DENDRITIC CELLS REGULATE THE INDUCTION OF EFFECTOR AND MEMORY $CD8^+$ T CELLS

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Dendritic cells (DCs) are key antigen-presenting cells in the immune system that can induce pathogen-specific T cell responses by presenting antigen (signal 1) to antigen-specific T cells in combination with co-stimulatory/inhibitory molecules (signal 2) and secretion of cytokines (signal 3). The ability of DCs to orchestrate CD8$^+$ T cell responses, combined with the ability to generate high numbers of DCs *in vitro* allows for their use in DC-based vaccination protocols. The success of DC-based vaccination protocols and other forms of immunotherapy of cancer is believed to depend on the successful induction of both effector CD8$^+$ T cell (CTLs), able to migrate into and kill tumors, and long-lived memory cells, able to generate a secondary response upon tumor recurrence. However, the signals that drive the differentiation of CD8$^+$ T cells into each of these T cell subsets and the role of DCs in this respect remain unclear. Studies have suggested that the same DC can induce effector cells early after maturation while inducing memory cells after prolonged maturation when the DCs have become exhausted.

Here, I analyzed the role of DCs matured under conditions mimicking acute/early inflammation ("inflammatory-DCs") or mimicking chronic/late inflammation ("non-inflammatory-DCs") on the differentiation of CD8$^+$ T cells. I observed that "inflammatory-DCs" produce high levels of IL-12p70 and induce the differentiation of naïve CD8$^+$ T cell into cytolytic effector cells with peripheral homing ability. Furthermore, I demonstrate the role of IL-

12p70 in this process. In contrast, “non-inflammatory-DCs” (exhausted DCs) do not produce IL-12p70 and induce the direct differentiation of naïve $CD8^+$ T cells into central-memory cells. The superior ability of “inflammatory-DCs” to induce anti-tumor responses guided me to develop an alternative, low-cost method of generating “inflammatory-DCs” with strong CTL inducing ability. Lastly, I show that modulation of the tumor-chemokine environment by IFNα, poly-I:C and indomethacin enhanced the attraction of tumor-specific CTLs while reducing regulatory T cell attraction.

Together, the presented data broadens our understanding of the mechanisms of DC-induced effector and memory cell differentiation and might lead to the improved DC-based cancer vaccines.

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PREFACE

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1.0 INTRODUCTION

Dendritic cells (DCs) play a crucial role in the induction of adaptive T cell responses needed for the defense against infections and tumors. The ability of DCs to take up, process and present antigen (Ag) to Ag-specific T cells, combined with the ability of DCs to selectively secrete polarizing cytokines and chemokines, allows for the generation of robust T cell responses and is the basis for DC-based vaccines. For protection against intracellular pathogens and tumors, the induction of Ag-specific cytolytic $CD8^+$ T cells (CTLs) capable of migrating to the sites of infections is essential, while the generation of long-lived Ag-specific memory $CD8^+$ T cells is crucial for long-term protection.

1.1.1 Dendritic cell subsets

DCs are generated from either myeloid or lymphoid bone marrow progenitors. The bone-marrow derived precursors migrate thought the blood and home to tissues where they reside in an immature state, scanning the environment for pathogens (1). There are distinct subsets of DCs which can be distinguished based on phenotype, function and localization (2), i.e. myeloid DCs and plasmacytoid DC (pDCs). However, within these subsets, other subsets can be distinguished based on the “pattern” of surface protein expression. For example, in the skin, the dermis hosts

two subsets, namely $CD1a^+$ DCs and $CD14^+$ DCs, while there is a distinct subset in the epidermis, the Langerhans cells (LC) (2).

One aspect in which these DCs differ is their expression of pattern-recognition receptors (PRRs), which respond to evolutionary conserved molecular patterns on pathogens, known as pathogen-associated molecular patterns (PAMPs) (3-4). The triggering of these PRRs on non-hematopoietic cells results in the secretion of inflammatory cytokines and chemokines, while the engagements of PRRs on the surface of DCs results in the activation and maturation of the DCs and their migration through the afferent lymph to draining lymph nodes (dLN), where they can interact with pathogen-specific T cells. Interestingly, the "same" PAMP (e.g. LPS) derived from different pathogens can activate DCs to secrete different cytokines (5).

Another aspect in which the DC subsets differ is their ability to secrete polarizing cytokines and to induce distinct T cell responses (*discussed below*). For example, pDCs express Toll-like receptor (TLR) 7 and TLR9, which recognize viral antigens. pDCs are recognized as the major source of type I interferons (IFNα/β) (6). Interestingly, pDCs express the cytolytic molecules granzyme B and TRAIL and activation of pDCs by Imiquimod (a TLR7/8 ligand) or IFNα induces the expression of TRAIL on the pDC surface, which enables them to kill target cells (i.e. Jurkat cells, melanoma cell lines) (7-8). On the other hand, dermal $CD14^+$ DCs express multiple TLRs (e.g. TLR2, TLR4, TLR5, TLR6, TLR8 and TLR10) and are able to produce a large set of cytokines, including IL-1β, IL-6, IL-8, IL-10 and IL-12 when stimulated through CD40 (9).

The observation that $CD8\alpha^+$ DC, found in T cell areas of the spleen and lymph nodes of mice, are capable of cross-presenting exogenous antigen to the TCR of $CD8^+$ T cells (10) has resulted in their use for priming $CD8^+$T cell responses to particulate antigens. Together with their

cross-presenting ability, CD8α$^+$DCs are also able to secrete large amounts of bioactive IL-12p70 upon maturation (11), allowing them to induce cytotoxic effector CD8$^+$T cells.

The ability of CD8$^+$ DCs to cross-present exogenous antigens is of great interest since it allows the loading of DCs with whole protein or cell lysates. However, the lack of CD8α expression by human dendritic cells has prevented the use of this subset for therapeutic purposes. Recent studies have therefore focused on finding a human homolog of mouse CD8α$^+$DCs. The newly described BDCA (Blood DC Antigen) surface proteins have allowed a better distinction of human DC subsets (12) and identified a BDCA3$^+$ DC population as potential homolog of the mouse CD8$^+$DC (13). This study showed that human BDCA3$^+$ DC and mouse CD8α$^+$ DCs had a very similar gene profile, setting them apart from other DC subsets. Further studies have identified other markers, XCR1 and CLEC9A (DNGR-1), that are conserved between mouse CD8α$^+$ DCs and a subset of human BDCA3$^+$ DC (14-15). An important finding for the use of these DCs in clinical settings, is the ability to generate these cells *in vitro* from cord blood derived hematopoietic stem cells, although the yield is very low (15). Like their mouse CD8α$^+$ counterparts, human BDCA3$^+$XCR1$^+$ DNGR-1$^+$ DCs are able to cross-present antigen and produce high levels of IL-12p70 upon maturation with poly-I:C (15).

1.1.2 DC maturation and polarization

While it was originally thought that DCs in lymphoid organs exists in an immunogenic state, it was observed that targeting of antigens to DCs *in vivo*, using the DC expression of the surface protein CD205 (formerly DEC-205), results in the expansion of antigen-specific T cells, which subsequently disappear yielding a state of specific immune tolerance (16-17). These

studies showed that under steady-state conditions, in the absence of acute infection sand the associated inflammatory factors, DCs function in inducing peripheral tolerance (5). The tolerance-inducing ability of "steady-state DCs" can be of use in the treatment of autoimmune disease or in tissue transplantation by the induction of regulatory T (T_{reg}) cells specific for auto-antigens (18). DCs, however, can be matured by pro-inflammatory cytokines, such as TNFα and type I interferons (IFNs), in the absence of PRR stimuli (19-20). DCs matured in this way exhibit elevated expression of co-stimulatory molecules (CD80, CD86 and CD40) and express CCR7, a chemokine receptor associated with lymphoid homing properties (20-22).

The ability to mature DCs using cytokines has facilitated the development of DC-based vaccines for immunotherapy. However, studies have suggested that these types of DCs are unable to provide a (type-1) polarizing signal 3 to the responding T cells (23) and are thus unable to induce Th1/CTL responses. Ag-specific naïve T cells interacting with these DCs are able to undergo proliferation and activation, but are unable to produce IFNγ and lack cytolytic ability. It has been suggested that DCs matured in the absence of PRR triggering play a fundamental role in the development and maintenance of T_{reg} cells (24). In accordance with this notion is the observation from human *in vitro* studies demonstrating that monocyte-derived (mo)DCs matured in the presence of cytokines and prostaglandin E_2 (without PRRs ligands) secrete higher levels of the T_{reg}-attracting chemokine CCL22 when compared to moDCs matured with pro-inflammatory cytokines and a TLR ligand (25). DC activation in the absence of PRR signaling *in vivo* could occur in the lymph nodes by diffusion of pro-inflammatory cyt okines from the site of infection or in a bystander fashion at later stages of the immune response when most pathogens have been cleared.

In vitro studies have suggested that the inability of DCs to provide signal 3 during T cell priming results in the expansion of non-polarized cells (3, 26). However, other studies have shown that the combination of IFNγ and CD40 ligation can induce the generation of DCs with a high IL-12p70 secretion ability. The priming of DCs for the production of bioactive IL-12p70 has been shown to require two signals (27). DCs matured by either TLR ligands alone or CD40L alone produce only marginal amounts of IL-12p70. In contrast, the production of IL-12p40, the inactive subunit of the IL-12 heterodimer which has an immune inhibitory function, could be induced by maturation with a single stimulus (4, 27).

It was shown that maturation of DCs by TLR signals alone, in the absence of inflammatory signals (e.g. IFNγ), induces the maturation of the DCs (4) and polarize them to preferentially secrete IL-23, which is associated with a Th17 T cell response (28). The combination of a pro-inflammatory cytokine (i.e. IFNγ) with a TLR ligand (i.e. LPS or R848) allows DCs to secrete IL-12p70 during maturation (spontaneous secretion) and after secondary stimulation via CD40 (DCs)-CD40L (T cells) interactions. In contrast, DCs matured by 2 TLR ligands in the absence of inflammatory cytokines produce IL-12 during maturation, but fail to produce additional IL-12p70 upon subsequent CD40 triggering (29). These results show the impact of different pathogen derived signals (direct through TLR ligands and indirect through cytokines) on the maturation of DCs and on controlling the induction of T cell responses.

1.1.3 Signal 3

As mentioned above, different DC subsets can elicit different T cell responses, depending on the cytokines they produce. DCs provide naïve (and memory) T cells in the lymph nodes 3 different "signals" about the pathogen/tumor: i) information about the molecular identity of the

pathogen/tumor (i.e. antigen presented to the T cells in the form of pathogen/tumor-derived peptide bound to MHC molecule complexes; "signal 1") ii) a signal about the pathogenicity of the pathogen/tumor, "instructing" the T cells how much to expand (i.e. by DC expression of co-stimulatory and co-inhibitory molecules; "signal 2")(3, 30), and iii) information about what type of immune response (e.g. Th1, Th2, Th17, Treg) is required (i.e. by the secretion of polarizing cytokines; "signal 3").

The T cell polarizing cytokines secreted by DCs during the DC:T cell interaction depend on the type of pathogen the DCs encounters (e.g. extracellular versus intracellular), the inflammatory milieu during which the DC is activated (e.g. acute inflammation versus late/chronic inflammation), the duration between DC activation and T cell encounter and possibly the antigen-dose (3-5, 26, 29, 31-35).

1.1.3.1 IL-12p70: a "signal 3" cytokine critical for type-1 immune responses

Type-1 immune responses (i.e. Th1 $CD4^+$ T cells and cytolytic $CD8^+$ T cells) are required for the clearance of intracellular infections and are believed to be optimal for the elimination of tumors (36). The effective induction of type-1 immune responses depends, in large part, on the production of IL-12p70 by DCs.

IL-12p70 is a heterodimeric cytokine that consist of the IL-12p35 and p40 subunits and is secreted by phagocytic cells in response to certain pathogens(37). Furthermore, the secretion of IL-12 by monocyte-derived DCs can be induced by their maturation in the presence of interferons and TLR-ligands (5, 27-28, 38). IL-12p70 binds to the IL-12R (consisting of 2 subunits: IL-12Rβ1 and IL-12Rβ2) on responding cells, resulting in the phosphorylation, dimerization and translocation of Signal Transducers and Activators of Transcription (STAT) 1,

3, 4 and 5 resulting in gene transcription. The majority of the effects of IL-12 signaling are mediated through STAT4 (39). IL-12p70 has been shown to induce IFNγ producing ability and cytolytic capacity in NK cells and $CD8^+$T cells (40-43). More recently, it has been shown that IL-12 regulates the expression of the transcription factor T-bet (44), which can directly regulate IFNγ production and granzyme B expression. Furthermore, IL-12 signaling represses Th2 T cell differentiation by inhibiting the expression of the Th2-associated transcription factor GATA-3 (45), suggesting that IL-12 function both to stimulate Th1 differentiation and simultaneously inhibiting Th2 development.

1.1.4 Antigen presentation by MHC class I molecules: Cross-presentation

The induction of $CD8^+$ T cell responses is dependent on the presentation of peptide antigens on MHC class I molecules. MHC class I molecules usually present peptides derived from the DC itself, by proteasomal degradation of endogenous antigens. Since most cell types express MHC class I molecules, the presentation of endogenous antigens allows $CD8^+$ T cells to recognize cell producing foreign (e.g. in the case of viral infection) or aberrant (e.g. in the case of transformation) peptides. One caveat of this system is that $CD8^+$ T cells need to be primed by MHC class I: peptide complexes presented by DCs, suggesting that the DCs itself would need to be infected or have developed a mechanism to present exogenous antigens on C class I molecules. The ability of DCs to take-up exogenous antigens, process them and load them on MHC class I molecules for presentation to $CD8^+$ T cells is termed" cross-presentation" (46).

The classical pathway for the presentation of peptides on MHC class I molecules occurs via the endogenous degradation of cytosolic proteins. Endogenous proteins are degraded by proteasomes (47) and the resulting peptides are transported into the endoplasmatic reticulum

(ER) via TAP1/TAP2 molecules (Transporters associated with Antigen Presentation). Within the ER, newly formed MHC class I molecules are retained and bound to chaperone proteins, such as calnexin, calreticulin and tapasin, which help in the correct folding of the MHC molecules and localizing of the molecules to TAP1/TAP2 complexes. The MHC class I molecules are retained in the ER until peptide is bound. Following peptide binding, MHC class I molecules are transported through the Golgi apparatus to the cell surface (47-48) where they are presented to $CD8^+$ T cells.

The ability of DCs to cross-present exogenous antigens is of great interest in the case of DC-based cancer therapies, since this allows DCs to be loaded *ex vivo* with tumor lysates or tumor-derived proteins(49). While it was originally observed that soluble protein antigens did not induce $CD8^+$ T cell immunity, particulate proteins antigens were able to elicit a $CD8^+$ T cell response, suggesting that whole proteins could be cross-presented (50-51). The method of cross-presentation requires three processes i) processing of antigen, ii) loading of peptides onto MHC class I molecules and iii) transport of the MHC class I molecules to the cell surface (52). There are two major tracks by which these processes can occur. During the endocytic pathway, the processing and loading of the antigens occurs in endosomal compartments with MHC molecules recycled from the cell surface (53). In the second pathway, cytolytic pathway, the antigens are translocated from the endosomes to the cytosol where they are processed and further follow the endogenous pathway (53).

1.1.5 Dendritic cells in immunotherapy: DCs in cancer

The ability of DCs to act as inducers of immune responses, via Th1, Th2, Th17, and CTL induction, as well as regulators via the induction of T_{reg}, has led to their use in immunotherapy against diseases such as cancer and autoimmunity as well as for the prevention of graft transplant rejection. One key aspect for the use of DCs in immunotherapies is the ability to produce large numbers of DCs *ex vivo* under clinically applicable conditions that can be injected into patients as therapeutic vaccine (54-55). The feasibility to generate DCs from blood-isolated monocytes using granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 has allowed for the study and use of DCs in clinical settings, since vast amounts of monocytes can be isolated from peripheral blood (19) while only few DCs are in circulation, although the injection of Flt3-ligand can increase the number of DC progenitors in circulation (56). Furthermore, tumors produce vast amounts of immune suppressive factors (e.g. IL-10, TGFβ, PGE_2), which affects the immune-stimulatory functions of endogenous DCs matured in the presence of these tumor-associated factors (e.g. reduced expression of co-stimulatory molecules, reduced secretion of factors required for T cell survival and CTL differentiation) (57-58). *Ex vivo* generated DC, in contrast, are not subjected to these immune-inhibitory factors and, when matured *ex vivo* are, partially, resistant to these factors (5, 59), suggesting that *ex vivo*-generated DCs are more suitable to be used as cancer vaccines.

In 2010, the Food and Drug Administration (FDA) approved Sipuleucel-T (Provenge), the first cellular therapy for the treatment of cancer. This DC-containing vaccine improved the overall survival of patients with hormone refractory prostate cancer by 4 months (60). However, Provenge does not induce tumor regression or prolong time to disease progression, possibly due to the immature state of the cells used. The results of this study show the feasibility of using DC-

containing, cellular vaccines to treat cancer, but also suggests the need to improve preparative methods for DC maturation *ex vivo*.

The ability of DC-based vaccines to be safely used in cancer trials and induce immune responses has been shown in clinical trials against melanoma, lymphoma and renal cell carcinoma (61-63). However, the clinical response rate is usually low (<15%), suggesting the need for optimization of DC maturation protocols (64).

Successful immunotherapy for the treatment of cancer and intracellular pathogens is believed to require the induction of Th1 $CD4^+$ T cell and effector CTL $CD8^+$ T cell responses. The generation of DCs that have a high IL-12p70 secretion following maturation and after CD40 ligation is pivotal to the induction of such effector T cell responses. The ability of IL-12p70 to stimulate Th1 and CTL differentiation has been known for some time (65-66). Recent data has highlighted the role of DC-secreted IL-12p70 in the induction of type-1 anti-tumor responses *in vitro* (67-69) and as a predictor of the therapeutic potency of DCs *in vivo* (70).The *ex vivo* maturation of DCs using interferons and TLR-ligands have been demonstrated to prime DCs for high IL-12p70 production following CD40-stimulation (71-73). These so-called DC1s have been shown to have superior anti-tumor T cell inducing capacity (68-69) when compared to DCs that do not produce IL-12p70. Besides their high IL-12p70 producing capacity, DC1s have other advantages that should ensure the effective induction of anti-tumor immune responses. Like DCs mature in the presence of PGE_2, DC1s have a high expression of the lymphoid chemokine receptor CCR7, which allows them to migrate in response to CCL19 and CCL21, two lymph node homing chemokines, suggesting these cells would be able to migrate to lymph nodes after injection (20, 74-75). Furthermore, unlike DCs matured in the presence of PGE_2, DC1s produce high levels of the chemokines CXCL10/IP-10 and CCL5/RANTES, which can attract CXCR3-

and CCR5-expressing Th1/CTL cells, while they do not produce CCL22, a chemokine which has been associated with the attraction of T_{reg} cells (25). This chemokine producing profile of DC1s, combined with their resistance to immune inhibitory factors produced by tumors, suggests that these DCs could attract Th1/CTL cells to tumor sites when injected into the tumor, which could result in enhanced anti-tumor effects(76).

1.1.6 Dendritic cells in immunotherapy: DCs in autoimmunity/transplantation

In the case of autoimmune diseases or to prevent rejection of transplanted tissues or organs, the induction of T_{reg} cells is preferred (77). Studies of transplant tolerance have provided insight into the role of DCs in the prevention of allograft rejection. Murine liver transplants models have shown that there is an increased frequency of pDCs in sustained liver grafts compared to the spleen and that this increased frequency correlates with an increase in T_{reg} cells (78-79). Infusion of mice with precursor (pre-) pDCs before heart transplantation resulted in prolonged cardiac allograft survival (80). Furthermore, human pDCs can induce T_{reg} cell *in vitro* (81-82). Currently there are protocols described for the generation of tolerogenic DCs from monocyte-derived DC by the addition of immune inhibitory factors (e.g. Vitamin D3, glucocorticoid, IL-10) to DC cultures (30-31). These tolerogenic DCs express reduced levels of co-stimulatory molecules and exhibit reduced production of inflammatory cytokines when compared to non-tolerogenic DCs and these DCs can inhibit (DC-induced) T cell expansion and cytokine production (30). Adoptive transfer of tolerogenic DCs continues to represent a promising experimental treatment for autoimmune diseases.

1.2 EFFECTOR CD8$^+$ T CELLS

The effective treatment of cancers with DC-based vaccines relies on the induction of large numbers of cytolytic effector cells able to recognize tumor antigens. Following the elimination of tumors by the type-1 polarized DCs-induced CD8$^+$ T cells, a population of memory cells should be formed which can provide long-term protection against disease recurrence.

1.2.1 Effector CD8$^+$ T cell differentiation

Naive CD8$^+$ T cells in the lymphoid organs scan resident DCs for the cognate antigen. Using time-lapse microscopy, it has been shown that the interaction of naïve T cells with irrelevant Ag-bearing DCs are stochastic and short lived (i.e. in the order of minutes). However, upon recognition of its cognate Ag, Ag-specific T cells form stable clusters with DCs, which can lasts for up to 24 hours (41). Using plate-bound antigen and co-stimulatory molecules (e.g. anti-CD28) or adherent cells transfected to express antigen and B7-1 (CD80), it was shown that short-term (2-24 hours) interaction between T cells and Ag/co-stimulation induces an "auto-pilot" response in which the cells proliferate and undergo differentiation (83-85). However, other studies have suggested that prolonged interactions are required for optimal delivery of signal 3 delivery to T cells and for the induction of cytolytic capacity in CD8$^+$ T cells (86).

1.2.2 DCs, signal 3 and the induction of effector $CD8^+$ T cells

Studies using artificial antigen presenting cells consisting of microspheres bearing pMHC complexes and co-stimulatory molecules (e.g. B7 ligands) and $CD8^+$ T cells from TCR transgenic mice, have suggested that the clonal expansion of naïve $CD8^+$ T cells requires the presence of IL-12p70 during priming (87). Hernandez et al., showed that injection of agonistic anti-CD40 antibodies resulted in the increased migration of DCs expressing high levels of co-stimulatory molecules to the lymphoid organs and that these DCs were able to induce $CD8^+$ T cell proliferation, but that the proliferating T cells did not acquire effector functions (88). In these studies, the addition of IL-12p70 to antigenic and co-stimulatory signals resulted in the acquisition of effector functions by the responding Ag-specific $CD8^+$ T cells. Interestingly, the priming of naïve $CD8^+$ T cells by high Ag-dose and co-stimulatory molecules in the absence of signal 3 (e.g. IL-12p70) induced tolerant or anergic $CD8^+$ T cells which were unable to subsequently develop cytolytic function upon re-challenge (89-90).

As mentioned before, the ability of DCs to provide signal 3 is dependent on their activation status and the duration of activation. Langenkamp *et al.*, have shown that moDCs produce the pro-inflammatory cytokine IL-12p70 after receiving maturation stimuli, which lasts for about 24 hrs. Priming of naïve $CD4^+$ T cells within this first period of DC maturation, when there is abundant IL-12p70 production, results in the generation of Th1-polarized T cells. However, these same DCs lose the ability to secrete IL-12p70 later after maturation (>24hrs) and preferentially induce Th2 and non-polarized T cell differentiation (26). These long-term matured DCs are classically considered exhausted.

The presence of IL-12p70 or IFNα during the priming period results in the activation of STAT 4 and the transcription of effector genes such as T-bet, IFNγ, granzyme B and perforin

(44, 91-92). Mouse studies have shown that the expression of the transcription factor T-bet is regulated by IL-12p70 (44). $CD8^+$ T cells from IL-$12p35^{-/-}$ mice exhibited reduced T-bet expression during the peak of the immune response after pathogenic infection. In contrast, a closely-related transcription factor, eomesodermin (eomes), which is increasingly expressed during the differentiation from effector to memory T cells (91), is elevated during the peak of the immune response in the IL-$12p35^{-/-}$ mice.

While IL-12p70 is recognized as the key cytokine for the induction of Th1 responses (66, 93), other cytokines have been shown to be able to induce Th1 differentiation in the absence of IL-12p70. A study using DCs over-expressing the transcription factor T-bet (required for type-1 polarization in T cells), showed the induction of Th1 T cell responses, which was largely IL-12p70- independent (94). Furthermore, priming of naïve $CD8^+$ T cells with anti-TCR antibodies in the presence of IL-27, an IL-12 family member, resulted in the increased expression of the cytolytic proteins granzyme B and perforin (95). More recently, it was shown that type-1 interferons (IFNα/β) could also provide signal 3 required for clonal expansion and effector and memory T cell differentiation (92, 96).

Klechevsky et al., have shown that *in vitro*-generated and epidermis-derived Langerhans cells (LCs) are potent inducers of cytolytic CTLs (9). In contrast to $CD14^+$ dermal DCs, LCs produce large amounts of IL-15 which can be secreted or "trans" presented to T cells in combination with IL-15Rα (97). Exogenous addition of IL-15 to co-cultures of $CD14^+$ dermal DCs and T cells induces strong T cell priming, suggesting that IL-15 can substitute for IL-12p70 as a Th1/CTL inducing signal 3 cytokine.

1.2.3 Chemokine-mediated effector $CD8^+$ T cell migration

Gradients of the chemokines CCL19 and/or CCL21, ligands for lymphoid-homing chemokine-receptor CCR7, allow naïve and central-memory $CD8^+$ T cell to migrate into the lymph nodes and interact with Ag-presenting DCs (98-99). During the activation and differentiation of naïve $CD8^+$ T cells into effector cells, the T cells down-regulate the expression of CCR7, allowing them to migrate out of the lymphoid organs and into the periphery. Furthermore, activated antigen-specific $CD8^+$ T cells up-regulate surface expression of peripheral-homing chemokine receptors such as CCR5 and CXCR3, that allow them to respond to chemotactic gradients of inflammation associated/peripheral chemokines (RANTES/CCL5 and Interferon-inducible protein-10 (IP-10)/CXCL10), respectively to traffic to infected tissues. In the inflamed tissue, effector $CD8^+$ T cells migration is halted by encounter of antigens (100). While these "effector" chemokine receptors are required for peripheral homing, it has been suggested that their expression in the lymph nodes are also necessary for interaction of the T cells with the DCs and the optimal priming of Th1 responses (101).

Secretion of CXCL10 by cells in the central-nervous system (CNS) of mice infected with *Toxoplasma gondii*, resulted in the enhanced migration of $CD8^+$ T cells to the infected tissue and accelerated the migration speed of $CD8^+$ T cells in the brain. The involvement of chemokine-chemokine receptors was shown by treatment of the infected animals with blocking anti-CXCL-10 antibodies or treatment with the general 7-trans membrane G-protein coupled receptors, pertussis toxin (PTX), both of which reduced the infiltration of Ag-specific $CD8^+$ T cells into the infected tissue and slowed the migration of cells in the brain (102).

Differentially matured DCs secrete different chemokines, allowing them to attract specific T cells and to elicit "pathogen-specific" responses (35). Type-1 polarized DCs are able

to produce CXCL10, allowing them to attract, and induce, Th1 and effector $CXCR3^+$ $CD8^+$ T cells. This ability of DCs has been shown to help in the DC-based vaccine treatment of malignant gliomas (76). The sub-cutaneous injection of type-1 polarized DCs, loaded with glioma-associated antigen (GAA)-derived epitopes, induced Ag-specific CTLs able to prolong survival of glioma-bearing mice, while intra-tumoral injection of the DCs resulted in the further enhancement of anti-glioma T cell responses, an effect that was absence in the case of injection of DCs derived from CXCL10 knockout mice (76).

The ability of T cells to migrate to and enter into tumors has been shown to correlate with disease progression and overall patient survival for various types of cancer(23, 77). The analysis of the type of immune cells infiltrating the tumors, as well as the number of cells and their localization has been shown to be a good predictor of patient survival in colorectal cancer (103). T cell infiltration in tumor islets was positively correlated with progression-free and overall survival in patients with ovarian carcinomas (104). Analysis of resected colorectal tumor specimens for the presence of early metastatic infiltration showed that those specimens without infiltration had increased infiltration of $CD8^+$ T cells and increased mRNA expression of type-1 immune response associated genes. The presence of T cell infiltrate and the absence of metastatic infiltration correlated with increased disease-free and overall survival. Interestingly, metastatic free tumors contained increased numbers of (effector-) memory $CD8^+$ T cells (105).

The importance of CXCR3 expression and proper migration by activated $CD8^+$ T cells was further highlighted by the finding that melanoma patients with many $CXCR3^+$ $CD8^+$ T cells in their tumor tissue have a better prognosis than patients with low numbers of CXCR3-expressing T cells (36). The ability of effector T cells to migrate into tumor tissue is pivotal as it has been shown that the ratio of T_{reg}:CTL in the tumor can be used as a diagnostic index. The

lower the T_{reg}:CTL ratio (low T_{reg} vs. high CTL) the better the disease prognosis (46). Therefore, it appears that the success of cancer immunotherapies may be related to the capacity to induce high numbers of $CXCR3^{+}$ tumor infiltrating lymphocytes (TILs)/CTLs that efficiently migrate into tumor tissues. This endpoint requires the expression of (tumor-) tissue specific chemokine receptors by the TILs/CTLs and a tumor chemokine milieu that favors the attraction of CTLs over T_{reg} cells.

Furthermore, the migratory abilities of T cells not only determine disease outcome, but can also affect the T cell differentiation. $CD8^{+}$ T cells activated in lymph nodes undergo expansion and migrate to the periphery under the influence of chemotactic gradients sensed by CXCR3 and CCR5, as previously mentioned. However, the programming of activated $CD8^{+}$ T cells into terminally-differentiated effector cells might occur at the site of infection under the influence of the pro-inflammatory environment. Mouse studies have shown that expression of CXCR3 and CCR5 by activated T cells plays a role in the subsequent induction of effector and memory $CD8^{+}$ T cells. In an influenza model, Kohlmeier et al., showed that $CCR5^{-/-}$ $CXCR3^{-/-}$ $CD8^{+}$ T cells migrate to different parts of infected lungs when compared to WT ($CCR5^{+/+}$ $CXCR3^{+/+}$) $CD8^{+}$ T cells. The WT $CD8^{+}$ T cells preferentially localize to tissues with high antigen density and inflammation where they may be induced to undergo further differentiation into effector $CD8^{+}$ T cells. In contrast, the $CXCR3^{-/-}$ $CCR5^{-/-}$ $CD8^{+}$ T cells tends to localize to tissues with less inflammation and lower Ag-density leading to a state of attenuated activation and enhanced memory cell development (106).

Although the expression of CCR5 (and CXCR3) is principally associated with peripheral tissue homing ability, the interaction of $CD4^{+}$ T cells with Ag-presenting dendritic cells induces the secretion of CCL3 and CCL4, two ligands for CCR5, and this helps in attracting naïve Ag-

specific $CD8^+$ T cells into DC-$CD4^+$ T cell clusters, allowing for the improved induction of CTL responses and subsequent memory T cell development (107). Furthermore, interaction of specific $CD8^+$ T cells with Ag-presenting DCs lead to the attraction and stable interaction of $CD8^+$T cells with DCs in an Ag-independent manner. This recruitment is dependent on the expression of CCR5 on the "non-Ag-specific" $CD8^+$ T cells (108). Since DCs that have taken up infected or transformed cells can present a range of antigenic epitopes, this CD8-CD8 help could provide a mechanism by which specific $CD8^+$ T cells could attract other ($CCR5^+$) $CD8^+$ that are reactive against other antigens that the DCs might be cross-presenting allowing for the development of poly-specific CTL responses. While naïve $CD8^+$ T cells do not express CCR5, the surface expression could be induced by inflammation (107).

Guarda *et al.,* showed that CXCR3-expressing $CD8^+$ T cells are also capable of migrating into "activated" lymph nodes where they can eliminate Ag-presenting DCs. This unexpected feature of $CXCR3^+$ $CD8^+$ T cells may limit the excessive induction of activated $CD8^+$ T cells and control the number of effector $CD8^+$ T cell in the periphery as a means to prevent unwanted auto-reactivity/autoimmunity (109).

DCs not only regulate T cell migration via secretion of chemokines, but they also regulate, as least in part, T cell expression of chemokine receptors (and other molecules involved in migration, such as integrins and lectins). T cells primed by DCs derived from Peyer's patches or the mesenteric lymph node, induce the expression of chemokines and integrins associated with gut-homing (CCR9). In contrast, DCs derived from the skin induce skin-homing properties in T cells (39). In line with these studies are the observations that the priming of T cells by DCs that have been stimulated with metabolites of either vitamin A (retinoic acid; found mostly in the intestines) or vitamin D ($1,25(OH)_2D3$; found in the epidermis of the skin) induce the surface

expression of the chemokines CCR9 (involved in migration to the gut) or CCR10 (involved in migration to the skin), respectively(37). While it is still unclear what mechanism underlies the induction of tissue-specific T cell homing by DCs, the ability of these APCs to instill tissue-specific homing abilities in responding T cells might be considered as "signal 4" (110). This could help in generating DCs that "direct" T cells to specific tissues by regulating there chemokine receptor expression.

1.3 MEMORY CD8$^+$ T CELL DEVELOPMENT

One of the key features of the adaptive immune response is the formation of memory cells. Following an infection, an effector response is elicited which results in the clearance of the infected cells and pathogens. When the pathogen is cleared, a contraction phase occurs during which the majority (>90%) of the Ag-specific CTL undergo apoptosis, leaving a small population of Ag-specific memory cells. These memory cells are long-lived cells that can provide life-long protection against subsequent infections with the same or cross-reactive pathogens in mice (111). The ability of the immune system to "remember" pathogens and mount rapid and robust responses to subsequent infection is the foundation for prophylactic vaccination strategies. Because of their essential role in vaccine development, many studies have examined the mechanism by which memory cells are generated and maintained as well as the basis for the enhanced capacity of TG cells to respond to subsequent infection.

Initial studies in mouse have suggested that the size of the memory T cell pool is dependent on the primary clonal burst size attained by Ag-specific CTLs following infection. The massive clonal expansion during the primary infection results in a substantial increase

(~1000-fold) in the precursor frequency of Ag-specific memory T cells when compared to naïve T cells (112). The higher precursor frequency allows for the accelerated generation of large numbers of Ag-specific CTLs following subsequent re-infection.

1.3.1 Effector-memory and central-memory T cells

Memory and naïve T cells also exhibit phenotypic and functional differences that allow for the more robust and accelerated response of memory T cells following re-challenge. Memory T cells are most commonly divided into two populations based on the expression of the CCR7 and L-selectin (CD62L), central-memory T cells (T_{CM}) and effector T cells (T_{EM}) (113). These two populations have distinct homing and cytolytic capacities. Central-memory cells are $CCR7^+$ and $CD62L^+$ and preferentially localize in lymphoid tissues. They lack direct cytolytic capability but exhibit proliferative ability. T_{CM} cells are thought to be responsible for the rapid second wave of CTLs development after re-infection. T_{EM} cells, in contrast, are $CCR7^-$ $CD62L^-$ and reside in peripheral tissues, where they express perforin and produce IFNγ in response to cognate-Ag stimulation. The direct cytolytic function and localization of T_{EM} cells allows them to respond acutely to re-challenge, explaining the more rapid clearance of secondary infections by memory T cells.

Analysis of resected colorectal tumors revealed that tumors that had elevated numbers of infiltrated T_{EM} cells had no histological sign of early metastatic invasion, while those tumors with signs of early metastatic invasion had reduced T_{EM} infiltration (105). The absence of early metastatic invasion was shown to correlate with increased disease-free and overall survival (105). These results suggest that the level of T_{EM} infiltration may have prognostic value. The

functionality of T_{EM} cells has also been studied in HIV/SIV infection models in non-human primates. The presence of HIV/SIV-Ag-specific T_{EM} cells at mucosal sites of viral entry was shown to provide protection against infection (49). Recently, it was shown that after clearance of a vaccinia virus infection in the skin, T_{EM} cells infiltrated these cutaneous sites and reside there for extended periods, providing long-term protection against reinfection of the skin, while T_{CM} cells instead,can be found in circulation (114). In accordance with this mouse study, Clark *et al.* showed that patients with leukaemic cutaneous T cell lymphoma (L-CTLC), a malignancy of T_{CM} cells, retained skin T_{EM} cells after T cell depletion therapy with alemtuzumab, without exhibiting an increased risk of infections (115).

Combined, these studies suggest that the presence of memory T cell populations with distinct localization abilities, self-renewal capacities and cytolytic properties have distinct functions in providing long-term immune protection to the host against pathogens/tumors.

1.3.2 Memory $CD8^+$ T cell differentiation

While memory T cell development is widely studied, the pathway by which naïve $CD8^+$ T cells differentiate into memory cells remains controversial. The notion that the clonal burst size of the primary Ag-specific CTL population affects the size of the subsequent memory pool suggests that memory cells are directly derived from CTLs that survive the initial contraction phase. Studies using transgenic mice in which the Ag-specific CTLs could be labeled at early time-points, suggest that these T cells were maintained in the memory pool (32). The mechanism(s) by which certain CTLs survive the contraction phase and become memory T cells remains an area of active research.

The observation that during the peak of an immune response there are $CD8^+$ T cells that have retained expression of IL-7Rα (a cytokine receptor involved in survival of naïve and memory T cells) suggests the existence of a memory precursor population (116). The expression of IL-7Rα by itself, however, is not enough for the generation of memory cells, since forced expression of the receptor by terminally differentiated effector cells did not alter contraction (117). Memory precursor T cells are IL-7Rα^+ T-bet^{low} and, in mice, these cells are KLRG1^{low} (Killer-cell lectin-like receptor subfamily G, member 1). The presence of a memory-precursor population suggests that effector and memory differentiation can occur independently. This notion is supported by the finding that naïve T cells undergo a primary asymmetric cell division during priming, in which the daughter cell closest (proximal) to the Ag-presenting APC acquires most of the effector molecules and eventually becomes a CTL while the more distant daughter cell are pre-disposed to become central-memory T cells (118). These two distinct daughter cells also have distinct T-bet expression, with the proximal cells having the highest T-bet expression and the more distant T cells typically have a T-bet^{low} expression profile (119). Interestingly, T-bet expression negatively regulates the expression of IL-7Rα, providing support for the concept that IL-7Rα^- CTLs are derived from the DC-proximal T cells (120). Using a transgenic mouse model in which T cells lose GFP expression upon differentiation into CTLs, it was shown that long-lived memory T cells may be generated without the requirement of evolving through an effector phase (32).

1.3.3 Inflammation and memory T cell differentiation

Studies using IFNγ-deficient mice showed a diminished contraction of $CD8^+$ T cells after infection with attenuated *L. monocytogenes* or LCMV (42) . In line with this finding is the observation that pre-treatment of mice with ampicillin before infection with *L. monocytogenes* results in memory formation without contraction, while the simultaneous induction of an inflammatory response by injection of a TLR9 agonist (CpG-containg DNA), results in contraction of the responding $CD8^+$ T cells (43). Furthermore, $CD8^+$ T cells lacking the IL-12 receptor showed reduced proliferation as well as reduced contraction following infection (121). Accelerated memory formation is also observed in cells lacking T-bet, which is regulated by IL-12 (122).

Badovinac et al., showed that the vaccination of mice with peptide-loaded DCs in the absence of overt inflammation resulted in the differentiation of the $CD8^+$ T cells into memory cells by day 5. These T cells were able to undergo secondary expansion upon Ag-restimulation and provided protection to subsequent infections. The addition of pro-inflammatory stimuli together with the peptide-loaded DCs resulted in the generation of CTLs (123).

These results highlight the role of inflammatory signals in the formation of memory T cells. It is, however, unclear how inflammatory signals affect the ability of DCs to induce effector versus memory differentiation.

1.3.4 The role of mTOR in effector versus memory $CD8^+$ T cell differentiation

Recently, the importance of inflammation on the regulation of CTL and memory formation was further underscored by the finding that rapamycin induced the formation of memory T cells. Rapamycin is a well-established immunosuppressive drug commonly used in organ transplantation settings to suppress immune reactions against transplanted tissues (124-127). Rapamycin inhibits the activation of the mammalian target of rapamycin (mTOR) complex I. IL-12 signaling during priming enhances and prolongs the activity of mTOR kinase resulting in the expression of T-bet and CTL differentiation, in an mTOR-dependent manner (128). Furthermore, the treatment of mice with rapamycin during the initial week of LCMV infection, results in normal expansion of primed T cells, but the contraction phase of the pathogen-specific T cell response is attenuated. Reduced contraction is associated with an increased population of $CD8^+$ T cells with a memory-precursor phenotype (129). mTOR kinase activity is also crucial for the development of memory cells under conditions of homeostatic proliferation (130). Furthermore, mTOR kinase activity regulates the migratory capacity of effector and memory T cells by modulating the expression of the transcription factor Kruppel-like factor 2 (KLF2), which regulates the expression of trafficking proteins such as CCR7 and CD62L. (131). High mTOR kinase activity in T cells results in reduced expression of KLF2 leading to down-regulation of the surface expression of CCR7 and CD62L, allowing these CTLs to leave lymphoid organs.

1.4 FEED-BACK INTERACTION BETWEEN T CELLS AND DENDRITIC CELLS

As described above, DCs interact with T cells dictates T cell fate but it also affects the maturation and polarization of the DCs in a reciprocal manner.

The binding of a CD28-Ig fusion protein to B7-1 and/orB7-2 (CD80 and CD86, respectively) on the DC surface results in the up-regulated expression of IL-6 and IFNγ by the DCs, which allows these APCs to elicit strong immune responses both *in vitro* and *in vivo* (132). In contrast, interaction of B7-1 or B7-2 with CTLA-4, an inhibitory receptor which competes with CD28 for binding of B7 molecules and inhibits T cell activation, results in the induction of indeolamine 2,3- dioxygenase (IDO) by the DCs (133). DC produced IDO inhibits T cell proliferation, induces the apoptosis of lymphocytes and drives immune tolerance. Since CD28 is expressed by naïve T cells, while CTLA-4, which binds B7 with a higher affinity than CD28, is only expressed after activation and enhanced by CD28 signaling (134), it appears that T cells can first help DCs elicit a type-1 immune response (via CD28 signaling), followed by inducing inhibitory factors (via CTLA-4 signaling) at later time points during the immune response as a negative feed-back loop.

CD4 Th cell interaction with antigen presenting DCs has been suggested to be required for the induction ("licensing") of the DCs to induce CTL responses. The licensing of DCs by CD4 Th cells is suggested to be dependent on the expression of CD154 (CD40L) on the $CD4^+$ T cells. Interaction of CD154 with its receptor, CD40, on the DC surface primes the DC for high IL-12p70 production (135-136). This interaction appears to be dependent on the cognate recognition of antigens on the same DC by $CD4^+$T cells and $CD8^+$T cells. DCs isolated from mice lacking MHC class II molecule expression are unable to induce full $CD8^+$ CTLs and have

impaired $CD8^+$ memory cell formation in response to herpes simplex virus-1 (HSV-1) infection (137).

Naïve $CD8^+$T cells, which do not express CD40L, produce IFNγ rapidly after interaction with DCs. In cooperation with CD40L-expressing $CD4^+$T cells, naïve $CD8^+$T cells are able to induce IL-12p70 production by DCs and help in their maturation (138). In contrast to the helper function of naïve $CD8^+$ T cells, CTLs have been shown to kill Ag-carrying DCs, a process which appears to prevent excessive T cell expansion and to limit tissue damage (139-140). Ag-loaded iDCs can be rescued from CTL-mediated killing by memory $CD8^+$ T cells, suggesting a helper function for memory T cells. While interaction of iDCs with CTLs results in the simultaneous release of TNFα and granzyme B by CTLs, interaction of memory cells with iDC results in the rapid release of TNFα, followed several hours later by granzyme B release (141). The rapid release of TNFα supports the up-regulation of the inhibitor of granzyme B, protease inhibitor PI-9 (analog of murine SPI-6), by the DCs, protecting them from the subsequent released of granzyme B by the CTLs (141). The ability of memory T cells to protect DCs from CTL-mediated killing can be used for the enhancement of DC-based vaccines. In a mouse model, it was shown that the incorporation of "heterologous" (tumor-irrelevant) recall Ags on tumor-peptide loaded DCs, helped in the induction of IL-12-dependent immune responses in tumor-bearing mice, which have tumor-specific CTLs in their circulation that would kill tumor-peptide loaded DCs (142). These data suggest that the simultaneous targeting of tumor-irrelevant memory T cells and tumor-specific T cells may have the potential to protect DCs from CTL mediated elimination before being able to elicit strong CTL, thereby enhancing the efficacy of DC-based anti-tumor vaccines.

1.5 SCOPE OF THIS THESIS

The scope of this thesis is to define the role of the maturation and polarization of DCs in the development of effector and memory $CD8^+$ T cells and to evaluate the ability of lymphocytes to induce polarized DC1s as vaccination tools in cancer. Furthermore, in an attempt to develop improved treatment of advanced cancer, I explored the feasibility of modulating the tumor chemokine microenvironment to enhance the entry of DC1-induced effector $CD8^+$ T cells into tumor tissues.

In ***Chapter 2*** I use a human *in vitro* model to analyze the effects of the differentiation status and IL-12 production ability of monocyte-derived DCs on the differentiation of naïve $CD8^+$ T cells. I demonstrate that DCs matured in the presence of mediators of acute inflammation (interferons and TLR ligands), favor the induction of cytolytic $CD8^+$ T cells with high expression of CXCR3 (receptor of CXCL9/MIG, CXCL10/IP10 and CXCL11/ITAC) and CCR5 (receptor for CCL5/RANTES) and peripheral homing abilities. In contrast, DCs matured under conditions mimicking chronic inflammation (matured in the presence of PGE_2) or matured for prolonged period of time (so-called exhausted DCs) induce the proliferation and activation of $CD8^+$ T cells, but do not imprint cytolytic abilities on them. The distinct ability of "inflammatory" type-1 polarized DCs (DC1s) to induce CTL functions is, in part, regulated by their elevated ability to secrete IL-12p70. Type-1 polarized DCs also induce $CXCR3^+CCR5^+GrB^+$ effector $CD8^+$T cells from melanoma derived $CD8^+$T cells, showing the superior ability of these DCs to induce type-1 anti-tumor responses.

The anti-tumor CTL inducing capacity of type-1 polarized DC suggests that these DCs would be favorable as vaccine in therapeutic cancer vaccines. The high expression of CXCR3 and CCR5 might allow the anti-tumor CTLs to migrate to tumor sites. Since CTLs induced by type-1 polarized DCs can revert to memory cells upon prolonged culture and can acquire cytolytic capacity upon restimulation with Ag-loaded type-1 polarize DCs, vaccination with this type of DCs would produce an "immediate" anti-tumor CTL response that could eliminate tumors, followed by the development memory cells that can provide protection against tumor recurrence.

The distinct $CD8^+$ T cell priming abilities by differentially matured DCs observed in chapter 2, the activation of $CD8^+$ T cells with cytolytic effector functions versus activation of $CD8^+$ T cells without cytolytic effector functions, prompted me in ***Chapter 3*** to examine the $CD8^+$ T cell differentiation pathway induced by the priming with low IL-12p70 producing "exhausted" DCs. I show that, in contrast to inflammatory DC1s, non-inflammatory, exhausted DCs lack expression of the transcription factor T-bet, which in T cells is associated with type-1 polarization, and lack IL-12p70 secretion. $CD8^+$T cells primed by these T-bet^-/$IL\text{-}12^-$ DCs show a reduced mTOR kinase activity when compared to (T-bet^+/$IL\text{-}12^+$) DC1-primed $CD8^+$ T cells and directly differentiate into central-memory cells which are able to undergo secondary expansion sooner than DC1-primed $CD8^+$ T cells and acquired cytolytic effector functions upon restimulation with DC1s. The data from chapter 3 suggest that exhausted DCs, which lack IL-12 production during T cell priming, might have a specialized function in immune responses by inducing the direct differentiation of naïve $CD8^+$ T cells into central-memory cells. This specialized central-memory inducing function of "non-inflammatory" exhausted DCs reconciles the observation that at later time points of an immune response there is a preferential induction of

memory T cells. The preferred induction of memory cells by these DCs would allow their use in prophylactic vaccine settings, in which the induction of large numbers of long-lived memory T cells is desired. Currently the induction of large numbers of central-memory cells is achieved by the priming of T cells in the presence of adjuvant, which elicits a strong inflammatory response, to ensure the induction of a large effector pool followed by memory cell formation. The memory cells are then "boosted" by vaccination to increase the number of specific memory cells. The interval between the priming and booster doses can take weeks or months. The accelerated generation of central-memory cells by non-inflammatory DCs might allow for the shortening of the interval between the prime-boosting. This would be favorable in situations where a rapid induction of large numbers of protective memory T cells is required (e.g. bioterrorism threats and epidemics).

The combined data from chapters 2 and 3 indicate that the inflammatory environment in which DCs are matured and the duration of maturation determine the preferential induction of cytolytic effector versus central-memory $CD8^+$ T cells. This differential T cell priming ability of DCs would allow for the optimization of the DC maturation methods for immunotherapy, depending on the T cell response (effector versus memory) that is desired.

Since the results of chapters 2 and 3 show a distinct ability of type-1 polarized DCs to induce cytolytic effector $CD8^+$ T cells, which is desirable in cancer immunotherapy, in ***Chapter 4*** I examine the feasibility of using lymphocytes to generate IL-12p70 secreting type-1 polarized DCs in order to limit the need for expensive recombinant clinical-grade cytokines. The ability of activated lymphocytes to secrete DC-polarizing cytokines allows for the maturation and type-1 polarization of autologous DCs, which produce elevated levels of CTL-promoting IL-12 and CTL-attracting CXCL10/IP10. Furthermore, I show that this DC maturation and polarization

protocol works equally well with PBMCs from healthy donors and melanoma patients, providing support for the use of this method in cancer immunotherapy settings. The supernatant-matured DCs are able to migrate in response to CCL21, suggesting that after injection these DCs would be able to migrate to lymph nodes. When loaded with tumor-associated peptides, supernatant-matured DCs are superior when compared to non-inflammatory DCs in inducing anti-tumor $CD8^+$ T cell responses from naïve $CD8^+$ T cells. These data show the feasibility of producing low-cost type-1 polarized DCs using autologous lymphocytes. While the use of cytokine-matured DCs in clinical settings might remain the preferred choice in countries where clinical studies have abundant funding, in many countries this might not be the case and the use of lymphocyte- or supernatant-matured DCs could provide an alternative method for type-1 polarized DC generation.

In chapters 2 and 3 and 4, I show that type-1 polarized DCs produce high levels of IL-12p70 and are required for the induction of $CXCR3^+$ $CCR5^+$ cytolytic anti-tumor $CD8^+$ T cells that have peripheral homing ability. However, tumors have developed multiple mechanisms to prevent elimination by T cells and most tumors do not produce chemokines (such as CXCL10 and CCL5) that attract tumor-specific CTLs. Since type-1 DC-primed $CD8^+$ T cells express CXCR3 and CCR5, in ***Chapter 5*** I examine the ability of manipulating the tumor-chemokine environment to enhance the production of CTL-attracting chemokines. I show that the combination of IFNα, COX-inhibitors (indomethacin), and poly-I:C, selectively enhances the production of CTL-attracting chemokines (CCL5 and CXCL10/IP10) while reducing the production of CCL22, a T_{reg}-attracting chemokine, by tumor tissues in an NF-κB-dependent manner. The enhanced production of CXCL10/IP10 and CCL5 by the "triple combination-" treated tumor cells results in the attraction of $CXCR3^+$ $CCR5^+$ cytolytic effector $CD8^+$ T cells,

while inhibiting the attraction of unfavorable regulatory T cells (T_{reg}). These data suggest that the unfavorable chemokine environment of tumors can be altered by the combined use of IFNα, indomethacin and poly-I:C. This treatment could be used as a cancer therapy by itself in which case it would allow for the attraction of spontaneously arising anti-tumor T cells. Alternatively, it could be used in combination with tumor (peptide)-loaded cytokine- (chapter 2 and 3) or lymphocyte-supernatant-(chapter 4) matured DC1s, which would induce large numbers of cytolytic $CXCR3^+CCR5^+$ tumor-specific CTLs that then can migrate to triple-combination treated tumors.

In ***Chapter 6*** I combine the findings of the work presented in this thesis with the findings from other groups to provide an overview of the role of DCs activated in different conditions in the induction of different subsets of $CD8^+$ T cells and their use in immunotherapy, alone or in combination with tumor-conditioning therapies.

2.0 INDEPENDENT REGULATION OF CHEMOKINE RESPONSIVENESS AND CYTOLYTIC FUNCTION VERSUS CD8$^+$ T CELL EXPANSION BY DENDRITIC CELLS

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Experiments performed by Erik Berk, contributed to figures 2, 3, and 4 and contributed to the preparation of the manuscript.

2.1 ABSTRACT

The ability of cancer vaccines to induce tumor-specific $CD8^+$ T cells in the circulation of cancer patients has been shown to poorly correlate with their clinical effectiveness. Here, we report that although antigens presented by different types of mature dendritic cells (DCs) are similarly effective in inducing $CD8^+$ T cell expansion, the acquisition of CTL function and peripheral-type chemokine receptors, CCR5 and CXCR3, requires antigen presentation by a select type of DCs. Both "standard" DCs (matured in the presence of PGE_2) and type-1-polarized DCs (matured in the presence of interferons and TLR-ligands which prevent DC "exhaustion") are similarly effective in inducing $CD8^+$ T cell expansion and acquisition of $CD45RO^+IL\text{-}7R^+IL\text{-}15R^+$ phenotype. However, Granzyme B expression, acquisition of CTL activity and peripheral tissue-type chemokine responsiveness are features exclusively exhibited by $CD8^+$ T cells activated by type-1-polarized DCs. This advantage of type 1-polarized DCs was observed in polyclonally-activated naïve and memory $CD8^+$ T cells and in blood-isolated melanoma-specific CTL precursors. Our data help to explain the dissociation between the ability of cancer vaccines to induce high numbers of tumor-specific $CD8^+$ T cells in the blood of cancer patients and their ability to promote clinical responses, providing for new strategies of cancer immunotherapy.

2.2 INTRODUCTION

Recent trials of cancer vaccines demonstrated that the induction of high numbers of circulating tumor-specific $CD8^+$ T cells is not necessarily accompanied by acquisition of an effector function (143-144), resulting in the limited ability of the current vaccines to induce tumor regression (145-148). This raises the question of whether the currently used vaccination strategies are optimal with regard to their ability to induce effector-type cytotoxic $CD8^+$ T cells (CTLs) with tumor-relevant homing potential.

In the case of $CD4^+$ T cells, extensive studies in human and mouse models have demonstrated that dendritic cells (DCs) maturing in different environments or pre-activated for different periods of time can instruct naïve $CD4^+$ T cells to selectively acquire Th1 or Th2 effector functions (3, 93, 149-150), leading to the concept of "signal 3" which selectively regulates the acquisition of T cell effector functions (3). While the role of the functional status of DCs in the development of effector $CD8^+$ T cells is less clear, in several *in vivo* mouse models of infections it was demonstrated that inflammatory cytokines, such as IL-12, IFNα and IFNγ, not only regulate the proliferation of $CD8^+$ T cells, but also their acquisition of CTL functions (121, 151-152).

In order to directly test if the induction of CTL functions and tumor-relevant chemokine responsiveness are differentially regulated by different DC types, we compared the phenotype and functions of human $CD8^+$ T cells primed by different types of mature, highly-stimulatory DCs, such as type-1-polarized DCs matured in the presence of IFNs and TLR ligands (including the clinically-used TNFα/IL-1β/Poly-I:C/IFNγ/IFNα-matured αDC1s; ClinicalTrials.gov: NCT00390338, NCT00099593, NCT00766753, NCT00558051 and NCT00970203) (71) and

non-polarized DCs matured in the presence of PGE_2 (including the clinically-applied TNFα/IL-1β/IL-6/PGE_2-matured "standard" (s)DCs) (20) that were previously shown to induce different numbers of tumor-specific T cells, as determined by IFNγ ELISPOT (71).

Our data indicate that while both type-1-polarized and non-polarized, DCs induce similar CD8+ T cell expansion, the induction of functional CTLs with peripheral homing capacity requires "non-exhausted" type-1-polarized DCs. In contrast, non-polarized DCs selectively induce CD8+ T cell expansion, without the accompanying development of CTL functions or peripheral homing potential.

2.3 MATERIAL AND METHODS

Cell lines, media and reagents. Serum-free AIM-V medium (Invitrogen, Carlsbad, CA) was used to generate DCs and IMDM (Invitrogen) with 5% human serum (Atlanta Biologicals, Norcross, GA) was used for *in vitro* sensitization (IVS) experiments. The following factors were used to generate mature DCs: *rhu*GM-CSF and IL-4 (gifts from Schering Plough, Kenilworth, NJ), IFNα (Intron A), *rhu*TNFα, *rhu*IFNγ, *rhu*IL-1β (all from Strathmann Biotech, Germany), *rhu*IL-6 (Genzyme, Cambridge, MA), lipopolysaccharide (Sigma, St. Louis, MO), PGE_2 (Sigma) and poly-I: C (Sigma). IL-2 (Chiron Corp, Emeryville, CA) and *rhu*IL-7 (Strathmann Biotech) were used to support the $CD8^+$ T cell expansion.

Generation and maturation of dendritic cells.

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors or melanoma patients using lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Monocytes were isolated on density gradients, with Percoll (Sigma), followed by plastic adherence. Monocytes were cultured for 6 days in 24-well plates (Falcon) at $5x10^5$ cells per well in *rhu*GM-CSF and IL-4 (both 1000U/ml). At day 6, maturation was induced by exposing the DCs to the following combinations of maturation stimuli: LPS (250 ng/ml) and IFNγ (1000 U/ml), LPS and PGE_2 (10^{-6} M), TNFα (100ng/ml) and IFNγ, TNF and PGE_2 for 48 hours (apart from Fig 1B, when 24-96 hours maturation was used, as indicated). In addition, as representatives of clinically-applicable polarized and non-polarized DC currently used as cancer vaccines, we used non-polarized standard (s)DCs matured for 48 hours in the presence of TNFα (100 ng/ml), IL-1β (25 ng/ml), PGE_2 (10^{-6} M), and IL-6 (1000U/ml) (20), and alpha-type-1-polarized DCs (αDC1) matured using the cytokine cocktail composed of TNFα (100 ng/ml), IL-1β (25 ng/ml), IFNγ (1000 U/ml), Poly-I:C (20 μg/ml); and IFNα (3000 U/ml) (71).

Isolation of peripheral blood $CD8^+$ T cell populations.

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors or melanoma patients using lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Naïve $CD8^+CD45RA^+CD45RO^-$ T cells were isolated from the lymphocyte fraction by negative selection with CD8 enrichment cocktail with the addition of biotinylated anti-CD45RO antibody, (StemCell Technologies Inc, Vancouver, Canada) as a uniform population of $CD8^+CCR7^+CD45RA^+CD45RO^-$cells (138, 153). $CD8^+CCR7^+CD45RA^-$ ($CD45RO^+$) memory T cell population was flow-sorted using MoFlo high-speed cell sorter (Dako Cytomation), after labeling with appropriate antibodies.

Flow cytometry.

Two- and three-color cell surface and intracellular immunostaining analysis was performed using Beckman Coulter Epics XL flow-cytometer, after staining with the antibodies against human Granzyme B (BD and CellSciences), CCR7 (R&D Systems), CCR5 (BD Pharmingen), or the corresponding isotypes IgG2a and IgG1. HLA-A2/MART1$_{27-35}$ tetramer staining (Beckman Coulter, Immunomics) was performed according to the manufacturer's instructions.

In vitro sensitization (polyclonal).

Naïve $CD8^+CD45RA^+CCR7^{high}$ T cells ($5x10^5$ cells/well) were activated with SEB-pulsed monocyte-derived DCs ($5x10^4$ cells/well), as described (138, 153). Autologous or allogeneic DCs were used with similar results. On day 5-6, expanded $CD8^+$ T cells were counted and analyzed for the expression of chemokine receptors and chemokine responsiveness, and for CTL phenotype and function (see Supplemental Fig. 1 for the kinetics of acquisition of CTL functions in the differentially-primed $CD8^+$ T cells). Alternatively, the cultures were fed with low dose IL-2 and IL-7 (10 ng/ml) every two days and analyzed for cell surface and intracellular markers on day 16-20. When indicated, neutralizing IL-12 antibody (R&D Systems; Clone 24910) was added at the beginning of the *in vitro* sensitization culture. In preliminary experiments, we compared the outcome of naïve $CD8^+$ T cell priming by polarized and non-polarized DCs in the additional presence of CD40L-expressing J558 cells. Since the presence of CD40L did not abolish the differences in the phenotype and function of the resulting T cells, all subsequent experiments were performed in the absence of CD40L.

In vitro sensitization (melanoma-specific).

Bulk $CD8^+$ T cells ($5x10^5$ cells/well) were activated with the HLA-A2-restricted peptide $MART1_{27-35}$ pulsed autologous DCs ($5x10^4$ cells/well). 3,000 rad-irradiated CD40L-J558 cells (5 x 10^4) were added as surrogates of CD40L-expressing $CD4^+$ Th cells, as described (71). On day 4 *rhu*IL-2 (50 units/mL) and IL-7 (10 ng/mL) were added. $CD8^+$ T-cell cultures were expanded by an additional stimulation (day 14) with irradiated peptide-pulsed autologous PBMCs. At day 24, the differentially induced $CD8^+$ T-cell lines were stained for CCR5, Granzyme B and MART1. CTL activity was determined by ^{51}Cr-release assays against HLA-$A2^+$ melanoma (Fem X), with HLA-$A2^{neg}$ 397 melanoma cells serving as negative specificity control.

Chemotaxis assay.

Chemotaxis assays were performed in 96 well transwell plates with a 3μm pore size polycarbonate filter (Corning Inc, Corning, NY). The lower chamber was filled with 200μl of *rhu*CCL19 (100-1000 ng/ml) or *rhu*CCL5 (100-1000ng/ml) in RPMI-1640+0.5% FBS (Chemotaxis media), 50 μl ($5x10^4$ cells) of differentially activated $CD8^+$ T cells were added in the upper chamber and migration chambers were incubated for 3 h at 37^{o}C. After 3 h, the cells from lower wells were harvested, and counted. The number of cells that migrated in media alone was subtracted to normalize for background migration.

CTL assay.

Cytolytic activity against HLA-$A2^+$ melanoma cells (Fem X) was determined by standard 4 h ^{51}Cr-release assays, with HLA-$A2^{neg}$ 397 melanoma cell line serving as negative control of specificity. The results were calculated and recorded as percent target killing at individual E:T ratios or percentage of cytolysis was converted to lytic units ($LU_{10}/10^7$) as described (154).

Statistical analysis.

The data was analyzed using student's t test (with paired tests being used for comparisons including αDC1- versus sDCs-induced responses from multiple donors). p values of less than 0.05 were considered significant.

2.4 RESULTS

2.4.1 Independent regulation of $CD8^+$ T cell expansion and acquisition of CTL functions by polarized and non-polarized DCs

In order to delineate the requirements for the effective expansion of $CD8^+$ T cells and their acquisition of effector functions, we compared the outcome of $CD8^+$ T cell priming by DCs induced to mature by mediators of acute inflammation (combination of interferons and TLR ligands) or by mediators of chronic inflammation (presence of PGE_2 (155-157)). While the DC maturation in the presence of PGE_2 is associated with an irreversible process of DC "exhaustion" manifested by reduced ability to produce IL-12, the key mediator of inflammatory-type responses (39), and reduced ability to induce Th1 responses of $CD4^+$ Th cells (5, 150, 158), type-1-polarized DCs (DC1s) induced in the conditions of early inflammation avoid the maturation-

associated DC “exhaustion”, retaining their ability to produce IL-12 and to induce Th1 responses of $CD4^+$ Th cells (3, 5, 71, 158).

As shown in Fig. 1A *(left)*, both polarized and non-polarized DCs induced similar rates of expansion of naïve $CD8^+$ T cells. However, only naïve $CD8^+$ T cells primed by the polarized DC1s in our previously-established model of priming of naïve $CD8^+$ T cells (138, 153) demonstrated an effective induction of Granzyme B (GrB; *right*), a marker of effector T cell differentiation (159). In sharp contrast, the low IL-12-producing non-polarized DCs (5, 71, 158) did not prime naive $CD8^+$ T cells to express GrB (Fig. 1A, *right*), despite inducing a similar or higher T cell expansion (Fig. 1A**,** *left*).

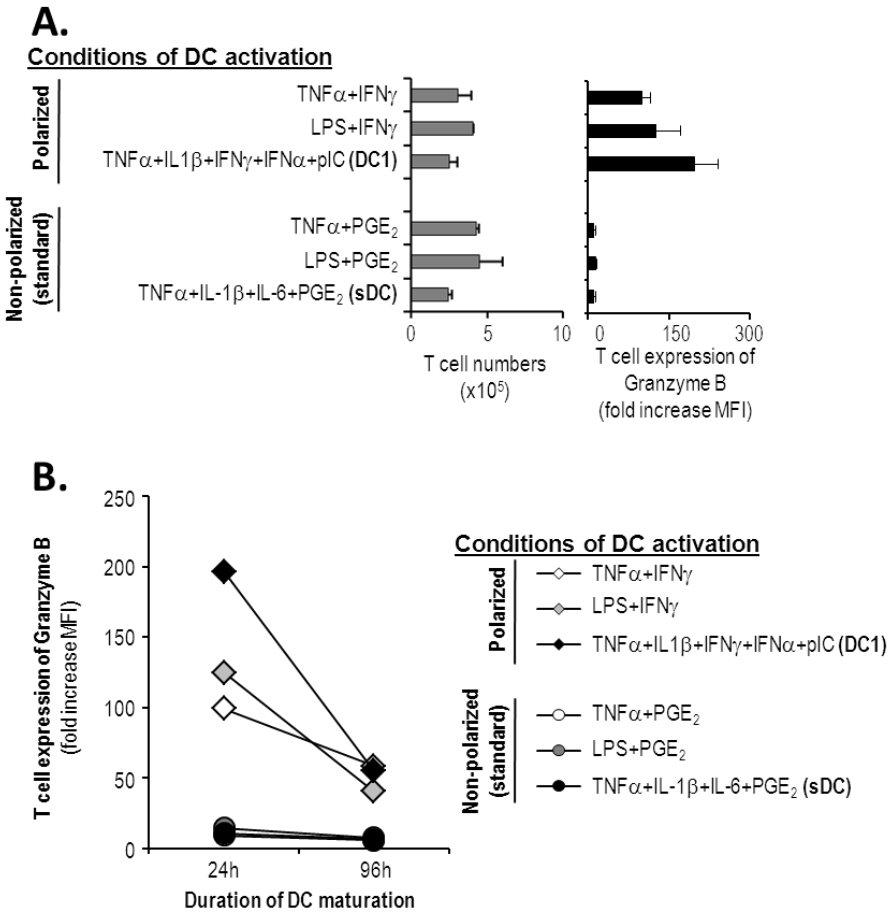

Figure 1. Differential regulation of CD8+ T cell expansion versus the induction of CTL granules by DCs matured in different inflammatory conditions.

Immature DCs were activated with different combination of cytokines (see Materials and Methods), resulting in different levels of IL-12p70 production (TNFα/IFNγ: 3610±160 pg/ml; LPS/IFNγ: 3960±30 pg/ml; αDC1: 1900±120 pg/ml; TNFα/PGE2: 330±80 pg/ml; LPS/PGE2 and sDC: both below 20 pg/ml). For priming of naïve CD8+ T cells, DCs were harvested after 48 hours (additionally, 24- and 96 hour-matured DCs were tested in Fig 1B), washed, pulsed with antigen (SEB) and co-incubated with naïve CD45RA+CD8+ T cells (triplicates). On day 5, CD8+ T cells were counted to assess cell expansion (A, left) and stained for intracellular Granzyme B (GrB) (A; right) The fold increase in MFI (mean fluorescent intensity) of GrB was calculated as the ratio of GrB MFI to isotype-control MFI (mean ± SE of three independent cultures). (A) Selective induction of GrB-expressing CD8+ T cells by polarized DCs. (B) Polarized DCs show persistent (although reduced) ability to induce Granzyme B-expressing CD8+ T cells even after 96 hrs of DC maturation.

Importantly for their use as therapeutic agents *in vivo*, type-1-polarized DCs retained a significant (although reduced) ability to induce GrB expression in expanding $CD8^+$ T cells, even at later times (96hrs) after the induction of their maturation (Fig. 1B). These latter observations indicate that the maturation of DCs in the conditions mimicking early inflammation allows them to at least partially avoid or delay the acquisition of an "exhausted" status (150, 158), previously shown to be associated with abrogated ability to induce functional Th1 responses in the population of $CD4^+$ T cells (150).

Using the clinically-relevant TNFα/IL-1β/Poly-I:C/IFNγ/IFNα-matured αDC1s (71) and TNFα/IL-1β/IL-6/PGE_2-matured "standard" (s)DCs (20) as representatives of type-1-polarized versus non-polarized DCs, we observed that the induction of GrB correlated with the superior cytolytic function of $CD8^+$ T cells primed by the polarized DCs (Fig. 2A). In contrast, priming of $CD8^+$ T cells by the PGE_2-matured sDCs led to low levels of GrB and poor ability to kill antigen-pulsed target cells (Fig. 2A-C), despite effective proliferation of T cells in these cultures and induction of CD45RO (Fig. 1, 2B). In accordance with the central role of IL-12 in the development of CTL activity in $CD8^+$ T cells, neutralization of that factor abrogated GrB induction by type-1 polarized DCs (Fig. 2D).

Since certain conditions of effector T cell induction can be associated with their irreversible differentiation into short-lived, terminally-differentiated effector cells (160), we tested the ability of the DC1-induced effector cells to respond to secondary activation and undergo secondary CTL differentiation. As shown in Fig. 3, after completing the effector phase of activation (more than 2 weeks after priming), the αDC1-primed $CD8^+$ T cells down-regulated the levels of GrB expression and their cytolytic activity. Consistent with the ability of polarized αDC1s to induce long-lived $CD8^+$ T cells (71), such resting αDC1-primed $CD8^+$ T cells

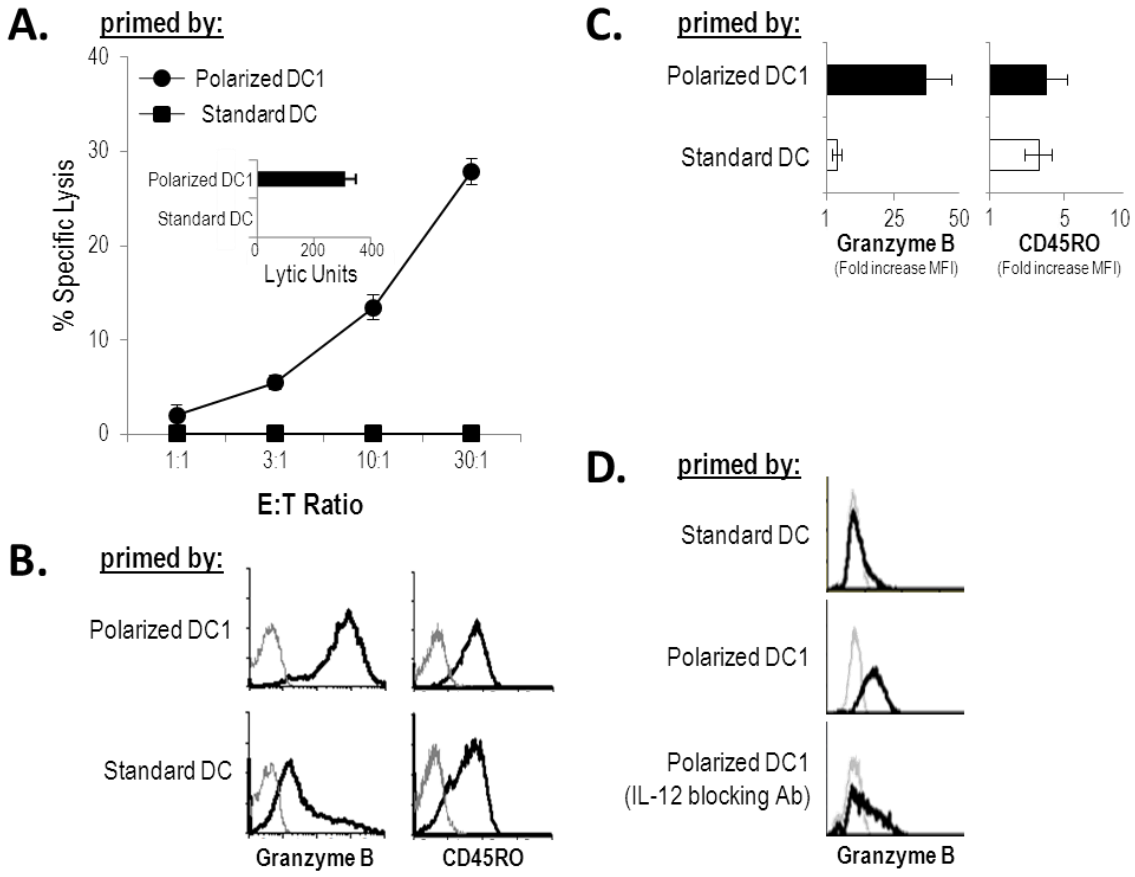

Figure 2. Induction of cytolytic and non-cytolytic pathway of CD8+ T cell differentiation by polarized DC1s and standard DCs.

Naïve $CD8^+$ T cells primed with either polarized or non-polarized DCs, using αDC1 (matured for 48 hours in TNFα, IL-1β, IFNα, Poly-I:C and IFNγ and sDCs (matured for 48 hours in TNFα, IL-1β, IL-6 and PGE_2 (20)), as the respective representatives. (A) Cytolytic function of day 5 primed $CD8^+$ T cells was assessed by standard ^{51}Cr-release assay using SEB pulsed JY cells as targets(153). *Inset,* data calculated as lytic units. (B-C) Intracellular expression of GrB and surface expression of CD45RO were determined by flow cytometry on day 5 (B) Data from a representative donor. Gray lines indicate isotype controls. (C) Summary of data from 3 different donors. Fold increase in MFI of GrB and CD45RO was calculated as in Figure 1. Data is shown as mean and SEM of three independent experiments that all showed advantage of polarized αDC1s in the induction of GrB ($p < 0.02$). (D) Neutralization of IL-12 abrogates the induction of GrB-positive $CD8^+$ T cells by polarized αDC1s. Representative data from 3 experiments that all yielded similar results

expressed high levels of IL-15Rα and IL-7Rα (CD127) (Fig 3A, see Supplemental Fig. 2 for the levels of both receptors in naïve $CD8^+$ T cells), the memory cell-associated receptors for the homeostatic cytokines mediating long-term survival of $CD8^+$ T cells (161-162), and were fully capable of rapidly re-acquiring high levels of CTL activity upon restimulation with polarized αDC1s (Fig. 3B).

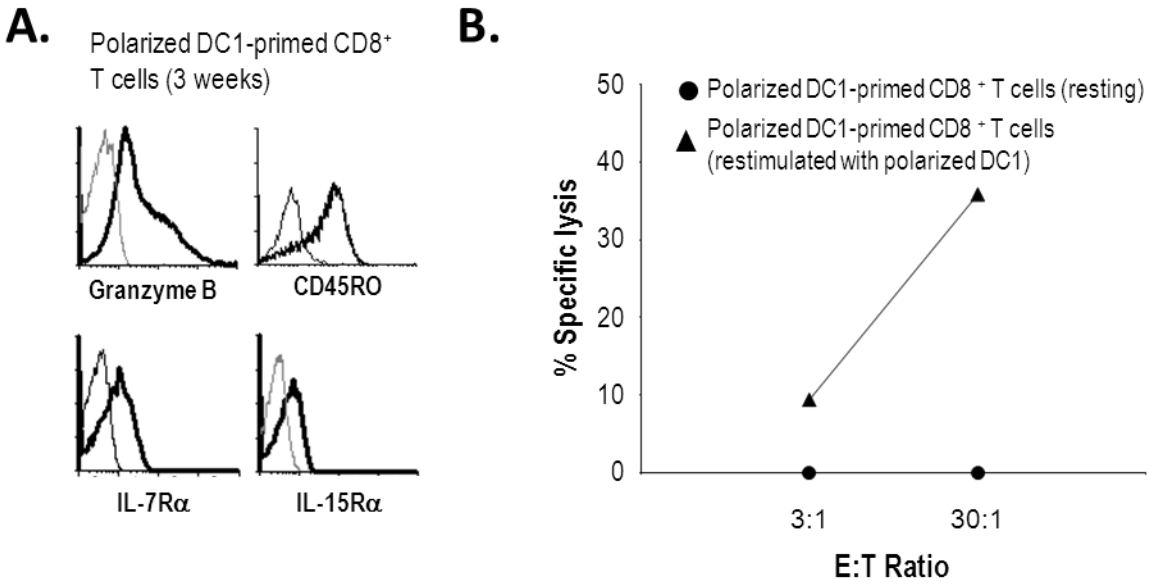

Figure 3. CD8+ T cells primed by polarized DC1s revert to memory status and can be reactivated to undergo secondary CTL differentiation.

(A) Induction of memory $CD8^+$ T cells at later stages of activation with αDC1. Naïve $CD8^+$ T cells were primed with αDC1. After 3 weeks, the cells were analyzed for the expression of GrB, CD45RO, IL-7Rα, and IL-15Rα. (B) Effective induction of secondary CTL function in αDC1-primed resting $CD8^+$ T cells. Three weeks after priming with αDC1s, resting $CD8^+$ T cells were (re)stimulated for 24 hours with αDC1s and re-assessed for CTL function. SEB pulsed JY cells were used as target population for Chromium release assay (153). Similar data was obtained in 3 independent experiments, with the observed range of killing between 0% and 8.9% (at the maximal 30:1 ratio) for the resting $CD8^+$ T cells and between 34.8% and 72% for the re-stimulated $CD8^+$ T cells.

2.4.2 Polarized DC1s induce a switch in chemokine receptor expression and peripheral tissue-associated chemokine responsiveness in expanding $CD8^+$ T cells: key role of IL-12.

Since polarized αDC1s and standard DCs both promoted the expansion of naive T cells but had a differential impact on the induction of their CTL function, we tested their influence on the $CD8^+$ T cell expression of CCR7 and CCR5, the respective lymphoid versus peripheral effector-type chemokine receptors, and the migratory responsiveness to their respective ligands, lymph-node-associated CCL19/MIP3β (163-165) and CCL5/RANTES, a ubiquitous peripheral tissue-produced chemokine (163-164) known to be over-expressed in cancer tissues (164, 166).

As shown in Fig. 4A and B, αDC1s effectively induced the expression of CCR5, the chemokine receptor typical for effector (and effector-memory) $CD8^+$ T cells (113, 167-168), with a concomitant loss of CCR7 on 50-70% of $CD8^+$ T cells. In contrast, $CD8^+$ T cells stimulated by sDCs retained high levels of CCR7 expression, and did not acquire CCR5.

In accordance with their differential expression of CCR7 and CCR5, the differentially-activated $CD8^+$ T cells showed reciprocal patterns of migratory responsiveness to the LN-associated versus peripheral tissue-associated chemokines (CCL19 and CCL5, respectively (163-164, 166, 168)) with αDC1-primed CTLs preferentially migrating towards the peripheral tissue chemokine CCL5 (RANTES), while the sDC-primed T cells preferentially responded to the lymphoid chemokine CCL19 (Fig. 4C).

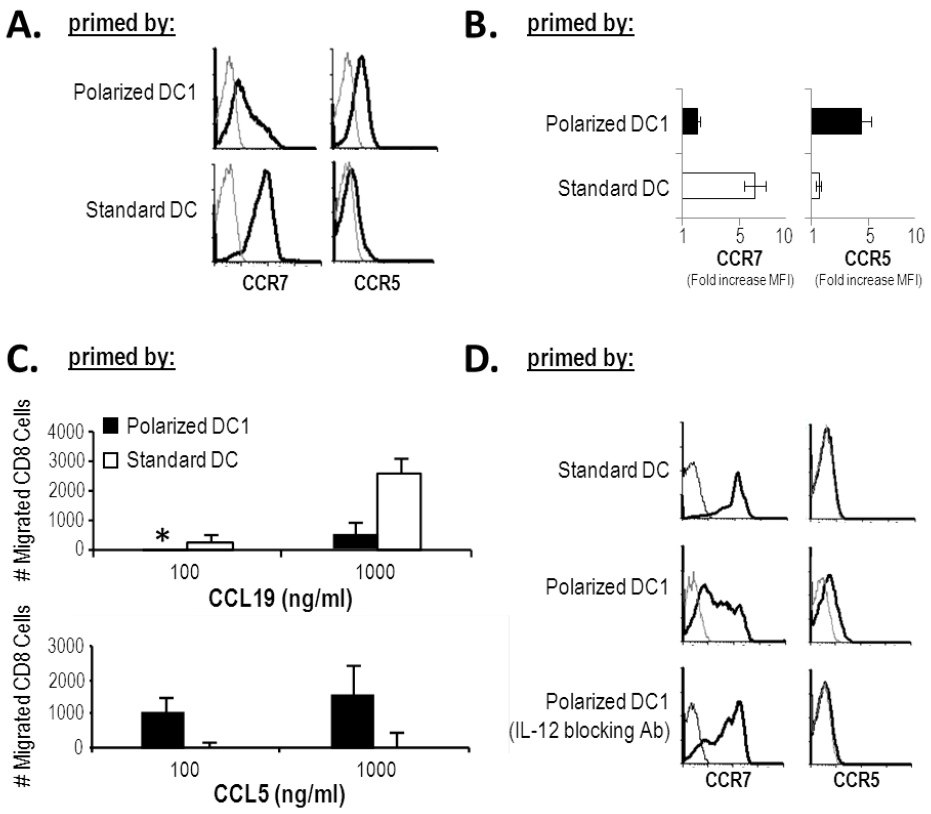

Figure 4. Polarized DC1s induce a switch in chemokine receptor expression and chemokine responsiveness.

Naïve $CD8^+$ T cells were primed by αDC1 or sDC. Differentially primed $CD8^+$ T cells were harvested on day 5 and analyzed for the expression of chemokine receptors. (A) Data from a representative donor: Levels of expression of CCR7 and CCR5 (black lines), compared to isotope controls (gray lines). (B) Cumulative data from 3 donors. Fold increase in MFI of CCR7 and CCR5 were calculated as in Figure 1. Data is shown as mean ± SEM of three independent experiments that all showed advantage of polarized αDC1s in promoting the loss of CCR7 expression ($p < 0.0005$) and induction of CCR5 ($p < 0.005$). (C) Differentially primed $CD8^+$ T cells were analyzed for their responsiveness to chemokine receptor ligands CCL19 and CCL5 by chemotaxis assay (mean ± SEM of three independent experiments) In the 3 donors tested, at the maximal concentrations of the two chemokines, the migration of αDC1s to CCL19 was 3.4-fold to 5.2-fold lower than the migration of sDCs to CCL19, while the migration of αDC1s in response to CCL5 was 3.6 to11.8- fold higher that the migration of sDCs. (D) IL-12 blocking antibody was added during the priming of naïve $CD8^+$ T cells by polarized αDC1s. CCR7 and CCR5 expression (black lines) was assessed by flow cytometry on day 5. Gray line indicates isotype control in all histograms. Similar data was observed in two additional experiments.

Since in the $CD4^+$ T cell system, the levels of DC-produced IL-12 were shown to be the key to the differential ability of DCs to induce a Th1 or Th2 pattern of differentiation in naïve $CD4^+$ T cells (3, 93, 150) and recombinant IL-12 was shown to directly affect the expression of Th1 and Th2-associated chemokine receptors (169-170), we tested the role of IL-12 in the DC-induced switch in chemokine receptor expression of $CD8^+$ T cells. As shown in Fig. 4D**,** the neutralization of IL-12 during T cell priming abrogated the above differences, preventing the down-regulation of CCR7 and elevation of CCR5 on $CD8^+$ T cells activated by the polarized DCs. These data indicate that IL-12, originally identified as a factor supporting killer activities of $CD8^+$ T cells and NK cells (reviewed in (39)), is also a key DC-produced factor responsible for the switch from central to peripheral chemokine receptor pattern in the differentiating naïve $CD8^+$ T cells.

2.4.3 Polarized and non-polarized DCs differentially regulate CTL activity and chemokine receptor expression on tumor antigen-specific $CD8^+$ T cells.

Prompted by the results of the experiments with polyclonally-activated naïve $CD8^+$ T cells (Fig 2A) and similar data obtained using memory cells **(**Supplemental Fig. 3), we have compared the outcome of *in vitro* sensitization (IVS) of HLA-A2-restricted melanoma-specific $CD8^+$ T cells using MART-1_{27-35}-loaded autologous αDC1 or sDCs , currently applied as cancer vaccines.

In contrast to the short-term experiments performed in the polyclonal system, the generation of high numbers of MART-1-specific T cells required prolonged cultures of the differentially-sensitized $CD8^+$ T cells. While in these long-term cultures we could not detect the

differences in CCR7 expression between the differentially-sensitized $CD8^+$ T cells (CCR7 was low on both populations, data not shown), exclusively the MART-1-specific (tetramer-positive) $CD8^+$ T cells sensitized with polarized αDC1s showed high GrB expression and high CTL activity against MART-1-expressing HLA-$A2^+$ melanoma cells (but not against HLA-$A2^-$ melanoma cells; Fig. 5A-C). While in contrast to their inability to induce CTL activity in naïve $CD8^+$ T cell population (see Figs 1-2), non-polarized DCs showed significant ability to induce CTL function in tumor-specific T cells from melanoma patients, though type-1 polarized DCs were clearly more efficient (Fig. 5C), with the level of advantage comparable to that observed in the polyclonal model of (re)activation of "bulk" (memory and naïve) $CD8^+$ T cells (Supplemental Fig. 4). In accordance with the data obtained in the polyclonal models (Fig 4), MART-1-specific $CD8^+$ T cells sensitized by polarized αDC1s also showed elevated levels of CCR5 (Fig. 5D).

In addition to CCR5, which shows high effectiveness in attracting mouse effector cells to melanoma lesions (171) and was recently implicated in the responsiveness of melanoma patients to immunotherapy (172), another CTL-associated chemokine receptor, CXCR3, has been recently implicated in melanoma regression (36) and prolonged survival of patients with advanced disease (23). Therefore, we compared the expression of CXCR3 on MART-1-specific $CD8^+$ T cells pre-sensitized with polarized DC1s and standard DCs. As shown in Fig. 5D, polarized αDC1 induced strongly elevated levels of CXCR3 in MART-1-specific $CD8^+$ T cells from melanoma patients.

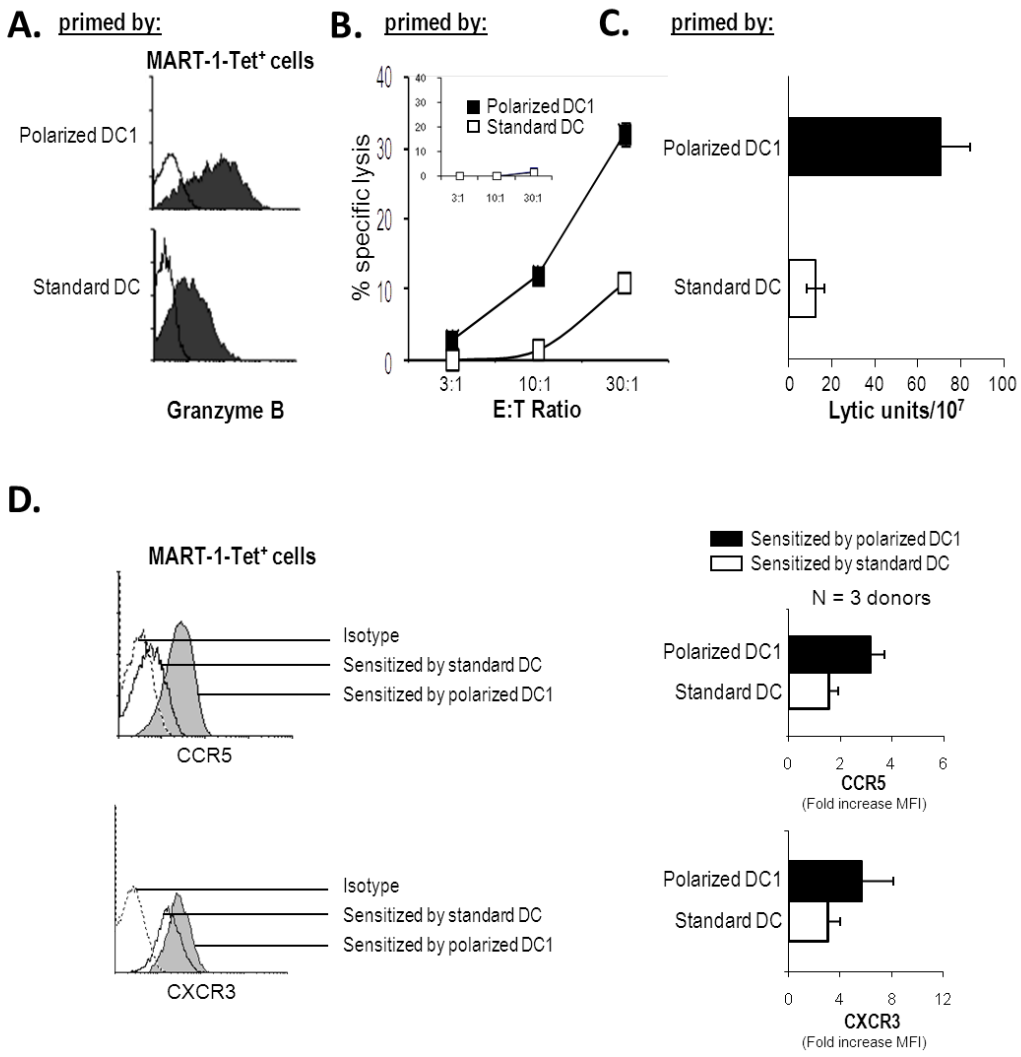

Figure 5. Polarized αDC1s and non-polarized sDCs induce differential expression of GrB and melanoma-relevant chemokine receptors on MART-1-specific CD8$^+$ T cells.

αDC1 and sDCs from HLA-A2$^+$ melanoma patients were pulsed with the HLA-A2-restricted MART-1 peptide and used to stimulate autologous CD8$^+$ T cells in an *in vitro* sensitization system (*see M&M*). (A) Intracellular expression of GrB in MART-1-tetramer$^+$ CD8$^+$ T cells. Similar data was obtained in case of two donors. (B-C) High CTL activity of αDC1-sensitized CD8$^+$ T cells against melanoma cells. Cytotoxic activity of the differentially primed CD8$^+$ T cells was measured against MART-1-expressing HLA-A2$^+$ melanoma cell line (Fem-X). *The inset:* HLA-A2$^-$ melanoma cell line (melanoma 397) was used as a negative control of antigenic specificity. (B) Representative data from one patient. (C) Combined data from 4 different patients expressed as lytic units *($p < 0.005$).* (D) Surface expression of CCR5 and CXCR3 were measured in MART-1-tetramer$^+$ CD8$^+$ T cells. *Left*:

Representative data from a single melanoma patient. *Right:* Cumulative data from 3 melanoma patients expressed as the mean ± SEM. αDC1-sensitized $CD8^+$ T cells show enhanced expression levels of both CCR5 and CXCR3 ($p<0.05$ in both cases).

2.5 DISCUSSION

Our data demonstrate that the ability of DCs to activate T cells and to efficiently induce their expansion does not predict their ability to induce CTL activity and the ability to respond to peripheral-type chemokines. In contrast, we observed that while the expansion of $CD8^+$ T cells can be driven efficiently by the DCs matured in a wide spectrum of inflammatory conditions, the induction of the $CD8^+$ T cell effector functions in naïve $CD8^+$ T cells and a switch in their chemokine responsiveness was a sole property of the "non-exhausted" IL-12-producing DCs matured in the conditions that mimic acute inflammation (presence of interferons and TLR ligands). This "inflammatory" pathway of activation of $CD8^+$ T cells, associated with the IL-12-dependent induction of GrB^{high} CTLs, eventually results in a resting population of memory-type ($CD8^+CD45RO^+GrB^{low}$) cells. In accordance with the previously-reported long-lived character of cells activated by the high IL-12-producing type-1-polarized DCs (71) and with the ability of recombinant IL-12 to promote CTL survival (173-174), the $CD8^+$ T cells undergoing such "inflammatory" pathway of differentiation expressed high levels of IL-7 and IL-15 receptors (Fig. 3), known to be essential for the homeostatic proliferation and long term survival of $CD8^+$ T cells *in vivo* (161-162), and could effectively re-acquire CTL function following restimulation with polarized DCs.

The effectiveness of polarized DC1s in inducing functional CCR5 (and CXCR3)-expressing CTLs suggest that these cells can be useful tools to direct the vaccination-induced T cells to tumors in therapeutic conditions. Since melanomas are known to over-express CCL5/RANTES (175-176), on which they rely as an autocrine growth factor (176-177), CCL5-responsive αDC1-induced T cells are likely to show improved therapeutic activity, not only due to their higher per-cell killer activity, but also due to their ability to preferentially home to tumor tissues. In support of the opposite roles of tumor-expressed CCR5 versus T cell expressed CCR5 in melanoma progression (respectively, tumor-promoting vs. tumoricidal), it was recently shown that while overall populations of melanoma patients lacking functional CCR5 (CCR5Delta32$^+$ individuals) and CCR5-competent melanoma patients have similar course of disease, functional CCR5 is needed for positive response to immunotherapy (172). Similarly, in accordance with high expression of CXCR3 ligands: CXCL9/MIG and CXCL10/IP10, in melanoma tissues (178) and the presence of CXCR3 on tumor-infiltrating lymphocytes in regressing melanoma lesions (36), high levels of CXCR3 on circulating CD8$^+$ T cells has been recently implicated in effective control of advanced melanoma (23).

In contrast to such "pro-inflammatory/effector" pathway of differentiation driven by polarized DCs, naive CD8$^+$ T cells activated by standard non-polarized DCs did not acquire CTL functions and remained responsive to LN-associated chemokines, even though they vigorously expanded. While our preliminary data indicate that such cells can be effectively reactivated by polarized DC1s (*data not shown*) to undergo secondary CTL differentiation, the identity and functional role of such "non-effector" CD8$^+$ T cells induced by standard "exhausted" DCs remains a subject of our follow up studies. Interestingly, while non-polarized DCs were unable to induce the *de novo* effector function in naïve CD8$^+$ T cells, they showed a significant (although

lesser than polarized DCs) ability to induce CTL function in (expectedly previously-primed) tumor-specific T cells from melanoma patients, type (Fig. 5A-C), and in the polyclonal model of (re)activation of "bulk" (memory and naïve) $CD8^+$ T cells (Supplemental Fig. 4).

The current demonstration that the ability of DCs to induce proliferation and expansion of tumor-specific $CD8^+$ T cells is independent from their ability to induce their tumor-relevant homing properties and tumoricidal effector functions, helps to interpret the limited effectiveness of cancer vaccines observed in recent clinical trials (145-148) and aid in designing corrective measures to enhance the efficacy of cancer immunotherapies. Several recently tested cancer vaccines involving antigenic peptides or tumor antigen-expressing viral vectors were shown to promote massive increase of blood-circulating tumor-specific $CD8^+$ T cells but not clinical responses (143-144, 146-148). Interestingly, at least one study indicated that such split effectiveness of cancer vaccines can be corrected by a follow up treatment of the vaccinated patients with IFNα (143). While our current data (Fig. 2D) demonstrate the key role of IL-12 in the induction of functional CTLs by type-1-polarized DCs, it remains to be tested if other factors may supplement or replace the function of IL-12 in differentially-matured DCs.

Our current data suggest that the limitations of current cancer vaccines, including "standard" DC-based vaccines (179), may result from their selective deficit in inducing the effector functions in tumor-specific T cells, and may be corrected by the modification of the current therapeutic vaccines, or their combination with pro-inflammatory factors, capable of inducing tumoricidal function and tumor-homing ability in tumor-specific T cells.

2.6 IMPLICATIONS

The goal of therapeutic (cancer) immunotherapies is the induction of large numbers of Ag-specific $CD8^+$ T cells followed by the development of long-lived memory T cells. The data presented in chapter 2 suggests that for the use in cancer vaccination strategies, type-1 polarized, inflammatory DC1s are superior in inducing the differentiation of naïve $CD8^+$T cell cytolytic effector cells when compared with DCs matured under late/chronic inflammatory conditions. Since type-1 polarized DCs are also able to induce the expression of peripheral homing chemokine receptors CXCR3 and CCR5 on primed $CD8^+$ T cells, these DCs could generate anti-tumor immune responses. However, $CD8^+$ T cells primed by DCs matured in the presence of PGE_2 proliferated and were activated (as observed by CD45RO expression), but failed to acquire cytolytic ability and peripheral homing ability. In the next chapter, I examine the cell fate of naïve $CD8^+$ T cells primed by these "PGE_2-matured" DCs.

3.0 DIFFERENTIALLY-ACTIVATED DENDRITIC CELLS INDUCE DIRECT VERSUS INDIRECT DEVELOPMENT OF MEMORY CD8$^+$ T CELLS

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The data presented here are reported as *Manuscript in Preparation*. Erik Berk produced the majority of the data which contributed to all the figures and prepared the manuscript.

3.1 ABSTRACT

The development of memory $CD8^+$ T cells is particularly effective at late stages of immune responses but the mechanism of this temporal effect remains unclear. Here, we show that human T-bet^-/IL-12^- dendritic cells (DCs) at late stages of activation or activated in the presence of the late-inflammatory mediator PGE_2, traditionally considered as "exhausted", induce direct transition of naïve $CD8^+$ T cells into central-memory cells (T_{CM}; T-bet^{low}/GrB^{low}/$CCR7^{high}$/IL-$7R\alpha^{high}$ with low mTOR activity). In contrast, T-bet^+/IL-12^+ DCs induced by short-term exposure to IFNs and TLR-Ls, promote IL-12-dependent development of cytolytic effector $CD8^+$ T cells (T_{EFF}; T-bet^{high}/GrB^{high}/$CCR7^{low}$/IL-$7R\alpha^{low}$/$mTOR^{high}$), followed by the subsequent development of both central-memory (T_{CM}) and effector-memory (T_{EM}) cells. T_{CM} cells induced by "exhausted" DCs share the phenotype and genetic profile with blood-isolated T_{CM} cells and can undergo accelerated secondary expansion and effector (T_{EFF}) differentiation when re-stimulated by the T-bet^+/IL-12^+ "effector" DCs. Our data indicate that T-bet^-/IL-12^- "exhausted" DCs represent specialized "memory-DCs" promoting the direct differentiation of naïve $CD8^+$ T cells into T_{CM} cells.

3.2 INTRODUCTION

Dendritic cells (DCs) are the key regulators of immune responses (1, 180-181). The environmental cues that DCs receive during their pathogen-triggered maturation and the duration of maturation, affect the immunostimulatory abilities of the DCs and the type of immune response (Th1, Th2, Th17 or Treg) that is elicited (3, 182-185). While short-time-activated DCs, particularly maturing in the presence of Toll-like Receptor Ligands (TLR-Ls) and interferons typical of early stages of inflammation (4, 186), produce high levels of the inflammatory cytokine IL-12p70 upon contact with T cells, allowing them to induce type-1 $CD4^+$ T helper (Th1) responses, as well as to activate the effector functions of CTLs and NK cells (5, 23, 187-189). In contrast, DCs at later stages of maturation or DCs maturing in conditions of chronic inflammation (typically associated with lack of interferons and predominance of PGE_2 and other IL-12-suppressing factors (190-193)), rapidly lose the ability to secrete IL-12 (26, 187) and become "exhausted", losing the capacity to induce Th1 and CTL responses (26, 189, 194).

Prompted by the high effectiveness of such nominally "exhausted" DCs in inducing the primary expansion of naïve $CD4^+$ (26) and $CD8^+$ T cells (189), and the documented negative impact of inflammation and inflammatory cytokines on the formation of $CD8^+$ T cell memory in mouse *in vivo* models (43, 122-123, 195-197), we tested the impact of such "late-inflammation-induced" ($_{LI}$)DCs on the development of memory $CD8^+$ T cells from human naïve precursors. Our data indicate that $_{LI}$DCs, are in fact specialized in the direct induction of central-memory $CD8^+$ T cells (T_{CM}), in sharp contrast to T-bet^+/IL-12^+ "early-inflammation-induced" ($_{EI}$)DCs

that induce a sequential development of $mTOR^{high}$/T-bet^{high}/GrB^{high} effector $CD8^+$ T cells (T_{EFF}), followed by the development of central-memory (T_{CM}) and effector-memory (T_{EM}) $CD8^+$ T cells.

3.3 MATERIALS AND METHODS

Cell lines, media and reagents. Serum-free AIM-V medium (Invitrogen, Carlsbad, CA) and IMDM (Invitrogen) with 10% FBS were used to generate DCs. IMDM with 5% human serum (Gemini Bio-products, West Sacramento, CA) was used for *in vitro* sensitization (IVS) experiments. The following factors were used to generate mature DCs: *rhu*GM-CSF and IL-4 (gifts from Schering Plough, Kenilworth, NJ), IFNα (Intron A), *rhu*TNFα, *rhu*IFNγ, *rhu*IL-1β (all from Strathmann Biotech, Germany), *rhu*IL-6 (Genzyme, Cambridge, MA), lipopolysaccharide (LPS), prostaglandin E2 (PGE_2) and poly-I:C (Sigma, St. Louis, MO). IL-2 and *rhu*IL-7 (Strathmann Biotech) were used to support the $CD8^+$ T cell expansion.

Generation and maturation of DCs. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (obtained from the blood bank) using lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Monocytes were isolated using density gradients made with Percoll (Sigma), followed by plastic adherence or by using $CD14^+$ isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to manufacturer's instruction. Both protocols yielded similar results. Monocytes were cultured for 6 days in 24-well plates at 5-6x10^5 cells per well in *rhu*GM-CSF and IL-4 (both 1000U/ml). At day 6, maturation was induced by exposing the DCs to the

following combinations of maturation stimuli: LPS (250 ng/ml) and IFNγ (1000 U/ml); TNFα (100 ng/ml), IL-1β (25 ng/ml), IFNγ (1000 U/ml), Poly-I:C (20 μg/ml); and IFNα (3000 U/ml);

LPS and PGE_2 (10^{-6} M); TNFα (100 ng/ml), IL-1β (25 ng/ml), PGE_2 (10^{-6} M), and IL-6 (1000U/ml) (20).

Isolation of naïve $CD8^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (obtained from blood bank) using lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Peripheral lymphocytes were obtained following density gradients with percoll. Naïve $CD8^+CD45RA^+CD45RO^-$ T cells were isolated from the lymphocyte fraction by negative selection with CD8 enrichment cocktail with the addition of biotinylated anti-CD45RO antibody, (StemCell Technologies Inc, Vancouver, Canada) as a uniform population of $CD8^+CCR7^+CD45RA^+CD45RO^-$cells. $CD8^+CCR7^-CD45RA^-CD45RO^{+/-}$ effector and $CD8^+$ $CCR7^+CD45RA^-CD45RO^+$ central-memory T cells were flow-sorted using MoFlo high-speed cell sorter (Dako Cytomation), after labeling with appropriate antibodies.

Flow cytometry

Fluorescein isothiocyanate (FITC)-labeled anti-human CD80, CD40, granzyme B, CD45RO, Phyocoerythrin (PE)-labeled anti-human CD83, PE-Cy5-labeled anti-human CD8, CD25, CD62L and Allophycocyanin (APC)-labeled anti-human CD86 were all purchased from BD Biosciences (San Jose, CA). FITC-labeled and APC-labeled anti-human CCR7 was obtained from R&D systems. PE-labeled anti-human CD127 (IL-7Rα) and purified anti-human/mouse T-bet were obtained from eBioscience. Alexa-Fluor 488-labeled rabbit-anti-human phosphorylated S6 (SER235/236) was purchased from Cell Signaling (Danvers, MA).

For surface staining, cells were transferred to a V-bottom 96-well plate and washed with FACS buffer (PBS/0.5%BSA/0.1% Azide). Cells were incubated with Abs for 45 minutes in the dark at RT. Following labeling, cells were washed twice and fixed with 2% para-formaldehyde and stored at 4°C until analysis.

For intracellular staining, cells were transferred to 5ml FACS tubes and washed with PBS. Cells were fixed for 20min at 37C with 2% PFA, washed with PBS and subsequently permeabilized using 100% ice cold methanol for 30min at -20C. Following permeabilization, cells were washed twice with FACS buffer and incubated with appropriate Abs for 30 min at RT in the dark. Following cell labeling, cells were washed with FACS buffer and either fixed (granzyme B and pS6 staining) or incubated for 30 min with FITC-labeled anti-mouse IgG-Fab_2 (T-bet staining), followed by washing with FACS buffer and fixation with 2% PFA. Fixed cells were analyzed using an Acurri C6 flow cytometer. Flow cytometry data was analyzed using FCS Express V3 software.

In vitro sensitization and restimulation. Naïve $CD8^+CD45RA^+CCR7^+$ T cells were activated with Staphyllococcus enterotoxin B (SEB)-pulsed monocyte-derived DCs in a 10:1 ratio. Where indicated, naïve $CD8^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) according to manufacturer's directions prior to co-culture with DCs. On day 6-7, expanded $CD8^+$ T cells were analyzed for phenotype and function. Alternatively, the cultures were fed with IL-2 (10 U/ml) and IL-7 (10 ng/ml) every two days and analyzed for cell surface and intracellular markers on day 18-21. When indicated, neutralizing IL-12 antibody (R&D Systems; Clone 24910) was added at the beginning of the *in vitro* sensitization culture.

In some experiments, day 7 or day 21 differentially primed $CD8^+$ T cells were collected, washed, counted and restimulated with SEB-loaded DCs for an additional 36 hours (for

granzyme B expression and CTL assay) or 4-5 days (for cell expansion). All cell counts were performed using a heamocytometer and trypan blue exclusion.

CTL assay. Cytolytic activity against SEB-loaded JY-1 cells was determined by standard 4 hour ^{51}Cr-release assays. Following incubation 65µl of supernatant was transferred to lunaplates (PerkinElmer, Boston, MA) The percentage specific lysis was calculated using the following equation: [(experimental release - spontaneous release) / (maximum-release – spontaneous release)]*100, where spontaneous release is measure from supernatant of untreated target cells and maximum release is obtained from target cells treated with 1% Triton solution. Data are represented as mean +/- SEM for 4 independent experiments.

Taqman analysis of mRNA expression.

$CD8^+$ T cell cultures and blood-isolated effector and central-memory cells were lysed using RLT buffer (RNeasy kit, Qiagen, Valencia, CA) and Total RNA was extracted using the RNeasy kit. 1µg of RNA extracted by above was used for cDNA synthesis and 25-50ng of subsequent cDNA was used to perform mRNA expression analysis by Taqman analysis on Step One Plus system (Applied Biosystems). All primer sets were purchased from Applied Biosystems.

Statistical analysis. The data was analyzed using student's t test (with paired tests being used for comparisons including "inflammatory" DC- versus "exhausted"-DC-induced responses from multiple donors). p values of less than 0.05 were considered significant.

3.4 RESULTS AND DISCUSSION

3.4.1 Late-inflammation-induced IL-12$^-$/T-bet$^-$ DCs induce primary expansion of naïve CD8$^+$ T cells but do not induce T_{EFF} differentiation

To analyze the role of "exhausted" DCs in the priming of naïve CD8$^+$ T cells we induced the maturation of human monocyte-derived DCs by the exposure to TLR ligands and interferons typical of mimicking early/acute inflammation (4, 186) resulting in high-IL-12-producing DCs (5, 71, 189) or in the presence of prostaglandin E2 (PGE_2), prevalent in chronic/late stage inflammation (190-193), for either 24 hours (early stage of maturation) or 96 hours (late stage of maturation). DCs activated by short-term exposure to the mediators of early inflammation were able of producing high amounts of IL-12 (Fig. 6A, top). As expected (187, 189, 198), the IL-12 secretion by long-term matured inflammatory DCs was strongly diminished (Fig. 6A, bottom). In contrast, DCs activated in the presence of PGE_2, demonstrated accelerated exhaustion, being unable to secrete IL-12, independently on the time-point of their activation (Fig. 6B). These differences in IL-12 producing capacity occurred despite the fact that the short- and long-term activated DCs all showed similar expression of maturation-associated co-stimulatory molecules (CD40, CD80, CD83 and CD86) as well as CCR7 (Fig. 6C-D). However, exclusively the DCs short-term activated in the early-inflammatory conditions expressed the transcription factor T-bet, a typical marker of type-1 effector T cells (199) which expression by DCs facilitates the induction of Th1 responses (94, 200). Strikingly, T-bet expression was absent in long-term matured DCs, independently on the conditions of their maturation (Fig 6E-F), indicating a

general role of T-bet as a marker absent in "exhausted" DCs, independently on the path of their induction.

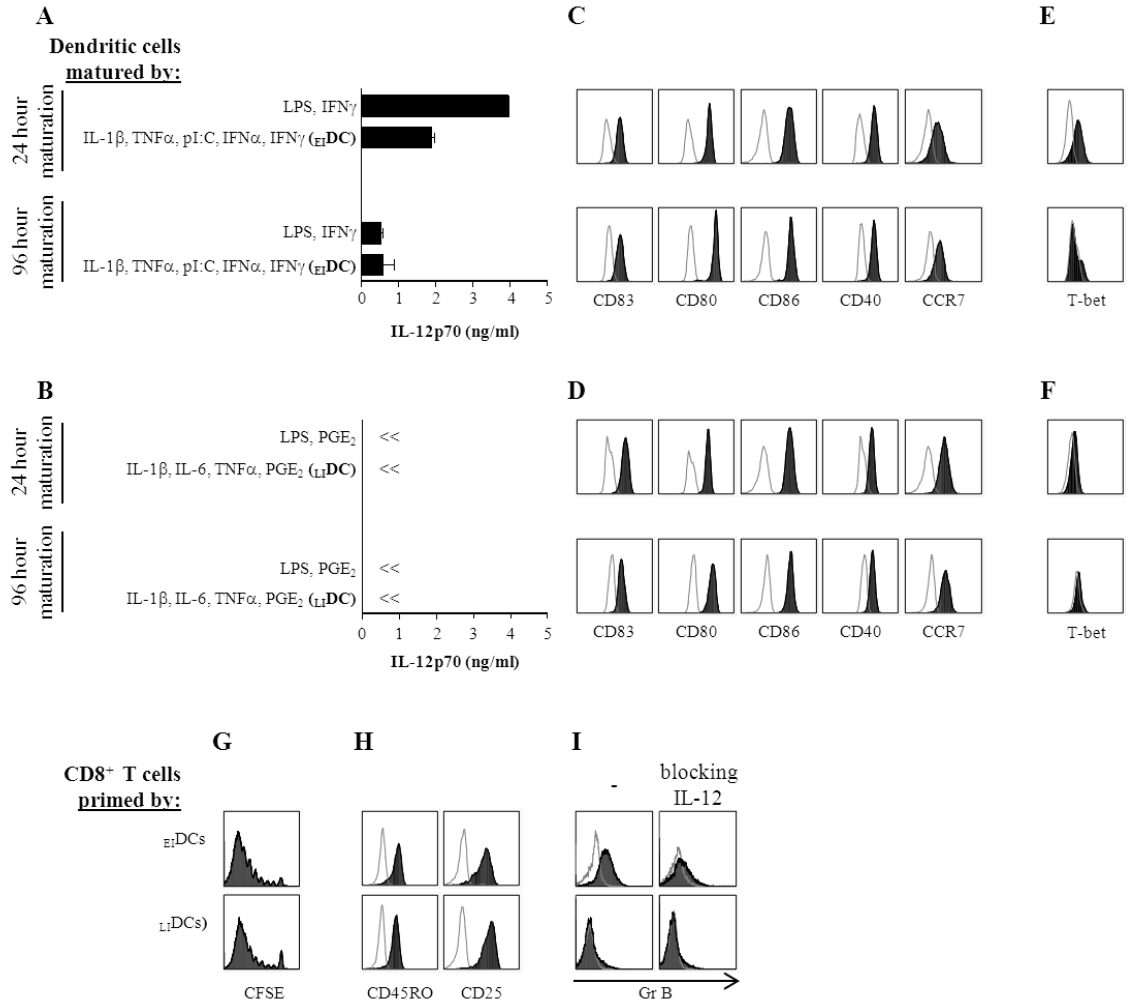

Figure 6. $_{LI}$DCs induce CD8$^+$ proliferation and activation but do not support effector cell differentiation.

Monocyte derived immature DCs were matured in different inflammatory conditions that either promote IL-12 production (LPS + IFNγ or IFNγ + IFNα + TNFα + IL-1β and poly-I:C) or late/chronic-inflammatory conditions (LPS + PGE_2 or TNFα + IL-1β + IL-6 and PGE_2) for 24 or 96 hours. (A-B) Following 24 hour and 96 hour maturation, DCs were collected and cultured for 24 hours with CD40L expressing J558 cells. 24 hour supernatant of co-cultures was collected and analyzed for IL-12p70 production by ELISA. Bars represent mean +/- SEM of triplicate wells of one experiment. Data are representative of 3 independent experiments that yielded similar results.

<<; below detection limit. (C-D) DC maturation status was determined by flow cytometry after 24 hours and 96 hours of maturation. (E-F) 24 hour and 96 hour matured DCs were analyzed for T-bet expression by intracellular flow cytometry. (G) 24 hour matured DCs were loaded with SEB and co-cultured with CFSE-labeled naïve $CD8^+$ T cells for 5 days. T cell proliferation was determined by flow cytometry. (H) Naïve $CD8^+$ T cells were primed by SEB-loaded $_{EI}$DCs or $_{LI}$DCs. Activation of day 6 primed $CD8^+$ T cells was determined by flow cytometry. (I) Naïve $CD8^+$ T cells were primed by SEB-loaded $_{EI}$DCs or $_{LI}$DCs in the presence of absence of anti-IL-12 blocking Ab. Day 6 $CD8^+$ T cells were analyzed for intracellular expression of granzyme B.

Using DCs short-term-matured in the presence of poly-I:C, IFNα and IFNγ as the model of IL-12^{high}/T-bet^{high} early-inflammation-induced($_{EI}$)DCs, and short-term PGE_2-matured DCs as the model of IL-12^{low}/T-bet^{low} late-inflammation-induced ($_{LI}$)DCs (which allowed for the parallel evaluation of their T cell-activating functions), we compared their ability to prime naive $CD8^+$ T cells in our established polyclonal *in vitro* sensitization model (189). Both types of DCs promoted a similar expansion of naïve $CD8^+$ T cells (Fig. 6G), and induced similar expression of CD45RO and CD25 on the differentially-primed $CD8^+$ T cells (Fig. 6H).

However, in contrast to the $CD8^+$ T cells primed by $_{EI}$DCs, that expressed high levels of the cytolytic effector protein granzyme B, the $_{LI}$DC-primed $CD8^+$ T cells did not acquire granzyme B expression (Fig 6I left). As expected, the differentiation of naïve $CD8^+$ T cells into cytolytic effector T cells (T_{EFF}) was IL-12-dependent, since blocking of IL-12 during the $_{EI}$DC-driven T cell priming prevented the induction of granzyme B (Fig. 6I right).

3.4.2 $CD8^+$ T cells primed by $_{LI}$DCs rapidly acquire central-memory phenotype and accelerated capacity to undergo secondary expansion

Since mouse studies have shown that IL-12-driven activation of mTOR kinase favors the development of T-bet^+ T_{EFF}, and that mTOR inhibition facilitates effective memory formation (128-129), we compared the phosphorylation of ribosomal protein S6 (pS6), an established marker of mTOR activity (128-129), in the $CD8^+$ T cells primed by the $_{EI}$DCs- versus $_{LI}$DCs. As shown in figure 7A, the $_{EI}$DC-primed T cells showed high levels of phosphorylated S6. In contrast, $CD8^+$ T cells primed by $_{LI}$DCs showed only marginal S6 phosphorylation (Fig. 7A). In contrast to $CD8^+$ T cells primed by $_{EI}$DCs, $_{LI}$DC-primed $CD8^+$ T cells had limited expression of T-bet (Fig. 7B), the downstream factor of IL-12 signaling (44, 199, 201) and mTOR activity (128). The addition of endogenous IL-12 during the priming with $_{LI}$DCs enhanced T-bet expression and S6 phosphorylation (data not shown).

Since reduced mTOR activity has been shown to favor memory development (128-129), we analyzed the differentially primed $CD8^+$ T cells for the expression of memory markers. As shown in figure 7C, $CD8^+$ T cells primed by the $_{LI}$DCs retained high expression of CCR7 and IL-7Rα (CD127), markers of central-memory cells, in a clear contrast to the $_{EI}$DC-primed $CD8^+$ T cells, which down-regulated both these markers at the same point of their activation. Such T-bet^{low}/$mTOR^{low}$/$CCR7^{high}$/IL-7Rα^{high} $CD8^+$ T cells and T-bet^{high}/$mTOR^{high}$/$CCR7^{low}$/IL-7Rα^{low} $CD8^+$ T cells induced *in vitro* by, respectively, the $_{LI}$DCs- versus "$_{EI}$DCs showed similar patterns of expression of the memory- and effector T cell-associated genes as *in vivo*-arising blood-isolated central-memory (T_{CM}) and effector (T_{EFF}) cells obtained by flow sorting (Fig. 7D-E), further supporting the notion that the $_{LI}$DC-primed $CD8^+$ T cells are T_{CM} cells.

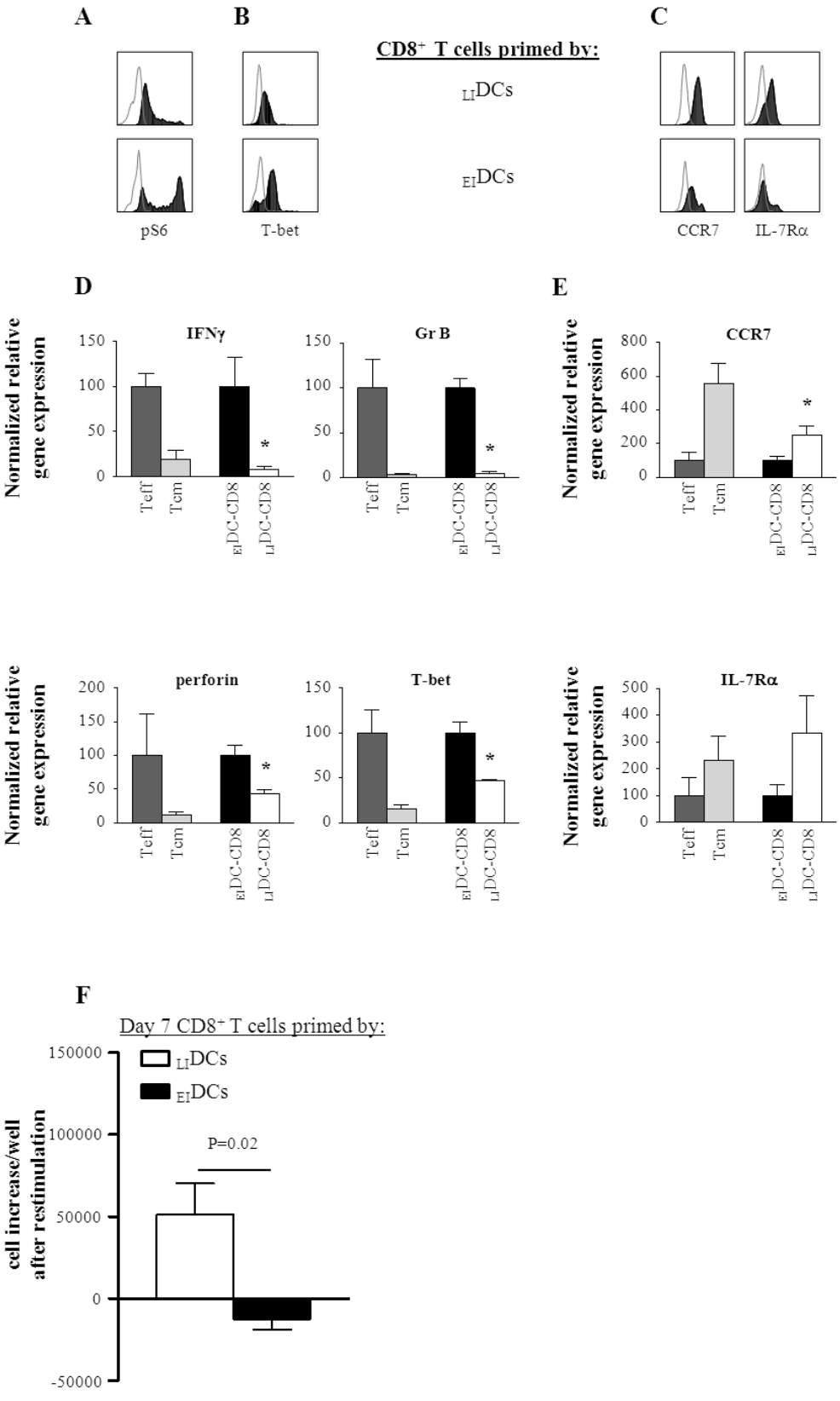
A
B
C
CD8+ T cells primed by:
LIDCs
EIDCs
pS6
T-bet
CCR7
IL-7Rα
D
E
Normalized relative gene expression
IFNγ
Gr B
CCR7
perforin
T-bet
IL-7Rα
Teff
Tcm
EIDC-CD8
LIDC-CD8
F
Day 7 CD8+ T cells primed by:
LIDCs
EIDCs
P=0.02
cell increase/well after restimulation
150000
100000
50000
0
-50000

Figure 7. $CD8^+$ T cells primed by $_{LI}$DCs have reduced mTOR activity and undergo accelerated central-memory cell differentiation with secondary expansion capacity.

Naïve $CD8^+$ T cells were primed for 3 (A-B) or 6 days (C-F) with SEB-loaded $_{EI}$DCS or $_{LI}$DCs. Day 3 differentially primed $CD8^+$ T cells were analyzed for the intracellular expression of (A) phosphorylated ribosomal protein S6 (pS6) and (B) T-bet by flow cytometry. (C) Day 6 differentially primed $CD8^+$ T cells were analyzed for surface expression of CCR7 and IL-7Rα (CD127) by flow cytometry. (D-E) Quantitative RT-PCR was used to analyze the expression of effector cell associated genes (D) or central-memory associated (E) by blood-isolated effector cells (T_{EFF}; dark grey bars), blood-isolated central-memory cells (T_{CM}; light grey bars), day 6 $_{EI}$DC-primed $CD8^+$ T cells ($_{EI}$DC-CD8; black bars) and $_{LI}$DC-primed $CD8^+$ T cells ($_{LI}$DC-CD8; white bars). Data are shown as ratios of the expression level of each gene in each T cell subset to the expression of the same gene in T_{EFF} cells or $_{EI}$DC-$CD8^+$ T cells and are the mean of 3 (blood-isolated) or 4 *in vitro* cultures +/- SEM. (F) Day 6 differentially primed $CD8^+$ T cells were restimulated for an additional 4 days (+/- 24 hours in 4 individual experiments) with SEB-loaded $_{LI}$DCs. The numbers of viable cells in restimulated cultures were determined using trypan blue exclusion. Data are represented as mean +/- SEM of 4 independent experiments.

A key functional feature of memory $CD8^+$ T cells is their ability to undergo effective secondary expansion upon challenge (202). As expected, while the $_{EI}$DC-$CD8^+$ T cells did not expand when restimulated at day 6 after priming, the T-bet^{low}/$mTOR^{low}$/$CCR7^{high}$/IL-$7R\alpha^{high}$ $CD8^+$ T cells primed by $_{LI}$DCs efficiently expanded when restimulated at the same time point in parallel cultures (Fig. 7F).

3.4.3 T_{CM} $CD8^+$ T cells induced by $_{LI}$DCs undergo secondary effector differentiation following their restimulation by $_{EI}$DCs

To directly compare the memory function of $CD8^+$ T cells primed by the $_{LI}$DCs versus $_{EI}$DCs, we cultured the $CD8^+$ T cells for 3 weeks in the presence of low dose IL-2 and IL-7, to allow the $_{EI}$DC-primed $CD8^+$ T cells to also differentiate into memory cells. Both populations of long-term cultured $CD8^+$ T cells expressed only low levels of granzyme B, independently of the conditions of their priming (Fig. 8A). However, while the $CD8^+$ T cells primed by $_{EI}$DCs contained both $CD62L^-$ $CCR7^-$ effector-memory (T_{EM}) and $CD62^+$ $CCR7^+$ T_{CM} cells (Fig. 8A, bottom), the $CD8^+$ T cells primed by $_{LI}$DCs consisted of only $CCR7^+$ $CD62L^+$ T_{CM} cells (Fig. 8A, top). In contrast to the strict differences in their ability to undergo secondary expansion at day 6, both populations of long-term cultured $CD8^+$ T cells were undergoing effective secondary expansion when restimulated at day 21 (Fig. 8B), although long-term cultured $_{EI}$DC-$CD8^+$ T cells expanded less than the $CD8^+$ T cells induced by $_{LI}$DC- cells, consistent with the lower proliferative capacity of the T_{EM} cells (203-204).

In contrast to naïve $CD8^+$ T cells, both populations of the differentially-primed $CD8^+$ T cells rapidly up-regulated the expression of granzyme B and acquired cytolytic function following restimulation with $_{EI}$DCs (Fig. 8C-D).

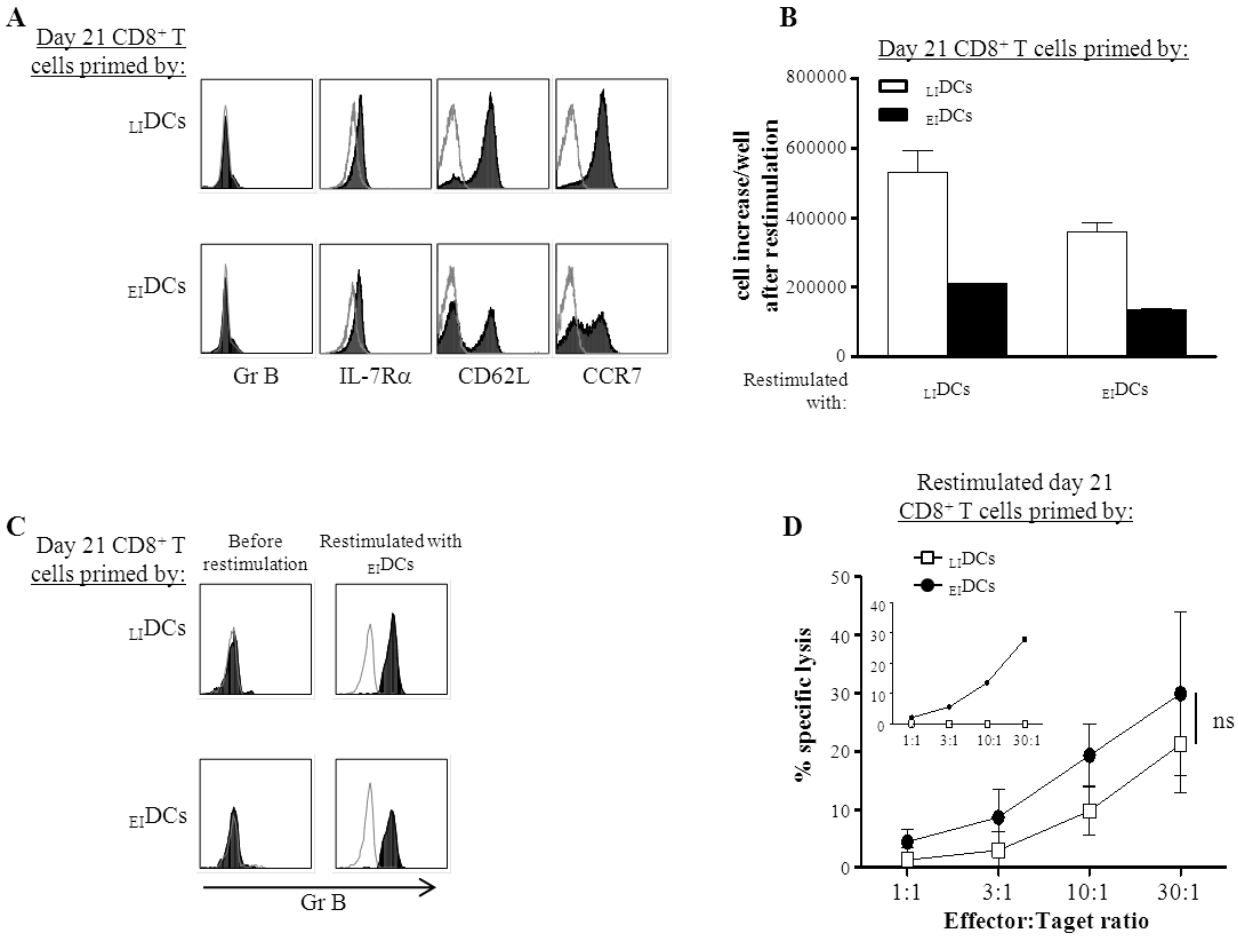

Figure 8. T_{CM} $CD8^+$ T cells induced by $_{LI}$DCs undergo secondary effector differentiation following re-stimulation by $_{EI}$DCs.

$_{EI}$DC- and $_{LI}$DC-primed $CD8^+$ T cells were cultured for 21 days. (A) Phenotype of long-term cultured differentially-primed $CD8^+$ T cells was analyzed by flow cytometry. (B) The secondary expansion (day 4) of resting $CD8^+$ T cells after restimulation with SEB-loaded DCs was determined by cell count and trypan blue exclusion. (C) The acquisition of granzyme B expression by $_{EI}$DC-restimulated $CD8^+$ T cells was determined by flow cytometry. (D) The cytolytic capacity of differentially-primed resting $CD8^+$ T cells restimulated with $_{EI}$DCs was determined by standard 4 hour ^{51}Chromium release assay using SEB-loaded JY-1 cells as targets. Insert: cytolytic capacity of day 7 differentially primed $CD8^+$ T cells. Data are represented as mean +/- SEM for 4 independent experiments. *Insert: cytolytic capacity of differentially-primed $CD8^+$ T cells on day 6 after priming.*

The current data demonstrate the unique role of DCs matured for extended periods of time or in the conditions of late/chronic inflammation, as specialized inducers of the direct differentiation of naïve $CD8^+$ T cells into T_{CM} cells, which are capable of accelerated secondary expansion and rapid T_{EFF} differentiation following secondary activation by $_{EI}$DCs. These data help to explain the preferential development of memory $CD8^+$ T cells at later stages of immune responses at the time when acute inflammation has ceased (195-196). In contrast to the acute inflammation dominated by the pathogen-derived- and exogenous distress signals, including TLR-ligands and interferons (186), the inflammatory process at the conclusion of immune responses is dominated by chronic inflammatory mediators that suppress IL-12 production and IL-12 responsiveness (190-193), including PGE_2 used in the current study.

The current data also helps to reconcile the apparent discrepancy between the relative patterns of the development of memory and effector $CD8^+$ T cells supported by different *in vivo* mouse models, which favor either a sequential (204-205) or parallel (to effector cells) mode (32) of memory T cell development (202). Our data suggest that in the conditions of active inflammation, where most of the antigen-carrying DCs are activated by interferons, TLR-ligands and other pro-inflammatory factors able of enhancing IL-12 production, the overall pattern of immunity will follow the sequential pattern when the originally predominant population of effector $CD8^+$ T cells (T_{EFF}) will gradually give rise to T_{CM} and T_{EM} subsets *(see Fig 8)*. However, in case of pathogens inducing a lesser degree of inflammation (or weaker adjuvants in the case of vaccination), the pattern of local inflammation may allow both types of DCs ($_{EI}$DCs and $_{LI}$DCs) to co-exist and to induce both effector and memory T cells simultaneously. In

addition to their effects via DCs, inflammatory cytokines generated during an infection can also affect T cell differentiation also in a direct or an indirect way (121).

The currently demonstrated new role of PGE_2 in promoting the development of T_{CM}-driving "memory" DCs also help to understand the paradoxical impact of PGE_2 on different aspects of the immune-stimulatory activity of DCs. While, similar to many other chronic inflammatory mediators, including IL-10, TGFβ or steroids, PGE_2 suppresses the IL-12p70 production by DCs (93, 194). In contrast to these additional factors which also suppress DC maturation and their ability to activate T cells (3, 194), PGE_2 uniquely promotes DC maturation (3, 20, 194), directing such IL-12-deficient mature DCs to the lymph nodes (206-207). In combination with this unique combination of features of PGE_2, our current data suggest a unique role of PGE_2 as the factor promoting the switch in DC activity from the induction of effector cells towards the preferential induction of memory $CD8^+$ T cells at later stages of immune responses.

The possibility of using this memory-promoting activity of the PGE_2-matured (or DCs "exhausted" by prolonged exposure to other maturation-inducing factors) may allow to shorten the duration of the vaccination schemas needed to achieve optimal protection against infective agents, by allowing shorter prime-boost cycles. While our data shows that the cells primed by the $_{EI}$DCs and $_{LI}$DCs both are able of effective secondary expansion at day 21 (*see Fig. 8B*), the memory cells primed by $_{LI}$DCs could be effectively restimulated already at day 6 after priming, at the time when restimulation of the $_{EI}$DC-primed T_{EFF} was clearly ineffective and resulted in contraction of the activated $CD8^+$ T cell pool (*see Fig. 7F*). However, potential advantages of such approach need to be considered in the context of the ability of PGE_2 and PGE_2-matured DCs to promote the preferential recruitment and activation of T_{regs} and MDSCs, while

suppressing the ability of DCs and tumor micro-environments to attract naïve, memory and effector cells and induce the T_{EFF} functions (25, 188-189, 208-211). These undesirable properties of PGE_2 and the documented ability of PGE_2-matured DCs to promote T_{reg} expansion in cancer patients (54), together with the currently-described ability to instruct $CD8^+$ T cells to sidestep the effector stage of activation, may constitute an obstacle in the utilization of PGE_2 in therapeutic (rather than preventive) settings, highlighting the need for tools to dissociate these desirable and undesirable features of PGE_2. Our ongoing work aims to evaluate the potential for the sequential application of $_{LI}$DCs followed by $_{EI}$DCs (to allow effector cell differentiation) in the induction of effective immunity.

The existence of a distinct memory $CD8^+$ T cell-inducing function of the T-bet^{low}/IL-12^{low} DCs activated for extended periods of time or activated in the presence of chronic inflammatory mediators, such as PGE2, suggests that the traditionally-used nomenclature of "exhausted DCs" does not fully reflect their nature and immune function. Their unique phenotype, function, and the kinetics of induction in relation to the T-bet^{high}/IL-12^{high} "non-exhausted" DCs, show strong analogy to the development of memory T cells, and indicate their role as a specialized "memory DC" subset, in contrast to T-bet^{high}/IL-12^{high} "effector" DCs induced at earlier stage of response and specialized in the sequential induction of T_{EFF} cells, followed by the development of T_{EM} and T_{CM} cells.

3.5 IMPLICATIONS

Effective cancer immunotherapy is believed to rely on the induction of both effector and memory T cell. In contrast, prophylactic vaccines are designed to generate large number of long-lived memory cells. The development of large numbers of memory cells is achieved by prime-boost strategies, which can take weeks or months. In chapter 3 I show that DCs matured under conditions that mimic late/chronic inflammation, do not produce IL-12p70 and induce the direct differentiation of naïve $CD8^+$ T cells into central-memory cells without passing through a cytolytic effector phase. These central-memory cells rapidly acquire cytolytic capacity upon restimulation with type-1 polarized DCs. The combined data from chapter 2 and 3 show that differentially matured DCs induce distinct differentiation pathways in naïve $CD8^+$ T cells. These data suggests that type-1 polarized DCs would be preferred for the use as cancer vaccines, when cytolytic effector cells are desired for the elimination of tumor cells, while the "non-inflammatory" DCs would be preferred for the use as prophylactic vaccines, in which the induction of large numbers of long-lived central-memory cells is required.

High IL-12p70 producing type-1 polarized DCs are superior to $IL\text{-}12^-$ "non-inflammatory" DCs in the induction of anti-tumor responses. However, the generation of large numbers of type-1 polarized DCs requires large amounts of clinical grade cytokines, which make the development of DC-based cancer vaccines expensive. In chapter 4 I examine the possibility to generate mature type-1 polarized DCs without the need for expensive clinical grade cytokines by making use of the ability of autologous lymphocytes to produce DC-maturing factors (IFNγ and TNFα).

4.0 LYMPHOCYTE-POLARIZED DENDRITIC CELLS ARE HIGHLY EFFECTIVE IN INDUCING TUMOR-SPECIFIC CTLS

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The data presented here are published in *Vaccine; 2012 (in press).* Erik Berk generated all of the data, all the figures and prepared the manuscript.

4.1 ABSTRACT

High activity of dendritic cells (DCs) in inducing cytotoxic T cells (CTLs) led to their application as therapeutic cancer vaccines. The ability of DCs to produce IL-12p70 is one of the key requirements for effective CTL induction and a predictive marker of their therapeutic efficacy *in vivo*. We have previously reported that defined cocktails of cytokines, involving TNFα and IFNγ- induce mature type-1 polarized DCs (DC1s) which produce strongly elevated levels of IL-12 and CXCL10/IP10 upon CD40 ligation compared to "standard" PGE_2-matured DCs (sDCs; matured with IL-1β, IL-6, TNFα, and PGE_2) and show higher CTL-inducing activity. Guided by our observations that DC1s can be induced by TNFα - and IFNγ-producing $CD8^+$ T cells, we have tested the feasibility of using lymphocytes to generate DC1s in a clinically-compatible process, to limit the need for clinical-grade recombinant cytokines and the associated costs. CD3/CD28 activation of bulk lymphocytes expanded them and primed them for effective production of IFNγ and TNFα following restimulation. Restimulated lymphocytes, or their culture supernatants, enhanced the maturation status of immature (i)DCs, elevating their expression of CD80, CD83 and CCR7, and the ability to produce IL-12p70 and CXCL10 upon subsequent CD40 ligation. The "lymphocyte-matured" DC1s showed elevated migration in response to the lymph-node-directing chemokine, CCL21, when compared to iDCs. When loaded with antigenic peptides, supernatant-matured DCs induced much high levels of CTLs recognizing tumor-associated antigenic epitope, than PGE_2-matured DCs from the same donors. These results demonstrate the feasibility of generation of polarized DC1s using autologous lymphocytes.

4.2 INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs), specialized in inducing T cell responses (180-181). Upon antigen encounter, DCs take up antigenic material, undergo maturation , and migrate from peripheral sites to draining lymph nodes where they are able to prime naïve antigen-specific T cells by providing them with processed antigen in the form of antigenic peptides complexed with MHC class I molecules (signal 1) (1, 212). In addition to this signal 1, mature DCs also express co-stimulatory molecules (signal 2) that regulate the magnitude of the T cells responses. They also to secrete different amounts of cytokines (signal 3), which determine the type of T cell response (i.e. Th1, Th2, Th17) that is elicited (3, 26, 213-214).

The ability of DCs to elicit T cell responses has been the rationale for the use of DCs as cancer vaccines in clinical trials (215-216). Furthermore, the Food and Drug Administration (FDA) recently approved the use of the first DC-containing cellular immunotherapy, Sipuleucel–T, (Provenge) for the treatment of hormone-refractory prostate cancer. While Provenge provides a 4 month survival benefit over placebo-treated patients, it does not affect the time to progression or tumor burden (60). These results indicate the feasibility of DC-based therapies for cancer, but also highlight the need for optimization of the methods of DC generation.

The production of high levels of interleukin-12 (IL-12) (217) by DC vaccines has been shown to correlate with their enhanced induction of anti-tumor responses (39, 67-68, 218) and predict their therapeutic benefit in vivo (70).Various maturation protocols are being used for the generation of type-1 polarized monocyte-derived DCs (DC1) that have high IL-12 production capacity in clinically-applicable conditions. These maturation protocols are based on the use of inflammatory cytokines (e.g. IFNγ, IFNα, and TNFα) and/or Toll-like receptor ligands (e.g. LPS

and polyI:C) (28-29, 71, 219-220). While the different maturation protocols are effective at polarizing DCs for high IL-12 production, for use in a clinical setting these maturation and polarization protocols require the use of clinical grade cytokines, which make the production of the DCs expensive.

We have previously shown that activated lymphocytes produce IFNγ and/or TNFα. Naïve $CD8^+$ T cells can perform a helper function, by producing IFNγ and TNFα, in co-activating DCs for the secretion of high levels of IL-12 (138, 221). The ability of lymphocytes to produce IFNγ and TNFα upon activation, and to use these factors to induce maturation and type-1 polarization of DCs (138, 141) prompted us to analyze whether this function could be used to induce DC1-based cancer vaccines in a clinically-compatible process, as alternative to immature DCs used in the currently-approved DC-containing Provenge.

Here we report on the ability to induce maturation and polarization of DCs using expanded autologous lymphocytes. The lymphocyte-matured DCs have a high expression of co-stimulatory molecules, migrate in response to the lymph node-associated chemokine CCL21, and produce high levels of bioactive IL-12p70 and interferon-inducible protein 10 (IP10/CXCL10). When lymphocyte-matured DCs were loaded with peptides representing various tumor-associated antigens (MART-1, gp100, PSA2 and PAP-3), they induce strong peptide-specific CTL responses in autologous naïve $CD8^+$ T cells.

4.3 MATERIAL AND METHODS

Cell lines, media and reagents

Serum-free AIM-V medium (Invitrogen, Carlsbad, CA) was used to generate DCs and Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) with 5% human AB serum (Gemini Bio-products, West Sacramento, CA) was used for CTL induction experiments. The following factors were used to generate mature DCs: *rh*GM-CSF and *rh*IL-4 (gifts from Schering Plough, Kenilworth, NJ), *rh*TNFα, *rh*IL-1β (both Strathmann Biotech, Germany), *rh*IL-6 (Genzyme, Cambridge, MA), Prostaglandin E2 (PGE_2; Sigma, St. Louis, MO). IL-2 and *rh*IL-7 (Strathmann Biotech) were used to support the $CD8^+$ T cell expansion.

Generation of DCs from healthy donors and cancer patients

Peripheral blood mononuclear cells (PMBCs) were obtained from healthy donors or from buffy coats (obtained from the blood bank) using lymphocyte separation medium (Cellgro, Mediatech, Herndon, VA). $CD14^+$ monocytes were isolated from PBMCs using $CD14^+$ isolation kit (Miltenyi Biotec, Auburn, CA, USA). Peripheral blood from melanoma patients was collected in accordance with the Institutional Review Board (IRB) of the University of Pittsburgh. All patients had given written consent. Blood was obtained by leukapheresis and cell subsets were separated by elutriation density gradient separation (Elutra) and stored in liquid nitrogen. In both cases, the resulting monocytes were cultured in serum-free AIM-V medium supplemented with *rh*GM-CSF (1000 U/ml) and *rh*IL-4 (1000 U/ml) in 24 well plates at 5-6 x 10^5 cells/ml for 5 days. On day 3, half the culture medium was replaced with fresh medium containing *rh*GM-CSF

and *rh*IL-4 (both at final concentration 1000 U/ml). On day 5, immature (i)DCs were matured for 24 hours by removing half the medium and adding fresh medium containing the maturation factors described below.

Expansion of bulk lymphocytes

Total CD14-negative cells from healthy donors, from $CD14^+$ isolation described above, or fraction 3 Elutra cells from cancer patients were washed and resuspended in IMDM supplemented with 5% human AB serum (Gemini Bio-products) at 1 x 10^6 cells/ml. Human T-Activator CD3/CD28 Dynabeads (Invitrogen Dynal AS, Oslo, Norway) were added to the lymphocytes at 5 µl/ml and cells were expanded for 6-7 days in 12 well plates with 2 ml/well. On day 3, fresh medium was added to the expanding cells.

Cytokine production by expanded lymphocytes

Day 6-7 expanded lymphocytes were collected in 50 ml conical tubes and CD3/CD28 beads were removed by placing the tube in a magnet for 15 minutes. Bead-free cell suspension was transferred to a fresh tube and cells were washed and resuspended at 2 x 10^6 cells/ml in serum-free AIM-V. Expanded lymphocytes were restimulated in either 96 well plates (for analysis of cytokine production) or 6 well plates (for DC maturation) with either plate-bound OKT3 Ab (1µg/ml; eBioscience, San Diego, CA) or 5 µl/ml CD3/CD28 beads. At the indicated times, supernatant was collected and stored at -20°C until analysis by ELISA. Briefly, ELISA plates were coated overnight at room temperature (RT) with 2 µg/ml monoclonal anti-human IFNγ Ab (Thermo Scientific, Rockford, IL) or 2 µg/ml monoclonal anti-human TNFα Ab (Endogen). After incubation, plates were blocked using PBS with 4% Bovine Serum Albumin

for 1 hour at RT. Plates were washed (50 mM Tris/0.2% Tween) and incubated with culture supernatants or appropriate cytokine standards for 1 hour. Supernatants were removed and plates were incubated for 1 hour with biotin-labeled monoclonal Abs against IFNγ or TNFα (both at 0.5 µg/ml). Following incubation, plates were washed and incubated with HRP-conjugated streptavidin (Thermo Scientific) for 30 minutes, after which the plates were washed and incubated with 100 µl TMB substrate solution (Pierce Biotechnology Inc, Rockford, IL). The color reactions were stopped by addition of 100 µl 2% H_2SO_4 and the absorbance was measured at 450nm.

Maturation of DCs

To induce the maturation of day 5 DCs, half the medium was removed and replaced with 500µl fresh serum-free AIM-V medium containing: (for iDC): *rh*GM-CSF and IL-4 (both 1000 U/ml) only; (for PGE_2-matured "standard" DC; sDC): *rh*GM-CSF (1000 U/ml) *rh*IL-1β (25 ng/ml) *rh*IL-6 (1000 U/ml) *rh*TNFα (50 ng/ml) and PGE_2; 10^{-6} M); (for lymphocyte-CD3/CD28-matured DCs): *rh*GM-CSF, 2 x 10^5 expanded lymphocytes/ml and αCD3/αCD28 beads (5 µl/ml); (for lymphocyte-OKT3-matured DCs), *rh*GM-CSF, 2 x 10^5 expanded lymphocytes/ml and OKT3 (1 µg/ml); or (for supernatant-matured DCs; $_{sup}$DC): *rh*GM-CSF and 500µl supernatant from 24 hour restimulated expanded lymphocytes. DCs were matured for 24 hours at 37°C. The recovery of supernatant-matured DCs was less than that of PGE_2-matured DCs but only slightly less than previously observed for other type-1 polarized DCs generated in our lab (αDC1). Typical cell yields were: PGE_2-matured DC: 1:3-5 (1 DC recovered per 3-5 monocytes plated); supernatant DCs: 1:9-12; iDC: 1:6-13). For the analysis of DC maturation markers, matured DCs were collected, resuspended in IMDM-5% HS and kept overnight at 37°C before

performing flow cytometric analysis. We have previously observed that the CCL19 production during type-1 polarized DC maturation results in transient internalization of surface CCR7, which is restored after removing the cells from the CCL19-rich maturation media, resulting in effective migration in vitro and in vivo (208). We therefore rested the matured DCs overnight in fresh media before performing phenotype and migration analysis. The morphology of the DCs was determined using an EVOS XL Core microscope (Advanced Microscopy Group, Bothell, WA).

Flow cytometry

3-color flow cytometry was performed using an Accuri C6 flow cytometer. Fluorescein isothiocyanate (FITC)-labeled anti-human CD80, CD40, CD3, CD40L, Phyocoerythrin (PE)-labeled anti-human CD83, CD16, PE-Cy5-labeled anti-human CD8, CD4, CD19, CD56 and Allophycocyanin (APC)-labeled anti-human CD86 were purchased from BD Biosciences (San Jose, CA). APC-labeled anti-human CCR7 was obtained from R&D systems. PE-labeled MART-$1_{(ELAGIGILTV)}$ tetramer was purchased from Beckman Coulter (Immunomics, Fullerton CA). Cells were transferred to a V-bottom 96-well plate and washed with FACS buffer (PBS/0.5%BSA/0.1% Azide). Cells were incubated for 45 minutes in the dark at RT. Following labeling, cells were washed twice and fixed with 2% para-formaldehyde and stored at 4°C until analysis. Flow cytometry data was analyzed using FCS Express V3 software.

Chemotaxis

The analysis of DC migratory function was performed using a 24-well transwell system with 5 μm pore size polycarbonate filter (Corning Inc, Corning, NY). 1 x 10^5 differentially

matured DCs were rested overnight to allow for re-expression of CCR7, as described above, loaded to the top chamber of the transwell system and allowed to migrate towards *r*CCL21 (100 ng/ml; Peprotech, Rocky Hill, NJ) over a 3 hour period at 37°C. Following incubation, the migrated cells from the bottom compartment were collected, the total volume of the cell suspension was measured and the cells in 100 µl of the suspension were counted using an Accuri C6 flow cytometer. The total number of migrated cells was calculated and is represented as percentage migrated cells of total cells. The number of cells that migrated in response to media (IMDM-5%HS) alone was subtracted from the number of chemokine-specific migrated cells to correct for background migration.

ELISA analysis of IL-12p70 and CXCL10 production by the differentially-matured DCs

Differentially matured DCs were harvested and resuspended in IMDM supplemented with 5% human AB serum at 2 x 10^5 cells/ml. 100µl of DC suspension (2 x 10^4 DCs) were transferred to a flat-bottom 96 well plate and stimulated for 20-24 hours with 5 x 10^4 CD40L-transfected J558 cells (kind gift of Dr. P. Lane, Birmingham, UK). After incubation, supernatant was collected and stored at -20°C until analysis. IL-12p70 and CXCL10 analysis was performed as described above for IFNγ and TNFα, using 2µg/ml primary Ab (mouse anti-human IL-12(p70), Thermo Scientific Pierce) and 0.5 µg/ml biotin-labeled Ab (rat anti-human IL-12(p70), Thermo Scientific Pierce) for IL-12p70 and 10 µg/ml primary Ab (rabbit anti-human IP-10, Peprotech) and 2.5 µg/ml biotin-labeled secondary Ab (rabbit anti-human IP-10, Peprotech) for CXCL10. HRP-conjugated streptavidin followed by TMB substrate development

was used for analysis and the colorimetric reaction was stopped with 2% H_2SO_4 and measured at 450nm.

Isolation of naïve $CD8^+$ T cells

For the induction of CTL responses, naïve $CD8^+$ T cells ($CD8^+CD45RO^-CD45RA^+$) were isolated from the $CD14^{neg}$ (lymphocyte) population by incubating the cells with anti-CD45RO-biotin Abs (StemCell Technologies Inc, Vancouver, Canada). Following incubation, cells were washed and incubated with tetrameric anti-biotin complex and CD8 T cell enrichment cocktail (StemCell). Cells were then incubated with magnetic colloid (StemCell) after which naïve $CD8^+$ T cells were isolated using a magnetic column (StemCell).

CTL induction

For the induction of CTL responses from naïve $CD8^+$ T cells, matured DCs were loaded with tumor-associated peptides (MART-1_{26-35}, $gp100_{209-217}$, PAP-$3_{135-143}$, and $PSA2_{146-154}$; all were used at the concentration of 10 μg/ml) for 2 hours at 37°C in IMDM-5% human AB serum. Peptide-loaded DCs were co-cultured with autologous $CD8^+$ T cells at a 1:10 ratio in 48 well plates for 12 days. $CD8^+$ T cells were supplemented with *rh*IL-2 (20 U/ml) on day 4 and with *rh*IL-2 and *rh*IL-7 (5 ng/ml) on day 7 and day 9. Day 12 $CD8^+$ T cells were analyzed for the presence of MART-1 specific CTLs or were stimulated with individual peptides for 24 hours for the analysis of the frequency of tumor-peptide specific $CD8^+$ T cells by IFNγ enzyme-linked immunospot (ELISPOT).

Statistical analysis

Data were analyzed using unpaired *Student's t test* (2-tailed). $P < 0.05$ was considered as statistically significant.

4.4 RESULTS

4.4.1 Expanded lymphocytes rapidly produce DC maturation- and polarization-inducing factors upon restimulation

The combination of a DC maturation factor and IFNγ has been shown critical for the induction of polarized DC1s (5) with additional need for IFNα for the complete development of polarized DC1s in the absence of FCS (71). Since superantigen (SEB)-activated T cells secrete IFNγ as well as TNFα and can use these factors to induce maturation and polarization of DCs in bovine-serum-supplemented cultures (138, 141), we tested whether activated bulk lymphocytes could be used to mature DCs in clinically-compatible protocols, involving serum-free media and clinically-relevant reagents. To test this, isolated lymphocytes were expanded for 6 days with αCD3 and αCD28 beads and analyzed to determine which cells were present. As expected, the expanded lymphocyte cultures consisted of mostly $CD4^+$ T cells (~65%) and $CD8^+$ T cells (~20%) while there were only low percentages (~5%) of NK cells and B cells present (Fig.9A).

The acquisition of high IL-12p70 producing ability by monocyte-derived DCs and counteracting DC exhaustion requires IFNγ signaling during maturation (5). To test whether the expanded lymphocytes were able to mature DCs and prime them for high IL-12p70 production, we restimulated day 6 expanded lymphocytes with αCD3/CD28 beads and analyzed cytokine secretion at various times after re-stimulation. As shown in figure 9B, the restimulated

lymphocytes started producing both IFNγ (left panel) and TNFα (right panel) within 2 hours of restimulation and continued to secrete these cytokine up to at least 24 hours after restimulation.

Since the expanded lymphocytes rapidly produced high amounts of both IFNγ and TNFα upon restimulation, we analyzed the ability of expanded lymphocytes to induce maturation and polarization of immature autologous DCs. For this, monocytes were isolated and cultured for 5 days in the presence of GM-CSF and IL-4 to generate immature DCs (iDCs). The co-cultures of iDC with expanded lymphocytes in the absence of a TCR stimulus did not result in any IFNγ or TNFα secretion by the lymphocytes (Fig. 9C). In contrast, when a TCR stimulus was added (i.e. αCD3/CD28 beads or soluble OKT3), the lymphocytes secreted both these cytokines (Fig. 9C).

4.4.2 Restimulated lymphocytes from healthy donors and melanoma patients induce DC maturation and polarization

In order to test whether the expanded and re-activated lymphocytes were able to induce the maturation of autologous iDC, we analyzed the morphology and phenotype of 24 hours matured DCs. As seen in figure 10A, DCs matured by re-activated lymphocytes or by the supernatant of 24 hour re-activated lymphocytes had a distinct morphology compared to iDCs or mature PGE_2-matured ”standard” DCs (sDC; matured in the presence of recombinant cytokines and PGE_2) (20) (Fig. 10A). To verify the maturation status of the DCs, we analyzed the expression of co-stimulatory molecules by the DCs. DCs exposed to restimulated lymphocytes had increased expression of the co-stimulatory molecules and maturation-associated markers CD83, CD80,

CD86 and CD40 compared to iDC and DCs co-cultured with lymphocytes in the absence of activation stimuli (Fig. 10B and 10C).

Lymphocyte-matured DCs also had elevated levels of the lymphoid-homing chemokine receptor CCR7, which allows for the migration of mature DCs to the lymphoid organs. Supernatant-matured DCs showed a mixed phenotype with a population of cells having an immature phenotype ($CD83^{low}$ $CCR7^{low}$) and another population being mature ($CD83^{high}$ $CCR7^{high}$) (Fig. 10B and 10C).

The induction of Th1 and cytolytic $CD8^+$ T cell (CTL) responses requires the secretion of IL-12p70 by DCs during the priming of naïve $CD4^+$ and $CD8^+$ T cells (222). Upon interaction with Ag-presenting mature DCs, $CD4^+$ T cells up-regulate the expression of CD40L, which interacts with CD40 on the DCs and induces IL-12p70 secretion. To determine the ability of the differentially matured DCs to produce IL-12p70, we activated them for 24 hours with CD40L-transduced J558 cells, as a surrogate for CD40L-expressing $CD4^+$ T cells (136). As shown in Figure 10D, while iDCs and PGE_2-matured sDCs produce only low levels of IL-12p70, DCs matured by restimulated lymphocytes or supernatant from restimulated lymphocytes have an elevated ability to secrete IL-12p70.

It has been shown that the optimal induction of Th1 responses by at least mouse DC vaccines relies on the secretion of CXCL10/interferon inducible protein 10 (IP-10) by mature DCs, which retain Th1 cells in the T cells area of the lymph nodes (76, 101). To determine if the lymphocyte-matured DCs were able to secrete CXCL10, we co-cultured 24 hour matured DCs with CD40L-expressing J558 cells and determined the levels of CXCL10 in the supernatant after 24 hours. As shown in figure 10E, iDC and PGE_2-matured sDCs lacked production of CXCL10 while both the supernatant- and lymphocyte-matured DCs produced high levels of the CXCL10.

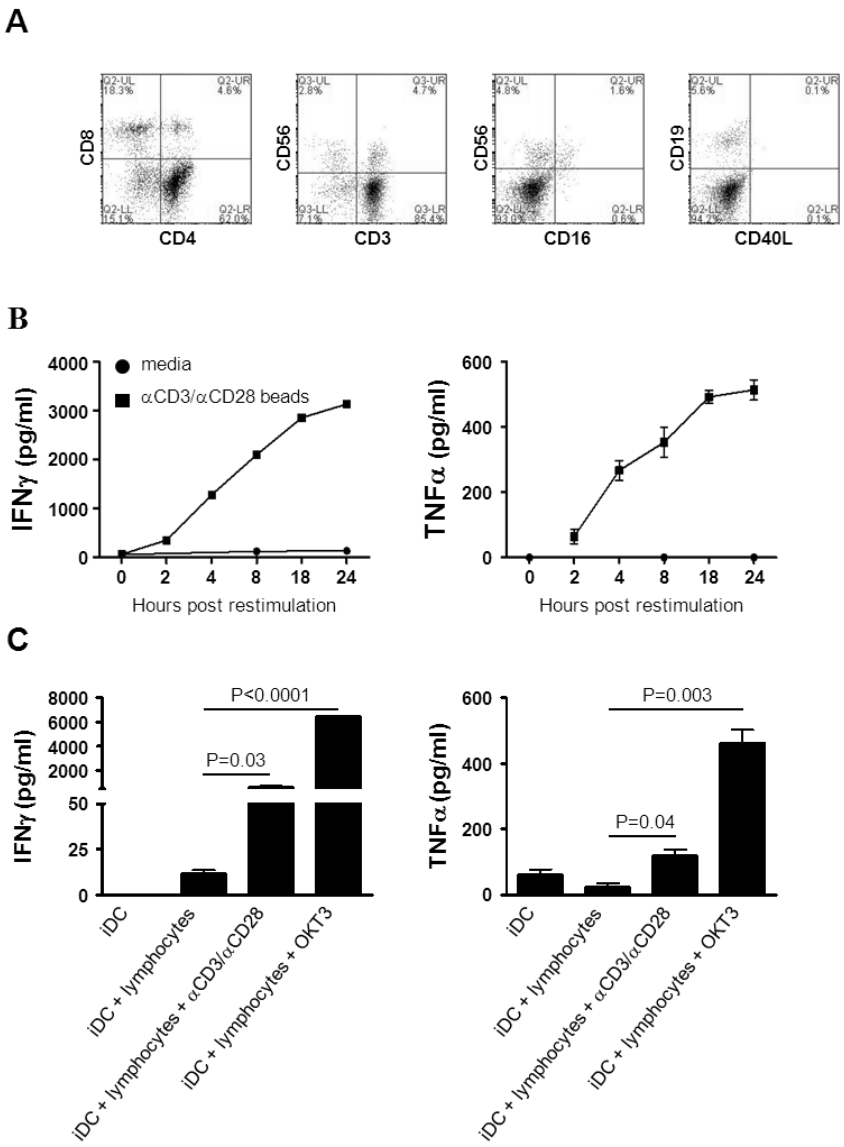
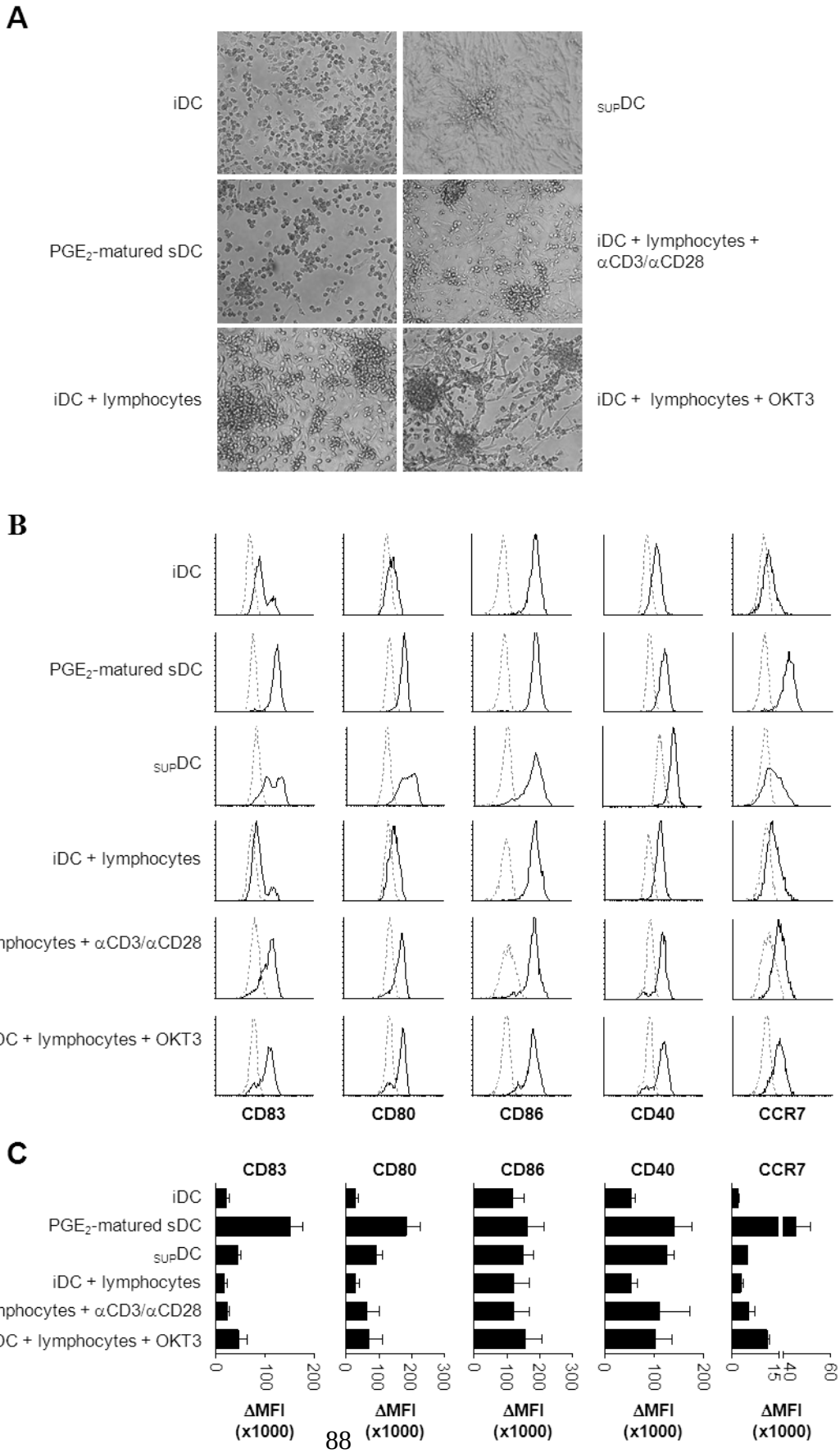


Figure 9. Ex vivo-expanded lymphocytes rapidly produce IFNγ and TNFα upon restimulation.

Isolated lymphocytes were expanded for 7 days with αCD3- and αCD28-coated beads. **A)** The phenotype of day 7 expanded lymphocytes was determined by flow cytometry. **B)** Day 7 expanded lymphocytes were harvested and either cultured in the absence of additional stimulation (circles) or restimulated (squares). At the indicated times, supernatants were collected to determine the secretion of IFNγ (left) and TNFα (right). **C)** Autologous day 5 iDCs were cultured in the presence of the day 7 expanded lymphocytes in the presence or absence of the indicated stimuli. After 24 hours of co-cultures, supernatants were collect and the production of IFNγ (left) and TNFα (right) was determined. Data shown from one of at least 2 independent experiments that yielded similar results.

In order to test the feasibility of using autologous lymphocytes to induce the maturation of DCs obtained from cancer patients, we compared the maturation- and polarization status of DCs from healthy donors with that of DCs derived from melanoma patients. Restimulation of patients' lymphocytes resulted in the secretion of similar levels of IFNγ and TNFα with similar kinetics (compare Fig. 9B and Supplemental Fig. 5A). Following co-culture of autologous lymphocytes with iDCs in the presence of a TCR stimulus the DCs from healthy donors and from melanoma patients showed similar levels of up-regulation of co-stimulatory molecules and maturation associated markers (Fig. 10F and supplemental Fig. 5B). Furthermore, CD40L stimulation of matured DCs from healthy donors and melanoma patients in both cases resulted in the elevated secretion of IL-12p70. There was no significant difference in IL-12 production between DCs from healthy donors and those obtained from PBMCs from melanoma patients (Fig. 10G).


A
iDC
SUPDC
PGE2-matured sDC
iDC + lymphocytes + αCD3/αCD28
iDC + lymphocytes
iDC + lymphocytes + OKT3
B
iDC
PGE2-matured sDC
SUPDC
iDC + lymphocytes
iDC + lymphocytes + αCD3/αCD28
iDC + lymphocytes + OKT3
CD83
CD80
CD86
CD40
CCR7
C
CD83
CD80
CD86
CD40
CCR7
iDC
PGE2-matured sDC
SUPDC
iDC + lymphocytes
iDC + lymphocytes + αCD3/αCD28
iDC + lymphocytes + OKT3
0
100
200
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15
40
60
ΔMFI (x1000)

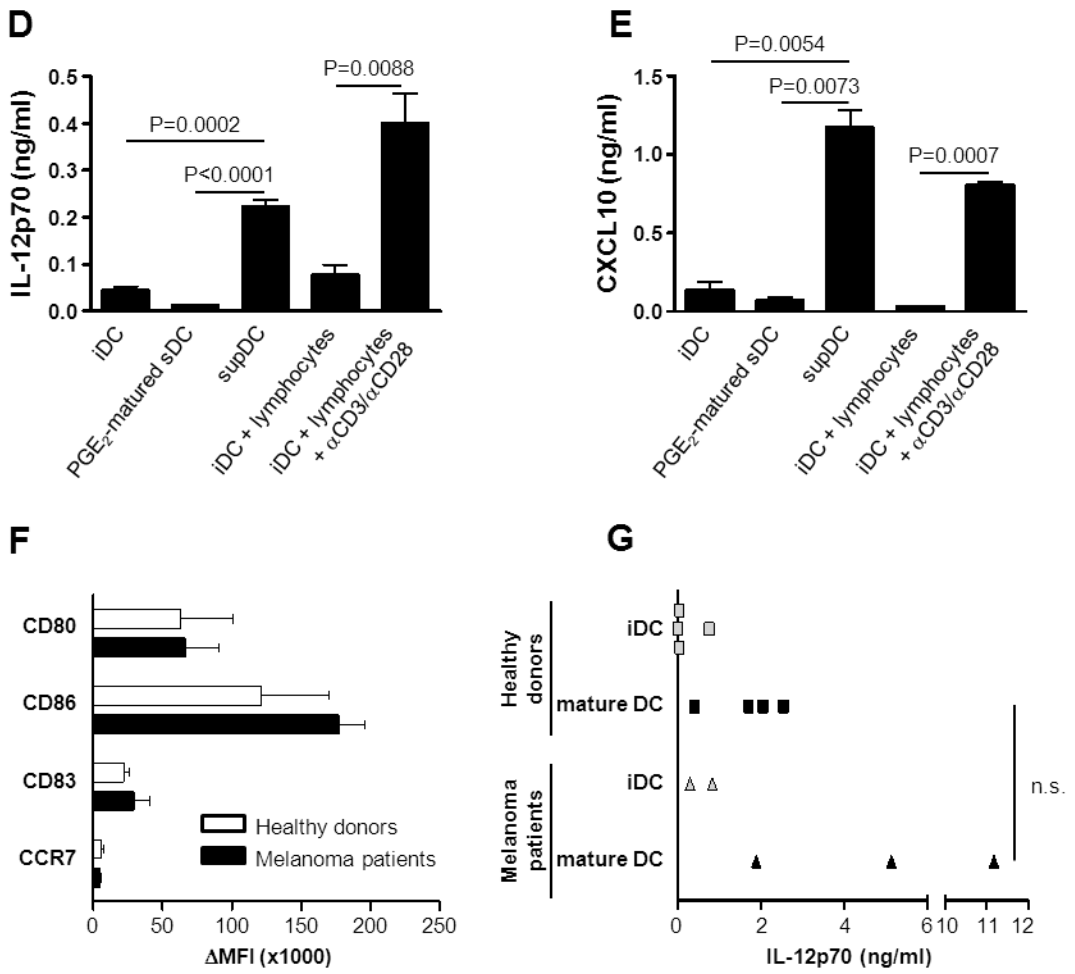

Figure 10. Restimulated expanded lymphocytes or supernatant induce the maturation of autologous DCs and primes them for high IL-12p70 and CXCL10 production.

iDC were cultured for 24 hours with autologous day 7 expanded lymphocytes in the presence or absence of the indicated stimuli or with supernatant from 24 hours restimulated lymphocytes. **A)** After 24 hours, the morphology of the DCs was analyzed using bright field microscopy. The morphology of lymphocyte-matured DCs was compared with that of iDCs and with that of DCs exposed to the non-polarizing maturation-inducing cytokine cocktail (PGE_2-matured sDC: IL1β, TNFα, IL-6 and PGE_2). **B)** 24 hour matured DCs were collected, washed and rested overnight at 37°C in fresh media to remove any CCL19 produced during maturation and allow for re-expression of chemokine receptor CCR7 on the cell surface (208) (see M&M). After resting, the phenotype of the DCs was determined by flow cytometry. **C)** Average Mean Fluorescence Intensity expression of DC maturation markers of 3-5 individual experiments. **D-E)** 24 hour matured DCs were collected, washed and subsequently cultured for 24 hour with

CD40L-expressing J558 cells ($2x10^4$ DC: $5x10^4$ J558). After co-culture, supernatant was collected and analyzed for the presence of **(D)** IL12p70 and **(E)** CXCL10. Data shown are representative results from at least 2 independent experiments. **F)** Comparison of average of Mean Fluorescence Intensity of DC maturation associated markers on lymphocyte-matured DCs from 4 healthy donors with those of 3 melanoma patients. **G)** Comparison of IL-12p70 production by immature and lymphocyte-matured DCs from healthy donors (4) and melanoma patients (3) after 24 hours of CD40L stimulation.

4.4.3 Lymphocyte supernatant-matured DCs migrate in response to CCL21

For clinical applications, the use of co-cultures of DCs and lymphocytes in the presence of TCR stimuli would be undesirable unless the DCs are subsequently separated from the poly-clonally-activated lymphocytes. Therefore, the use of supernatant-matured DCs may be more feasible for clinical applications. For this reason, we decided to focus on these cells for the subsequent experiments. Also, since there was no difference between DCs from healthy donors and from melanoma patients, we only used DCs from healthy donors for the remaining experiments.

Since the ability of mature DCs to migrate into lymph nodes is dependent on the expression of the lymphoid-homing chemokine receptor CCR7 (74-75), the elevated expression of CCR7 on supernatant-matured DCs prompted us to determine their capacity to migrate in response to CCL21, the chemokine involved in attracting DCs to lymph nodes (21). Using a transwell system we found that, in contrast to immature DCs, PGE2-matured sDCs migrated efficiently towards CCL21. Supernatant-matured DCs also migrated towards CCL21 albeit to a lesser extent than the cytokine-matured PGE_2-matured sDCs (Fig. 11), which correlates with the difference observed in surface expression of CCR7 (Fig. 10B).

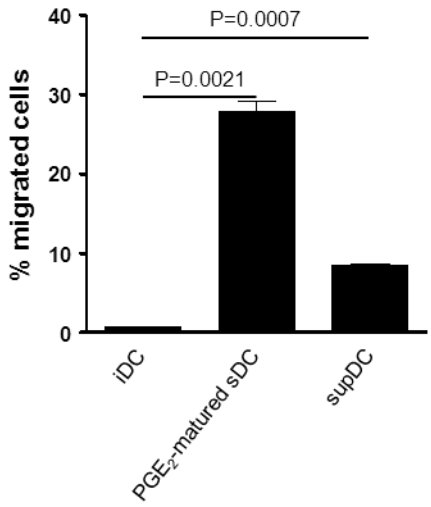

Figure 11. Supernatant-matured DCs efficiently migrate in response to CCL21.

24 hour matured DCs were collected, washed and rested overnight at 37°C. After resting, DCs were placed in the top chamber of a transwell system and allowed to migrate for 3 hours towards CCL21 (in the bottom chamber) in a chemotaxis assay. Following incubation, the migrated cells in the bottom chamber of the transwell were collected and counted using a flow cytometer (see M&M). Migrated cells are represented as percentage of total cells. Migrated cells were counted twice and data is shown as mean +/- SD. Data shown are representative results from 2 independent experiments.

4.4.4 Lymphocyte supernatant-matured DCs induce strong anti-tumor peptide CTL responses

Since the DCs matured by supernatant from restimulated lymphocytes showed a distinctly-activated phenotype, we compared the ability of the supernatant-matured DCs to induce Ag-specific CTL responses with that of the PGE_2-matured sDCs. DCs from a healthy HLA-$A2^+$ donor were matured for 24 hours and loaded with a panel of 4 peptides (MART-1, gp100, PSA2, PAP-3) that are associated with different cancers (melanoma and prostate cancer)

and used to prime autologous naïve $CD8^+$ T cells. As shown in Figure 12A, peptide-loaded supernatant-matured DCs generated a similar expansion of MART-1-specific $CD8^+$ T cells as the PGE_2-matured sDC. The $CD8^+$ T cells were then tested for their ability to recognize the individual peptides in an IFNγ-ELISPOT, which has been shown to be a good correlate of CTL function (223). Naïve $CD8^+$ T cells primed by peptide-loaded supernatant-matured DCs induced strong IFNγ responses against all 4 peptides, in contrast to naïve $CD8^+$ primed by peptide-loaded PGE_2-matured sDC (Fig. 12B).

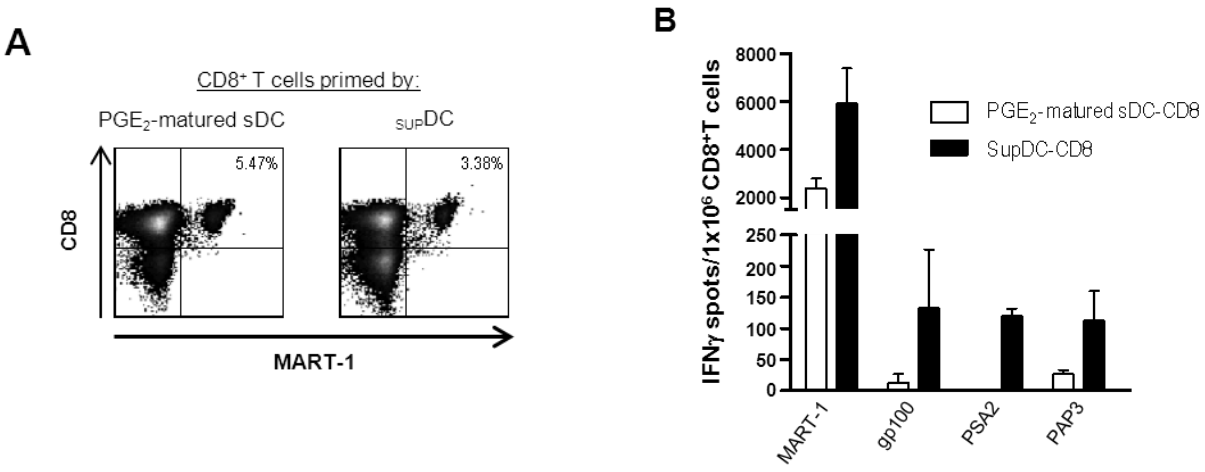

Figure 12. Tumor-peptide-loaded supernatant-matured DCs induce strong anti-tumor CTL responses from autologous naïve CD8+ T cells.

24 hour-matured DCs (supernatant-matured or PGE_2-matured sDC) from healthy donors were collected, washed and loaded for 2 hours with tumor peptides (MART-1, gp100, PAP3, and PSA2). Following peptide loading, DCs were cultured with autologous naïve $CD8^+$ T cells at a 1:10 ratio. **A)** On day 12, $CD8^+$ T cells were collected and the percentage of MART-1-specifc $CD8^+$ T cells was determined by tetramer staining. **B)** Day 12 $CD8^+$ T cells were collected, washed and used in a 24 hour IFNγ-ELISPOT analysis against tumor-peptides. Data are shown as mean +/- SEM of triplicate spot counts of one of 3 independent experiments that yielded similar results.

4.5 DISCUSSION

The ability of dendritic cells to induce strong adaptive immune responses has resulted in the use of DCs to treat malignancies in clinical trials. Since the generation of clinically-approved DCs requires the use of expensive cGMP cytokines, we analyzed the possibility of using activated lymphocytes to mature and polarize autologous iDC. We found that re-stimulation of expanded lymphocytes resulted in rapid secretion of inflammatory cytokines IFNγ and TNFα, which led to type-1 polarization of iDCs. These lymphocyte-matured DCs were able to migrate in response to the lymphoid-homing chemokine CCL21 and, when loaded with peptides, prime naïve $CD8^+$ T cells for strong anti-tumor peptide responses.

Animal studies and *in vitro* studies have shown that the ability to induce strong anti-tumor responses is dependent on the inflammatory cytokine IL-12 (67, 224). Currently, the use of a mixture of IL-1β, IL-6, TNFα and PGE_2 for the maturation of DCs is commonly used in the preparation of DCs for vaccinations. As we present here and have shown before, these cells have a limited IL-12 production capacity compared to type-1 polarized DCs and the use of these "exhausted" DCs as cancer vaccines in clinical trials might be a possible explanation for the lack of clinical responses in these trials. In contrast, the use of type-1 polarized DCs that have a high IL-12 production upon CD40L ligation (71) have been shown to be very effective in the treatment of malignant gliomas (70).

The ability of monocyte-derived DCs to produce high levels of IL-12 requires maturation in the presence of exogenous inflammatory factors, mimicking the conditions of acute (e.g. viral) infection (5, 29, 222). It is known that activated lymphocytes are able to produce such inflammatory signals when interacting with Ag-presenting DCs. Naïve $CD8^+$ T

cells help CD40L-expressing $CD4^+$ T cells, by producing IFNγ and TNFα (138). As shown here, this ability of lymphocytes to produce cytokines that induce type-1 polarization of (autologous) iDCs could be used to circumvent the need for expensive cGMP cytokines for the generation of clinically applicable DCs.

It was recently shown in a mouse model, that the ability of DCs to produce CXCL10/IP-10 at a tumor site allows them to attract effector T cells (76). This ability allowed for enhanced anti-tumor responses and increased survival. Furthermore, the CXCL10 producing ability of DCs was shown to be pivotal for retaining Th1 cells in the T cell areas of the lymph nodes for optimal induction of Th1-mediated immune responses (101). Unlike the PGE_2-matured sDCs, supernatant- and lymphocyte-matured DCs were able to secrete high levels of CXCL10.

The presence of non-matured DCs in the supernatant-matured DCs may indicate that the soluble factors in the supernatant were less concentrated compared to the DC-lymphocyte co-cultures due to the proximity of secretion of the factors. Since the DCs that did not mature with the supernatant are still immature (and not partially matured) the involvement of surface molecule interaction in the DC maturation is unlikely.

The ability of DCs to migrate in response to lymph node-homing chemokines is a key feature of matured DCs and required for induction of T cell responses. Although DCs matured in the presence of PGE_2 have a high expression of the lymph node homing chemokine receptor CCR7, the DC1s induced by restimulated lymphocytes (or their supernatants) also acquire CCR7 expression (and enhance its expression upon prolonged culture) and were able to migrate towards CCL21 in a transwell system. While supernatant-matured DCs contained both mature and non-matured cells, the expression of CCR7 is only observed on the matured ($CD83^+$) population. The lower migration of these cells, when compared to PGE_2-matured sDCs,

correlates with the mixed character of the supernatant-matured DC population. We anticipate that a subcutaneous or intradermal application of such cells in clinical settings would result in the $CCR7^+$ subset of DCs to migrate to the lymph nodes to interact with naïve and memory T cells, while the $CCR7^-$ iDCs are more likely to stay at the site of injection, interacting with effector- and effector-memory-type T cells. The presence of iDCs at the peripheral effector site could be beneficial since it has been shown that in the presence of tumor cells, iDCs protect tumor-specific CTLs from tumor-mediated activation-induced cell death (AICD) (225). The iDC at the site of injection could also take up tumor-Ag and possibly continue to infiltrate the nodes due its continued process of maturation following the injection and the inflammatory milieu at the effector site. However, the injection of Ag-loaded iDCs has also been linked to Ag-specific inhibition of CTL functions (226), suggesting the need for optimization of DC maturation protocols. In regard of this, we have previously shown that in media without FCS the induction of type-1 polarized DCs could be enhanced by the addition of IFNα (71). Since IFNα (Intron A) is relatively inexpensive, the addition of IFNα to optimize the DC maturation by supernatant from restimulated lymphocytes would not greatly increase the production costs. An alternative approach to further enhance the maturation status of the lymphocyte-matured DCs may be the addition of TLR ligands, such as poly-I:C used in our earlier studies with cytokines or NK cells (71).

Whether the same protocol can be used for the large scale production of type-1 polarized DCs in cancer patients still needs to be verified. Our preliminary comparison between DCs from healthy donors and melanoma patients show the elevation of the maturation status and priming for elevated of IL-12 production, independently on the source of the cells (Fig. 10F, 10G and supplemental Fig. 5). Since T cells constitute high proportion in PBMCs and can also be

efficiently expanded using CD3/CD28 beads (available as clinical grade reagent), the current method is likely to generate sufficient numbers of DCs needed for repetitive cycles of vaccination. Importantly for the clinical application of this method, we have shown the feasibility of using the cell-free supernatants from expanded T cells, eliminating the logistic concerns related to the presence of T cells in DC preparations, and the risk associated with the contact of immature DCs with T cells , which can lead to DC killing or suppression (141, 223, 227).

While the proposed approach is likely to reduce the overall cost of generation of type-1 polarized DCs, due to the elimination of the expensive recombinant cGMP cytokines, these advantages need to be balanced against the higher complexity of the whole process of DC1 generation, which involves an additional step of T cell expansion and generation of T cell conditioned media, and need to decide if the quality control process may need to include additional T cell-relevant variables, apart from the quality of the final DC product. However, since the times of T cell expansion and generation of immature DCs can at least partially overlap, the overall duration of generating polarized DC1s may be comparable to the process involving recombinant cytokines.

These data show that activated lymphocytes can be used to induce maturation and priming of autologous DCs, that these DCs produce high levels of cytokines associated with type-1 polarization (IL-12p70 and CXCL10) and are efficient in inducing anti-tumor responses by naïve $CD8^+$ T cells when loaded with peptides. The use of expanded lymphocytes could eliminate the use of expensive clinical grade cytokines and reduce the cost for generating DCs for immunotherapies.

4.6 IMPLICATIONS

Type-1 polarized DCs which produce high levels of IL-12p70 induce strong anti-tumor $CD8^+$ T cell responses. However, the development of DCs for clinical use as cancer vaccines is expensive due to the need for clinical grade cytokines. The data presented in chapter 4 shows that type-1 polarized DCs can be generated using restimulation of activated lymphocytes. These restimulated cells rapidly produce DC-maturing factors IFNγ and TNFα, which induce DC maturation and primes them for high IL-12p70 production compared to immature and "non-inflammatory" DCs. The lymphocyte-matured DCs are able to migrate in response to CCL21, suggesting they would be able to migrate to lymph nodes and they induce strong anti-tumor $CD8^+$T cells responses. These data provide an alternative method for the generation of type-1 polarized DCs in a clinical relevant setting.

In chapters 2, 3 and 4 I have shown that type-1 polarized DCs are strong inducers of anti-tumor $CD8^+$ T cell responses and that these T cells express the peripheral homing chemokine receptors CXCR3 and CCR5, which would allow them to migrate to inflamed tissues. However, the majority of tumors do not produce chemokines that attract cytolytic effector $CD8^+$ T cells (e.g. CXCL10/IP10 and CCL5). Therefore, in chapter 5 I examine the ability of modulating the tumor chemokine environment to enhance the attraction of type-1 DC-induced $CD8^+$T cells.

5.0 NF-κB HYPER-ACTIVATION IN TUMOR TISSUES ALLOWS TUMOR-SELECTIVE REPROGRAMMING OF CHEMOKINE MICROENVIRONMENT TO ENHANCE THE RECRUITMENT OF CYTOLYTIC T EFFECTOR CELLS

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Erik Berk contributed to the generation of the data reported in figure 16 and 1, and contributed to the preparation of the manuscript.

5.1 ABSTRACT

Tumor infiltration with effector $CD8^+$ T cells (T_{eff}) predicts longer recurrence-free survival in many types of human cancer, illustrating the broad significance of T_{eff} for effective immunosurveillance. Colorectal tumors with reduced accumulation of T_{eff} express low levels of T_{eff}-attracting chemokines such as CXCL10/IP10 and CCL5/RANTES. In this study, we investigated the feasibility of enhancing tumor production of T_{eff}-attracting chemokines as a cancer therapeutic strategy, using a tissue explant culture system to analyze chemokine induction in intact tumor tissues. In different tumor explants, we observed highly heterogeneous responses to IFNα or poly-I:C (a TLR3 ligand) when they were applied individually. In contrast, a combination of IFNα and poly-I:C uniformly enhanced the production of CXCL10 and CCL5 in all tumor lesions. Moreover, these effects could be optimized by the further addition of COX inhibitors. Applying this triple combination also uniformly suppressed the production of CCL22/MDC, a chemokine associated with infiltration of T regulatory cells (T_{reg}). The T_{eff}-enhancing effects of this treatment occurred selectively in tumor tissues, as compared to tissues derived from tumor margins. These effects relied on the increased propensity of tumor-associated cells (mostly fibroblasts and infiltrating inflammatory cells) to hyper-activate NF-κB and produce T_{eff}-attracting chemokines in response to treatment, resulting in an enhanced ability of the treated tumors to attract T_{eff} cells and reduced ability to attract T_{reg} cells. Together, our findings suggest the feasibility of exploiting NF-κB hyper-activation in the tumor microenvironment to selectively enhance T_{eff} entry into colon tumors.

5.2 INTRODUCTION

The ability of $CD8^+$ T cells to infiltrate cancer lesions is essential for anti-tumor immunity, as evidenced by studies highlighting the prognostic value of effector T (T_{eff}) cells in multiple cancer types, including colorectal cancer (CRC) (77, 103, 105, 228). In contrast, tumor infiltration with regulatory T cells (T_{regs}) predicts poor outcomes (229-232). Chemokines and their respective receptors are critical for T cell migration and homing (170, 233-237). High levels of CCL5/RANTES (CCR5 ligand) and CXCL9/MIG and CXCL10/IP10 (ligands for CXCR3) in tumor tissues are associated with enhanced infiltration of $CD8^+$ T cells in CRC (238), melanoma and gastric cancer (178, 239). In contrast to the benefits of intra-tumoral expression of CCL5 and CXCL9-11 (240), high levels of CCL22/MDC, the CCR4 ligand preferentially attracting T_{regs}, can be associated with reduced survival, as shown in ovarian cancer patients (47).

Several studies have indicated the propensity of colorectal tumors to over-express COX2 and its product PGE_2 (241-242), the factor shown to promote the induction of CCL22 in dendritic cell (DC) cultures (25). Prompted by these reports, and by our observations of the reciprocal impact of IFNα versus PGE_2 on the production of T_{eff}- and T_{reg}-attracting chemokines in isolated DCs (25), we tested the feasibility of using these factors to manipulate tumor microenvironment to enhance the production of T_{eff}-attracting chemokines in intact human tumor tissues. We used an *ex vivo* tumor/tissue explant culture system previously applied to study migration of DCs (243), to avoid spontaneous activation of the chemokine-producing cells in the process of tumor dissociation.

Guided by reports showing common hyper-activation of NF-κB in cancer tissues (244-247), and the requirement for this factor in the induction of both T_{reg}- and T_{eff}-attracting classes of chemokines (248-250), we tested whether the selected PGE_2- and IFNα-targeting strategies can be used to selectively enhance the production of T_{eff}-attracting chemokines in tumor tissues, rather than marginal tissues, in order to selectively direct T_{eff} cells to tumors.

5.3 MATERIALS AND METHODS

Patients.

72 colorectal patients were involved in the study. Tumors and marginal tissues were harvested during routine surgery. The patient profile is presented in Table 1. All patients signed a consent approved by the Institutional Review Board of the University of Pittsburgh for collection of tumor samples (UPCI 02-077).

Culture of macrophages, fibroblasts, HUVEC cells and colon cancer cell lines.

For preparation of macrophages, monocytes were cultured in AIM-V with GM-CSF for 6 days. Fibroblasts (Cascade Biologicals, Portland, Oregon) and colon cancer cell lines CACO-2, HCT116, HT29, SW480 and SW620 (ATCC, Manassas, VA) were grown in IMDM+10% FBS, while HUVECs (AllCells, Emeryville, CA) were cultured in HUVEC complete media (Basal media supplemented with HUVEC stimulatory supplement; AllCells). All were washed, reseeded at 20,000 cells in 300μL in 96 well plates and treated with IFNα, poly-I:C, and/or indomethacin as indicated for 48hrs, and supernatants were analyzed for chemokine production by ELISA.

Ex vivo cultures of tumor- and marginal tissue explants.

Using a 4mm biopsy puncher, the cubes of tumor or marginal tissue were prepared and placed in antibiotic-containing IMDM/10% FBS (typically 3 cubes/well in 24 well plates) for 24-48 hours, as indicated. When indicated, the tissues were treated with 10,000 units of IFNα, 20μg/mL of poly-I:C, 50μM of indomethacin or 10μM of celecoxib. Biopsies were harvested at 0 and 24hrs for mRNA analysis and confocal microscopy analysis. Culture supernatants were harvested at 24-48 hours, as indicated (all groups in a single type of experiment were harvested at the same time point) for ELISA and chemotaxis assays. The detailed work flow is depicted in Supplementary Fig. 6A. The system used, based on our previously-developed *ex vivo* whole tissue culture system (243), allowed us to avoid spontaneous induction of chemokine production by the process of tumor dissociation (Supplementary Fig 6B and data not shown).

Taqman analysis of mRNA expression in tumors and marginal tissues.

4mm biopsies were placed in lysing Matrix E tubes (MP Biologicals, Solon, OH) containing RLT buffer (RNeasy kit, Qiagen, Valencia, CA), and agitated using a FP120 homogenizer (MP Biologicals). Debris-free supernatant from the lysis matrix tubes were transferred into new tubes and the total RNA was extracted using the RNeasy kit. 1μg of RNA extracted by the above described method was used for cDNA synthesis, and 25-50 ng of subsequent cDNA was used to perform mRNA expression analysis by Taqman analysis on the Step One Plus system (Applied Biosystems). All the primers used for the analysis were standard, purchased from Applied Biosystems.

ELISA analysis of chemokines in tumor ex vivo culture supernatants.

Culture supernatants from tumor *ex vivo* cultures were analyzed by ELISAs for the presence of chemokine proteins CCL5, CCL22 and CXCL10, using primary and secondary antibodies from Peprotech, Rocky Hill, NJ. Detection was done using Streptavidin-HRP conjugate and TMB substrate from Pierce Biotechnology Inc, Rockford, IL.

Isolation of tumor infiltrating $CD8^+$ T cells.

Tumor infiltrating lymphocytes were isolated as described by Dudley et al (251), with the following modifications: Tumor was cut into 4mm cubes using a biopsy punch, and each 4mm tumor piece cultured in 1mL of IMDM + 5% human AB serum with 1000U/mL IL-2 for 2 weeks. Medium was changed twice a week, until lymphocytes were extruding from tumor and formed proliferating clusters.

Chemotaxis.

Chemotaxis assays were performed in 24 transwell plates with 5μm pore size polycarbonate filters (Corning Inc, Corning, NY). The lower chambers were filled with 600μL of tumor supernatants. As indicated, $2x10^5$ of either isolated tumor-infiltrating lymphocytes or αDC1-activated $CD8^+$ T_{eff} cells (189), in 200μL of IMDM 10% FCS, were added to the upper chambers and incubated for 3hrs at 37^oC. Migrated cells were harvested from the lower chambers and stained for CD8. Cell counts were performed by a 60 second limited run on a BD Beckman Coulter XL cytometer. For analysis of T_{reg} cell migration, bulk $CD4^+$ T cells were isolated by negative selection using EasySep CD4 enrichment kits (StemCell), and $1x10^6$ of the isolated cells in 200μL were allowed to migrate towards 600μL of tumor supernatants in the

bottom chambers. The migrated cells in the bottom chambers were harvested and FOXP3/GITR frequencies were determined by Taqman analysis or flow cytometry.

In situ hybridization.

Tissue specimens were fixed in 4% para-formaldehyde, processed and pre-treated as described (252), except that tissues were sectioned on a cryostat at 5µm. Gene-specific riboprobes were synthesized by *in vitro* transcription using a Maxiscript SP6/T7 kit (Ambion) and unincorporated nucleotides were removed using RNA Mini Quick Spin Columns (Roche). In situ hybridization with ^{35}S-labeled riboprobes was performed as described (252-253), with 0.1M dithiothreitol included in the hybridization mix. Hybridizations were performed at 50^{o}C overnight. Tissue sections were coated with NTB emulsion (Kodak) and exposed at 10^{o}C for 7-14d. Simultaneous *in situ* hybridization and immunohistochemistry were performed as described (252-253), except that the dithiothreitol concentrations were 0.01M in the hybridization mix and 1mM in the washes. An antibody against HLA-DR (Dako) was used at a dilution of 1:25.

Confocal microscopy analysis of tumor and marginal tissues.

4mm tumor punches, either untreated or treated, were embedded in OCT medium-containing cryomolds and immediately frozen in 2-methyl-butane. 6µm frozen sections of the tissues were made using the cryostat and layered on superfrost® plus slides (Thermo Scientific, Rockford, IL). The slides were incubated in 4% para-formaldehyde for 15 minutes, washed and blocked for 60 minutes at RT. The slides were then stained for 3hrs at RT with antibodies for P65 (ab16502) or for CD8 (ab4055), CXCL10 (ab8098) and CCL5 (ab10590; both AbcamCambridge, MA). The slides were washed 3 times with 1x PBS and incubated with

secondary antibodies anti-rabbit (Alexa 647), anti-mouse (Alexa 488; both Cell Signal, Danvers, MA) and anti-goat (Alexa 488; Invitrogen, Carlsbad, CA) for 30 minutes at RT. The slides were washed 3 times with 1x PBS and once with high salt PBS. Cover slips were mounted on the sections using prolong gold anti-fade solution (Invitrogen). Confocal analyses of stained slides were performed using a LEICA TCS SL DMRE Microsystems. To quantify the numbers of cells showing nuclear NF-κB translocation, images were taken of 10 different fields (63x magnification) of the tumor and marginal tissue sections (untreated or treated). To identify the cells showing P65 translocation and chemokine production in response to treatment, tumor tissues were stained with CD45 (H130, BioLegend, San Deigo, CA), CD326 (9C4, BioLegend), fibroblast marker (TE-7, EMD Millipore, Billerica, MA) and CCL5 (ab9679, Abcam) and CXCL10 (ab9807, Abcam), and cells were enumerated as described above.

Statistical analysis.

Pearson rank correlations between the chemokine genes and T cell markers were calculated on logarithmically transformed data. In situations where significant between-batch variation was observed, the correlation was adjusted for the batch effect by performing a multivariate analysis of variance (MANOVA) of each pair of variables on batch, and deriving the correlation from the MANOVA residual matrix. Comparisons of continuous variables between groups were performed by two-tailed paired *t* tests. $P < 0.05$ was considered significant. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC) or GraphPad Prism 5 software.

5.4 RESULTS

5.4.1 The expression of effector T cell (T_{eff})-recruiting chemokines in colorectal tumor samples correlates with effector $CD8^+$ T cell markers.

Using resected tumor material from 72 patients with advanced colorectal cancer (metastatic in 68 patients), we observed that local expression of two T_{eff} cell markers (CD8 and Granzyme B; GZMB) is strongly correlated with the expression of two T_{eff}-attracting chemokines, CCL5 and CXCL10 (Fig. 13A). In contrast, the T_{reg} markers FOXP3 and GITR were correlated with CCL22 (Fig. 13B), a known T_{reg} attractant (25, 47). Additional correlations were observed between CXCL9 (alternative CXCR3 ligand) and T_{eff} markers and between CCL22 and the CCL22- inducing factor (22) COX2 (Supplemental Fig. 7A-B). Confocal microscopy analysis of the tumor sections revealed that all CXCL10-producing cells (Supplemental Fig. 7D; right panel) and a significant proportion of CCL5-producing cells (left panel) were CD8-negative, arguing against the possibility that the above correlations result from the production of these chemokines by $CD8^+$ T cells themselves, instead suggesting their causative role in mediating $CD8^+$ T cell infiltration.

The phenotypic analysis of $CD8^+$ tumor-infiltrating lymphocytes (TIL) obtained from colon cancer patients (see M&M) revealed that the majority of $CD8^+$ TILs are $CCR5^+$ $CXCR3^+$ (Supplemental Fig. 7E) and Granzyme B^+ (Supplemental Fig. 7F), which further indicates that the intra-tumoral expression of the CCR5- and CXCR3-ligands was responsible for recruiting the effector T cells into the tumor.

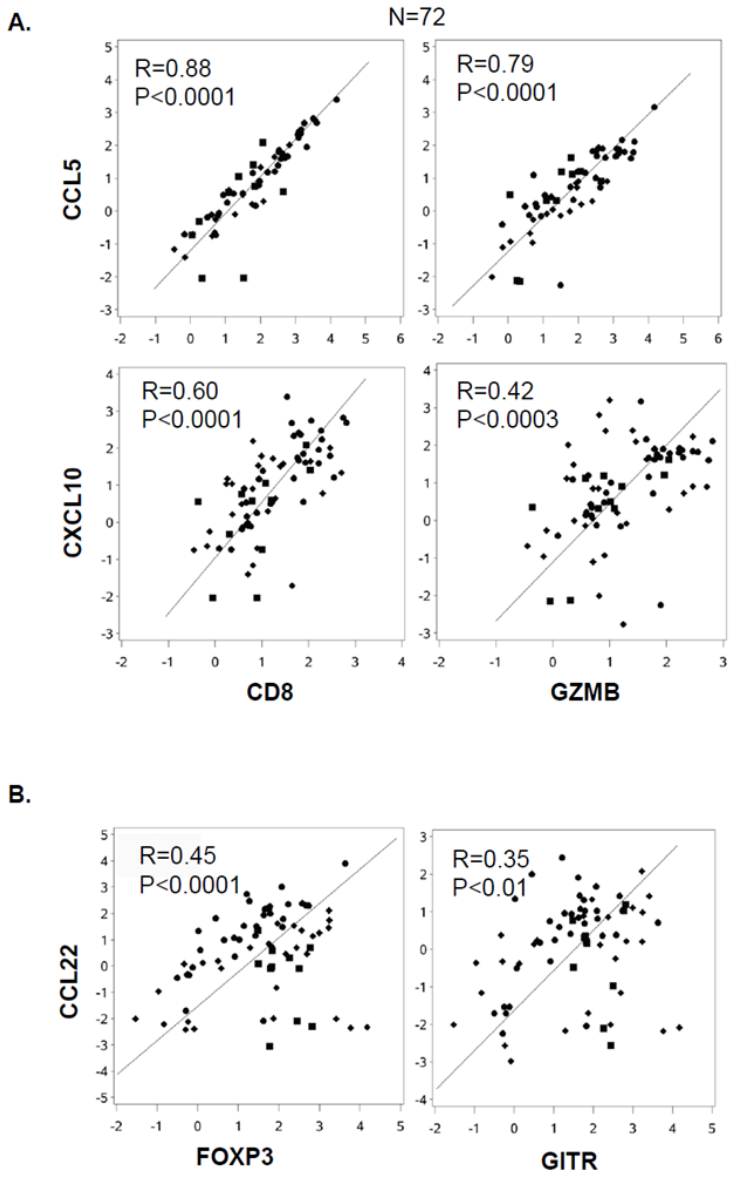


Figure 13. Presence of T_{eff}- and T_{reg} markers in tumors correlates with intra-tumoral expression of, respectively, T_{eff}- or T_{reg}-attracting chemokines.

Tumor biopsies from colon cancer patients were lysed, RNA extracted and Taqman analysis of various markers was performed. (A) Correlation between T_{eff} markers (CD8 and Granzyme B; GZMB) and T_{eff}-attracting chemokines (CCL5 and CXCL10) in tumor lesions. (B) Correlation between T_{reg} markers (FOXP3 and GITR) and the chemokine CCL22 in tumor lesions.

5.4.2 Combination of IFNα, indomethacin and poly-I:C selectively enhances the production of T_{eff}-recruiting chemokines in tumor tissues and suppresses T_{reg}-recruiting chemokines.

In order to test the possibility of correcting the chemokine environment in the tumors with low ratios of T_{eff}- to T_{reg}-attracting chemokines, we tested in pilot studies the feasibility of modulating their production using different combinations of IFNα, indomethacin (COX1/2 inhibitor) and poly-I:C in individual populations of tumor-relevant cells, such as colon cancer cells, macrophages, fibroblasts and HUVECs. We observed strong synergy between IFNα and poly-I:C in the induction of CCL5 and CXCL10, and a strong suppressive effect of IFNα on the production of CCL22 in macrophages and fibroblasts (Supplementary Fig. 8A-B). These desirable effects were further potentiated in the presence of indomethacin (Supplementary Fig. 8B). In contrast, none of the long-term-cultured colon cancer cell lines tested (CACO-2, HCT116, HT29, SW480 and SW620) or HUVECs produced any detectable CCL5, CXCL10 or CCL22 (data not shown).

In order to test the feasibility of using these factors to manipulate the complex microenvironment of whole tumor tissues, involving all the above cell types and their interactions, we used an *ex vivo* tumor/tissue explant culture system previously developed to study migration of DCs (243). This system allowed us to avoid nonspecific activation of the chemokine-producing cells during tumor dissociation (see Supplementary Figure 6B and data not shown).

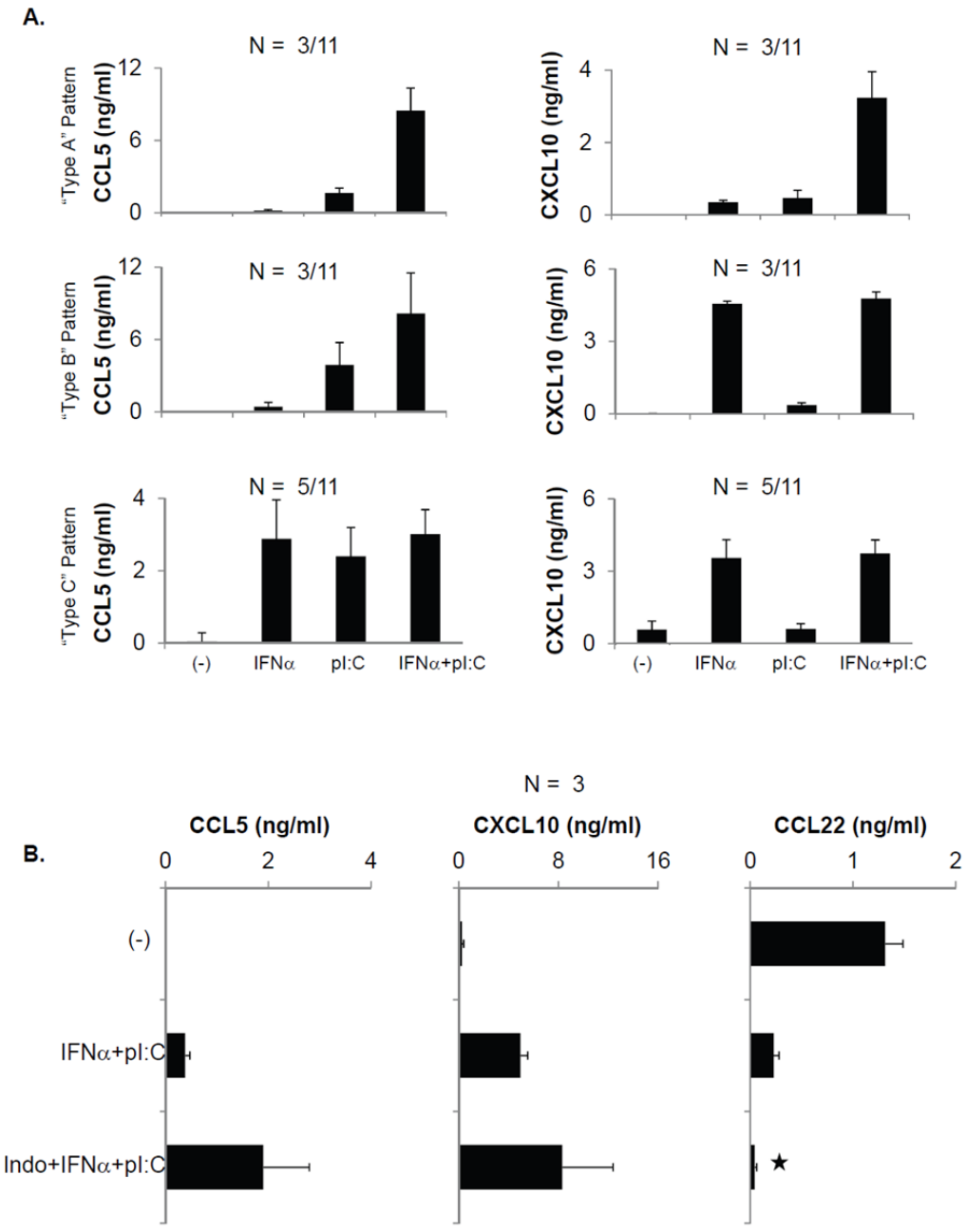


Figure 14. Heterogenous response pattern of different tumor tissues to individual chemokine modulators and their uniform response to the combination of IFNα, poly-I:C and indomethacin.

(A) Fresh tumor samples from 11 patients with metastatic colorectal cancer were untreated or treated with IFNα and poly-I:C either individually or in combination for 48 hours. The release of CCL5 and CXCL10 into culture media was analyzed by ELISA. Numbers indicate the prevalence of tumors with each chemokine pattern (respective

patterns A, B or C). (B) ELISA analysis of CCL5, CCL22 and CXCL10 in tumors untreated or treated with IFNα+ poly-I:C, with or without indomethacin. *denotes P<0.05 (the presence or absence of indomethacin).

As shown in Figure 14, different tumor tissues treated with IFNα or poly-I:C alone showed variable chemokine expression, falling into three different patterns: minimal induction of CCL5 and CXCL10; minimal induction of CCL5 but significant induction of CXCL10; or significant induction of both CCL5 and CXCL10 (Fig. 14A). This heterogeneity was observed between tumors from different patients, and even between different lesions within a single patient (Fig. 14A and Supplemental Fig. 8C). However, combining IFNα and poly-I:C resulted in uniformly high expression of both CCL5 and CXCL10 in all tumors tested (Fig. 14A and Supplemental Fig. 8C).

Additional exposure to indomethacin (which blocks COX1 and COX2) further enhanced the production of CCL5 and CXCL10 induced by the combination IFNα and poly-I:C and reduced CCL22 in whole tumor tissues (Fig. 14B), with similar results obtained using a selective COX2 blocker, celecoxib (Supplemental Fig. 8D).

Based on these data, we selected the triple combination of IFNα, poly-I:C and indomethacin as the preferred treatment for all subsequent experiments. This combination consistently enhanced CXCL10 and CCL5 production and suppressed the production of CCL22 in all tumor samples, as shown by individual chemokine gene expression at the single cell level using in-situ hybridization (ISH; Fig. 15A) and at the level of chemokine secretion, using ELISA (Fig. 15B). Similar observations were also made in case of CXCL9 (data not shown).

The dual staining for HLA-DR (immunohistochemistry) and chemokine mRNA (ISH) demonstrated that CCL22 was expressed predominantly by HLA-DR$^+$ APCs, while

CXCL10 and CCL5 were expressed by both HLA-DR$^+$ and HLA-DR$^-$ cells (Supplemental Fig. 9), indicating the contribution of multiple tumor-associated cell types to the production of T_{eff}-recruiting chemokines within the tumor microenvironment.

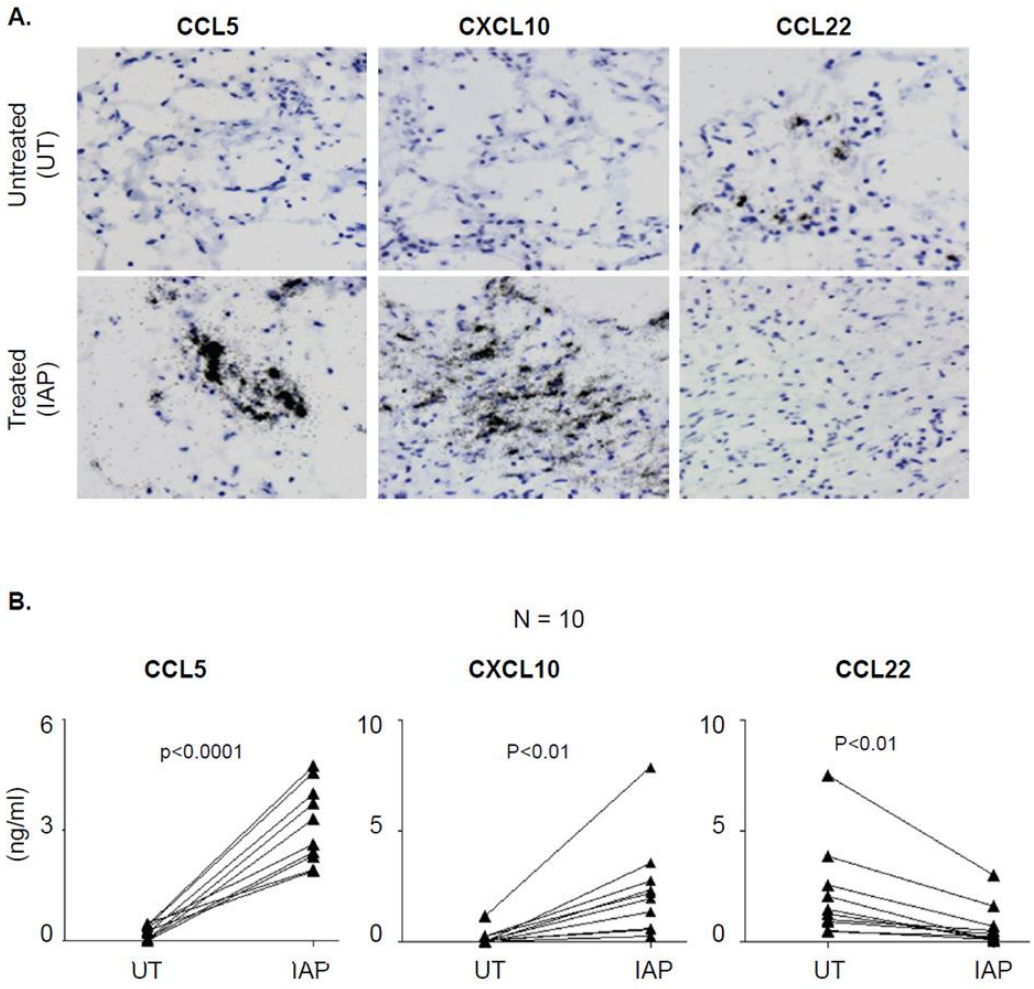


Figure 15. Combination of IFNα, poly-I:C and indomethacin, consistently up regulates Teff-attracting chemokines and suppresses Treg-attracting chemokines in tumor tissues.

(A) *In-situ* hybridization for respective chemokine mRNA (black grains) in tumor biopsies which were either left untreated or treated with the combination of indomethacin, IFNα and poly-I:C (IAP). (B) ELISA analysis of the chemokine contents in the supernatants of 48 hour-cultured tumor tissues (untreated or treated) from 10 different patients.

5.4.3 Enhanced activation of tumor-associated NF-κB by the chemokine-modulatory regimen results in preferential induction of CXCL10 in tumors, rather than marginal healthy tissues.

Using matched tissue samples from 10 patients with metastatic colon cancer, we compared the responsiveness to the chemokine-modulating regimen between liver-metastatic tumor tissues and marginal tissues. As shown in Fig. 16A (and Supplemental Fig. 10), while the baseline differences in chemokine production between the untreated liver-metastatic tumors and marginal liver tissues did not reach significance (P=0.12), tumor treatment with the combination of IFNα, poly-I:C and indomethacin induced much more pronounced secretion of CXCL10 by tumor tissues compared to the marginal tissues (P<0.01). Similar observations at the protein and chemokine gene expression level were made in the case of CCL5 (Supplemental Fig. 10A, B). This increased responsiveness of tumors compared to marginal tissues was not due to decreased survival of the marginal tissues, as determined by undisturbed expression levels of glycogen phosphorylase (Supplemental Fig. 10C). Similarly, the differences in the responsiveness to the chemokine-modulatory regimen between tumors and marginal tissues could not be explained by potential differences between their expression of the IFNα receptor, TLR3, IRF1, IRF3, or the differential infiltration with APCs or NK cells, which were all similar between tumors and marginal tissues (data not shown).

Driven by the previously reported key role of NF-κB in the induction of CXCL10 and other chemokines (248-250), and the ubiquitous enhancement of the NF-κB signaling in cancer lesions critically needed for tumor survival and growth (244-247), we tested whether

potential differences in NF-κB activation could be responsible for the differential ability of the tumors versus marginal tissues to respond to the chemokine modulatory regimen.

In accordance with this possibility, we observed that the colorectal cancer tissues showed not only elevated baseline levels of NF-κB activation (measured by the rate of its nuclear translocation; see Fig. 16B, left), but an even more pronounced ability to further activate NF-κB after the IFNα/poly-I:C/indomethacin treatment (Fig. 16B, right). The key role of NF-κB in CXCL10 production by tumor tissues was validated by using an NF-κB inhibitor, CAY10470, which completely abrogated CXCL10 induction (Fig. 16C).

CCL5 regulation showed a similar pattern (treatment-induced up-regulation in tumors, rather than in marginal tissues) and was also blocked by CAY10470 (Supplemental Fig. 10B), showing the general role of the tumor-associated NF-κB deregulation in the selective induction of T_{eff}-attracting chemokines by the chemokine-modulating regimen. CAY10470, used in these experiments (at 20μM), was non-toxic, as shown by similar expression of glycogen phosphorylase mRNA in untreated and treated tissues (Supplemental Fig. 10C).

Interestingly, our confocal microscopy analysis revealed that most of the cells that showed nuclear translocation of NF-κB and produced CCL5 and CXCL10 represented $CD45^+$ infiltrating inflammatory cells and (TE-7-binding) tumor-associated fibroblasts, with only some of the $CD326/EpCAM^+$ cancer cells producing CCL5 (Fig. 16D; also see Supplemental Fig. 10D for example of single-color analyses).

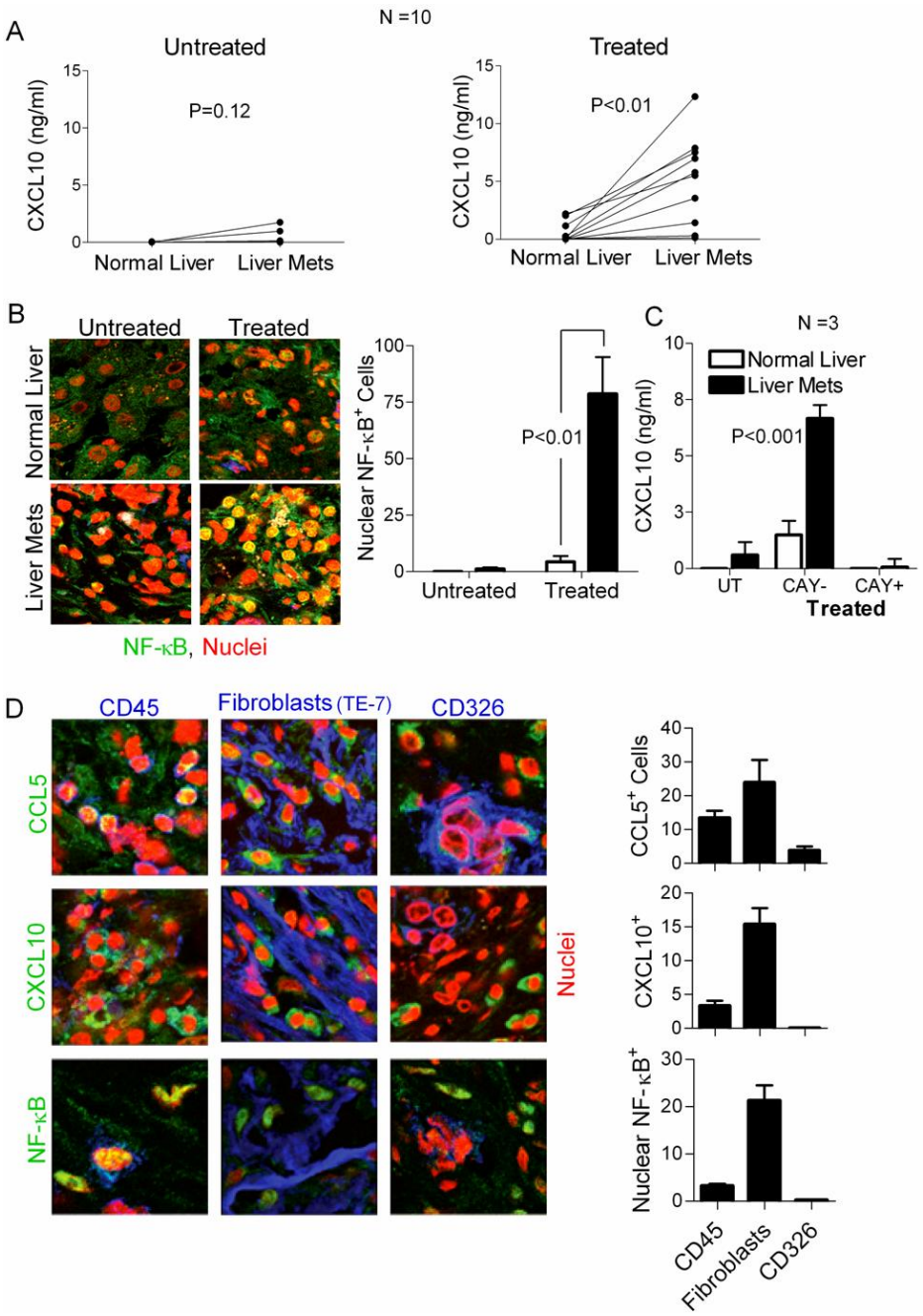


Figure 16. NF-κB-dependent selective enhancement of CXCL10 production in tumor tissues following exposure to the combination of IFNα, poly-I:C and indomethacin.

(A) ELISA for CXCL10 expression in matched normal liver and liver metastatic tissues from 10 different patients either untreated (left panel) or treated (right panel). (B) Average number of cells counted per field (confocal microscopy; in a total of 10 fields) showing nuclear translocation of NF-κB in normal liver or liver metastatic tissues either untreated or treated (right panel). Representative images of each condition are shown in the left panel.

(C) ELISA analysis of CXCL10 production by the matched normal liver and liver-metastatic colorectal cancer tissues, either untreated or treated (IFNα, poly-I:C and indomethacin), in the absence or presence of 20μM CAY10470 (NF-κB inhibitor). D) Colorectal cancer tissues from three colorectal cancer patients were treated with indomethacin + IFNα + Poly-I:C for 30 minutes (p65 translocation) and 24 hours (chemokine production) and analyzed by confocal microscopy for the translocation of p65 and production of CCL5 and CXCL10 by infiltrating inflammatory cells ($CD45^+$), tumor-associated fibroblasts (TE-7-binding cells) and cancer cells (CD326). Representative data from one of three experiments. *Left panel:* representative sections of the activated tumor tissue. *Right panel:* Numbers per vision field of the individual cells types showing p65 translocation and chemokine production. Data from 10 vision fields is expressed as average +/- SEM.

5.4.4 IFNα/poly-I:C/indomethacin-treated colorectal tumors preferentially attract effector $CD8^+$ T cells.

In order to demonstrate that the modulation of chemokine achieved by the combination of IFNα, poly-I:C and indomethacin is indeed sufficient to affect the ability of tumors to attract different subsets of T cells, we used an *ex-vivo* chemotaxis assay involving the supernatants from differentially-treated tumors and either expanded tumor-infiltrating $CD8^+$ T cells (TILs; see Supplemental Fig. 7F) or polyclonal *ex-vivo*-induced effector $CD8^+$ T cells induced by superantigen-loaded αDC1s (189). As shown in Fig. 17A, each type of effector $CD8^+$ T cell showed strongly enhanced migratory responsiveness uniformly to all the IFNα/poly-I:C/indomethacin-treated tumors. In contrast, $CD4^+FOXP3^+$ T cells preferentially migrated to untreated tumors, as determined by Taqman analysis of the migrated blood-isolated $CD4^+$ T cells (Fig. 17B), or flow cytometry (Supplemental Fig. 11A). As expected, Taqman analysis of another T_{reg} marker, GITR, yielded similar results (Supplemental Fig. 11B).

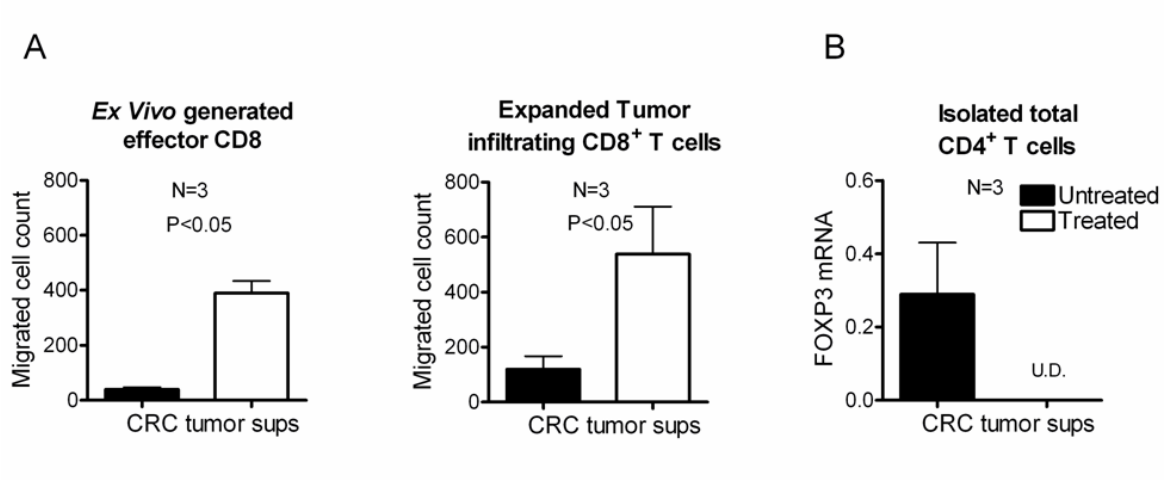


Figure 17. IFNα, poly-I:C and indomethacin-treated tumors show enhanced ability to attract Teff, but strongly-reduced ability to attract Tregs.

(A) *Ex vivo* generated T_{eff} (left) or isolated $CD8^+$ tumor-infiltrating lymphocytes (right) (see Materials and Methods) were allowed to migrate towards supernatants from either untreated or treated tumors from 3 different patients in transwell chemotaxis assays. (B) Negatively-isolated total $CD4^+$ T cells were allowed to migrate towards the treated- or untreated tumor supernatants. Migrating cells were lysed and analyzed for FOXP3 expression by Taqman. U.D.: undetectable.

5.5 DISCUSSION

Our data demonstrate the feasibility of tumor-selective modulation of the chemokine environment, using clinically applicable combinations of pharmacologic and biologic factors to correct the balance between tumor-infiltrating T_{eff}- and T_{reg}- cells, the types of immune cells known to differentially affect the clinical course of cancer (77, 103, 105, 228-232). Importantly for the clinical application of this strategy, we observed that while the responses of the individual tumor lesions (even in the same patient) to the individual chemokine-modulators were highly variable (consistent with the limited clinical effectiveness of such factors applied individually), the combination of IFNα, poly-I:C and cyclooxygenase inhibitors allowed for highly consistent and selective enhancement of T_{eff}-attracting chemokines (CCL5 and CXCL9-10) within tumor lesions tested, with the concomitant uniform suppression of local CCL22, the T_{reg}-attracting chemokine.

The IFNα/poly-I:C/indomethacin-induced production of T_{eff}-attracting chemokines was highly tumor-selective, suggesting that even systemic administration of these chemokine-modulating factors can preferentially direct effector cells to tumors. While the attraction of different subsets of T cells to different tumor types is known to be regulated by a complex network of additional chemokines not included in our current analysis (254-255) and can be subject to regulation at the level of chemokine receptor expression, for example by CCR5 polymorphism (172), our current functional data (Fig 17) indicate that the proposed regimen can uniformly promote the influx of effector $CD8^+$ T cells (both spontaneously-arising TILs and αDC1 vaccine-induced CTLs). The known role of CXCR3 and CCR5 in the attraction of Th1

cells and NK cells (170, 236, 256-257) suggests that the proposed regimen may also be able to promote the entry of these additional types of desirable cells into tumors.

We observed that the tumor-selectivity of the proposed regimen depends on the propensity of tumor-associated fibroblasts and infiltrating inflammatory cells (with lesser involvement of tumor cells themselves) to not only spontaneously hyper-activate NF-κB, but also respond to treatment with further-enhanced levels of NF-κB activation. Since NF-κB activation, critically involved in tumor survival and growth, represents an intrinsic feature of many tumor types (244-247), the current data suggest that the currently-described NF-κB-targeting modulation of the tumor microenvironment may be applicable to multiple types of cancer.

The currently-developed chemokine-modulating regimen consists of the combination of IFNα (type 1 interferon), poly-I:C (TLR3 ligand), and either indomethacin (COX1 and COX2 inhibitor) or a selective COX2 inhibitor, celecoxib. While our data demonstrate that interferons and prostanoids differentially regulate the NF-κB-driven production of $T_{eff-,}$ and T_{reg}-attracting chemokines, the specific mechanisms and the molecular level of interplay between these factors remain subjects of our current research. Our analyses performed so far did not reveal any differences between the expression of the IFNα receptor, TLR3, IRF1, or IRF3 between tumors and marginal tissues (data not shown), but our current work focuses on the differential regulation of each of the pathways (poly-I:C, IFNα and PGE_2 responsiveness) in whole tumor tissues and different types of tumor-associated cells. Similarly, we are also evaluating the mechanisms underlying the increased sensitivity of tumor-related cells to activate NF-κB and the relative heterogeneity of different tumors with regard to the requirement for poly-I:C activation, which

may help us to identify new strategies of chemokine regulation and of targeting NF-κB in tumor therapy.

The combination of IFNα, poly-I:C and COX inhibition will be evaluated in clinical trials in patients with metastatic colorectal cancer, as a standalone treatment or in combination with αDC1 vaccines (71, 189), to enhance the numbers of circulating effector-type tumor reactive $CD8^+$ T cells that respond to CCR5 and CXCR3 ligands (189) and enter tumor tissues. Our follow-up analyses will also allow us to determine whether the observed differences in the expression of chemokines and T_{eff} markers in patients with metastatic colorectal cancer also translate into differences in clinical course of the disease and patient survival, as predicted by studies in primary colon cancer (77, 103, 105, 228).

5.6 IMPLICATIONS

In chapter 5 I show that the treatment of colorectal tumor tissues with IFNα, indomethacin and poly-I:C results in the modulation of the chemokine environment to favor the production of effector $CD8^+$ T cell attracting chemokines CXCL10/IP10 and CCL5/RANTES. The triple combination treatment appears to be tumor specific, which would prevent the infiltration of cytolytic effector $CD8^+$ T cells into healthy tissues and is mediated by NF-κB translocation. These data, combined with the data from chapters 2, 3 and 4, which show that high IL-12p70 producing, type-1 polarized DCs induce large numbers of $CXCR3^+$ $CCR5^+$ anti-tumor $CD8^+$ T cells, suggests that a combination of type-1 polarized DCs vaccines and the triple combination treatment would enhance the efficacy of DC-based cancer vaccines.

6.0 SUMMARY OF THE THESIS AND IMPLICATIONS

I examined the effects of inflammatory cytokines on the maturation and polarization of DCs and on their ability to prime naïve $CD8^+$ T cells, and developed a novel method of polarizing DCs using inflammatory cytokines derived from activated lymphocytes. Furthermore, I explored the feasibility to enhance DC1-based cancer immunotherapies by increasing the ability of tumor-specific CTLs to migrate into tumors by altering the tumor-chemokine-environment.

The induction of cytolytic effector $CD8^+$ T cells is essential for the clearance of virally-infected and transformed cells, while the induction of memory T cells is necessary for long-term protection against reinfection or tumor recurrence. While it is well established that mature DCs play a key role in inducing cytolytic T cell responses, less is known about their role in inducing memory T cell differentiation. It has been shown that long-term matured (type-1 polarized) DCs become exhausted and induce non-polarized $CD4^+$ T cells responses (26), but whether the same is true for $CD8^+$ T cells and whether short-term matured DCs can be instructed to induce memory $CD8^+$ T cells was unknown. In chapters 2 and 3 I show that *in vitro* generated monocyte-derived DCs can be matured to either induce CTL development or central-memory T cell development. The short-term (24-36 hour) maturation and polarization of DCs using factors associated with early/acute inflammation (i.e. interferons and TLR-ligands) results in the generation of type-1 polarized DCs (DC1s or inflammatory-DCs) that induce the differentiation of naïve $CD8^+$T cells into cytolytic effector cells. These CTLs express the peripheral-homing

chemokine receptors CXCR3 and CCR5, while down-regulate the lymph node-homing chemokine receptor CCR7 and are thus able to leave the lymph nodes and migrate to sites of inflammation (infected areas or tumors). In contrast, DCs matured by prostaglandin E2 (PGE_2; sDC or non-inflammatory-DCs) or matured for a prolonged duration (96 hours) induce $CD8^+$ T cell expansion and activation, as observed by a switch in expression of CD45RA to CD45RO and CD25 expression, but do not instill cytolytic capacity in them. Instead, non-inflammatory DC-primed $CD8^+$ T cells undergo a direct differentiation into central-memory cells. These cells would be retained in the lymph nodes due to expression of CCR7 and can rapidly undergo secondary expansion and give rise to a CTL population upon restimulation with inflammatory DC1s. The data described in chapters 2 and 3 prompted us to propose a model (see figure 18) in which the inflammatory status of Ag-presenting DCs determines whether naïve $CD8^+$ T cells develop into CTLs, followed by differentiation into effector-memory and central-memory cells, or differentiate directly into central-memory cells.

The effect of an inflammatory environment on the development of effector and memory T cells has been extensively evaluated in mouse models (195-196, 258). However, in most cases, these models do not allow for the direct analysis of the inflammatory milieu on DC maturation and polarization and the role of such DCs in directing T cell differentiation. In cases where DCs are used, inflammation is induced by injection of a strong adjuvant and the resulting effect on T cell differentiation could be, in part, due to an effect of inflammatory factors directly on the T cells and not through the DCs. Our human *in vitro* model of DC maturation, using different maturation cocktails and a variable period of maturation and T cell priming allowed us to analyze the role of DCs on the induction of effector and memory T cells. One benefit of our *in vitro* model is that we can employ homogenous populations of DCs, which are matured for

approximately the same duration and exhibit a defined phenotype. We can thus determine T cell differentiation induced by the priming with one type of DC versus another, instead of trying to interpret the impact of heterogeneous DC population, as is the case *in vivo*, where different DC subsets, matured for different lengths of time might contribute to the overall characteristics of the resulting T cell response.

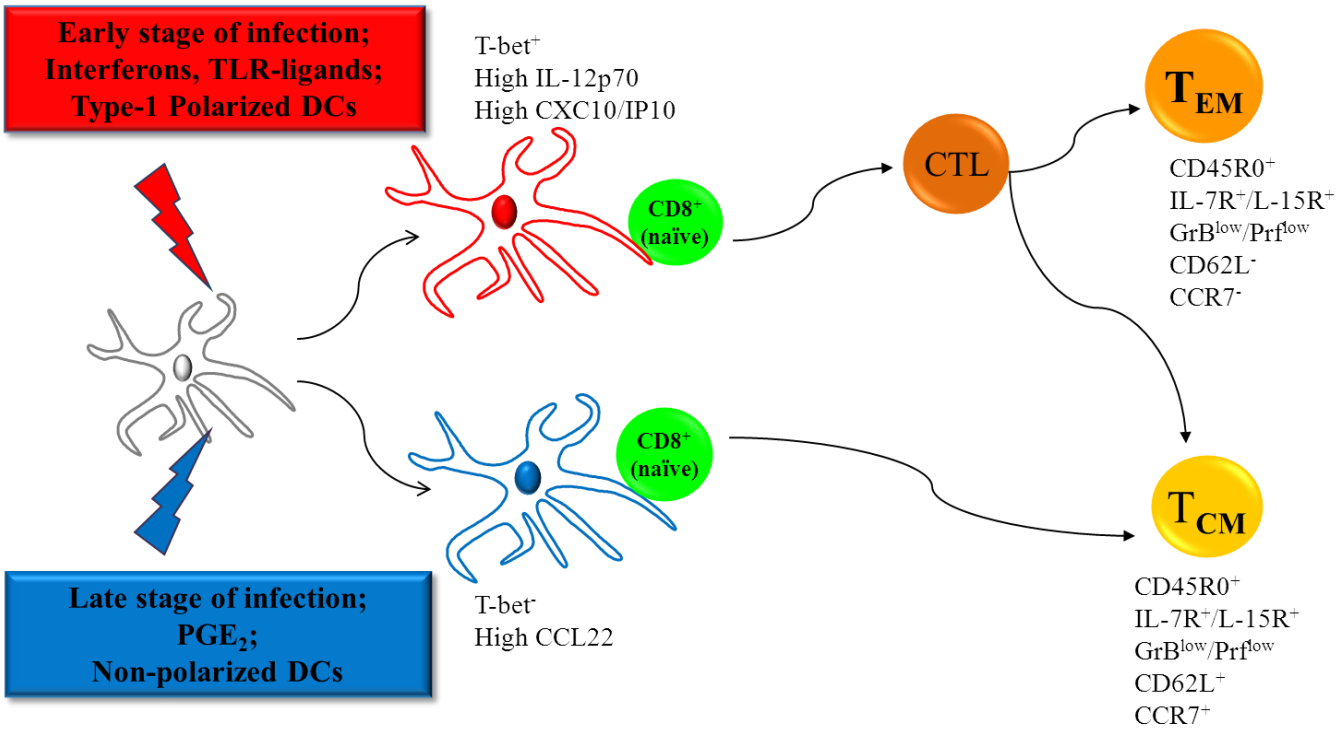


Figure 18. The regulation of effector and memory CD8$^+$ T cells by DCs: the model.

Short-term maturation of DCs by mediators of early/acute inflammation (i.e. interferons and TLR ligands) results in type-1 polarized DCs that express T-bet and secrete high amounts of IL-12p70 and the CTL-attracting chemokine CXCL10/IP10. Naïve CD8$^+$ T cells primed by these type-1polarized DCs undergo differentiation into cytolytic effector cells, followed by the formation of effector-memory and central-memory cells. In contrast, maturation of DCs by a mediator of chronic infection (PGE_2; or long-term maturation of type-1 polarized DCs) results in DCs that do not express T-bet and do not secrete IL-12p70 (previously considered exhausted) and produce the Treg-attracting chemokine CCL22. Naïve CD8$^+$ T cells primed by these non-inflammatory DCs undergo a direct differentiation into central-memory cells without passing through a cytolytic effector phase.

The current data help to elucidate the pathways by which memory T cells can form. While some mouse models suggest a linear differentiation pathway (205), in which naïve T cells first differentiates into CTLs and then, a portion of the effector cells, further develop into memory T cells, other models suggest that memory T cells arise during the initial "effector phase" simultaneously with the effector T cells and do not need to pass through a cytolytic effector phase (32). My data suggest that both pathways can occur and depend on the ability of DCs to provide signal 3 to responding T cells. Recently, a model of effector and memory T cell differentiation based on asymmetrical cell division has been proposed (118). In this model, a naïve T cell undergoing its first cell division during interaction with an Ag-presenting DC, gives rise to a daughter cell with effector properties and a daughter cell with central-memory T cell properties. Our observation that non-inflammatory DCs generate only central-memory T cells would suggest that there is no asymmetrical cell division in our model. It could be that there is asymmetrical cell division in our inflammatory DC1-primed $CD8^+$ T cells, since there appears to be a population of $CCR7^+$ IL-7Rα^+ cells present during the effector phase, but preliminary experiments examining this pathway did not reveal any sign of asymmetrical cell division.

It has been suggested that the duration of interaction of T cells with DCs affects the balance of effector versus memory T cell formation (45). Longer duration would allow for more signaling through the TCR and co-stimulatory molecules, favoring effector T cell differentiation. I did not detect any difference in the expression of MHC class I and class II molecules or in co-stimulatory molecules between inflammatory DC1s and non-inflammatory-DCs which would suggest a difference in the interaction of DCs with the T cells that could account for the distinct differentiation pathway. Furthermore, the acquisition of granzyme B expression and the down-regulation of CCR7 by DC1-primed $CD8^+$ T cells could be blocked by neutralizing IL-12p70,

which would presumably not affect the duration of T cell interaction with the DCs. Conversely, preliminary experiments in which either exogenous IL-12p70 or IL-27, a IL-12 family member which has been shown to have Th1/CTL-inducing properties, was added to non-inflammatory DC-$CD8^+$ T cell cultures, showed the upregulation of granzyme B by the T cells, suggesting that in our model, CTL differentiation depends primarily on the ability of DCs to provide signal 3 (i.e. IL-12). However, the duration of interaction of T cells with differentially matured DCs could still play a role and would need to be examined. The dependence on signal 3, is in accordance with previous observations made in mouse models, in which the differentiation of $CD8^+$ T cells into CTLs is regulated by IL-12p70 via the modulation of T-bet expression in T cells (44). High levels of IL-12p70 up-regulate T-bet expression, while in the absence of IL-12p70 signaling, T-bet expression was diminished and memory Tcell formation was favored. Interestingly, IL-12p70 signaling has been shown to enhance mTOR kinase activity, which up-regulates T-bet expression (128). Furthermore, T-bet down-regulates the expression of IL-7Rα, which is a hallmark of central-memory cells. In our model, $CD8^+$ T cells primed by IL-12-deficient non-inflammatory DCs express reduced levels of mTOR kinase activity and T-bet and they retain IL-7Rα expression.

In the case of DC-based cancer immunotherapy, the use of short-term matured type-1 polarized DCs loaded with tumor antigen would be favorable, since this would generate cytolytic effector T cells capable of killing tumor cells and indeed, the use of type-1 polarized DCs in clinical trials suggests the ability to prolong the survival of patients with malignant gliomas (70). The subsequent development of memory cells against the tumor would aid in the prevention of tumor recurrence. A combination of DC-types for use in cancer vaccination protocols could be envisioned, in which a patient first receives a dose of non-inflammatory DCs, which migrate to

the lymph nodes and induce central-memory T cell development, and they then subsequently receive a second vaccination with inflammatory DCs, which would induce CTL differentiation. The generation of central-memory T cells by the non-inflammatory-DCs would allow for the generation of higher numbers of CTLs by the "booster" inflammatory-DCs. However, these potential advantages need to be considered in the context of the undesirable attraction and expansion of T_{regs} by the PGE_2-matured DCs (25, 54).

The induction of a large pool of memory T cells is the basis for prophylactic vaccination therapy (as in the case of "childhood diseases" and influenza infection). In order to generate a large pool of memory T cells capable of rapidly responding to infection, these vaccinations must be given multiple times, in so-called prime-boost regiments (259). Since the number of memory T cells that is formed is primarily dependent on the initial clonal burst size, the prime-boosting strategy consists of a first dose of antigen in the presence of a strong adjuvant in order to ensure a robust expansion of Ag-specific T cells and specific effector T cell differentiation. Following the effector T cell generation and subsequent contraction phase a small population of memory T cells remains. The boosting dose of the vaccination regiment allows for the secondary expansion of the remaining memory T cells as well as new naïve T cells, allowing for a more robust expansion than observed during the priming phase, and the development of a larger pool of memory cells. Also, it has been suggested that the booster vaccines may skew the memory T cell population towards higher affinity clones (260). Since terminally-differentiated effector T cells are resistant to secondary expansion, a relatively long time interval (weeks or months) between the priming and subsequent booster dose is required. Furthermore, circulating CTLs have been shown to eliminate Ag-presenting DCs in a granzyme B- and perforin-dependent manner (139, 141). Since, DCs present in the region of the vaccination injection would need to take up antigen

and transport it to the lymph node to cross-prime specific T cell responses, the ability of CTLs to eliminate DCs would limit the number of DCs that reach the lymph nodes, and thus, prevent the induction of a secondary immune response.

The long time interval between the priming and boosting is acceptable in certain situations (e.g. "childhood diseases"), but whenever rapid/sustained protection is desired (e.g. during epidemics or bioterrorism threats), the accelerated generation of a large pool of memory T cells is favorable. The observation that DCs induced in the absence of acute inflammation could rapidly generate central-memory T cells allows for the optimization of current vaccination protocols. However, the use of autologous, *ex vivo* matured DCs would be a costly process and is unlikely to be used for a large population of patients. In a recent mouse study, Pham et al., used cell-associated antigens or antigen-coated biodegradable microspheres to cross-prime $CD8^+$ T cells (261). The injection of the microspheres in the absence of adjuvant, result in the rapid generation of $CD8^+$ memory T cells responsive within days to a booster dose with secondary expansion and CTL development. The use of clinically-applicable microspheres for the induction of memory cells would strongly reduce the cost of the vaccination process compared to the generation of personalized DC-based vaccines.

The induction of strong anti-tumor CTL responses by DCs is dependent on the provision of inflammatory signal 3 (e.g. IL-12) (67-69, 98). Type-1 polarized DCs are able to secrete high levels of IL-12p70 and to induce strong CTL responses. However, the generation of large numbers of type-1 polarized DCs in a clinical setting requires the use of expensive clinical-grade cytokines, making DC production costs high. While this might not be an issue for many of institutes in the US and Western-Europe, which have sizeable resources for conducting clinical trials using GMP cytokine-matured DCs, for institutes in most countries, this process would be

too expensive. In previous studies, we have shown that NK cells can promote the type-1 polarization of autologous DCs and that these "$_{NK}$DC1s" can generate strong anti-tumor CTL responses (262-263). The NK-induced type-1 polarization was dependent on NK-secretion of IFNγ and TNFα. Since activated lymphocytes can produce these same DC-polarizing factors, I decided to analyze the feasibility to use lymphocytes to induce DC maturation and polarization. During the isolation of monocytes for the generation of DCs, lymphocytes are obtained as a "by-product". By expanding the bulk lymphocytes, using relatively cheap expander beads, without further separation, I was able to obtain high numbers of lymphocytes, which mostly consisted of $CD4^+$ and $CD8^+$ T cells. These cells produce high levels of IFNγ and TNFα upon reactivation and are able to mature autologous DCs *in vitro*. The lymphocyte-matured DCs produce IL-12p70 and CXCl10/IP10 upon CD40 ligation and induce stronger anti-tumor T cell responses than non-inflammatory DCs. While these data show the feasibility of using bulk lymphocytes for the type-1 polarization of DCs I did not perform an extensive cost analysis. In part because this would not be representative for the actual cost, since different countries have different regulatory requirements and the costs will thus be different. Also, before the suggested protocol of DC1 generation can be used in clinical settings, more extensive research is required. For instance, the amounts of inflammatory cytokines produced by the lymphocytes would be expected to fluctuate. It would need to be established what the minimum concentration of cytokines is for effective DC maturation and polarization, resulting in additional testing of the supernatant of restimulated lymphocytes, thus increasing the cost of DC production for clinical use. This is especially important in the case of using supernatants from restimulated lymphocytes for the DC maturation, since we observed an additional iDC population in our cultures. When used in clinical settings, the iDC population would be unwanted since it has been shown that Ag-

presentation by resting/steady state (immature) DC induces the formation of T_{reg} cells and immune tolerance (17, 226). Furthermore, in the analysis of matured DCs, I focused on IL-12p70 and CXCL10, since previous studies by our group and other groups have shown that these factors are required for optimal CTL development. However, these DCs could also produce inhibitory factors, such as IL-10 or TGFβ, which could support T_{reg} cell development and thereby inhibit immune responses. While the functional data suggest that the lymphocyte-matured DCs induce strong anti-tumor $CD8^+$ T cell responses, I did not examine its effect on other immune cells. However, from a practical standpoint, since the lymphocyte activation and restimulation can be performed simultaneously with the generation of immature monocyte-derived DCs, the additional work involved when compared to cytokine-matured DC1 production would be minimal.

My observation that DC1-primed $CD8^+$ T cells acquire peripheral-homing ability (based on expression of CXCR3 and CCR5) is of interest since it would allow them to migrate into inflamed tissues (as in the case of sites of infection or cancer). The presence of tumor-infiltrating $CD8^+$ T cells has been shown to have positive prognostic value (264). However, only a relatively small percentage of tumor tissues exhibit high numbers of $CD8^+$ TILs. Tumors create an environment that favors attraction of T_{reg} cells and exclusion of Th1 and CTLs (47) and cancer patients have increased percentage of T_{reg} cells in their peripheral blood (48). The ratio of tumor infiltrating $CD8^+$ T cells to $CD4^+$ $Foxp3^+$ T_{reg} in tumor lesions has been shown to have good prognostic value for disease outcome (46). This suggests that one hurdle to overcome in cancer immunotherapy is to produce tumor-reactive CTLs that have a high cytolytic capacity and can infiltrate tumor tissue. The use of adoptive T cell transfer of *ex vivo* expanded tumor infiltrating lymphocytes (TILs) has shown some promise as immunotherapy. These cells are able to

repopulate patients after non-myeloid ablative regiments and to traffic into tumor tissue (53). However, since these TILs were obtained from tumors that thus already contained lymphocytes and thus produce factors (e.g. chemokines) that already favor T cell attraction. In the case of tumors that do not contain TILs, the chemokine micro environment could be such that it prevents CTL attraction. Indeed, genetic profiling of melanoma metastases revealed that tissues that had infiltrated $CD8^+$ T cells, expressed the chemokines, CCL3, CCL4, CCL5 (all ligands for CCR5), CXCL9, and CXCL10 (ligands for CXCR3), all of which attract $CXCR3^+CCR5^+$ CTLs, while tumors without T cell infiltration do not express these cytokines (265). Our data show a positive correlation between CCL22 and Foxp3 mRNA expression message in colorectal tumor tissues, suggesting the attraction of T_{reg} cells by certain (CCL22 producing) tumors (see chapter 6).

In order to increase the tumor infiltration of (DC1-induced) CTLs and to prevent the unwanted attraction of T_{reg} cells, I analyzed the feasibility to alter the tumor chemokine environment. Since we have previously observed the induction of CCL22 production by PGE_2-treated DCs while treatment with IFNα increased expression of CXCR3 and CCR5 ligands (25), I analyzed the effects of treating colorectal tumor tissue with either IFNα or with inhibitors of COX-2, which is the key regulator of PGE_2 synthesis. I found that a combination of IFNα, poly-I:C and indomethacin (COX-1 and COX-2 inhibitor) or celecoxib (a selective COX-2 inhibitor) results in reduced expression of the T_{reg}-attracting chemokine CCL22 by tumor tissues, while simultaneously increasing the expression of the T_{eff}/CTL-attracting chemokines CXCL10/IP10 and CCL5/RANTES. The enhanced expression of CXCL10 and CCR5 was associated with enhanced nuclear translocation of NF-κB in tumor cells, a transcription factor known to be involved in chemokine production (51). Of importance is the observation that the triple treatment appeared to be selective for the tumor tissue, since the marginal stromal tissues didn't

shown enhanced NF-κB translocation. In a chemotaxis assay, the increased production of CTL-attracting chemokines by treated tumor tissues enhanced the recruitment of *ex vivo* expanded TILs and of DC1-induced CTLs. Furthermore, the treated tissues showed a reduced recruitment of $Foxp3^+$ T cells, when compared to untreated tissues. This ability to alter the chemokine environment in tumor tissue could help in optimizing the success of adoptive T cell transfer trials and DC-based vaccination protocols. High numbers of tumor-specific T cells, either developed via *ex vivo* expansion of TILs using current protocols or by *in vitro* priming of $CD8^+$ T cells with tumor-loaded autologous DC1s could be generated and screened for expression of CCR5 and CXCR3 before their adoptive transfer into patients that have previously received the triple combination treatment to alter the chemokine environment. This could allow for more efficient migration of the T cells into the tumor tissue. In the case of DC-based vaccines, one could envision that delivery of tumor-loaded DC1s, capable of induce tumor-specific $CXCR3^+$ $CCR5^+$ CTLs (see chapter 2), followed several days later by treatment with the triple combination treatment, could enhance the migration of the induced anti-tumor CTLs into tumor tissues. Since the triple treatment appears to be specific for the tumor tissue, and not for the marginal healthy tissue, I assume that there would be specific migration to the desired tissue and limited "off target" infiltration and autoimmunity.

The reduced attraction of T_{reg} cells into tumors would be expected to benefit the function of the infiltrating anti-tumor $CD8^+$ T cells making the combined treatment even more effective. Furthermore, the application of a COX-2 inhibitor in the triple combination treatment might have additional beneficial effects, other than modulating CCL22 production and T_{reg}-attraction. Recent studies have shown that the presence of PGE_2 in tumor tissues can drive the differentiation of monocytic precursors towards myeloid derived suppressor cells (MDSCs),

which can inhibit the cytolytic function of $CD8^+$T cells (52). Inhibition of COX-2 appears to reverse the immune-suppressive functions mediated by MDSCs in cancer patients. Also, tumor-produced PGE_2 regulates the expression of CXCR4 on MDSCs, and possibly other immune-suppressive cells, as well as the CXCR4 ligand, CXCL12 (50). Thus, COX-2 inhibition could prevent the PGE_2-induced CXCR4 and CXCL12 expression and reduce MDSC tumor infiltration. Therefore, the triple combination treatment may bias effector-over-regulatory T cell function in the tumor, leading to superior therapeutic. While these data are promising and might allow us to improve our current DC-based vaccination strategies, we have not fully analyzed whether the treatment actually allows CTLs to gain entry into tumor tissues and whether the treatment indeed reduces the presence of inhibitory cell types in the tumor tissue. Mouse studies with spontaneously developing tumors would be required to test these aspects. One could assume that the use of spontaneously developing tumors would be favorable over transplantable tumors, since transplanted tumors would be less vascularized, which would negatively affect the ability of T cells to migrate to the tumors. Also, transplanted tumors would already have fully developed immune inhibitory mechanisms in place, while in early spontaneously arising tumors these might be less established, which could allow for clearer differences between treated and untreated conditions.

In summary, our data show that the inflammatory status of DCs regulates the differentiation pathway of naïve $CD8^+$ T cells into $CXCR3^+$ $CCR5^+$ cytolytic effector with peripheral-homing properties, and central-memory cells that retain expression of the lymph node-homing receptor $CCR7^+$ and can rapidly undergo secondary expansion and CTL development upon restimulation with inflammatory DCs. Furthermore, the induction of effective anti-tumor responses requires the priming of naïve $CD8^+$ T cells by inflammatory-DCs/DC1s,

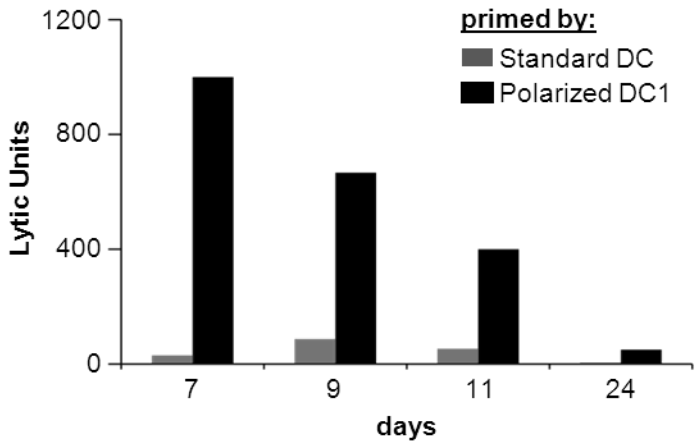
which can be generated in a low cost manner by using autologous activated lymphocytes, and the efficacy of DC-based anti-tumor vaccinations may be enhanced by selectively modulating the tumor chemokine environment, using a combination treatment with IFNα, poly-I:C and COX-2 inhibitors.

The combined data from chapters 2, 3, 4 and 5 show that DCs can be instructed to preferentially induce either type-1 anti-tumor responses useful for therapeutic vaccinations when large numbers of cytolytic effector $CD8^+$ T cells are desired, or to induce the direct differentiation into $CD8^+$ T_{CM} cells, useful for prophylactic vaccines where the rapid generation of large numbers of long-lived memory cells are desired. By manipulation of the tumor-chemokine environment, the attraction of type-1 DC-induced $CXCR3^+CCR5^+CD8^+$ T cells by tumors can be enhanced, which could lead to an improved efficacy of DC-based cancer vaccination strategies. Furthermore, type-1 polarized DCs can be generated by autologous lymphocytes which could reduce the cost of the development of DC-based cancer treatments, making it more accessible in countries with less funding.

Also these data broadens our understating of the role of inflammation and of DCs in the induction of effector versus central-memory development, which could help in the improvement of the clinical effectiveness of DC-based vaccines.

APPENDIX A

SUPPLEMENTAL FIGURES



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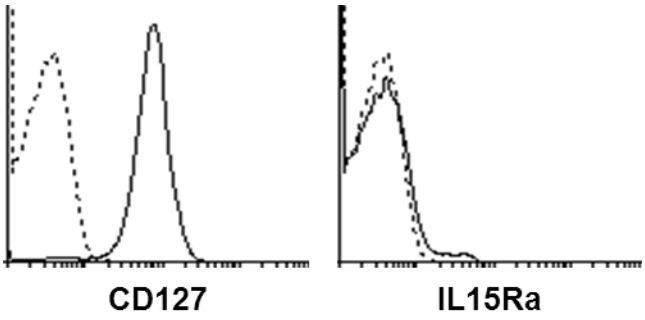
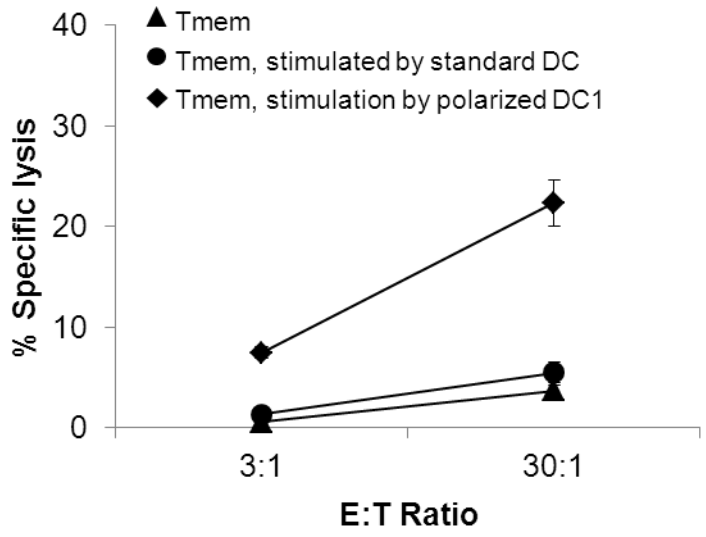
Supplementary figure 1. Kinetics of induction and disappearance of killing capacity of differentially primed CD8$^+$ T cells.

The cytolytic activity of DC1- and standard DC-primed CD8+ T cells against SEB pulsed JY-1 cells was determined at specified time points after priming, using a standard 4 hour ^{51}Cr release assay.



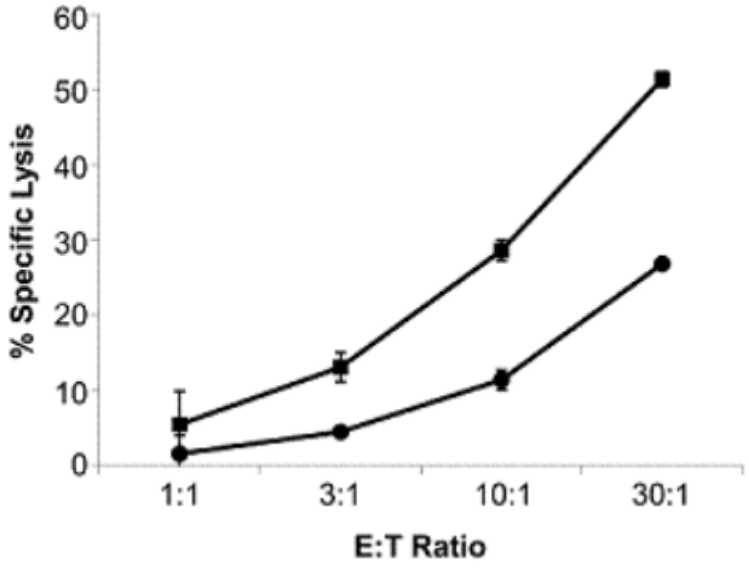
Supplementary figure 2. Expression of CD127 (IL-7Rα) and IL-15Rα on naïve $CD8^+$ T cells.

The expression of CD127 and IL-15Rα (solid lines) on $CD45RO^-$ $CD8^+$ T cells was determined by flow cytometry. Dotted lines are mouse IgG1 isotype controls.



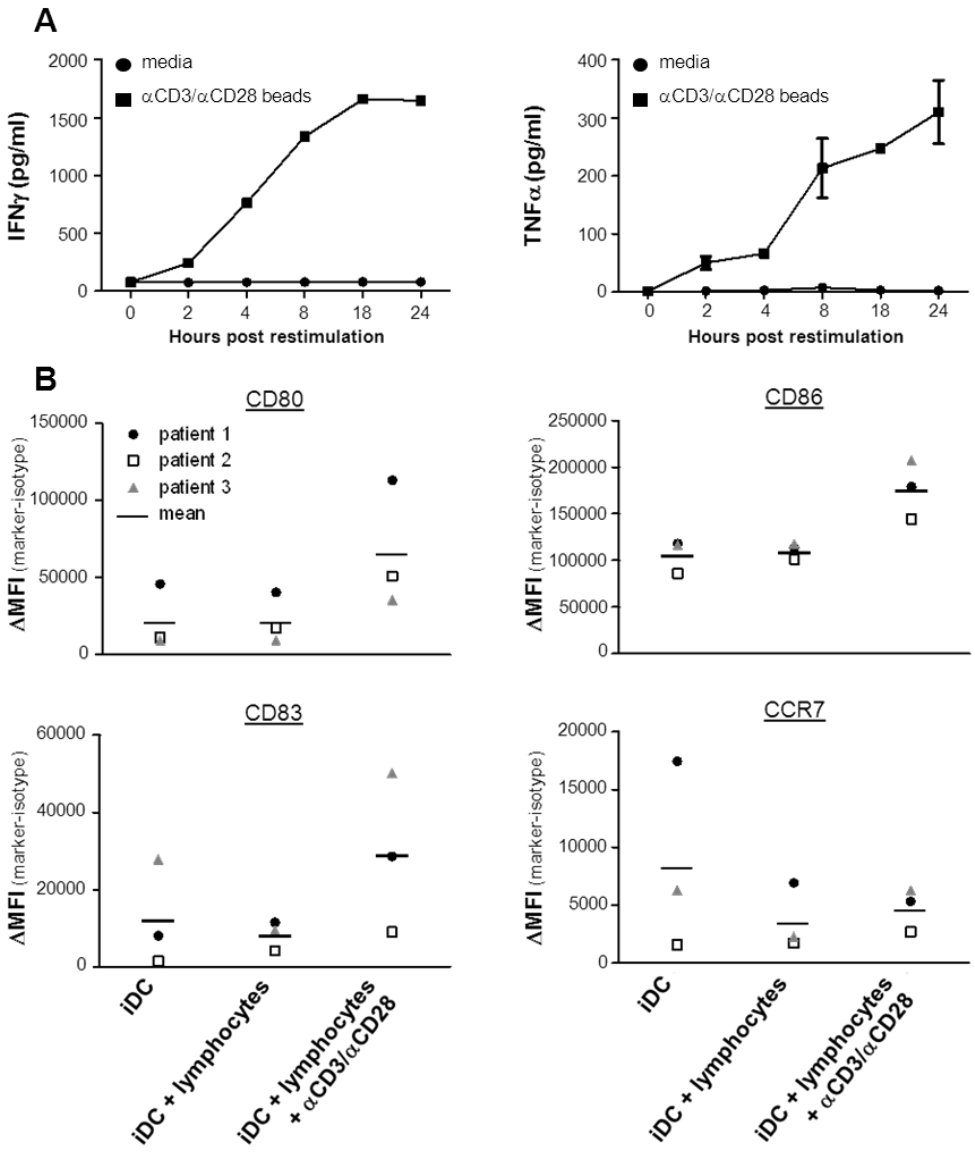
Supplementary figure 3. Blood isolated memory CD8$^+$ T cells require stimulation by polarized DC1s for secondary CTL differentiation.

Circulating memory CD8$^+$CD45RO$^+$CCR7$^+$ T cells were flow sorted from peripheral blood and stimulated with polarized DC1s or standard DCs. After 3 days, the cells were analyzed for cytolytic potential, using a standard 4 hour 51Cr release assay. Similar data was obtained in 3 independent experiments.



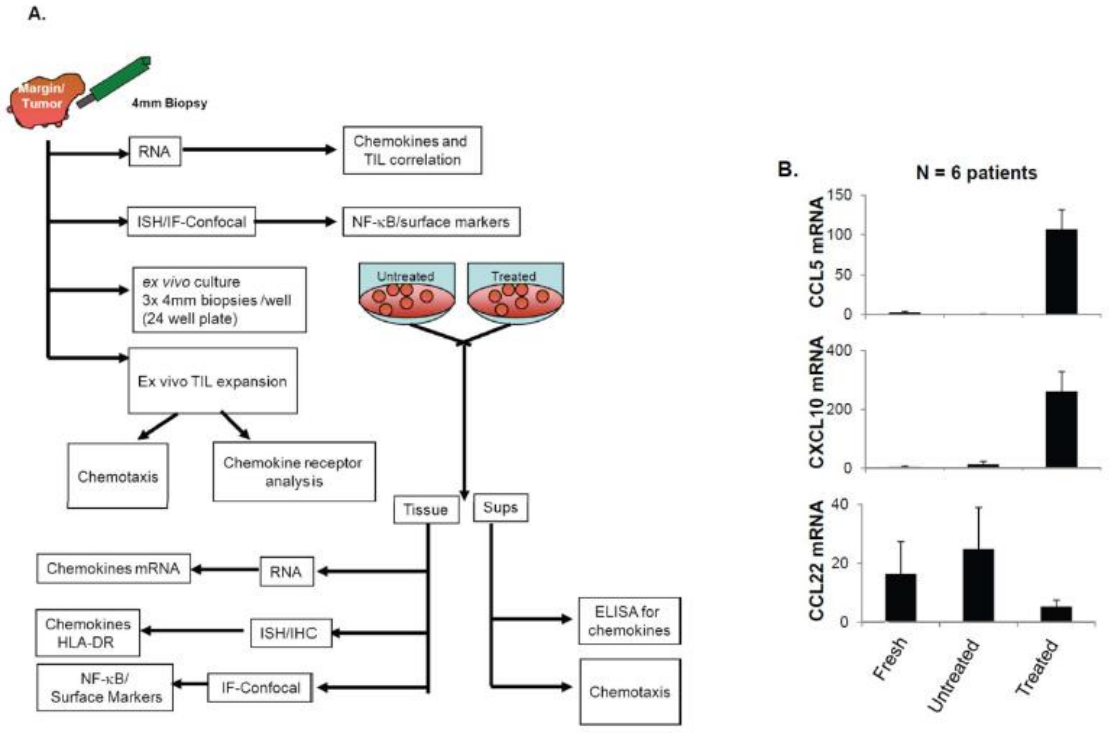
Supplementary figure 4. Activation of bulk (previously-primed and naïve) $CD8^+$ T cells by differentially matured DCs results in the induction of killing capacity.

Bulk $CD8^+$ T cells, normally consisting of 60% $CD45RA^+$ $CD8^+$T cells and 40% $CD45RO^+$ $CD8^+$ T cells, were isolated from blood using negative selection. Cultures of dendritic cells and T cells were supplemented with 50U/ml IL-2. The cytolytic capacity of DC1- and standard DC-primed bulk $CD8^+$ T cells against SEB-pulsed JY-1 cells was determined 5 days later using a standard 4 hour ^{51}Cr release assay. Data from 1 of 2 experiments that yielded similar results.



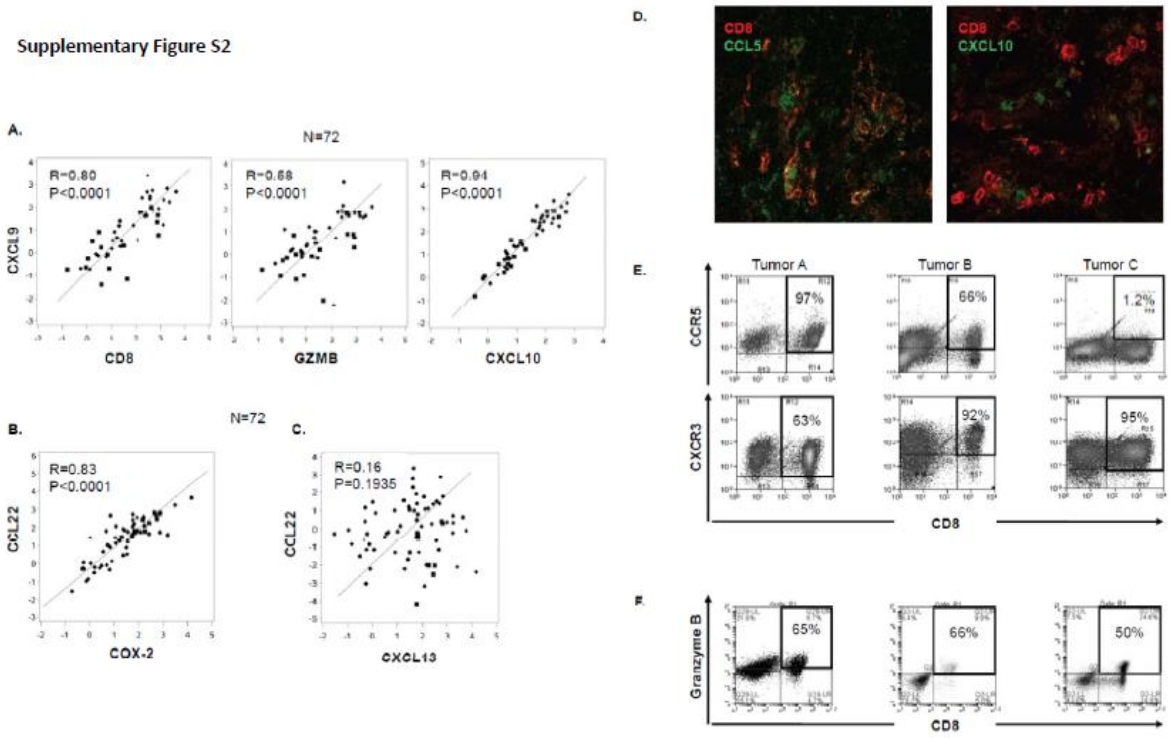
Supplementary figure 5. Re-stimulated lymphocytes from melanoma patients induce DC maturation and polarization.

Lymphocytes isolated from the blood of melanoma patients were expanded for 7 days with αCD3 and αCD28 coated beads. A) Day 7 expanded lymphocytes were harvested and either left in complete media (circles) or were re-stimulated (squares). At the indicated times, supernatant was collected to determine the secretion of IFNγ (left) and TNFα (right). B) iDCs were cultured for 24 hours with autologous day 7 expanded lymphocytes in the presence or absence of re-stimulation stimuli. 24 hour matured DCs were collected, washed and rested overnight in fresh media at 37C. After resting, the phenotype of the DCs was determined by flow cytometry.



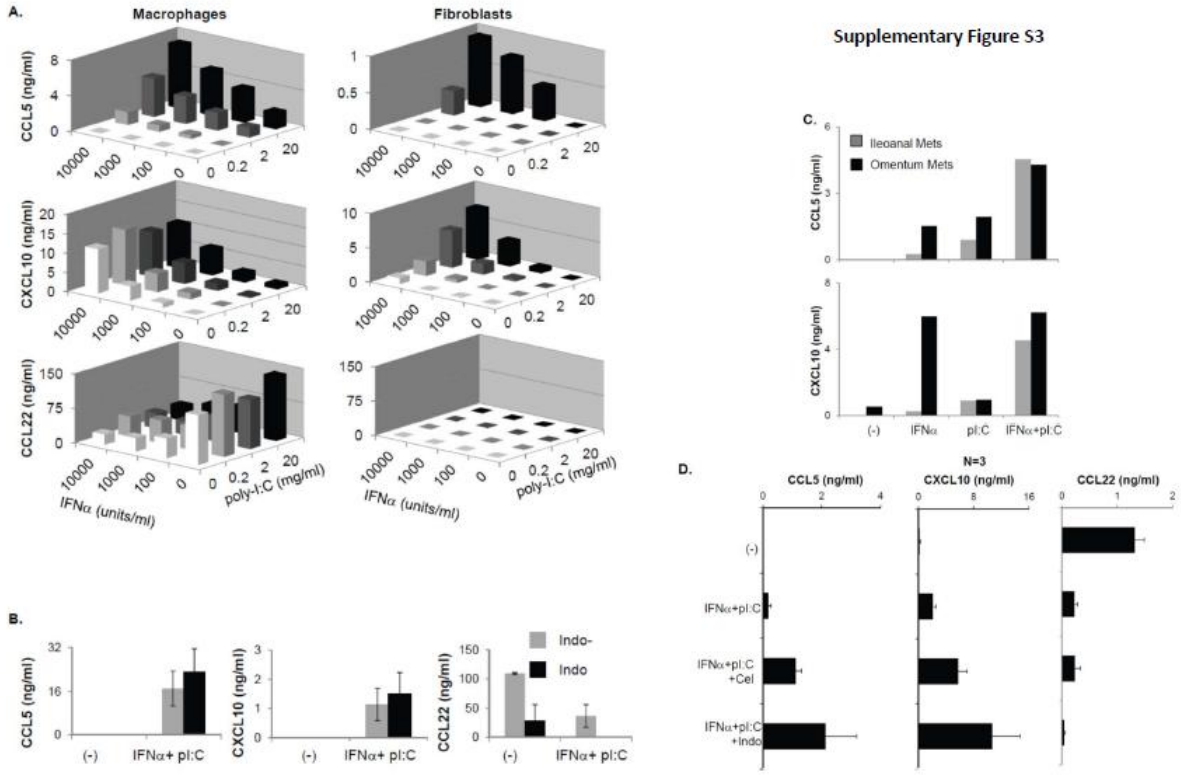
Supplementary figure 6. Processing of tumors and marginal tissues.

(A) Workflow of tissue processing for *ex vivo* explant culture and subsequent functional analyses. Ex vivo culture was done for 30 min for NF-κB, analysis, 24hours for RNA and 48hours for ELISA (B) CCL5, CXCL10 and CCL22 mRNA expression in tumors (n = 6 tumors from 6 different patients) was analyzed in freshly-harvested tumor tissue (fresh) or in tumor tissues cultured for 48 hrs in medium alone (untreated) or in the presence of indomethacin + IFNα + poly-I:C (treated). The treated and untreated tumor samples were harvested simultaneously at 48 hrs of culture. Note that the 48 hr-long cultures did not significantly affect the spontaneous pattern of chemokine production in the (untreated) tumor tissues compared to fresh tumor samples ($P>0.05$ for all three chemokines).



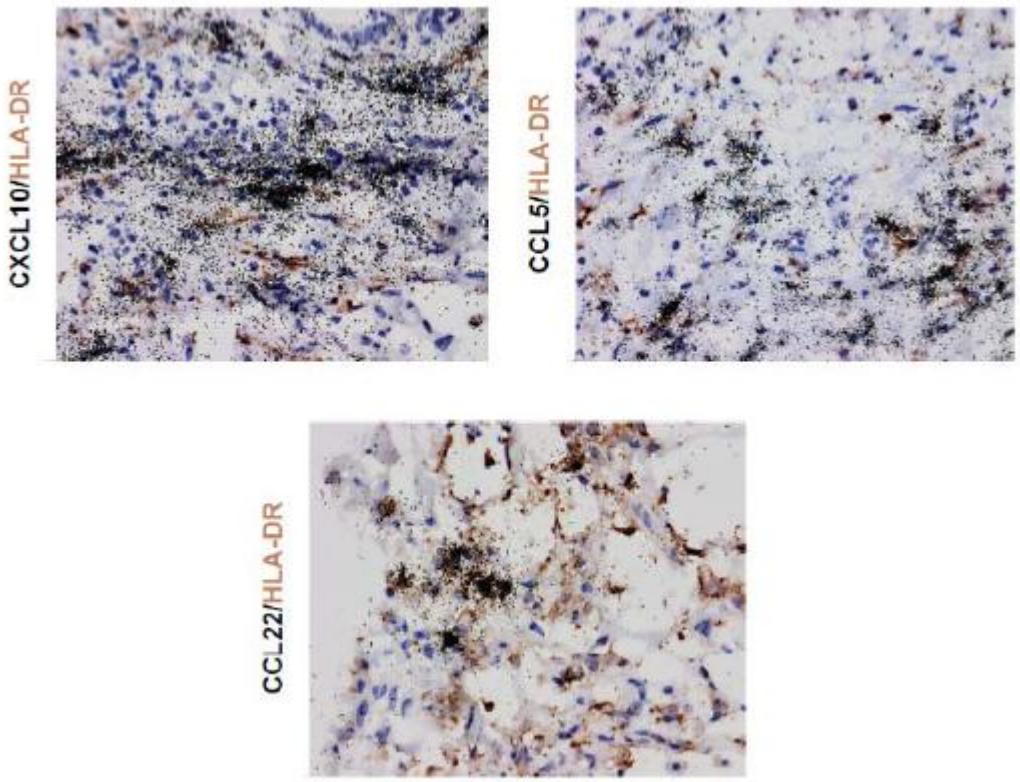
Supplementary figure 7. Presence of T_{reg} and T_{eff} markers in tumors correlate with intra-tumoral expression of T_{eff}- and T_{reg}-attracting chemokines.

(A) Expression of an alternative CXCR3 ligand, CXCL9, is correlated with local expression of CXCL10 and with T_{eff} markers, CD8 and GZMB. (B) Correlation between CCL22 and COX-2 (C) Example: Lack of correlation between CCL22 and CXCL13. (D) Confocal analysis of CD8, CCL5 and CXCL10 expression in colon tumor sections. Expression of CXCR3, CCR5 (E) and Granzyme B (F) in CD8+ TILs grown from tumor biopsies of 3 different patients. The biopsies were cultured for 2-3 weeks in 1000 units of IL-2 to obtain TILs (See M&M).



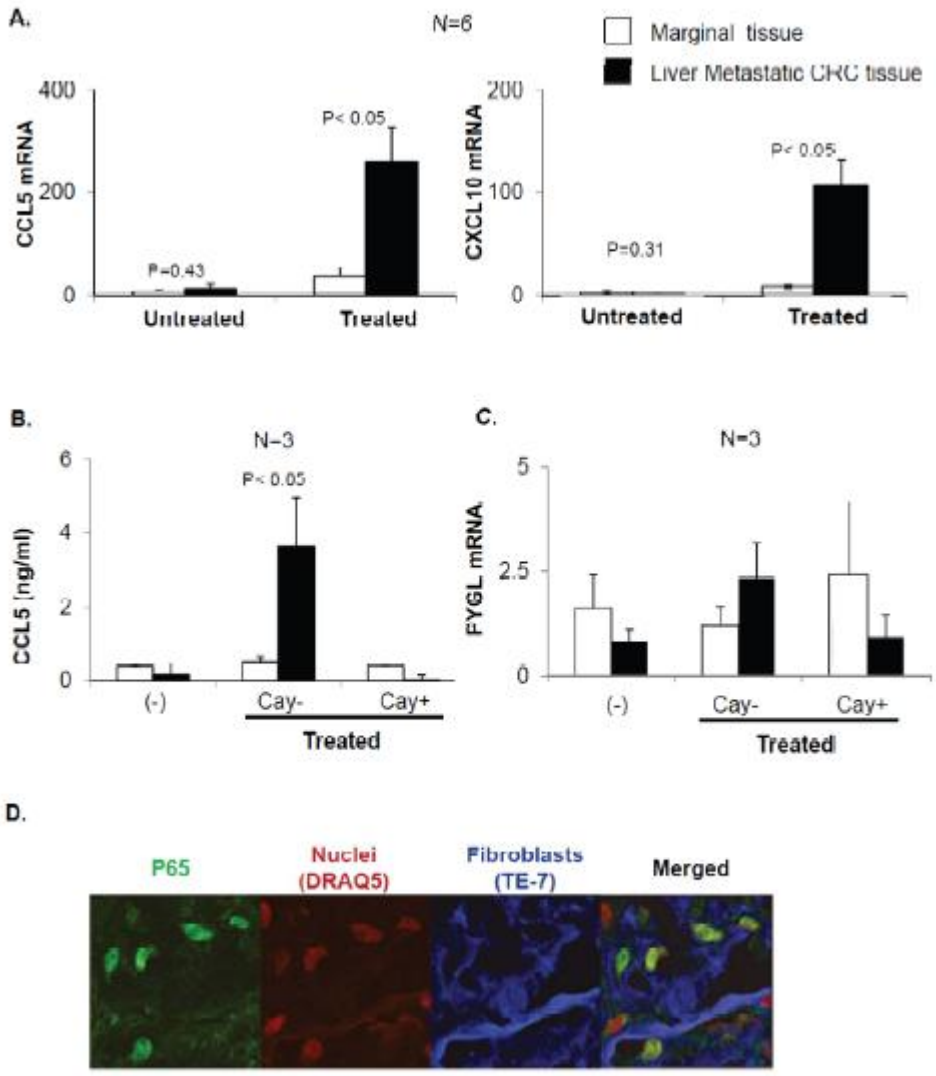
Supplementary figure 8. Combination of indomethacin, IFNα and poly-I:C induces the optimal pattern of chemokine expression in isolated cell cultures.

(A) Dose-dependent impact of IFNα and poly-I:C on the production of T_{eff}-, and T_{reg}-attracting chemokines by *in vitro* generated macrophages (see M&M) and in fibroblasts (obtained from Cascade Biological). Data from one representative experiment of three. (B) Effects of indomethacin on T_{eff}-, and T_{reg}-attracting chemokines produced by macrophages (N=3) by ELISA analysis. The concentrations of chemokines in 48 hr cultures were analyzed by ELISA. Note the suppression of CCL22 production by indomethacin. (C-D): Combination of IFNα poly-I:C and cyclooxygenase blockade is needed for the optimal and consistent modulation of chemokine production in metastatic colorectal cancer lesions. (C) Heterogeneous response of different tumor lesions from the same patient (CCL5 and CXCL10 production) to the individual components of the chemokine-modulating cocktail. (D) Indomethacin and celecoxib enhance the IFNα/poly-I:C-induced T_{eff}-attracting chemokine expression, but suppress T_{reg}-attracting chemokine expression in colorectal cancer lesions. All cultures were for 48hr. Combined data from the tumors of 3 different patients (n=3).



Supplementary figure 9. CCL22 is predominantly expressed by HLA-DR^{+} APC whereas CXCL10 and CCL5 are expressed by both HLA-DR positive and negative cells.

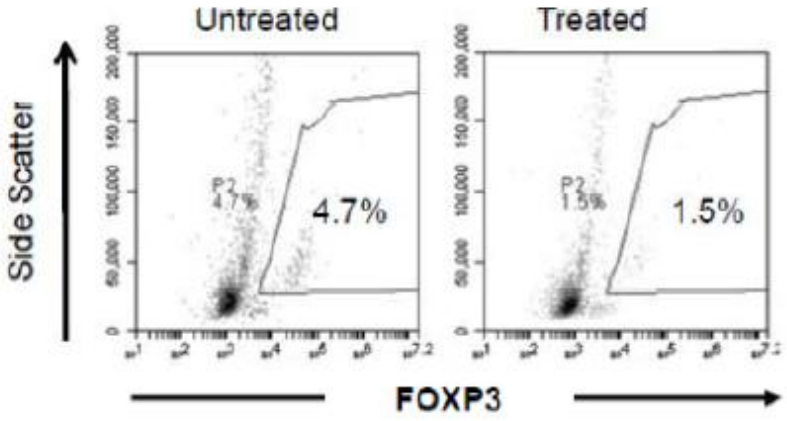
Immunohistochemistry analysis of HLA-DR+ protein (brown staining) and in-situ hybridization analysis of chemokine mRNA (black silver grains) in tumor tissues. To achieve the optimal levels of expression of each of the three chemokines, CXCL10 and CCL5 expression was analyzed in the treated tissues, while CCL22 expression was in untreated tissues.



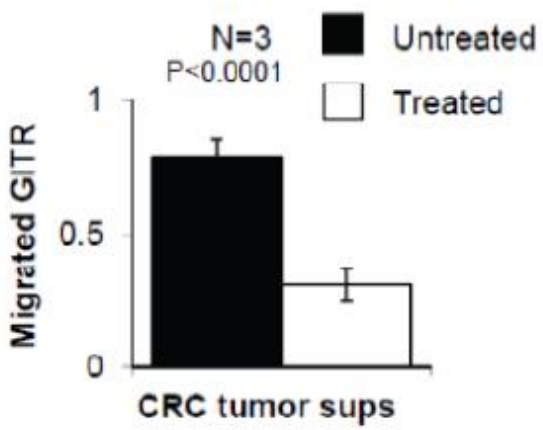
Supplementary figure 10. Elevated expression of CXCL10 and CCL5 in liver metastases compared to normal liver tissues: role of NF-κB.

Matched samples of marginal liver tissues and liver-metastatic colorectal cancer tissues (3 biopsies in 1ml in 24 well plate), were cultured for 24hrs either untreated or treated with IFNα+ poly-I:C + indomethacin and (A) analyzed for CCL5 and CXCL10 expression by Taqman (see matched protein data in Fig 4). (B) Tissues were untreated or treated with IFNα+ poly-I:C + indomethacin in absence or presence of 20μM CAY10470 (NF-κB inhibitor). The supernatants were analyzed for CCL5 production by ELISA (see matched CXCL10 data in Fig 4). (C) CAY10470 (20μM) effects on liver glycogen phosphorylase mRNA expression in matched marginal liver tissues and liver-metastatic colorectal cancer. (D). Example: single and composite images of p65 nuclear translocation in fibroblasts.

A.



B.



Supplementary figure 11. Tregs are preferentially attracted by untreated tumors.

Negatively-isolated total CD4+ T cells were allowed to migrate towards the supernatants from either untreated or treated tumor tissues. Treg migration was analyzed either by (A) flow cytometry analysis of the percentage of $FOXP3^+$ cells or (B) Taqman analysis of GITR mRNA in migrated cells.

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