb (R&D Systems), and anti-hGITR pAb (Biolegend), were used at a final concentration of 10 μg/ml. Additionally, D-, L-, and DL-1 methyl-Tryptophan (Sigma) was used at a final concentration of 250nM. Briefly, DC.Foxp3 or control DC were plated with naïve or memory T cells at a DC:T cell ratio of 1:10 in triplicate in 96-flat bottom plates in the presence or absence of neutralizing antibodies\reagents. On day 3, cell-free supernatants were collected and evaluated using IFN-γ ELISA. Alternatively, cells were cultured for a total of 14 days in the presence of neutralizing antibodies\reagents, with restimulation on day 5 with DC supplemented with IL-2 and IL-7. Neutralizing antibodies\reagents were supplemented every other day at the dictated concentration. T cells were restimulated with PMA\Ionomycin, prior to analysis by flow cytometry for IFNγ production.

3.4. Results

3.4.1. Phenotype of Foxp3 expressing DC

Adenoviral encoding human Foxp3 was transduced into monocyte-derived DC for 48 h prior to analysis of phenotype and Foxp3 expression in DC. Foxp3 expressing DC (DC.Foxp3) transduction efficiency was evaluated by flow cytometry and found to be greater than 50% on average $CD11c^{+}Foxp3^{+}$, whereas the control DC, which contained non-transduced DC (DC.null) or DC transduced with the empty adenoviral backbone (DC.ψ5), contained no Foxp3 co-expression (**Fig 9A**). Annexin-V staining was additionally performed 48 h after transduction to confirm that ectopic Foxp3 expression in DC was non-toxic (**Fig 9B**). Fluorescent staining of DC.Foxp3 or control DC revealed that Foxp3 localizes to the nucleus in transduced cohort (**Fig 9C**). This data identifies that Foxp3 expressing DC are viable and that Foxp3 localizes to the nucleus.

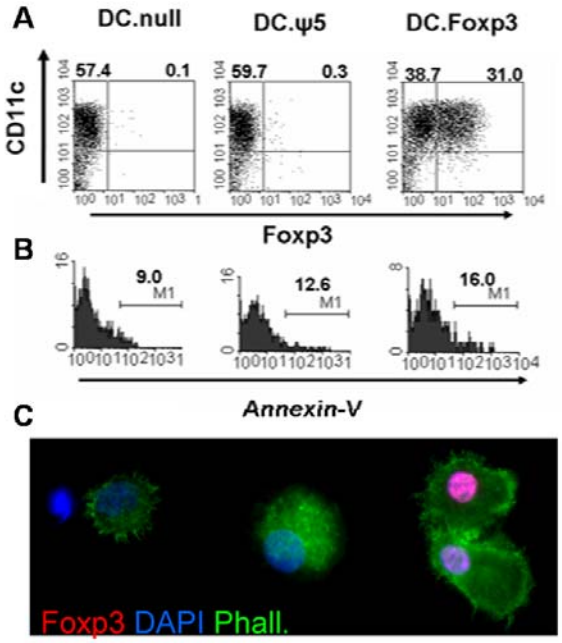

Figure 9. Foxp3 expression in DC. Dendritic cells were left untransduced (DC.null) or transduced with an empty adenoviral vector (DC.ψ5) or an adenoviral vector encoding human Foxp3. After 48h, DC were costained with either anti-CD11c and -Foxp3 or (B) with Annexin-V prior to analysis by Flow cytometry. (C) DC were cytospun and fixed to slides prior to staining with Foxp3, DAPI, and Phalloidin

3.4.2. Modulation of cytokine profile in Foxp3 expressing DC

Peripheral Blood Monocytes (PBMC) monocytes were cultured with IL-4 and GM-CSF, prior to transduction with mock or hFoxp3 vectors. Additional control groups included monocytes cultured with IL-4 and GM-CSF in the presence of TGFβ (DC.TGFβ) or IL-10 (DC.IL-10). All DC subsets were $CD11c^{+}CD14^{--}$. Widescreen mRNA analysis revealed that DC.Foxp3 had decreased levels of the shared IL-12\23 p40 subunit, IL-12p35 (IL-12α), IL-27p28 subunit (**Fig 10A and 10B**). Addition of TGFβ (DC-TGFβ), IL-10 (DC-IL-10), or VEGF (**data not shown**) throughout the immature DC generation phase did not induce Foxp3 expression (**Fig 10B**). Interestingly, a small appreciative increase was seen in IL-9 and a concomitant reduction in VEGFR2 mRNA expression. DC.Foxp3 was impaired in soluble secretion of IL-12p70, IL-6, and IL-23p19, (as well as IL-1β and TNFα (**data not shown**)) cytokines important in the generation or maintenance of Type 1 or Type 17 T helper cell differentiation, respectively (**Fig 10C**). Interestingly, IL-6 abolished protein expression did not correlate directly with mRNA expression levels, suggesting that DC.Foxp3 may exert post-transcriptional regulation of cytokine production (Fig. **10A and 10C**). Densitometer analysis revealed that no significant differences between TGFβ band expression in DC.Foxp3 compared to control DC (**data not shown**). There were no significant changes in co-stimulatory molecule expression (**data not shown**).

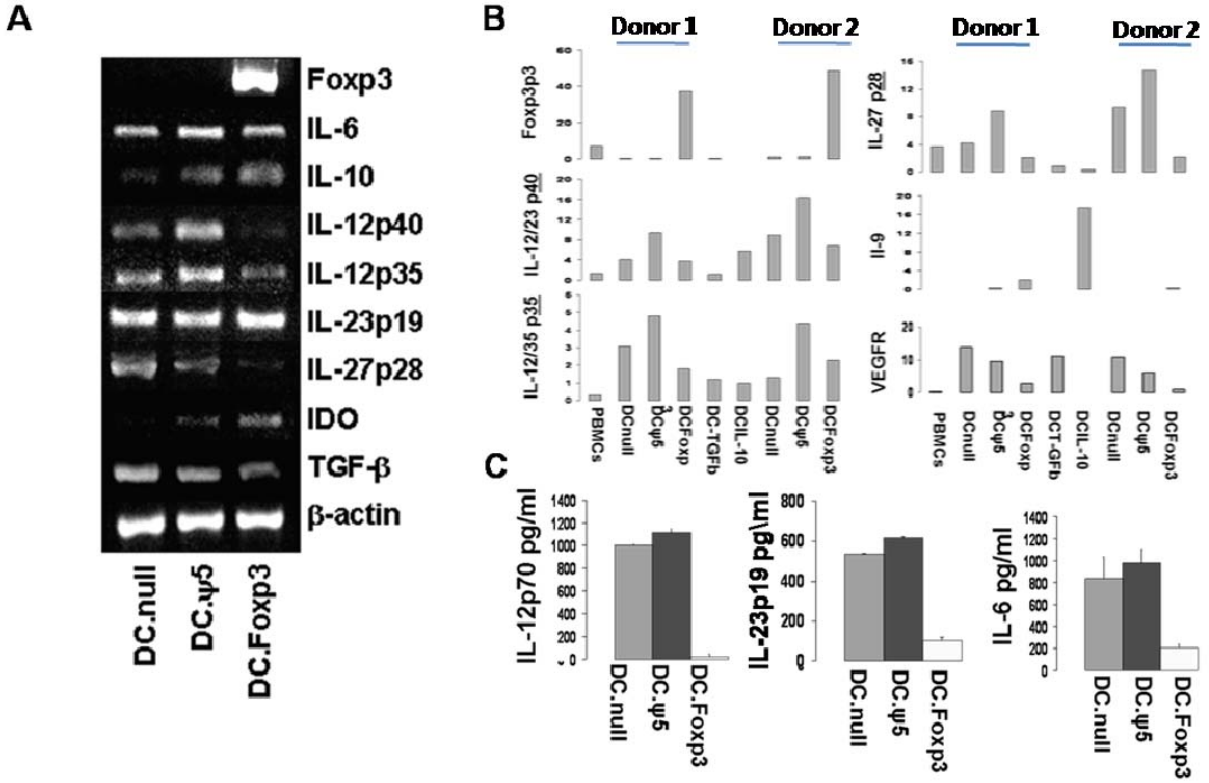

Figure 10. Foxp3 expression in DC modulates cytokine profiles. DC.Foxp3 or control DC, along with TGFβ or IL-10 Treated DC, DC.TGFβ or DC.IL10, respectively, were assessed for transcriptional and protein levels of cytokines and regulatory markers. (A) DC were analyzed for indicated transcriptional levels by reverse transcriptase PCR and (B) densitometry analysis was performed against β-actin of each cohort. (C) DC were treated with CD40L-expressing fibroblasts for 24h prior to collection and analysis of supernatant for indicated protein levels.

3.4.3. Impairment of early T cell responses

Significant changes in DC.Foxp3 cytokine profile prompted investigation of T cell responses. Briefly, DC.Foxp3 or control DC were cultured with autologous or allogenic naïve or memory T cell subsets at a ratio of 1:10, respectively. For autologous studies, DC.Foxp3 or control DC were pulsed with 1ng/ml of the superantigen SEB for 3h prior to culturing with T cells. DC.Foxp3 had less total expanded naïve, but not memory, T cells than control DC primed naïve T cells (**Fig 11A**). Further investigation revealed that DC.Foxp3 generated much fewer total T cells per round of replication than in control DC expanded T cells (**Fig 11B**). In each

round of replication, there was no increased fold in expansion, unlike what was seen in control DC expanded naïve T cells. This prompted investigation of measuring cell death. Thus, DC.Foxp3 or control DC expanded T cells were evaluated on day 5 for apoptosis and cell death by co-staining with Annexin-V and phosphotidol inositol (PI). DC.Foxp3 expanded naïve $CD4^+$ T cell subsets had a 3-5 fold increase in levels of apoptosis compared to control DC expanded naïve $CD4^+$ T cell subsets (**Fig 11C**). There was a little-to-no difference in apoptotic levels of naïve $CD8^+$ T cells primed by DC.Foxp3 or control DC **(data not shown**). Identifying that DC.Foxp3 was inducing apoptosis in naïve $CD4^+$ responding T cells, we next performed mRNA expression analysis of T cell differentiation transcription factors, which included T-bet (T_H1), GATA-3 (T_H2), RORγt (T_H17), and Foxp3 (T regulatory) in the $CD4^+$ T helper subsets. To ensure pure population of $CD4^+$ T cells, MACS microbeads were used to isolate $CD3^+CD4^+$ cell subsets from the negative selection, yielding > 99% purity of $CD4^+CD3^+$ T cell subsets. Analysis of transcription factors revealed a significant increase in the ratio of GATA-3 to T-bet and Foxp3 to RORγt **(Fig 11D**). This suggested the T_H1 and T_H17 subsets were preferentially induced to cell death by DC.Foxp3.

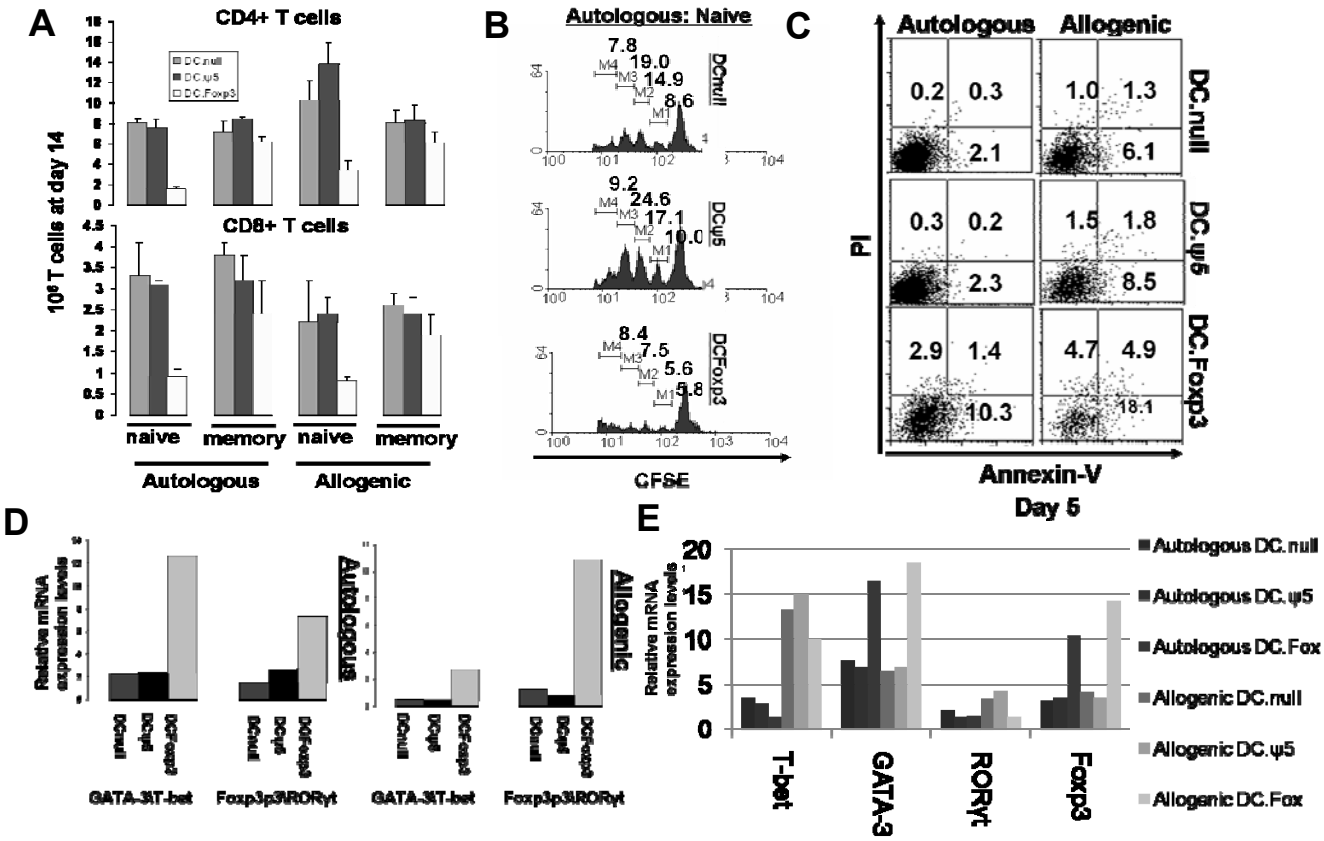

Figure 11. Impairment of early T cells by Foxp3 expressing DC. DC.Foxp3 or control DC were cultured with autologous or allogenic naïve or memory T cells at a 1:10 DC:T cell ratio. (A) T cells were counted on day 14. (B) T cells were stained with CFSE prior to culture with DC cohorts and assessed by flow cytometry on day 4. (C) CD4+ T cells were stained with Annexin-V and phosphatidyl inositol (PI) after 4 days of culture with DC prior to flow analysis. (D, E) T cells were cultured with DC for 3 days prior to isolation of T cells by CD3 microbeads using MACS™. Total RNA was extracted and subjected to RT-PCR

3.4.4. Suppression of Type 1 and Type 17 T cell responses

Further analysis of Type 1 and 17 polarization was evaluated upon fully committed and differentiated T cell subsets. Briefly, DC.Foxp3 or control DC were cocultured with autologous or allogenic naïve or memory T cells for 14 days, with restimulation of T cells with respected DC supplemented with IL-2 and IL-7 on day 5. T cells were isolated and co-stained with CD4 or CD8 and assessed for T cell phenotypes. For cytokine evaluation, T cells were stimulated with PMA\Ionomycin for 4h, with monensin added into the culture for the final 2h. DC.Foxp3 expanded autologous and allogenic naïve CD4 and CD8 T cells were restrained in production of

the Type 1 associated cytokine IFNγ with a concomitant increase in IL-4 production, indicative of T_H2 subsets, compared to control DC expanded naïve T cells (**Fig. 12A)**. This may suggest that these T_H2 cell subsets were under less influence of apoptosis by DC.Foxp3, and that T_H1 cells were the principle targets of DC.Foxp3 direct (or indirect) induction of apoptosis. Again, supporting the mRNA expression profile of $CD4^+$ T cells, DC.Foxp3 impaired IL-17 production from (T_H17) subsets, most notably identified in the allogenic setting (**Fig. 12B**). Increased IL-10 production was observed in $CD4^+$ and $CD8^+$ T cells from the naïve pool primed by DC.Foxp3 compared to control DC, which was further corroborated by ELISA analysis (**Fig. 12C and data not shown**). Further investigation of Type 1 associated markers revealed that DC.Foxp3 restrained CXCR3 expression, which is associated with T cell trafficking to sites of inflammation, in naïve CD4 and CD8 T cells, of both the autologous and allogenic settings (**Fig. 12D**).

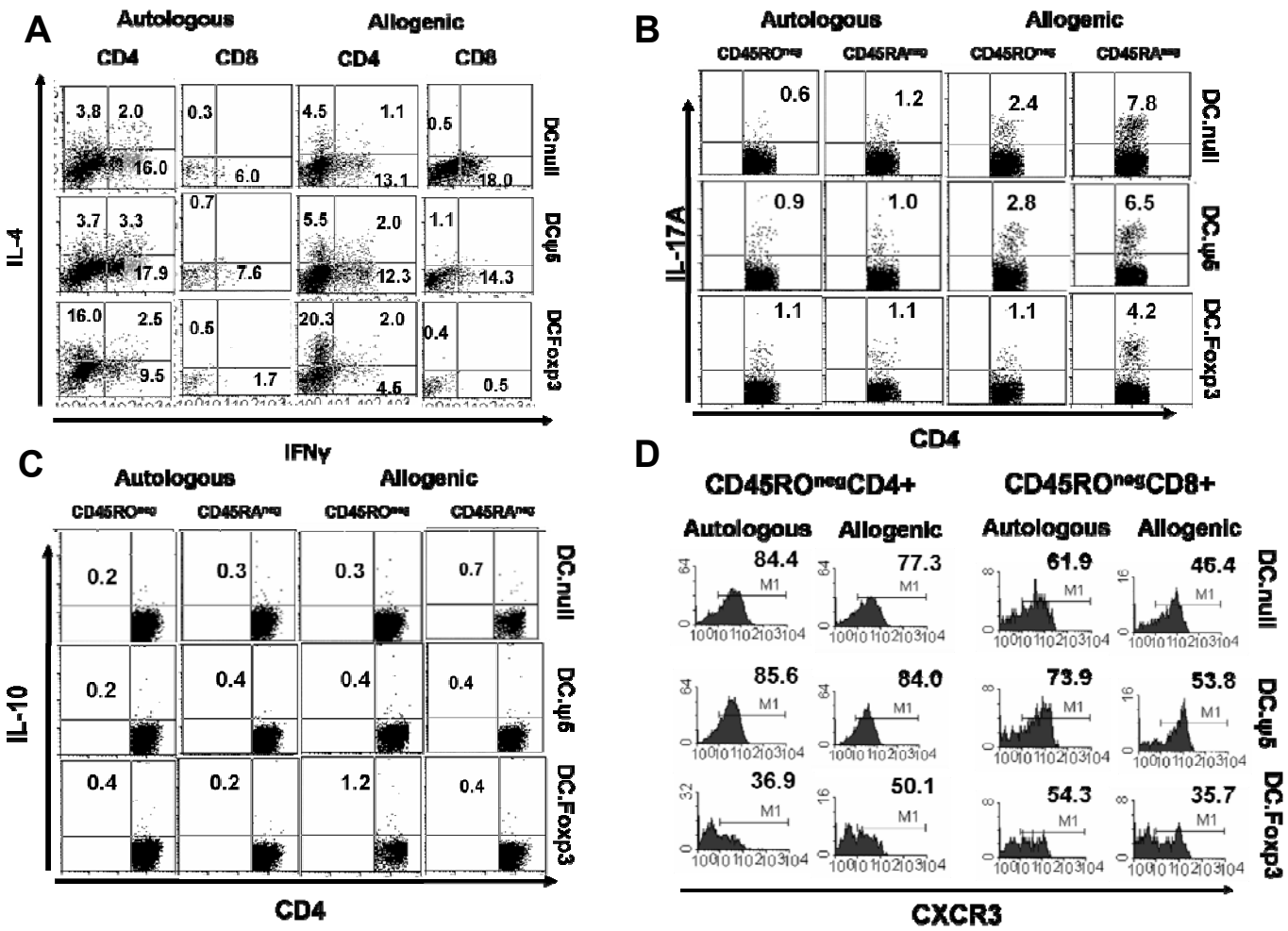

Figure 12. DC.Foxp3 skews functional polarization of T cells. Naïve or memory autologous or allogenic T cells were cultured with DC.Foxp3 or control DC for 14 days, with restimulation on day 5 with respected DC supplemented with IL-2 and IL-7. (A-C) T cells were restimulated with PMA\Ionomycin for 4h with monensin added the final 2h of culture and subsequently stained with antibodies to IFNγ and IL-4, or IL-17A or IL-10. (D) T cells were co-stained with anti-CXCR3.

3.4.5. DC.Foxp3 expands CD4+CD25+Foxp3+ T regulatory cells

Mutual exclusion of Foxp3+ T regulatory cell subsets and T_H17 subsets has been Identified and shown in both the mouse and human systems [21; 23; 151]. Therefore, I next investigated whether depressed T_H17 generation was due to preferential selection of $Foxp3^+$ T regulatory cell subsets. Briefly, DC.Foxp3 or control DC were cultured with naïve or memory

autologous or allogenic T cells for a total of 21 days, with restimulation by respected DC on day 5 supplemented with IL-2 and IL-7. T cells were then co-stained with antibodies to CD4, CD25 and Foxp3 and assessed by flow cytometry. DC.Foxp3 selected for a 5 to 10-fold increase in CD4+CD25+Foxp3+ T cells from naïve, but not memory, precursors compared to control DC expanded naïve T cells **(Fig 13A**). DC.Foxp3, but not control DC, naïve primed $CD4^+$ T cells co-expressed elevated levels of CD25, CTLA-4, GITR, and Neuropilin-1 (NRP-1; **Fig 13B**). Interestingly, although Foxp3 was found in the control DC expanded naïve T cells, its expression did not co-express with CTLA-4, GITR or NRP-1, suggesting that Foxp3 expression in these T cells was not an indicator of T regulatory cells (**Fig 13B**). Bulk $CD45RO^{neg}$ naïve T cells were used as detailed in afore mentioned studies, with T cells gated on (for flow cytometry) or isolated (for PCR or protein levels) for $CD4^+$ T cells. To confirm DC.Foxp3 direct modulation of $CD4^+$ (or $CD8^+$ T cells), naïve $CD4^+$ or $CD8^+$ T cells were isolated by depleting memory T cells using CD45RO microbeads and subsequently isolating CD4 or CD8 positive cells from the depleted group using specific microbeads. Flow cytometry was performed to identify the purity of each fraction, to which we generated >99% $CD45RA^+CD4^+$ or $CD45RA^+CD8^+$ allogenic cohort to culture with DC.Foxp3 or control DC **(Fig 13C**). Studies revealed analogous results to the bulk naïve studies, where DC.Foxp3 expanded a 5-10 fold increase in $CD4^+CD25^+Foxp3^+$ T regulatory cells (**Fig 13D**) and suppressed $CD8^+$ T effector function (**data not shown**), as assessed by reduction in Granzyme-B, compared to control DC expanded T cells.

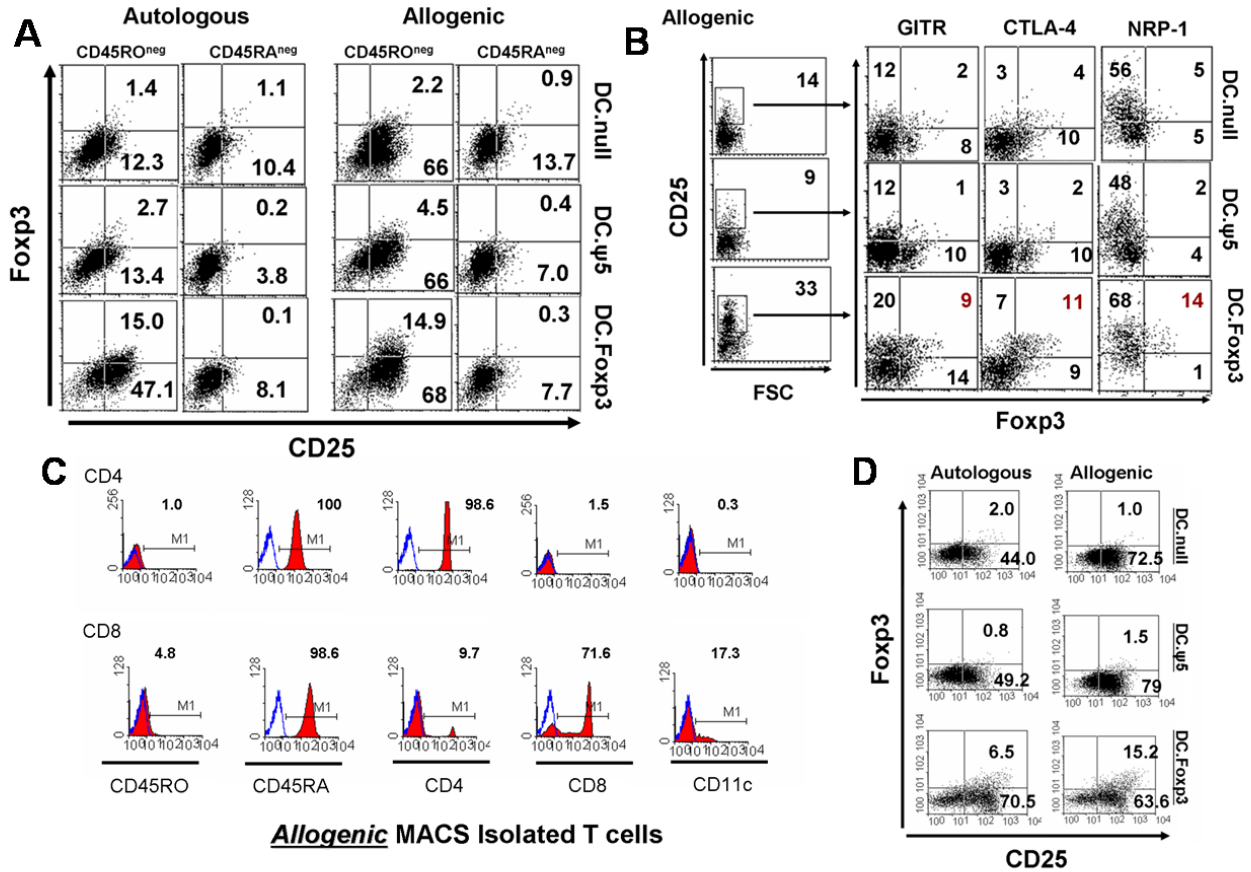

Figure 13. DC.Foxp3 expands pehenotypical, prototypical T regulatory cell subsets. DC.Foxp3 or control DC cultured with naïve or memory T cells, restimulation with respected DC on day 5 supplemented with IL-2 and IL-7, and evaluated on day 21. (A) Day 21-T cells were collected and costained with anti-CD4, CD25, and Foxp3. Plots represent the CD4+ gated population. (B) T cells were further co-stained with anti-GITR, CTLA-4, NRP-1. Plots are depicted as CD25+ populations. (C) MACS™ isolation of naïve (CD45RAposCD45RO^{neg}) CD4+ and CD8+ T cells was confirmed by flow cytometry. (D) Analgous studes to the afore mentioned procedure and staining of antibodies to CD4, CD25, and Foxp3 on day 21 to MACS™ isolated naïve CD4+ T cells

3.4.6. DC.Foxp3 expands functionally suppressive CD4+CD25+Foxp3+ Tregs

The next series of studies were designed to investigate XiTreg ability to suppress T cell proliferation and effector responses. Briefly, autologous or allogenic naïve CD45RO^{neg}CD4^{+} T cells were cultured with DC.Foxp3 or control DC for 21 days, with restimulation on day 5 with respected DC supplemented with IL-2 and IL-7. On day 21, CD25^{+} T cells were isolated using CD25 microbeads (miltenyi; MACS^{TM}). Naïve or memory CD8^{+} T cells, originating from the

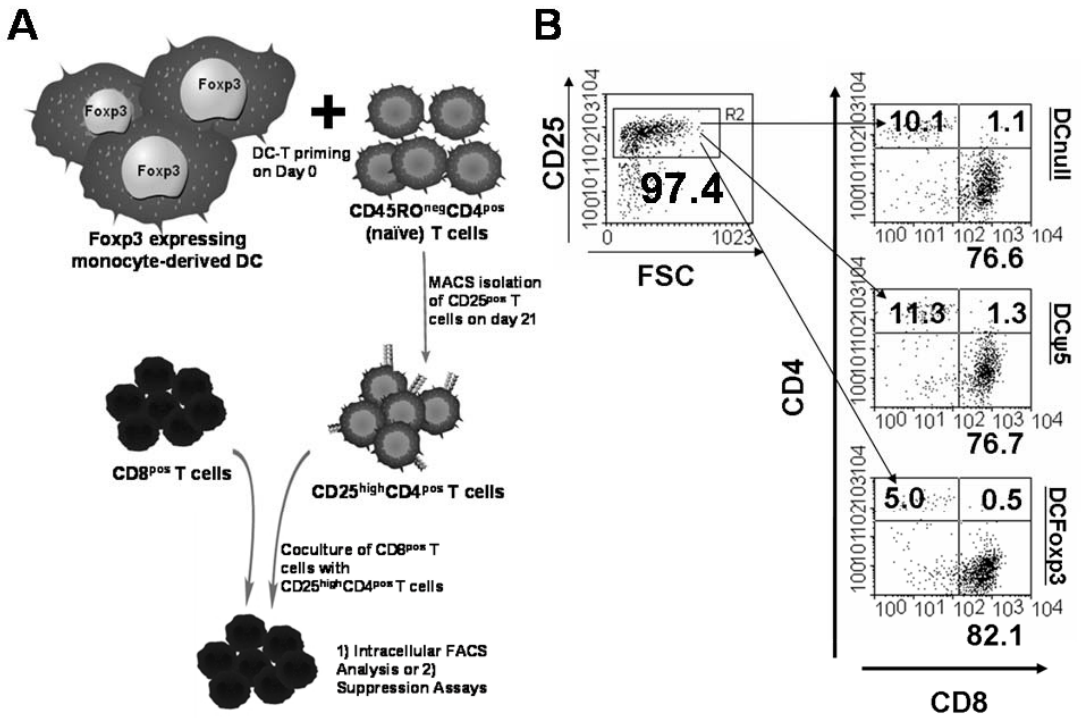

Figure 14. Schematic diagram for Treg generation from DC.Foxp3 or control DC and subsequent culture with responding naivce CD8+ T cells. (A) Schematic diagram of DC and CD45RO^{neg}CD4^{+} T cell initial culture on day 0, subsequent isolation of CD25^{+} T cells on day 21 and culture with fresh CD8^{+} responding T cells. (B) Flow analysis of day 21-CD25^{+} T cells isolated from each respected initial DC primed cohort and cultured with, at a 1:10 ratio, CD8^{+} T cells.

same donor as autologous DC, were isolated by depletion of CD45RO or CD45RA, respectively. Subsequently, CD8^{+} T cells were isolated from the depleted fraction to yield CD45RO^{neg}CD8^{+} or CD45RA^{neg}CD8^{+} T cells (**Fig. 13C**). Next, XiTreg or control DC expanded and isolated CD4^{+}CD25^{+} T cells were plates at a 1:10 ratio with naïve or memory CD8^{+} T cells in the presence of anti-CD3\CD28 (mitogen stimulating) microbeads (dynabeads, Invitrogen; **Fig. 14A**). This method allowed for gating of CD8^{+} fraction of T cells for changes in proliferation and effector functions via flow cytometric analysis. The ratio of CD4^{+}CD25^{+} T cells to CD8^{+} T cells was approximately 1:8 in the control DC expanded CD4^{+}CD25^{+} T cells and 1:16 for XiTregs **(Fig 14B**). Greater than 95% of CD4^{+} T cells plates with the CD8^{+} T cells co-expressed

CD25 (**Fig 14B**). After 3 days in culture, it was apparent by light microscope imaging that XiTreg, both derived from autologous and allogenic naïve $CD4^+$ T cells, impaired cluster formation (**Fig 15A**), suggesting that these (Xi)Tregs limited T cell activation. Labeling of the $CD8^+$ (naïve or memory) T cells with CFSE identified that the $CD4^+CD25^+$ T cells expanded from the DC.Foxp3 (XiTregs) culture greatly suppressed proliferation of naïve and memory $CD8^+$ T cells compared to control DC $CD4^+CD25^+$ T cells (**Fig 15B)**. Next, to assess restrained T effector functions, $CD8^+$ T cells, of the same donor as the autologous DC, were stimulated with anti-CD3\CD28 microbeads in the presence of IL-12p70, IFNγ, and anti-IL-4 for 10 days. Cells were washed thoroughly, counted and plated at a 10:1 ratio with DC.Foxp3 or control DC expanded $CD4^+CD25^+$ T cells, respectively, in the presence of anti-CD3\CD28 microbeads for 6h in the presence of 2 μM monensin. XiTreg suppressed IFNγ production by 60% of autologous and 90% of allogenic $CD8^+$ T effector cells (**Fig 15C**).

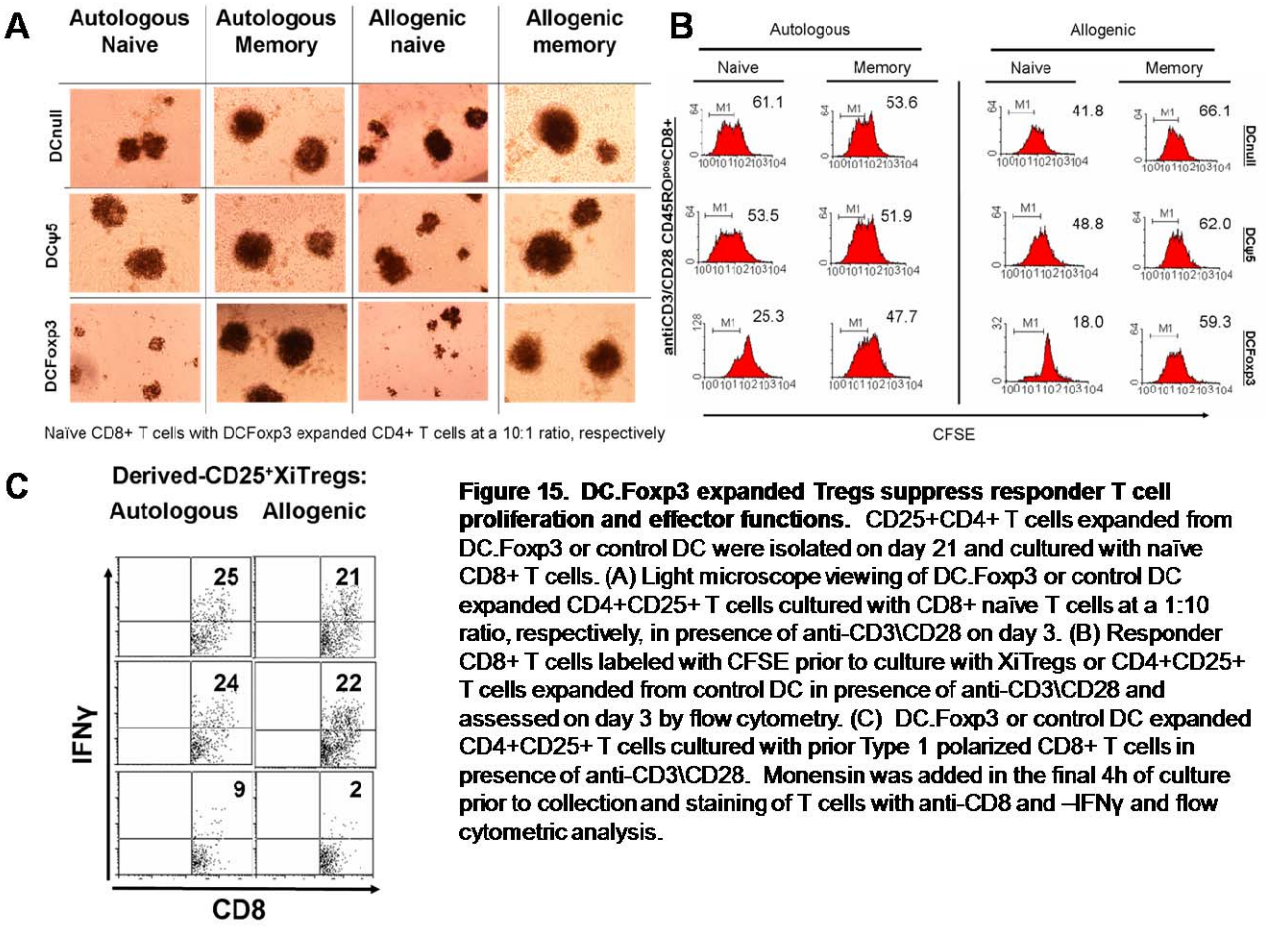

Figure 15. DC.Foxp3 expanded Tregs suppress responder T cell proliferation and effector functions. CD25+CD4+ T cells expanded from DC.Foxp3 or control DC were isolated on day 21 and cultured with naïve CD8+ T cells. (A) Light microscope viewing of DC.Foxp3 or control DC expanded CD4+CD25+ T cells cultured with CD8+ naïve T cells at a 1:10 ratio, respectively, in presence of anti-CD3\CD28 on day 3. (B) Responder CD8+ T cells labeled with CFSE prior to culture with XiTregs or CD4+CD25+ T cells expanded from control DC in presence of anti-CD3\CD28 and assessed on day 3 by flow cytometry. (C) DC.Foxp3 or control DC expanded CD4+CD25+ T cells cultured with prior Type 1 polarized CD8+ T cells in presence of anti-CD3\CD28. Monensin was added in the final 4h of culture prior to collection and staining of T cells with anti-CD8 and –IFNγ and flow cytometric analysis.

3.4.7. CD8$^+$ T cells are hyporesponsive

DC.Foxp3 directly suppressed CD45RO^{neg}CD8$^+$ T cells in the absence of CD4$^+$ T cells, showing that the DC.Foxp3 did not work solely through the generation of Tregs to suppress CD8$^+$ T cell responses (**Fig 12D and data not shown**). Thus, it remained to be seen if DC.Foxp3 induced hyporesponsiveness in CD8$^+$ T cells or if DC.Foxp3 generated CD8$^+$ T *suppressor* cells. Although still unclear, some evidence has been published that CD8$^+$ T *suppressor* cells can mediate direct suppression of T cell responses, co-express Foxp3, CTLA-4, GITR, and are absent of the CD28 costimulatory molecule (64; 67; 182). Briefly, DC.Foxp3 or control DC were

cultured with autologous or allogenic CD45RO^{neg}CD8^{+} T cells, restimulated on day 5 with respected DC in the presence of IL-2 and IL-7. Cells were harvested on day 21 and co-stained with antibodies to CD8, CD25, GITR, CTLA-4, CD28, and Foxp3. DC.Foxp3 primed CD8^{+} T cells from naïve, not memory, pools were diminished in CD25 expression, giving a strong reason as to why these Tregs had reduced proliferation responses and less total CD8^{+} T cell numbers (**Fig. 16A, 16B and data not shown**). Foxp3 expression was not found in any of the CD8^{+} T cell groups primed by DC.Foxp3 or control DC (**data not shown**). Interestingly, GITR expression was also restrained in DC.Foxp3 expanded CD8^{+} T cells (**Fig. 16B**). We ruled out the possibility that all these cells were dying, as staining of CD8^{+} T cells expanded by DC.Foxp3 had no changes in Annexin-V vs. PI levels upon flow cytometric analysis (**data not shown**), unlike what was observed in CD4^{+} responder T cells **(Fig. 11B**). CD28 or CTLA-4 had no changes in expression level between DC.Foxp3 or control DC stimulated groups (**Fig 16C**). Collectively, the data purposes that DC.Foxp3 does not induce CD8^{+} T *suppressor* cells, but rather generates hyporesponsive CD8^{+} T cell subsets that lack the high affinity IL-2Rα chain necessary for proliferative responses.

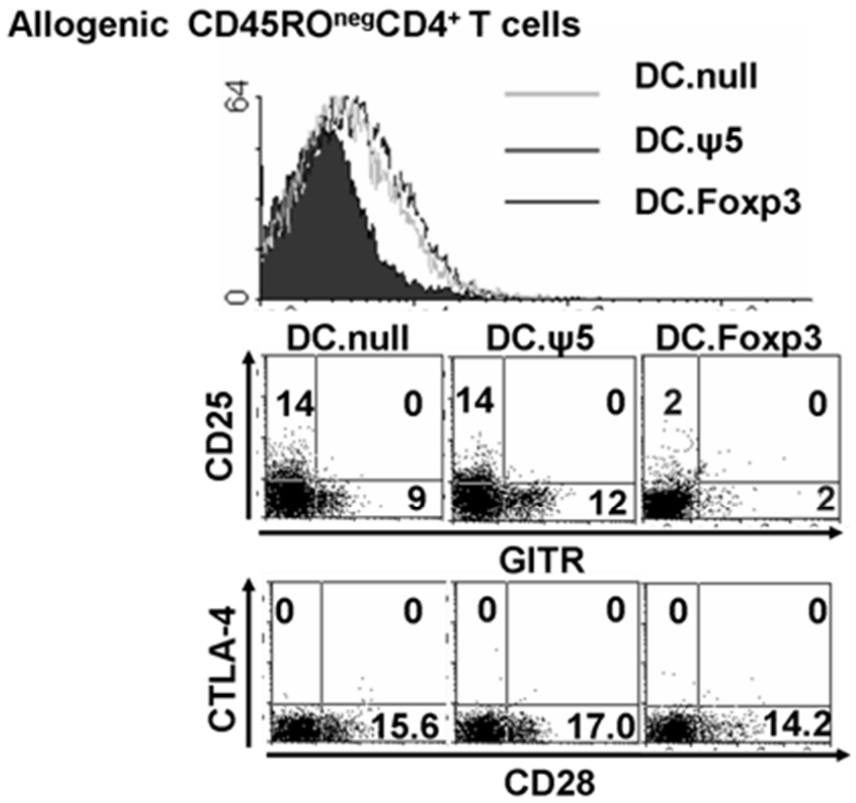

Figure 16. DC.Foxp3 induces hyporesponsiveness in responding CD8+ T cells. DC.Foxp3 or control DC were cultured with allogenic naïve CD8+ T cells for 14 days, with restimulation with respected DC supplemented with IL-3 and IL-7 on day 5. T cells were restimulated with PMA\Ionomycin for 4h with monensin and co-stained with antibodies to (A-B) CD25, (B) GITR, and (C) CTLA-4 and CD28. All plots are represented as the CD8+ gated subpopulation

3.4.8. Role of IDO and TGFβ in DC.Foxp3 induced tolerance.

To identify whether DC.Foxp3 mediated-immunosuppression was soluble or cell-contact, we first performed transwell studies. DC.Foxp3 plated in the lower chamber of a transwell plate was unable to suppress IFNγ production from activated responding naïve T cells in the upper chamber, as compared to control DC (**data not shown**), suggesting that DC.Foxp3 exerted immunosuppression via a direct cell-cell contact or close proximity mediated-mechanism(s). Next, a panel of inhibitory antibodies and agents were supplemented in cultures of DC.Foxp3 or

control DC with naïve (CD45RO^{neg}) CD4^{+} T cells, which included antibodies to TGFβ, IL-10, CD95 (Fas), programmed death ligand-1 (PDL1; B7H1), glucocorticoid-induced tumor necrosis factor receptor (GITR), and the neutralizing agent 1-methyl-tryptophan (1MT) that inhibits Indoleamine 2,3-dioxygenase (IDO) activity. DC.Foxp3 immunosuppressive functions were alleviated upon addition of anti-TGFβ or 1MT as indexed by total T cell count (**Fig 17A**) and Annexin-V^{+} CD4^{+} T cells on day 5 (**Fig 17B**). DC.Foxp3 suppression mediated by IDO and TGFβ were confirmed in dose response experiments to which IFNγ production was restored (**Fig 17C**) and the CD25 (IL2Rα), as an index to Tregs, was concomitantly restrained (**Fig 17D**).

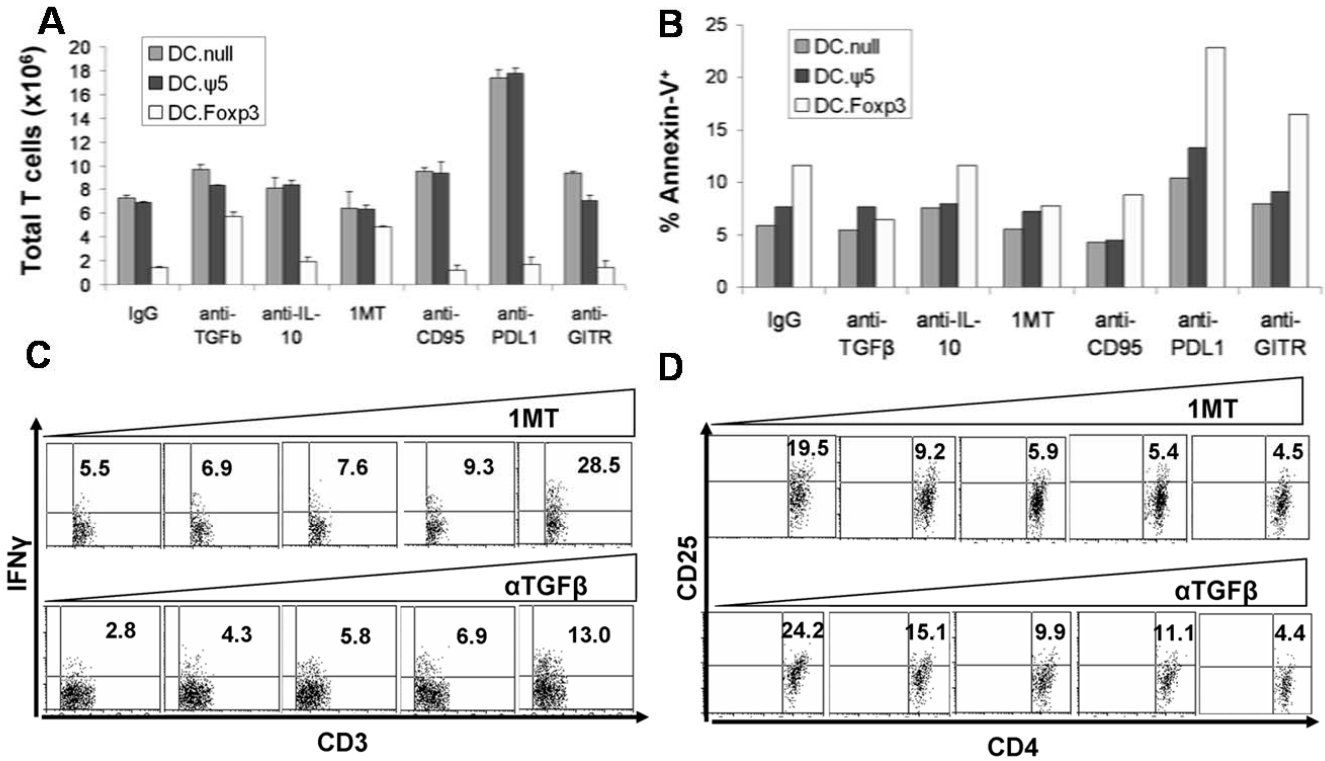

Figure 17. IDO and TGFβ exert partial dominant roles in induction of Tregs and suppression of Type-1 immune responses. DC.Foxp3 or control DC were cultured with allogenic naïve T cells in the presence of neutralizing antibodies or agents, as depicted. (A) Total T cells were counted on day 5 or (B) stained with Annexin-V and represented as a bar graph of percentage Annexin-V^{+}. Additionally, T cells were primed by DC.Foxp3 in the presence of varying concnetrations of IDO or TGFβ, restimulated on day 5 with respected DC supplemented with IL-2, IL-7 and respective concentration of IDO or TGFβ. (C) T cells were then restimulated on day 14 with PMA\Ionomycin and monensin for intracellular IFNγ analysis. (D) T cells were assessed on day 21 for co-expression of CD4 and CD25, as an index for Treg generation

CD25 (IL-2Rα) in these studies is an index (marker) for frequency of Tregs and not of activation, as T cells were assessed at a resting point over 15 days after last antigenic stimulation with DC.Foxp3 or control DC. Expression of IDO_1 and IDO_2, Neuropilin-1 (183) and the TGFβ-associated proteins Latency Associated Peptide (LAP) and Furin revealed no increased levels compared to control DC (**data not shown)**. Lack of functional conversion of L-tryptophan into kynurenine assessed by high performance liquid chromatography (HPLC) corroborated lack of DC.Foxp3 expression of IDO (**data not shown**).

3.4.9. DC.Foxp3 induces a state of hyporesponsiveness

In previous studies, T cells primed by DC.Foxp3 were restimulated with DC.Foxp3 on day 5, supplemented with IL-2 and IL-7. However, it remained to be identified whether these initial DC.Foxp3 primed T cell effector functions could be recovered upon restimulation with "normal" DC (non-gene modified immature DC). Briefly, DC.Foxp3 or control DC (donor A) was cultured with bulk naïve T cells ($CD45RO^{neg}$; donor B). After 7 days, T cells were collected and kept as bulk (labeled $CD45RO^{neg}$; indicating that the cells were initially stimulated from naïve precursors) or collected and further isolated by sorting for populations of $CD4^+$ or $CD8^+$ T cell subsets. T cells ($CD45RO^{neg}$, $CD45ROnegCD4^+$, or $CD45ROnegCD8^+$) were then counted and replated at 10:1 ratio to be restimulated with "normal" DC from initial donor (donor A) or a third, unrelated, donor (donor C). After an additional 7-9 days (on day 14-16), total T cells numbers were 5 fold less if initially primed by DC.Foxp3 compared to control DC (**Fig 198**). Additionally, supernatant collected 3-5 days after restimulation with donor A or donor C "normal" DC resulted in impaired IFNγ production from the initially primed DC.Foxp3 T cells **(Fig 18B)**. To accurately depict the frequency of responding T cells to produce IFNγ, we used intracellular staining after stimulation with PMA\Ionomycin in the presence of monensin to detect cytokine production in

the CD4^{+} or CD8^{+} cohorts on day 14. In both the CD4^{+} and the CD8^{+} (where the CD8^{+} group is represented as CD4^{neg} in flow diagrams), T cells initially primed by DC.Foxp3 were unable to produce IFNγ production upon recall after restimulation with "normal DC", showing that initial priming by DC.Foxp3 induced a long-lasting state of hyporesponsiveness in T cells (**Fig 18C**).

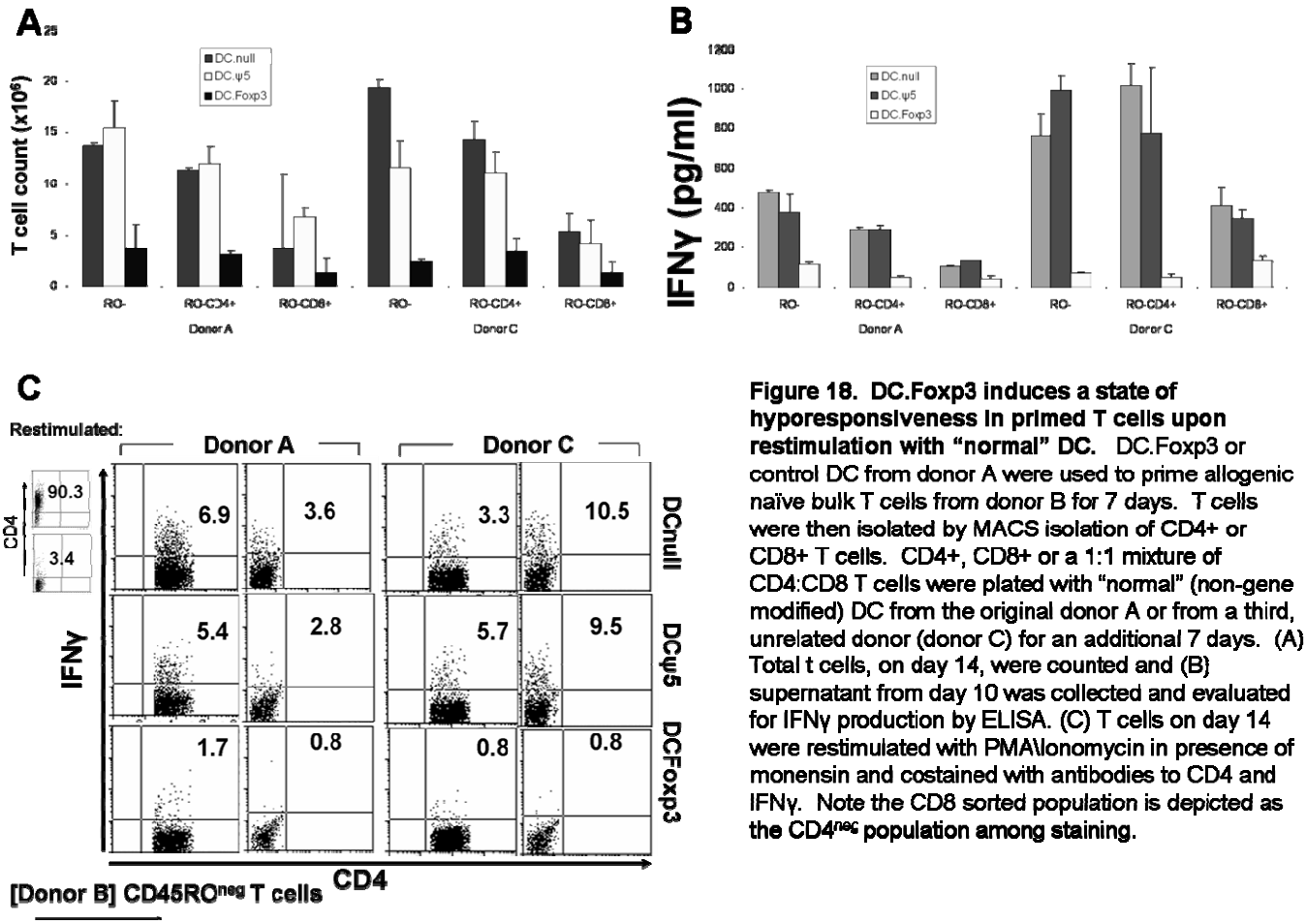

Figure 18. DC.Foxp3 induces a state of hyporesponsiveness in primed T cells upon restimulation with "normal" DC. DC.Foxp3 or control DC from donor A were used to prime allogenic naïve bulk T cells from donor B for 7 days. T cells were then isolated by MACS isolation of CD4+ or CD8+ T cells. CD4+, CD8+ or a 1:1 mixture of CD4:CD8 T cells were plated with "normal" (non-gene modified) DC from the original donor A or from a third, unrelated donor (donor C) for an additional 7 days. (A) Total t cells, on day 14, were counted and (B) supernatant from day 10 was collected and evaluated for IFNγ production by ELISA. (C) T cells on day 14 were restimulated with PMA\Ionomycin in presence of monensin and costained with antibodies to CD4 and IFNγ. Note the CD8 sorted population is depicted as the CD4^{neg} population among staining.

3.5. Discussion

In this study, I examined the role of ectopic Foxp3 expression in monocyte-derived DC and evaluation of T cell responses as an index to potential immunoregulatory potential of the gene-modified DC. The impact of FOX family transcription factors in suppressing the immune system has been attributed to the lymphocyte (13), epithelial (25), and myeloid, specifically DC (181), lineages. Foxp3 expression has correlated directly with immune suppression and\or evasion in lymphocyte and epithelial cells; however, its expression in myeloid cells has yet to be uncovered. Interestingly, another member of the FOX family, Foxo3, has been shown to modulate DC towards a prototypical tolerogenic state by impairing cytokine production and regulating the magnitude of T cell responses (181).

Ectopic Foxp3 expression in DC (DC.Foxp3) impaired expansion of both autologous and allogenic naïve, but not memory, $CD4^+$ and $CD8^+$ T cells. Furthermore, DC.Foxp3 was found to have differential mechanisms of immunosuppression on $CD4^+$ vs. $CD8^+$ T cell subsets. Specifically, DC.Foxp3 expanded T cells proliferated as many rounds as control DC expanded T cells, however, upon each subsequent division, there was no net increase in T cell number, suggesting that at each division round a proportion of T cells underwent apoptosis (or necrosis). I confirmed that there was higher cell death induced in predominately the naïve $CD4^+$, as opposed to the $CD8^+$, T cell subsets, suggesting that DC.Foxp3 does impart some element of selectivity in T helper cell populations. Alternatively, due to the heterogeneous population of Foxp3 expressing and non-expressing DC in the Adenoviral Foxp3 transduced cohort, it could be argued that the small proliferating cells are promoted by the non-expressing Foxp3-DC subsets. Further evaluation of DC.Foxp3 expanded naïve T cells revealed that Type 1, as assessed by IFNγ, T-bet, CXCR3, and Granzyme-B expression, and Type 17, as measured by IL-17A and RORγt expression, were restrained. It can be partially attributed to the lack of (polarizing) cytokine support, as DC.Foxp3 had abolished production of the cytokines IL-12p70,

IL-23, IL-6 and absence of the transcript to IL-27p28, lead to impaired Type 1 and 17 effector functions. Interestingly, IL-10 expression by DC.Foxp3, which suppresses Type 1 T cell responses by inhibiting proliferation and expression of IL-2, IFNγ, TNFα, IL-4, and IL-5 (184), was completely restrained. I reasoned that DC.Foxp3 may shut down IL-10 to prevent complete inhibition of T cell responses to allow for selective expansion and\or retention of differentiated $CD25^+Foxp3^+$ T cells and T_H2 from naïve T cell precursors. The mutual exclusion of the T_H1-T_H2 (18; 49) program in T helper subsets was further supported by increased frequency of IL-4 from DC.Foxp3 over control DC expanded T cells from the naïve precursors. Alternatively, this increased frequency of IL-4 positive cells could be due to specific T_H1 subsets undergoing selective apoptosis, whereas T_H2 subsets were spared; although there is currently no known mechanism for *DC selective- or modulated*-apoptosis of T_H1 over T_H2 cells.

DC.Foxp3 supported the selected generation (or retention) of $CD4^+CD25^+Foxp3^+$ T cells (as much as 10-fold) compared to control DC. There is a very low probability that Foxp3 is inadvertently transduced into T cells. Aside from extensive washing and isolation of DC prior to adenoviral transduction, naïve T lymphocytes do not express the required coxsackie\adenovirus receptor necessary for adenovirus cellular-mediated entry (185; 186). Furthermore, memory T cell subsets primed by DC.Foxp3 or control DC were unaltered in any indexed profile. DC.Foxp3 was unable to induce T *suppressor* cells from the $CD8^+$ naïve or memory T cell pools, or register any elevated levels of Foxp3. In turn, DC.Foxp3 repressed expression of CD25 (IL-2Rα) and GITR compared to control DC expanded $CD8^+$ T cell subsets, suggesting that DC.Foxp3 mediated $CD8^+$ T cell suppression in part by regulating responses to IL-2 via downregulation of the IL-2Rα chain.

Recently, early activated and proliferating human T cells have been shown to express Foxp3 concomitantly with CD25 chain (139; 187). My data showed that DC.Foxp3, but not control DC, expanded $CD4^+CD25^+Foxp3^+$ T cells co-expressed T regulatory-associated membrane-bound molecules CTLA-4 (188), GITR (189), and Neuropilin-1 (190) on day 21, over

15 days after last antigenic stimulation. Finally, DC.Foxp3 expanded $CD4^+CD25^+Foxp3^+$ T cell were functionally competent in their ability to suppress responding ($CD8^+$) T cell proliferation and effector functions; proper evaluation of suppressive responses were assured by isolating DC.Foxp3 or control DC expanded $CD25^+$ cells generated from the naïve precursors on day 21 (15 days after last stimulation) and using only $CD8^+$ T cells as readouts. Limited studies were performed using TGFβ or IL-10 culturing with GM-CSF and IL-4 during the 5-6 day monocyte differentiation into iDC. Culturing of these naïve T cell with these DC led to marginal skewing of Type 1 towards Tr1-like or T_H2 responses (**data not shown**). However, with the identification of Foxo3 in DC as a modulator of T cell responses, future studies will want to compare and contrast the differential roles of Foxo3 to Foxp3 in DC as a governer to T cell responses.

Transwell studies revealed that DC.Foxp3 suppression of Type 1 was dependent on cell-to-cell contact (*or close proximity*). Further investigation of mechanisms exerted by DC.Foxp3 to suppress Type 1 $CD4^+$ T cell responses and concomitantly induce functional $CD4^+CD25^+Foxp3^+$ T regulatory cells revealed roles for IDO and TGFβ. I found no increased protein or direct functional activity of IDO_1 or IDO_2 as assayed by western blot and HPLC from DC.Foxp3 compared to control DC, suggesting that DC.Foxp3 may impart early activated naïve T cells to act back on the DC (both DC.Foxp3 and neighboring DC) to induce IDO, possibly through CTLA-4:B7.2 engagement. Thus, it can by hypothesized that DC.Foxp3 utilizes other mechanism(s) to expand Tregs, which then functionally act back to perpetuate tolerance by inducing IDO in neighboring DC to aid in suppression of T cell responses. Thus, a proposed mechanisms may be that DC.Foxp3 exert early properties on responding naïve T cells that ensure skewing away from immunity and towards tolerance, in part by inducing T cells to provide TGFβ for support of Tregs, which may then act back on DC to induce IDO to impair Type 1 polarized responses and further support generation of Tregs (**Fig 19**).

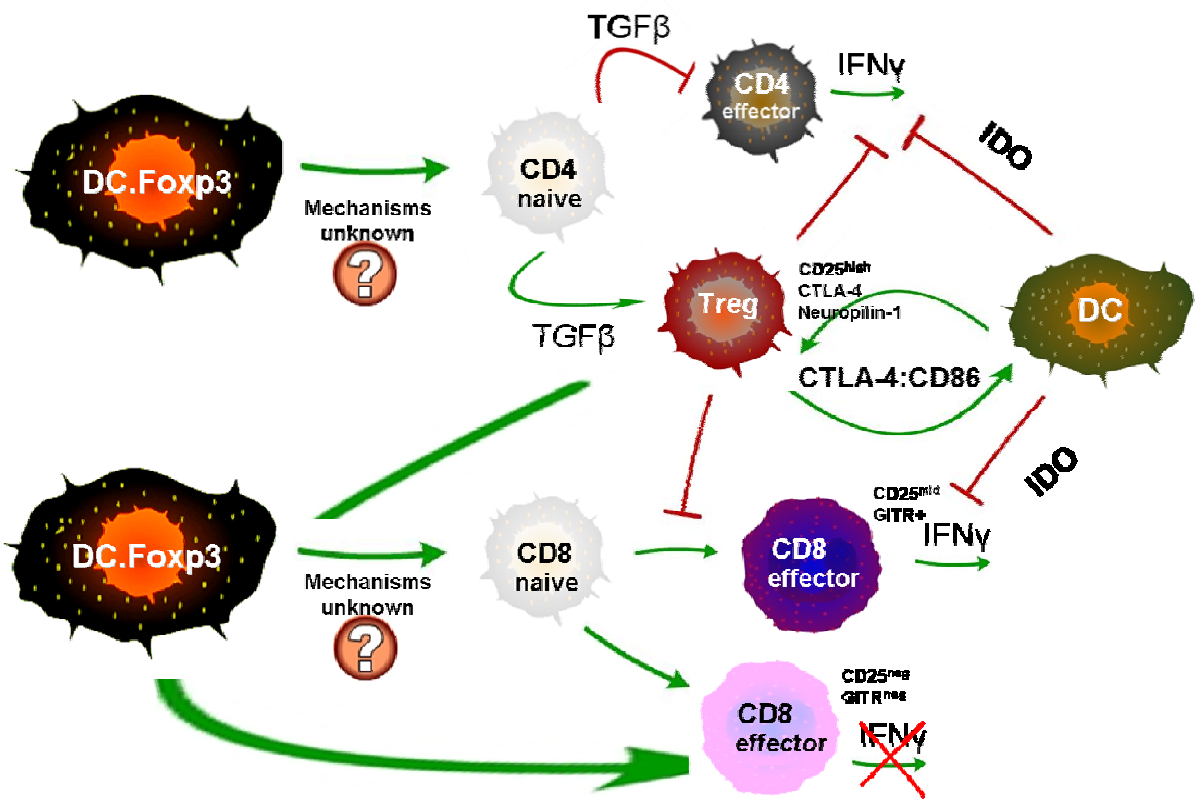

Figure 19. Possible mechanism for IDO and TGFβ in DC.Foxp3 suppression of T cell responses

It remains to be resolved what additional mechanisms of regulation Foxp3 exerts in DC; as well as how those translate into induction of Tregs and\or suppression of Type 1 polarized responses. However, identification of Foxo3 transcription factor expression in DC does lead us to believe that other FOX family members may play supportive roles in regulating DC mediated-immune tolerance. Additionally, Foxp3 complexing with other transcription factors in the lymphocyte lineage, including histone deacetylases and methylases, may provide a foothold for investigation into FOX roles in DC as modulators of T cell responses. Translationally, this data supports a strong candidate DC based-vaccine for both autoimmunity and transplant clinical settings, to which a pleiotropic cycle results were Type 1 T cell responses are restrained and Tregs are generated.

GENERAL DISCUSSION

T cell polarization state, which is mainly attributed to the $CD4^+$ T cell helper lineage, is primarily identified by the strict cytokine profile of differentiate T cells, with regulation at the epigenetic level. T helper (T_H) subsets currently consist of IFNγ (T_H1,), IL-4 (T_H2), or IL-17 (T_H17) polarization types [191]. $CD8^+$ T cell subsets, or cytotoxic T lymphocytes (CTL), are the principle mediators of direct cytolytic activity and are predominately associated with Type 1 polarization, as they predominately secrete IFNγ. Polarization states are additionally reflected in other membrane-bound and cytokine profiles that aid in T cell specific induction of humoral or cell-mediated immunity. For example, CTL express Granzyme-B and perforin for antigen-specific targeted release of cytotoxic granules, and both T_H1 and CTL express CXCR3 for trafficking to sites of inflammation [192]. Alternatively, T_H2 cells express little or no CXCR3, and are notably retained in the lymph nodes to prime B cell responses [77; 193], whereas T_H17 subsets have been shown to co-express CCR6 associated with their ability to home into intestine and associated lymphoid tissue [194]. Immunosuppressive T helper subsets have been clearly identified, designated T regulatory cell subsets (Tregs), which have been shown to exert immunosuppression in a both cell-contact and cytokine mediated manner [87; 195]. These cells have also been shown to co-express high levels of the cell surface molecules CD25, CTLA-4, GITR, and Neuropilin-1, each of which has been shown to play a distinct, yet possibly synergistic role, in suppressing immune responses [190; 195-197]. However, it was the identification of the master regulators of the T helper subsets that clarified the rigid differentiation or polarization of T cells. Expression of T-bet (T_H1, CTL), GATA-3 (T_H2), RORγt (T_H17), and Foxp3 (Tregs) by polarized subsets of T cells have recently been described [14; 21; 57; 198]. Most interestingly, these transcriptional factors appear functionally antagonistic in a

given T cell clone. Hence, T-bet antagonizes GATA-3 expression [18], impairing T_H2 responses and reinforcing Type-1 immunity, and the absence of T-bet, allows GATA-3 expression to go unopposed, and yields a Type-2 functional program [14]. Similar antagonizing is seen and has helped clarify the mutual exclusivity between T_H17 and Tregs subsets, where Foxp3 antagonizes RORγt expression [21].

As interesting as this is, it is critical to remember that important role played by polarized DC in orchestrating corollary T cell polarization. In such a fashion, micro-environmental cues that serve to shape DC polarity in the periphery may be imparted upon responder T cells in tissue-draining lymphoid organs [36; 90; 94; 158; 168; 169; 199; 200]. Glimcher et al [24] was the first to show that absence of T-bet expression in DC led to impaired Type-1 T cell responses, as indexed by IFNγ expression, but that T cell proliferation was largely unaffected. Follow up studies by the Glimcher group and others have confirmed that both conventional and monocyte-derived DC endogenously express T-bet. These published and preliminary studies prompted me to evaluate the impact of ectopically overexpressing T-bet in DC on consequent T cell responses. Similarly, even though no published reports exist to support the natural expression of Foxp3 by DC, unpublished anecdotal data from a mouse tumor model suggested that $CD11c^+$ DC within the tumor microenvironment may express Foxp3, suggesting the physiologic relevance of studying Foxp3 expression in DC (**Storkus et al, unpublished data**). If validated, both DC.T-bet and DC.Foxp3 would have clear translational value for the development of vaccines targeting either the augmentation or eradication of Type-1 immune responses, respectively.

Adenoviral insertion of T-bet into DC (DC.T-bet) yielded little-to-no changes in any costimulatory molecules evaluated; however, these DC were impaired in their ability to produce the cytokines IL-12, IL-23, IL-27, and TNFα, which are key mediators of Type 1 polarization in responder naïve and/or memory T cells. Nevertheless, naïve T cells primed using DC.T-bet

exhibited an average 3-fold increase in their levels of IFNγ produced when compared to control DC primed T cells. Light microscopy images showed significant increases in the microcluster sizes formed between DC.T-bet and proliferating naïve, but not memory, T cell responders as early as day 3 in culture, suggesting that the dynamic interplay between DC.T-bet and T cells might be reinforced at the immunological synapse level. It is important to note that there were no differences in performed assays and results generated upon DC.T-bet and control DC primed memory T cells (i.e., DC.T-bet did not *repoloarize* or *reprogram* memory T cells). Further investigation revealed that the T-bet transcription factor was increased in responder naive T cells, concomitant with decreased GATA-3 expression by day 3 of DC.T-bet-T cell cultures. This bias in transactivator protein expression by responder T cells was recapitulated in the balance of T cell elaborated cytokines, with increased IFNγ production, and restrained IL-4 and IL-10 expression. Interestingly, IL-17 expression from responder T cell, despite RT-PCR data suggesting that changes in T cell expressed RORγt were marginal, was increased. As Foxp3 antagonizes the RORγt induction of T cells, I believe this is a by-product of DC.T-bet restraining Foxp3 expression in T cells, allowing for existing levels of RORγt to function unopposed in DC.T-bet-driven T cells. Notably, IL-17-producing responder T cells did not co-produce IFNγ (i.e. IFNγ^{+}IL-17^{+} double positive T cells), suggesting that DC.T-bet did not promote the cross-conversion of Type 1 cells into T_H17 cells, or *vice versa* [16; 201].

Reiner et al first showed that Type 1 polarization, as indexed by T-bet, was instigated prior to IL-12/IL-12R engagement in T cells [45]. My data sets suggests that DC.T-bet induces an IL-12-independent Type 1 polarization in T cells, as DC.T-bet were unable to produce IL-12 (or any cytokine investigated) upon TLR or CD40 engagement. Use of neutralizing antibodies to IL-12, IL-23, or IL-27 confirmed that DC.T-bet induces elevated levels of IFNγ in responding naïve T cells. Furthermore, usage of antibodies to block the IFNγR1 failed to impair DC.T-bet induction of Type 1 polarization in T cells, suggesting that DC.T-bet did not rely on the autocrine

feedback of IFNγ production by activated T cells to drive Type 1 polarization. Several other reports have suggested IL-12 independent mechanisms of Type 1 polarization engendered in T cells by DC, which included both delta like 4 (DLL4) engagement with notch ligands and CD70 engagement with CD27 on responding T cells. Blockade of these factors also did not alleviate DC.T-bet induction of Type 1 polarized responses. My studies thus propose a novel mechanism(s) by which transgene T-bet drives DC to induce Type 1 T cell polarization, further reinforcing work by the Reiner group that additional mechanism(s) prior to IL-12 secretion and IFNγ feedback (for stabilization of the Type 1 profile) are critical for Type 1 T cell induction. Furthermore the ability to break operational tolerance in $CD8^+$ T cells reactive against melanoma-associated antigens using DC.T-bet-based stimulation may have translational merit in the development of melanoma vaccines for clinical application.

Although thoroughly investigated, I have been unable to as yet identify the specific mechanism(s) exerted by DC.T-bet in driving Type-1 T cell polarization. I have, however, ruled out many contenders. Transwell assays suggest that DC.T-bet exerts its Type 1 inducing function in a cell-cell contact-dependent manner or that this effect requires the close proximity of DC.T-bet and T cells in order to occur. There were no significant changes in levels of known co-stimulatory or co-inhibitory molecules expressed by DC.T-bet vs. control DC, nor differences in CD70 or DLL4 expression that have previously been linked to IL-12-independent promotion of Type-1 immunity by DC. Although, in regards to costimulatory molecules, it could be that they remain constitutively. Hence, the logical suspects have failed to be incriminated in my studies thus far, and this may warrant the prospective use of microarray/proteomic analyses to define molecular differences downstream of T-bet in DC that are required for the observed DC.T-bet functional attributes. The dependency of DC.T-bet function on the identified candidates would then need to be confirmed via the use of Ab-blocking or mRNA knock-down in vitro experiments, or the development and use of specific knockout mouse models.

In complementary studies, I investigated the potential ability of Foxp3 gene insertion to yield regulatory DC. Despite the lack of publications documenting endogenous Foxp3 in DC, one recent report by Dejean et al that showed absence of Foxo3, another member of the FOX-related family of transcription factors, conferred sustained T cell immune responses [181]. Additionally, previous unpublished data generated in the CMS4 tumor model in Balb/c mice (by Dr. Aklile Berhanu while a GSR in the Storkus laboratory) suggested that a subset of $CD11c^+$ DC in the tumor microenvironment co-expressed Foxp3 protein based on immunofluorescence microscopy analyses. Since DC in the progressive tumor microenvironment have been typically shown to be regulatory APC, this provided some degree of confidence that DC.Foxp3 could represent regulatory DC; also highlighting the translational relevance in the autoimmunity and transplant settings.

Autoimmunity is characterized by aberrant, autoreactive T cells that cause pathology by targeting host tissues for destruction. Transplantation, similarly, is plagued by undesirable alloreactive T cells that target the recipient grafts for rejection. Numerous studies have shown that immunosuppressive strategies that limit T effector cell responses and\or expand the population of T regulatory cell subsets can control both auto- and alloreactive responses. More interestingly, it has been shown that modulation of DC *ex vivo* or *in vivo* can skew their polarization state to a more suppressive, regulatory fate that can effectively limit T effector cell responses and\or induce T regulatory cells [106]**.** It was the goal of this secondary project to assess if DC.Foxp3 are indeed tolerogenic (regulatory) DC.

Priming of naïve, but not memory, T cells with Foxp3 expressing DC (DC.Foxp3) led to impaired early T cell responses. More importantly, restrained T cell responses were observed in both the autologous (using superantigen model system) and allogenic (different donor T cells to DC), suggesting that DC.Foxp3 could provide therapy benefits in both the autoimmunity and transplant settings. Interestingly, total T cell numbers resulting from stimulation with DC.Foxp3

were limited, which was partially attributed to restrained T cell proliferation and the selective apoptosis of Type-1 polarized $CD4^+$ T helper subsets in these assays. This suggested that DC.Foxp3 preferentially selected against Type 1 polarization in the $CD4^+$ T helper subset of responder T cells. There was much less of a pronounced affect on $CD8^+$ T cells activated using DC.Foxp3, suggesting that suppression of proliferation was a principle mechanism underlying restrained CTL development guided by DC.Foxp3. Analysis of polarized T cells expanded from the naïve pool by DC.Foxp3 revealed a dramatic impairment of Type 1-associated IFNγ, CXCR3, and Granzyme-B gene product expression, with a concomitant increase in Type 2-associated IL-4 production. T cell cytokine profiles corroborated early transcription factor profile analysis, and also highlighted the slight decrease in IL-17 production (T_H17 subsets) that was not as dominant when screening for comparative levels of RORγt mRNA using RT-PCR. These data sets clearly show that DC.Foxp3 effectively restrains T effector Type 1 and Type 17 cell responses.

My studies revealed the DC.Foxp3 (vs. control DC) preferentially expand and differentiate functionally-suppressive, $Foxp3^+$Tregs that co-expressed CD25, CTLA-4, GITR, and Neuropilin-1. I took numerous steps to ensure the quality of the results, in particular by evaluating T cells for Foxp3 expression over 15 days after last stimulation, to alleviate concerns that Foxp3 is transiently expressed and carried through the activation states of human T cells (and that only Tregs, upon T cell rest, maintain elevated levels of Foxp3). Furthermore, the fact that these T cells coexpressed $Foxp3^+CD25^{high}$ along with CTLA-4, GITR, and Neuropilin-1 suggested that these were classical Treg. After performing suppressor functional studies, I concluded that these resultant T cells were indeed inducible ($Foxp3^+$) Tregs. Identification of $Foxp3^+$Tregs activated by DC.Foxp3 from the $CD4^+$ naïve pool led me to next investigate whether these APC could also develop $Foxp3^+CD8^+$ T suppressor (regulatory) cells in vitro. Some reports suggest that $CD8^+$ T cell suppressor cells also co-express CTLA-4, GITR, and

CD25, but are deficient in expression of CD28 [64]. However, DC.Foxp3 primed naïve $CD8^+$ T cells did not express higher levels of Foxp3, CD25, CTLA-4, or GITR compared to controls. Alternatively, CD25 and GITR expression in DC.Foxp3 primed $CD8^+$ T cells was suppressed compared to control DC expanded $CD8^+$ T cells. It can be hypothesized that absence of the IL-2Rα chain constrains $CD8^+$ T effector responses, and also argues why DC.Foxp3 may not need to depend on differential apoptosis as a mechanism to suppress $CD8^+$ T effector cells. Whereas in the case of $CD4^+$ T cells, DC.Foxp3 utilizes apoptosis to selectively weed out T_H1 and T_H17 responses, allowing T_H2 and Tregs to persist/expand. Additionally, GITR signaling on $CD8^+$ T cells has been shown to induce potent T effector functions, effectively overcoming immune tolerance in the cancer settings [202; 203]. Furthermore, DC.Foxp3 induces $CD4^+Foxp3^+$ Tregs cells in the absence of $CD8^+$ T cells and can conversely impair $CD8^+$ T cells in the absence of Tregs, suggesting that direct mechanisms are employed by DC.Foxp3 to restrain $CD4^+$ or $CD8^+$ T cells.

IDO and TGFβ (based on neutralization/blocking studies) play prominent roles in DC.Foxp3-associated regulatory functions. Investigation of DC.Foxp3 for direct expression suggested that Foxp3 did not directly induce IDO or TGFβ. IDO is produced primarily by DC, playing important roles in limiting immune responses by starving T effector cells of free tryptophan[108], whereas TGFβ is primarily expressed by T cells (as well as supporting mesenchymal\stromal and epithelial cells in draining lymph nodes). I hypothesized that DC.Foxp3 primed early activated T cells to secrete TGFβ to aid in Treg generation and impair Type 1 (IFNγ) responses. Concomitantly, these early activated T cells were engendered by DC.Foxp3 to, possibly through CTLA-4:B7 engagement [107], upregulate IDO expression. The culmination of these studies proposes that DC.Fop3 skews naïve T cells towards Tregs, and away from Type 1 T effector cells, to effectively suppress immune responses, to which these early activated T cells are promoted to secrete TGFβ to reinforce Treg generation and engage

B7 molecules on DC in order to induce IDO-mediated suppression of T effector cells and support of Tregs. Additionally, the phenotype of DC.Foxp3 expanded Tregs also suggests that CD25 acts as a cytokine sink for free IL-2, leading to IL-2 starvation of T effector cells, and Neuropilin-1 serves to allow Tregs to outcompete naïve or activated T cells for access DC, thereby limiting effector functions. In the case of $CD8^+$ T cells cultured with DC.Foxp3, antagonism of IDO, and to a lesser extent, TGFβ, served to mitigate the function of regulatory aspects of DC.Foxp3. I believe this again contributes to the dynamic interplay at the immunological synapse between DC.Foxp3 and responding T cells that acts back on DC to invoke multiple mechanisms to suppress T effector responses. A simple model system using Foxp3 knockout mice would remove T regulatory cell subsets from the equation, allowing us to identify what DC.Foxp3 does directly without T regulatory cell subsets. Additionally, IDO knockout DC and TGFβ knockout DC and\or T cells would help to identify the importance of one or the other molecule to DC.Foxp3-associated function(s).

Future studies utilizing knockout studies and expression vectors in mouse model systems will help to characterize the roles of T-bet and Foxp3 in DC, in both regulating T effector and T regulatory cell responses. Additionally, given the pleiotropic and redundant biology of transcription factors, large scale phenotypic analysis will be required to accurately identify changes in DC, including microarray and proteomic analysis. These studies will undoubtedly reveal novel mechanism(s) of action for transgene-altered DC function(s). I believe that these results will ultimately highlight the intricacies and importance of early events occurring within the DC-T cell immunological synapse that serve to enforce corollary T cell functional polarization. I believe DC may then subsequently respond to T cell-delivered signals by expressing cytokines that support polarized T cell differentiation process after DC-T cell disassociation. Such DC-produced factors would remain in the local microenvironment. This makes conceptual sense, as it allows T cells to continue their differentiation process while not

occupying a “docking” spot on the DC surface, thus allowing for access of alternate naïve T cells.

Beyond the confines of basic science, both of these systems define novel and potentially powerful immunotherapeutic DC-based vaccines. The necessity to induce antigen-specific, or polyclonal suppression in the case of allograft rejection, is best engendered by DC, the principle orchestrators of immunity. *Ex vivo* modulation of these DC to overexpress T-bet or Foxp3 and adoptive transfer will in turn perpetuate desirable T cell responses in the cancer/infectious disease vs. autoimmunity/transplant settings. The prospective use of murine model systems will highlight the pre-clinical efficacy of these vaccines, for which preliminary data already appears favorable in the case of a DC.T-bet model system.

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