COMBINATIONAL CYTOKINE THERAPY OF CANCER: PLEIOTROPIC IMPACT WITHIN THE TUMOR MICROENVIRONMENT

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Studies demonstrating the ability of *in vitro* generated dendritic cells (DCs) to successfully mediate anti-tumor efficacy when used as therapeutic vaccines suggest that treatments capable of promoting *in situ* DC-mediated cross-priming events may exhibit at least a comparable degree of clinical effectiveness. As a result, I assessed whether optimizing the number of DCs within the tumor microenvironment would improve the cross-priming of tumor-reactive T cells, resulting in improved therapeutic benefit. I observed that the treatment of CMS4-bearing BALB/c mice with the combination of Flt3 ligand (FL) and GM-CSF for five sequential days is sufficient to optimize the number of tumor-infiltrating DCs and to result in the enhanced systemic priming of tumor-specific CD8+ T cells that are consequently recruited into the tumor microenvironment. Despite these preferred immunologic endpoints, combinational FL and GM-CSF treatment failed to impact the growth of established CMS4, RENCA or CT26 tumors. The observation that large numbers of CD4+ T cells also infiltrate tumors in mice treated with combinations of FL and GM-CSF prompted me to explore whether CD4+ regulatory T cells might play an active role in mediating the suppression of co-infiltrating tumor-specific CD8+ T cells. Indeed, I found that nearly half of the tumor-associated CD4+ T cells expressed the Foxp3 protein and significantly suppressed the proliferation of naïve allo-reactive CD4+ T cells and the IFN-γ production by tumor-specific CD8+ T cells *in vitro.* Moreover, I found that combinational FL and GM-CSF treatment induced significant expansion of Foxp3+CD4+ T cells in the spleens of treated animals, regardless of tumor-bearing status. Consistent with the

suppressive effects of tumor-associated CD4+ T cells on combined FL and GM-CSF-based therapy, the *in vivo* depletion of CD4+ T cells resulted in a marked inhibition of tumor growth in treated mice that was dependent on the presence of CD8+ T cells. My findings have important implications in cancer therapy as they demonstrate that suppression mediated by regulatory T cells can present a major roadblock for the successful implementation of immunotherapeutic approaches to treat cancers.

TABLE OF CONTENTS

AKNOWLEDGEMENTS ix
1. INTRODUCTION 1
1.1. Dendritic Cells 1
1.1.1. Subsets 2
1.1.2. Origin and Development 3
1.1.3. Recruitment and Migration 5
1.1.4. Antigen Capture 7
1.1.5. Antigen Processing 8
1.1.5.1. MHC Class I Antigen Processing Pathway 9
1.1.5.2. MHC Class II Antigen Processing Pathway 10
1.1.6. Maturation 12
1.1.7. T-Cell Priming 15
1.2. DC Dysfunction in Cancer 18
1.3. Role of CD8+ T Cells in Anti-Tumor Immune Responses 22
1.4. Role of CD4+ T_H1/T_H2 Cells in Anti-Tumor Immune Responses 26
1.5. Role of CD4+ Regulatory T Cells (Tregs) in Anti-Tumor Immune Responses 31
1.5.1. Initial Characterization 31
1.5.2. Subsets 32
1.5.3. Phenotype of Treg Cells 35
1.5.4. Mechanisms of Treg-mediated immune suppression 39
1.5.5. Role of Treg cells in tumor immunity 46
1.6. DC-Based Immunotherapy for Cancer 50
2. Combinational rFlt3-Ligand and rGM-CSF Treatment Promotes Enhanced Tumor Infiltration by Dendritic Cells and Anti-Tumor CD8+ T Cell Cross-priming, but is Ineffective as a Therapy 55
2.1. ABSTRACT 55
2.2. INTRODUCTION 56
2.3. MATERIALS AND METHOD 58
2.3.1. Mice 58
2.3.2. Tumor Establishment 58
2.3.3. Combinational Cytokine Therapy 59
2.3.4. Analysis of Single-cell Preparations from Tumor and Spleen 59
2.3.5. Flow Cytometry 60
2.3.6. Confocal Immunofluorescence Staining and Metamorph Quantitation 60
2.3.7. In Vitro Activation of DC 61
2.3.8. Analysis of DC stimulation of alloreactive T cells in vitro 62
2.3.9. DQ-Ovalbumin Uptake Assay 62
2.3.10. In Vitro Stimulation (IVS) of C8+ T Cells 63
2.3.11. ELISA 63
2.3.12. Statistical Analysis 64
2.4. RESULTS 65
2.4.1. Combined treatment of mice with rFL + rGM leads to an increase in systemic DC numbers in vivo 65

2.4.2. Significantly enhanced infiltration of tumor tissues by DC is observed in mice treated with both rFL + rGM 66
2.4.3. Optimal levels of DC infiltration into the tumor or spleen tissues occur with slightly different kinetics 67
2.4.4. The enhancement of CD8α+ dendritic cells in the spleen of rFL + rGM treated mice is not mirrored in the tumor 68
2.4.5. rFL + rGM induced TIDC exhibit apparently normal phenotype and function ... 69
2.4.6. TIDC produce higher levels of IL-10 rather than IL-12p70 upon activation in vitro 71
2.4.7. TIDC mediate soluble antigen uptake and processing and effectively stimulate allogeneic T cell responses in vitro 72
2.4.8. rFL + rGM treatment results in the systemic cross-priming of anti-tumor T cells and enhanced T cell infiltration of tumor lesions in situ 73
2.4.9. The therapeutic impact of rFL and rGM treatment is minimal in controlling tumor growth in vivo 75
2.5. DISCUSSION 76
3. Treatment-Enhanced CD4+Foxp3+GITR^{Hi} Regulatory Tumor Infiltrating T Cells Limit the Effectiveness of Cytokine-Based Immunotherapy 82
3.1. ABSTRACT 82
3.2. INTRODUCTION 84
3.3. MATERIALS AND METHOD 87
3.3.1. Mice 87
3.3.2. Tumor Establishment 87
3.3.3. Combinational Cytokine and Depletion Therapy 88
3.3.4. Preparation of Single-Cell Suspensions from Tumor and Spleen 88
3.3.5. CD4+ and CD8+ T Cell Isolation 89
3.3.6. Flow Cytometry 89
3.3.7. Immunofluorescence Staining and Imaging 90
3.3.8. Allogeneic Mixed Leukocyte Reaction (MLR) 91
3.3.9. In Vitro Stimulation (IVS) of C8+ T Cells 92
3.3.10. ELISA 92
3.3.11. RNA Isolation 93
3.3.12. Reverse Transcriptase PCR (RT-PCR) 93
3.3.13. Statistical Analysis 94
3.4. RESULTS 95
3.4.1. Significantly enhanced infiltration of CMS4 tumors by CD4+T cells after treatment with rFlt3-L (rFL) + rGM-CSF (rGM) 95
3.4.2. A high proportion of CD4+ TILs isolated from rFL + rGM treated mice display a regulatory T cell phenotype 96
3.4.3. CD4+ TILs isolated from rFL + rGM treated mice express an activated/memory phenotype consistent with that of regulatory T cells 97
3.4.4. rFL + rGM treatment significantly expands CD4+Foxp3+ regulatory T cells in the spleen and results in high level infiltration of the tumor by CD4+Foxp3+ TILs 98
3.4.5. CD4+ TILs isolated from rFL + rGM treated mice suppress alloreactive CD4+ T cell proliferation and IFN-γ secretion by tumor-specific CD8+ T cells 100

3.4.6. CD4+ TILs from rFL + rGM treated mice express higher levels of TGF-β and IL-10 mRNA transcripts. 101
3.4.7. In vivo depletion of CD4+ T cells results in significantly improved anti-tumor efficacy of rFL + rGM therapy that is mediated by CD8+ T cells 103
3.5. DISCUSSION 106
4. SUMMARY AND CONCLUSIONS 120
5. FIGURES 130
APPENDIX A 151
ICOS ligand (B7RP-1) and CD40 expression on dendritic cells 151
APPENDIX B 152
Indoleamine 2,3-dioxygenase (IDO) expression by TIDCs 152
APPENDIX C 153
The effect of TLR7 agonist imiquimod on tumor growth 153
APPENDIX D 154
Cytokine secretion profile of TIDCs and SPDCs 154
APPENDIX E 155
Chemokine secretion profile of TIDCs and SPDCs 155
BIBLIOGRAPHY 156

LIST OF FIGURES

Figure 1. Combined treatment with rFlt3 Ligand (FL) and rGM-CSF (rGM) expands more splenic CD11c+ dendritic cells (DC) than either treatment alone. 130
Figure 2. Treatment of mice with combinations of rFL and rGM also increases tumor infiltration by CD11c+ dendritic cells (TIDCs). 131
Figure 3. Five days of treatment with rFL + rGM results in optimal TIDC numbers. 132
Figure 4. The vast majority of CD11c+ TIDC co-express the CD11b, but not the CD8α or B220 markers. 133
Figure 5. CD11c+ TIDCs exhibit the surface phenotype of mature dendritic cells and do not exhibit a "suppressed" or highly apoptotic phenotype. 134
Figure 6. Immunofluorescence staining of tumor sections confirms the CD11b+CD11c+ (Figure A) and I-A^d+CD11c+ (Figure B) phenotype of TIDCs. 135
Figure 7. Both TIDCs and SPDCs isolated from rFL + rGM treated mice produce higher levels of IL-10 rather than IL-12 p70 when cultured with maturation stimuli. 136
Figure 8. TIDCs are more efficient than SPDCs in mediating soluble antigen uptake and processing and are capable of inducing alloreactive T cell proliferation. 137
Figure 9. rFL + rGM treatment results in enhanced infiltration of specific CD8+ T lymphocytes within CMS4 tumors. 138
Figure 10. The impact of rFL+ rGM-CSF treatment on the growth of tumors *in vivo* is minimal. 139
Figure 11. Tumors in mice treated with rFL + rGM display increased infiltration by CD11c+ dendritic cells, CD8+ and CD4+ T cells. 140
Figure 12. The majority of CD4+ TIL in rFL + rGM treated mice express a $GITR^{HI+}CD4^+$ regulatory T cell phenotype and half of these cells co-express CD25. 141
Figure 13. Most CD4+ TIL in rFL + rGM treated mice exhibit an activated/memory phenotype consistent with Treg cells. 142
Figure 14. rFL + rGM treatment significantly expands CD4+Foxp3+ regulatory T cells in the spleen and the infiltration of the tumor by CD4+ TILs of which more than 50% are Foxp3+. 143
Figure 15. CD4+ TILs from cytokine-treated mice suppress alloantigen-specific T cell proliferation and are functionally antagonized by anti-GITR mAb. 144
Figure 16. CD4+ TIL from cytokine-treated mice suppress IFN-γ secretion by anti-CMS4 CD8+ T cells isolated from rFL + rGM treated, tumor-bearing mice. 145
Figure 17. CD4+ TIL in cytokine-treated mice express transcription factor *foxp3*, IL-10 and TGF-β mRNA and *in situ*, IL-10 protein is detected in tumor sections. 146
Figure 18. *In vivo* depletion of CD4+ T cells results in slowed tumor growth in rFL + rGM treated mice (but not untreated mice) only during the depletion period. 147
Figure 19. The maintenance of the *in vivo* depletion of CD4+ T cells results in a prolonged, significant inhibition of tumor growth in rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent. 148
Figure 20. The maintenance of the *in vivo* depletion of CD4+ T cells results in a prolonged, significant inhibition of tumor growth in rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent. 149
Figure 21. The maintenance of the *in vivo* depletion of CD4+ T cells results in prolonged survival of rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent. 150

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1. INTRODUCTION

1.1. Dendritic Cells

The ability of the immune system to provide antigen-specific protective immunity is dependent on its capacity to appropriately recognize antigens and orchestrate the activation of the necessary effector cells. This recognition is dependent on the antigen's uptake, processing and presentation by professional antigen presenting cells (APCs). Though dendritic cells (DCs), B-cells, and macrophages can serve as APCs both in vivo and in vitro, DCs have been identified as having the unique and most potent capacity in priming antigen-specific naïve T lymphocytes (1, 2). DCs are far more efficient than other APCs in stimulating T cells because of their ability to express high levels of major histocompatibility complex (MHC)-peptide complexes and co-stimulatory molecules, to secrete high levels of stimulatory cytokines and to form stable clusters with T cells (1, 2). Since T cell priming occurs in secondary lymphoid organs, such as the spleen and the lymph nodes, DCs further serve the essential role of antigen transporting cells by conveying antigens from their "residence" in peripheral tissues of the body to the T cell rich areas of the regional lymphoid organs. The recognition of peptides presented in the context of major histocompatibility complex (MHC) molecules on the DC surface by T lymphocytes that express the T cell receptor (TCR) specific for the peptides leads to T cell activation, proliferation, and subsequent differentiation into effector and/or memory cells that are suitably equipped to carry out their immune defense duty. As a result, DCs play a central function in initiating the immune system's response required to defend and cleanse the body from the dangers posed by diverse bacterial, viral, parasitic or fungal infections, as well as other diseases, such as cancer. However, in addition to their protective role against pathogens and cancer, DCs

are also responsible for antigen presentation that results in the undesired activation of the immune system in cases of autoimmunity, allergy, and graft rejection.

1.1.1. Subsets

Dendritic cells are scarcely (<1%) found cells that are identified by their characteristic morphology of hair-like cytoplasmic projections—called dendrites—extending from their plasma membrane surfaces (3). They are a heterogeneous population of cells found in different areas of the body: in non-lymphoid tissues, such as the skin, in lymphoid tissues, such as the spleen, lymph node, and Peyer's patches, in interstitial spaces of organs, such as the heart and kidney, and in afferent lymph and peripheral blood (3). DCs may be identified by different names based on their anatomic localization. DCs that are resident in the epidermal layer of the skin are known as Langerhans' cells and they migrate to the regional lymph nodes when induced by inflammation or infection. DCs that are found in the blood or are in the process of migrating to lymph nodes through the afferent lymph are termed veiled cells because of their "extensively ruffled membrane" (3). DCs that are localized in the T cell rich regions of lymphoid organs are called interdigitating cells.

Murine DCs are identified by their surface expression of CD11c in combination with CD8α, DEC-205 (CD205), CD4 and CD11b markers. Based on the expression of these markers, the heterogeneity of DC populations found in the mouse varies among the thymus, spleen, and lymph nodes. Though the level of CD8αα expression is widely variable, mouse thymus appears to contain a major homogenous population of CD8α+CD205+CD11b− DCs and a minor population of B220+CD11c^{medium} cells known as plasmacytoid dendritic cells, pDCs (4, 5). The spleen contains B220+CD11c$^{intermediate/low}$ pDCs (6-8), CD8α+CD205+CD11b− (previously

known as “lymphoid” DCs) and CD8α−CD205−CD11b+ (previously known as “myeloid” DCs) DC subsets although the CD8αα−CD205−CD11b+ DCs are further divided into two populations that are either positive or negative for the CD4 molecule (9, 10). In addition to these DC subsets in the spleen, most lymph nodes also contain dendritic cells that are CD4−CD8α−CD205^{low}CD11b+ (11). Apart from other lymph nodes, subcutaneous lymph nodes further contain a DC population that is CD8α$^{-/low}$ CD4−CD205^{High}CD11b+, which is considered to be Langerhans’ cells that have migrated from the epidermis via the lymphatics (11). In addition to the DC subsets described above, lymph nodes draining certain internal organs, such as the lung, (but not subcutaneous or mesenteric lymph nodes) contain DCs that are CD4−CD8α−CD205+ CD11b− (12). In humans, two general populations of dendritic cells can be identified in the blood based on the expression of the CD11c and CD123 molecule on their cell surface. DCs that have a monocytoid appearance and are CD11c+CD123^{low} are called “myeloid” DC (or DC1) while those that exhibit antibody-producing plasma cell-like morphology and are CD4+CD11c−CD123^{High} are called “plasmacytoid” DC (or DC2) (13, 14).

1.1.2. Origin and Development

Despite their phenotypic heterogeneity, all known DC lineages in vivo are believed to be derived from hematopoietic progenitors in the bone marrow and migrate to peripheral tissues via the blood. Traditionally, murine DCs were subdivided into at least two general populations based on precursor origin and cell surface phenotypes: “lymphoid” and “myeloid”. “Lymphoid” DCs were thought to share a common lymphoid-committed precursor with T-, B-, and natural killer (NK) cells (15, 16). This was because the earliest ‘low-CD4’ T cell precursors isolated from adult mouse thymus were shown to give rise to T-, B-, and dendritic cells but not myeloid

cells when transferred into irradiated mice (15). Further, only DCs and T cells continue to be generated upon the transfer of the next down stream CD4–CD8–CD44+CD25+ precursor population suggesting that lymphoid DCs might be more closely related with T cells than with B or NK cells (16). On the contrary, "myeloid" DC subsets were so-called because they were shown to be derived from myeloid-committed progenitors that also had the capability to give rise to granulocytes and macrophages (17). However, this broad classification for murine DCs is no longer accepted to indicate hematopoietic origin since it has recently been demonstrated that both "lymphoid" and "myeloid" DCs can arise from both myeloid and lymphoid progenitors within the bone marrow (18, 19). Additional recent studies have also shown that early precursors for all DC subtypes are found within fms-like tyrosine kinase-3 (Flt-3) receptor expressing precursor populations within the bone marrow regardless of their common lymphoid or common myeloid lineage origin (20, 21). In these studies, it was shown that the administration of Flt-3 ligand (FL) results in the expansion of both conventional dendritic cells (CD8α+ and CD8α− DC) and plasmacytoid pre-dendritic cells (p-preDC) from the bone marrow Flt3+ myeloid and lymphoid precursor populations (20, 21) although the lymphoid precursors were the most efficient precursors for the generation of DCs (21). Even though surface markers are important in distinguishing DC subset populations, there seems to be some plasticity in terms of their expression on the surface of the DCs during their differentiation from precursors to immature DCs to terminally differentiated mature DCs (22). Some studies suggest that as DCs mature, they might gain the expression of the CD8α molecule, thus making it a marker of maturation rather than lineage specification (23-25). For example, Merad et al. showed that CD8α− Langerhans' cells generated in vitro from a CD8 wild-type mouse and injected into the skin of a CD8α knockout mouse upregulated CD8α expression when they reached the draining

lymph node (24). Martinez del Hoyo et al. also showed that intravenously-injected, highly-purified splenic CD8α– DCs upregulated CD8α, CD205, and CD24 expression while down-regulating CD11b, F4/80, and CD4 expression (23).

In vitro, murine CD8α–B220–CD11b+ DCs can be generated from bone marrow and peripheral blood mononuclear cells (PBMC) in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) or GM-CSF and interleukin-4 (IL-4) (26, 27). Plasmacytoid DCs can be generated from bone marrow cells in the presence of Flt3 ligand (25, 28), which upregulate CD8α when treated with interferon-α (IFN-α) or lipopolysaccharide (LPS) during the final 24 hours of culture (25, 28). In humans, myeloid DCs can be generated from peripheral blood monocytes with GM-CSF and IL-4 (29, 30) or from bone marrow or umbilical-cord blood CD34+ hematopoietic progenitor cells cultured in the presence of GM-CSF and TNF-α (31). Large numbers of plasmacytoid DC can also be generated from CD34+ hematopoietic stem cells by the synergistic effect of Flt3-L and thrombopoietin (32).

1.1.3. Recruitment and Migration

Dendritic cell recruitment into peripheral tissues and secondary lymphoid organs is regulated by chemokines. Chemokines are low molecular weight (8-10 kDa) proteins that direct the migration and chemotaxis of cells that express the G-protein coupled receptors towards cells/tissues that express those chemokines. They are generally classified into two functional groups: inflammatory and lymphoid (33). Inflammatory chemokines, such as RANTES (CCL5) and MIP-1α (CCL3), are not normally expressed in normal tissues but can be induced during tissue injury or infection in order to recruit cellular infiltrates to those sites (33). In contrast, lymphoid chemokines, such as MIP-3β (CCL19) and SLC (CCL21) are constitutively expressed

within lymphoid organs and are involved in lymphocyte trafficking and the maintenance of lymphoid organ structure (33). During their different stages of differentiation, DCs may express varying sets of chemokine receptors on their cell surface and thus be responsive to different chemokine gradients. Immature DCs express "inflammatory" chemokine receptors, such as CCR5, CCR2, CCR6, CXCR1, and CXCR2, and are recruited to inflamed tissues in response to inflammatory chemokines released by non-immune cells, such as keratinocytes in the skin, due to tissue injury or infectious assault (34). As immature DCs are induced to mature due to exposure to inflammatory cytokines, such as TNF-α, and microbial products, such as LPS, the expression of CCR5 is down-regulated while the expression of CCR7 is up-regulated (35-38). CCL19 and CCL21, the two chemokine ligands of CCR7 expressed on mature DC, are involved in mediating DC migration from inflamed tissues to lymph nodes and to their co-localization with T cells (35-38). In addition to maturation status differences, DC migration patterns might also differ based on the type of the DC subset. Colvin et al have reported that mature CD8α+ DCs appear to be inferior compared to mature CD8α− DCs in their ability to migrate *in vitro* to the CC chemokines CCL19 and CCL21 (39). Further, a recent study has shown that plasmacytoid DC exhibit a distinct trafficking pathway that differs from conventional (CD8α+ and CD8α−) DCs in that they can enter lymph nodes directly from the blood and not through the afferent lymphatics (40). This entry was shown to occur through transmigration across high endothelial venules (HEVs) in a CXCL9- and E-selectin-dependent manner (40).

In addition to inflammation- and infection-induced migration, DC also undergo steady-state migration into lymph nodes under normal, non-inflammatory conditions and may contribute to immune tolerance to self antigens (41). The upregulation of CCR7 on immature DC—without

inducing maturation by the upregulation of MHC class II or CD86 molecules— following uptake of apoptotic cells might play a role in the migration of "tolerogenic" DC to lymph nodes (42).

1.1.4. Antigen Capture

Immature DCs are uniquely equipped to sample the tissue environment of their residence and to acquire antigens that can be presented to T cells in lymphoid organs. To this end, DCs employ a variety of mechanisms to capture and internalize antigens for processing and presentation on MHC class I and/or class II molecules. In a constitutive process known as macropinocytosis, immature DCs can continuously acquire soluble proteins and fluid from the external environment (43). DCs can ingest large particulate antigens, such as bacteria (17), viruses or virus-like particles (44) and intracellular parasites (45), in a process known as phagocytosis. DCs can also employ receptor-mediated uptake for the internalization of exogenous antigens. DC endocytic receptors include C-type lectin receptors, such as mannose receptor and DEC-205, for glycosylated protein uptake (46, 47), immunoglobulin F_c receptors (Fcγ I and II) for uptake of antigen-antibody immune complexes (48), and scavenger receptors, such as CD91 and LOX-1, for capturing heat shock proteins (HSPs) and associated peptides or proteins (49, 50).

Furthermore, immature DCs possess the capacity to obtain cell-associated antigens directly from other cells. Harshyne et al have demonstrated that DCs can acquire cellular material from live non-apoptotic cells, including other DCs, by a membrane "nibbling" process that appears to be mediated by a class A scavenger receptor (51, 52). Apoptotic and necrotic cells can also be taken up by DCs (53, 54) via the αvβ3 or αvβ5 integrins and CD36 receptors (55, 56). Recently, it has been reported that DCs can induce apoptosis of tumor cells via

multiple cytotoxic TNF family ligands—TNF, lymphotoxin-α1β2, Fas ligand, and/or TNF-related apoptosis inducing ligand (57, 58)—and enhance cross-presentation of tumor-associated antigens (59). DCs have also been shown to efficiently take up exosomes—60-90-nm membrane vesicles—constitutively released by tumor cells, and to capture the tumor antigens contained in these structures (60). Immature DCs generated *in vitro* express abundant empty MHC class II molecules on their cell surfaces that do not appear to contain bound peptides (61). These empty MHC II molecules are stable at physiological temperature and are receptive to exogenous peptide binding, allowing for the collection of peptides directly from the extracellular medium for presentation to T cells without internalization or further processing (61). The peptides that are loaded on the empty molecules are believed to be released to the extracellular medium due to cellular lysis or to be generated at sites of inflammation by proteases released by DCs, macrophages and/or granulocytes that process intact proteins into peptides outside of cells (61, 62). However, many of the antigen capture mechanisms described above were demonstrated to occur in *in vitro* settings and it is not yet clear which mechanisms actually contribute to DC antigen presentation function *in vivo*.

1.1.5. Antigen Processing

Once DCs have acquired antigens, they process them for presentation to CD4+ and/or CD8+ T cells. Since CD8+ and CD4+ T cells recognition is dependent on peptides presented in the context of MHC class I and II molecules, respectively, acquired antigens are processed in the following two major pathways for loading onto the corresponding MHC molecules.

1.1.5.1. MHC Class I Antigen Processing Pathway

DCs process endogenous antigens—synthesized within DCs themselves—in a TAP (transporter associated with antigen presentation) dependent processing pathway for "direct presentation" of antigens to CD8+ T cells, also generally termed cytotoxic T lymphocytes (CTLs). Since most cells can also process endogenously synthesized proteins for presentation on their cell surface, direct presentation is not unique to DCs. However, DCs can also capture and process exogenous antigens (such as bacterial, viral, or tumor antigens) into the MHC class I pathway via a process called "cross-presentation" or "cross-priming" (63). Even though cross-presentation has been reported to occur in B cells, endothelial cells, and macrophages, the major cell type involved in this process is the DC (63).

The first step in the direct presentation pathway is the processing of endogenously-produced proteins into peptide fragments by the proteasome in an ATP- and ubiquitin-dependent mechanism. These peptides, generated in the cytosol, are then transported into the endoplasmic reticulum (ER) through ATP-dependent TAP proteins located in the ER membrane. Newly-synthesized MHC class I molecules are retained in the ER in a partially folded state in a coordinated process that involves the accessory proteins calnexin, calreticulin, and tapasin (64). However, their folding gets completed once peptides that have been translocated by the TAP complex into the ER bind to their peptide-binding cleft and stabilize the assembly of the α (heavy) chain with the invariant β_2-microglobulin (light) chain (64). MHC class I-peptide complexes are then competent to exit the ER and to be transported to the cell surface through the Golgi apparatus. In normal, uninfected DCs, peptides derived from self-proteins are involved in the folding and thus are presented at the cell surface. However, when DCs are infected with pathogens that replicate in the cytosol (65) or exogenous antigen is introduced directly into their

cytosol (66), in addition to self peptides, peptides derived from pathogens or introduced antigens will also bind to MHC class I molecules in the ER and ultimately be presented at the cell surface.

The mechanisms involved in DC cross-presentation are not yet as clear as that for direct presentation described above. Recent studies suggest that cross-presentation may occur in a compartment resulting from the fusing of ER-derived vesicles with phagosomes containing phagocytosed extracellular antigen(s) (67-69). These ER-phagosome compartments are self-sufficient for antigen presentation in that they contain nascent MHC class I molecules, TAP, and the accessory proteins (calreticulin calnexin, and ERp57) involved in peptide loading (67-69). It appears that the targeting of newly-synthesized, empty MHC class I molecules to the phagosome is dependent on a tyrosine residue in the cytoplasmic tail of the molecules since cross-presentation (but not direct presentation) is affected by tyrosine mutation (70). The compartments also contain a protein complex known as Sec61 that seems to play the important role of translocating the phagocytosed antigens from the ER-phagosome compartments into the cytosol for proteosomal degradation into peptides (67, 71). These peptides are then transported back into ER-phagosome compartments through the membrane-associated TAP complex (67-69).

1.1.5.2. MHC Class II Antigen Processing Pathway

The function of this processing pathway is the presentation of exogenous antigens and microorganisms acquired from the extracellular milieu by macropinocytosis, phagocytosis, and receptor-mediated endocytosis. These internalized antigens are contained within vesicles called phagosomes (or endosomes) and fuse with lysosomes—vesicles that contain acid proteases—to form vesicles called phagolysosomes. During the formation of phagolysosomes, the pH of the

vesicles decreases and results in the activation of the acid proteases, which function optimally at low pH (72). The activated proteases, such as cathepsin S, then degrade the internalized protein antigens contained in vesicles into peptide fragments that can be loaded onto MHC class II molecules (72). MHC class II molecules are synthesized in the rough ER but are typically prevented from binding peptide by the Invariant chain (Ii) that binds in the peptide binding groove of MHC class II molecules (73). The Ii also plays the additional role of directing the vesicles containing the MHC class II molecules to the endosomal pathway that results in their fusion with phagolysosomes (73). This fusion results in the cleavage of the Ii into short fragments by proteases, such as cathepsin S, to allow for peptide binding to the MHC class II molecules in what are called MHC class II compartments (MIIC) (74). Since Ii cleavage leaves it's residual small peptide fragment called CLIP (class II-associated invariant-chain peptide) in the peptide binding groove, CLIP removal and antigenic peptide binding is catalyzed by an MHC class II-like molecule called H-2M in mice (HLA-DM in humans) (75). It is not yet known whether H-2M removes the CLIP directly out of the groove or induces a conformational change in the MHC class II molecule that allows for CLIP to fall out of the groove. Finally, the MHC class II-peptide complexes are transported to the plasma membrane for recognition by CD4+ T cells. This transport to the cell surface appears to occur via tubular endosomes extending intracellularly and polarizing toward the interacting T cell (76, 77).

In addition to the two major protein processing pathways, DCs also possess the capability to process and present endogenous (such as sphingolipids and phosphatidylinositols) or pathogen-derived (such as mycolic fatty acids expressed by *Mycobacterium tuberculosis*) lipid antigens on MHC class I-like CD1 molecules (78). Mouse DCs express one CD1 protein (i.e. CD1d) whereas human DCs can express five (i.e. CD1a-e) (79). This processing of fatty acids,

glycolipids, and lipopeptide antigens for recognition by CD1-restricted CD8+ and CD4+ T cells, as well as NKT cells, is believed to occur in specialized intracellular compartments similar to that of MHC class II antigen processing pathway in a TAP-independent manner (78). This CD1 presentation pathway appears to be involved in not only in microbial immunity (80), but also in anti-tumor immunity (81) and autoimmune diseases (82).

1.1.6. Maturation

Generally, DCs are found in two functional states: immature and mature DCs. Immature DCs are highly-efficient at antigen endocytosis, but express low levels of MHC class II, co-stimulatory (such as CD80, CD86, CD40), and adhesion molecules (such as CD54) on their cell surfaces. In these DCs, MHC class II molecules are found in intracellular compartments rather than at the cell surface (83). Thus, immature DCs are comparatively poor at mediating antigen presentation and co-stimulation and lack strong immunostimulatory potential. On the contrary, the maturation of DCs is accompanied by the loss of phagocytic/endocytic capability (43), but increased cell surface expression of MHC class II, co-stimulatory, and adhesion molecules. Upon maturation, DCs also undergo dramatic morphological changes from being "stellate" in appearance to having multiple, large processes (dendrites) protruding from their cell body, yielding an increased surface area on which T cells can sample MHC-presented peptides (2). The pattern of chemokine receptor expression is also altered upon the maturation of DCs. That is, the expression of CCR1 and CCR5 is down-regulated and the expression of CCR7 is up-regulated (37, 38). In addition to these changes observed at the cell surface, DC maturation is also marked by the synthesis and secretion of high-levels of cytokines (such as IL-12p70, and TNF-α) that can regulate the magnitude and functional type of T cell immune responses being

generated as a consequence of DC-mediated stimulation. However, the profile of cytokine produced by DCs depends on the type of activating stimuli being used as well as the subtype and origin of the DC being stimulated (78). For example, in mice, the CD8α+ DCs are the major producers of IL-12 p70, whereas plasmacytoid DCs are the predominant producers of type I IFN (IFNα/β) upon stimulation (6, 84, 85). All the changes described above converge to transform the immature DCs into highly-immunostimulatory mature DCs.

Several stimulatory factors have been shown to induce DC maturation. Microbial and viral products characterized by pathogen-associated molecular patterns (PAMPs) stimulate DCs by engaging their pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) (86). To date, DCs have been shown to express ten TLRs (TLR1-TLR10) that differ in their expression pattern among DC subsets and in their recognition of different sets of ligands (86). For example, TLR9 mediates the recognition of bacterial and viral DNA containing unmethylated CpG motifs (87); TLR4 mediates the recognition of LPS (88); and TLR3 mediates the recognition of double-stranded RNA (89). Ligation of TLRs triggers cascades of intracellular signaling pathways that result in the induction of proinflammatory cytokines, chemokines, type I interferon (IFN-α or -β) and maturation of DCs (90).

Inflammatory cytokines, including TNF-α (91), IL-1β/-1α (91), and IFN-α (92), have also been shown to activate DCs by acting through the corresponding receptors expressed by immature DC. In addition to microbial stimuli, inflammatory cytokines can also serve as "danger signals" in inducing increased migration of DCs from peripheral tissues to the lymph nodes in addition to their undergoing the process of maturation (93, 94). Activation through the ligation of CD40 molecules is also a strong inducer of DC maturation and immunogenecity both *in vitro* (95, 96) and *in vivo* (97, 98). The ligation of CD40 by either anti-CD40 antibody or

CD40L has been shown to be very effective in inducing high-level production of bioactive IL-12p70 and a marked up-regulation of adhesion and co-stimulatory molecules (95, 96). Furthermore, activated CD4+ T cells expressing CD40L can activate DCs through CD40 cross-linking (99-101). The ligation of CD40 by CD40L expressed by CD4+ T cells "conditions" DCs to acquire powerful T cell stimulatory capability (99-101). In addition to T cell-derived activation signals, there is also a bi-directional cross-talk between DCs and NK cells in which DCs can activate NK cells and NK cells in return can induce DC activation and production of IL-12 (102, 103). NK cell mediated DC activation seems to be dependent on soluble factors such as TNF-α and IFN-γ as well as cell contact through surface receptors such as NKp30 in human NK cells (104). Moreover, Sauter et al have shown that exposure to necrotic tumor cell lines (but not apoptotic tumor cells or necrotic lysates of primary cells) or their supernatants can provide maturation signals to DCs (105). Further analysis demonstrated that supernatants isolated from necrotic tumor cell lines (but not supernatants of primary cells) are enriched in the heat shock proteins (hsp)70 and gp96, and that purified hsp70 (106) and gp96 induce human DC maturation (107). The ability of HSPs to activate DCs has also been observed in mice (108), where it was shown that immunization of mice with gp96 induces DC maturation and their migration to draining lymph nodes (109). The DC receptors for HSPs have not yet been clearly defined, but may include CD40 (110) and TLRs (111, 112).

In addition to the immature and mature states of DC described above, Lutz and Schuler have proposed a stage of DC development that they termed "semi-mature" (113). These "semi-mature" DCs are MHCII^{high}, costimulation^{high}, cytokines^{low} compared to the "fully mature" DCs that are MHCII^{high}, costimulation^{high}, cytokines^{high} (113). This is distinction is necessary to make because DC activation stimuli yield considerably different impacts on DCs and thus both

phenotypic changes and IL-12 p70 production associated with the maturation of DCs may not occur upon stimulation with varying stimuli. Menges et al reported that DCs matured in the presence of TNF-α and administered *in vivo* acted in a tolerogenic fashion by inducing CD4+IL-10+ regulatory T cells (Tregs) and blocking autoimmune encephalomyelitis (EAE) (114). On the contrary, the administration of DCs matured in the presence of LPS and anti-CD40 did not block EAE and resulted in the induction of CD4+ T_H1 cells (114). Furthermore, Akbari et al (115) and McGuirk et al (116) reported the induction of Tregs *in vivo* by DCs that appear to be mature. In all three cases, the tolerogenic DCs exhibited high-levels of expression of MHC class II and co-stimulatory molecules but low/no production of proinflammatory cytokines, such as IL-12 p70, TNF-α, IL-1β and IL-6 (114, 116, 117). However, these DCs produced the anti-inflammatory cytokine IL-10 (116, 117).

1.1.7. T-Cell Priming

In order to be activated to proliferate and differentiate into effector cells, CD8+ and CD4+ T lymphocytes require at least two independent signals delivered by DCs. Signal one is delivered to T cells through the engagement of their T cell receptor (TCR) by peptide presented in the context of the appropriate MHC molecules on the surface of DCs. CD8+ T cells are activated by MHC class I-peptide-complexes where as CD4+ T cells are activated by MHC class II-peptide complexes. Signal two is provided to T cells by costimulatory molecules expressed on DCs. T cells express the CD28 molecule that serves as a co-receptor for the CD80 and CD86 costimulatory molecules expressed by DCs. Adhesion molecules, such as ICAM-1 expressed by DCs and its ligand LFA-1 on T cells, also play a significant role in forming stable interactions between the DC and T cells. Once a T cell recognizes the peptide presented by the DC and

receives a signal through the TCR, the TCR-peptide-MHC complexes form a structure known as the immunological synapse by segregating into discrete areas of the membrane (118). In the immunological synapse, the TCR-peptide-MHC complexes localize in the central area while adhesion and costimulatory molecules localize in the peripheral area and form what is known as supramolecular activation clusters (SMAC) (118). This clustering leads to the activation of intracellular protein tyrosine kinases that transmit the activation signal downstream of the cell surface. If a T cell receives signal one but not signal two by the same DC, the T cell generally fails to be activated and becomes anergic (119). This anergic state is marked by a lack of T cell responsiveness even when the specific antigen recognized by the TCR is presented on DCs that are capable of providing signal two (119). In cases where a T cell recognizes peptide with high avidity on a DC expressing low levels of co-stimulatory molecules, the T cell might undergo a brief period of proliferation without developing effector function (120, 121). In this case, the T cell might be deleted or the small numbers that survive stimulation might become anergic (120, 121).

As a result, antigen presented on immature DCs in the absence of activation signals leads to T cell tolerance rather than immunity (97, 98). Hawiger et al and Bonifaz et al showed that *in vivo* antigen delivery to DCs via targeting through the DEC-205 receptor (without DC activation stimuli) induced transient antigen-specific T cell activation that was followed by the deletion and unresponsiveness of the activated T cells (97, 98). However, when DC activation was induced by the co-administration of anti-CD40 agonistic antibody, the outcome of the immune response was marked by prolonged T cell activation and immunity rather than tolerance (97, 98). Further, immature DCs can also induce antigen-specific peripheral tolerance by expanding regulatory T cells that suppress the responses of effector cytotoxic and helper T lymphocytes (122, 123).

Mahnke et al. reported that DEC-205 targeted delivery of antigen to steady-state DCs led only to short-lived proliferation of antigen-specific T cells but it also induced CD4+CD25+ T cells with regulatory properties (123). In another study Dhodapkar et al. reported that immunization of healthy subjects with antigen-pulsed immature DCs led to the inhibition of antigen-specific CD8+ T cell effector function and the appearance of antigen-specific IL-10 producing cells (122). In contrast to the results with immature DCs, injection of mature DCs induced the rapid expansion of antigen-specific and functional effector CD4+ and CD8+ T cells (124). In summary, the maturation of DCs through exposure to activating stimuli results in the upregulation of MHC-peptide complexes and co-stimulatory and adhesion molecules and leads to the acquisition of a powerful capacity to prime and stimulate T cell immunity.

In addition to signal 1 and 2, DCs are also known to provide a third signal (i.e. polarization signal) to responder T cells that directs their functional polarization towards T_H1 or T_H2 cells (125, 126). The presence or absence of IL-12p70 production is considered to be the major factor responsible for the ability of stimulating DCs to drive this helper T cell polarization (125, 126). The presence of IL-12 polarizes toward T_H1, whereas its absence, polarizes toward T_H2 (125, 126). This is likely due to IL-12p70's known capacity to serve as the most efficient inducer/enhancer of IFN-γ production, which promotes the growth and differentiation of T helper precursors into T_H1 cells and antagonizes T_H2 cell development (127). In mice, CD8α DCs, but not CD8α− DC or pDC have been shown to be the major producers of bioactive IL-12p70 (84, 85). The i*n vivo* administration of CD8α+ DCs (84) or their dramatic expansion *in vivo* by Flt3-L treatment (128) has been shown to preferentially induce T_H1-type immune responses. In contrast, the i*n vivo* administration of CD8α− DCs (84) or their selective expansion by polyethylene glycol-modified GM-CSF (128) preferentially induces T_H2-type immune

responses. Consistent with the requirement for the presence of IL-12p70 in T_H1 polarization, the injection of CD8α+ DCs obtained from IL-12-competent but not IL-12-deficient mice induced a polarized T_H1 response when injected into wild-type animals (84). Furthermore, the administration of antigen-pulsed CD8α− DCs resulted in the development of T_H1 polarized immune response if recombinant IL-12p70 was co-administered (84). In humans, myeloid DCs are the major producers of IL-12p70 and these APCs preferentially induce T_H1 differentiation, whereas pDC preferentially drive T_H2 responses (129).

1.2. DC Dysfunction in Cancer

Despite the expression of antigens by tumor cells that can potentially be recognized by T lymphocytes that are capable of mediating their rejection, many murine and human tumors continue to grow uncontrolled in the absence of discernable immune responses (130). In light of the high efficiency of DCs to present exogenous and cell-associated antigens and prime T cells, several studies have suggested that defects in full DC function in tumor-bearing mice and cancer patients may contribute to the ability of tumors to escape from immune recognition by interfering with DC-mediated presentation of tumor associated antigens to tumor-specific T cells (131-133). Consistent with this view, Gabrilovich et al (131, 132) and others (134, 135) have shown that when DCs were directly isolated from cancer patients or tumor-bearing mice and compared with DCs from healthy controls for their ability to simulate peptide-specific CTLs or allogeneic T cells, they exhibited a significantly reduced antigen presentation and T cell stimulation capability. However, if T cells were isolated from the same cancer patients or tumor bearing mice and stimulated with DCs isolated from normal controls, they mounted the same level of T cell responses as normal control T cells (131, 132). Furthermore, several studies have reported

that cancer patients and tumor-bearing mice have decreased numbers of mature DCs than normal controls (131, 136-139). In tumor-bearing mice, the reduction in the number of mature DCs was observed in the skin, lymph nodes, and spleen (131, 136, 137). In humans, patients with squamous-cell carcinoma of the head and neck (HNSCC), breast cancer, and lung cancer patients exhibited dramatically reduced numbers of DCs in their peripheral blood (138, 139). This decrease was shown to be associated with the stage and duration of the disease, with the surgical removal of tumors resulting in partial reversal of the observed deleterious effects (138). These observations suggest that the growth of tumor *in vivo* might dramatically alter the number and functionality of DCs in such a way as to shield the tumor from effective immune attack.

Due to the heterogeneity of malignancies, there are a variety of underlying mechanisms by which tumors can induce DC dysfunction through the production of immunosuppressive factors. One such mechanism is interference with the normal hematopoietic development of DCs. The presence of vascular endothelial growth factor (VEGF)(140), IL-10 (141), macrophage colony-stimulating factor (M-CSF) (142), gangliosides (143), and IL-6 (135)—all of which have been shown to be secreted by tumors—dramatically reduce the number of murine or human DCs that can be generated *in vitro* from progenitors or precursors isolated from bone marrow or the blood. In addition, an inverse correlation has been reported between the expression of VEGF and the density of DCs in gastric adenocarcinoma tissue and in connection decreased numbers of DCs correlated with poor patient prognosis (144). A second mechanism of DC dysfunction in cancer is the inhibition of the maturation of DCs. IL-10 and TGF-β (transforming growth factor β) are two cytokines that have been implicated in down-regulating the expression of the B7 molecules and thus reducing the ability of DCs to deliver co-stimulatory signals (Signal 2) necessary for productive activation of T cell immunity (145). Shurin et al. have shown that tumor-derived IL-

10 can further down-regulate CD40 expression on DCs and preventing them form receiving T cell-derived maturation signal (146) as well as reducing their resistance to tumor-induced apoptosis (147). A third mechanism associated with DC dysfunction in cancer appears to involve interference with IL-12p70 production by DCs. DC stimulation with activation signals that are known to induce IL-12p70 production, such as CD40 ligation, in the presence of IL-10 results in the lack of bioactive IL-12 production by DCs but can be abrogated by addition of neutralizing anti-IL-10 antibody (146). Furthermore, tumor-associated MUC1 glycoprotein appears to mediate a similar effect by altering the balance of DC production of IL-12p70 and IL-10 in favor of DCs expressing an IL-10^{high}IL-12^{low} phenotype (148). The absence of IL-12p70 production, in turn, results in the lack of T_H1 lymphocyte priming (149) that is critical for control of growth or eradication of tumors. A fourth mechanism of DC dysfunction in cancer is the induction of apoptosis in DCs, leading to deficiencies in tumor associated antigen presentation either within the tumor microenvironment or in the tissue-draining secondary lymphoid organs. Gangliosides (150) and as yet unknown tumor-secreted factors (151) have been reported to induce DC apoptosis.

Consistent with the negative impact the tumor environment can have on DCs, the analysis of tumor-infiltrating DCs (TIDCs) in tumors *in situ* has generally revealed that they exhibit an immature, non-activated DC phenotype. Typically, both rodent and human TIDCs are defective in expressed or inducible B7 molecules and lack the ability to drive T cell proliferation and differentiation (133, 152, 153). However, it appears that the effects of tumor-secreted immunosuppressive factors are limited to immature DCs and that fully-matured DCs seem to be resistant to these factors (141, 154). Allavena et al. reported that the addition of IL-10 at the initiation of the culture of human monocytes promoted their irreversible differentiation into

macrophages, but not into DCs (141). However, when added to already differentiated DCs, IL-10 did not induce the differentiation of DC into macrophages (141). In addition, Beissert et al. demonstrated that *in vitro* incubation of Langerhans cells in IL-10 before exposure to rGM-CSF completely inhibited their antigen presentation capacity, whereas neither co-incubation of LC in IL-10 and GM-CSF (without pre-incubation in IL-10) nor IL-10 treatment after GM-CSF incubation affected antigen presentation (154). When evaluated for their ability to induce a secondary immune response *in vivo*, only the LCs incubated in IL-10 before pulsing with tumor-associated antigen (TAA) significantly inhibited Type-1 delayed-type hypersensitivity responses (154).

In addition to inducing DC dysfunction, tumors can also employ a variety of other mechanisms to evade the immune response (155). One such mechanism is the loss/down-regulation of MHC class I molecules or the loss/decrease of tumor antigen expression so that CD8+ CTLs will not be able to recognize and attack the tumors (155, 156). This can result from genetic alterations of genes encoding the heavy or light chain of the MHC class I molecule, interference with the antigen processing pathway responsible for loading peptides on nascent MHC class I molecules in the ER and transporting them to the cell surface, or mutation/loss of genes that encode tumor antigens during tumor progression (155). However, the absence of MHC class I expression on the surface of tumor cells can also be a disadvantage to tumors since it can make them susceptible to NK cell attack because MHC class I molecules serve as ligands for killer inhibitory receptors (KIRs) expressed by NK cells (157). Another mechanism of immune evasion is the production of immunosuppressive cytokines, such as TGF-β and IL-10, by tumors to block the expansion and/or effector function of tumor-specific T cells (155). Tumors can also affect the effector function of T cells by altering the expression of signal

transduction molecules within the T cells, including induction of a significant decrease in the expression of CD3ζ chain or $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases (155, 158). As a result, T cell internal signaling can be disrupted and lead to decreased tyrosine phospohorylation, calcium flux, and the translocation of nuclear transcription factors such as NF-kB into the nucleus to activate various genes, including proliferation and cytokine genes (155, 158). Moreover, some tumors may express Fas ligand on their cell surface and thus induce apoptosis in activated tumor-infiltrating T lymphocytes that express Fas (155, 159).

1.3. Role of CD8+ T Cells in Anti-Tumor Immune Responses

CD8+ T cells or cytotoxic T lymphocytes (CTLs) are critical players in the cellular arm of the adaptive immune response. They recognize short 8-10 amino acid long peptides presented by MHC class I molecules through their TCR and the co-receptor glycoprotein CD8, which specifically recognizes and binds to an invariant part of the MHC class I (but not MHC class II) molecules (160). CTLs mediate their effector function by recognizing, attacking, and lysing pathogen infected cells, as well as, malignant cells. In their pathogen control role, CTLs provide protection against bacteria, viruses, and parasites that replicate in the cytoplasm of the host cell, since these intracellular pathogens are not accessible to antibody-mediated destruction. CTLs can distinguish cells infected by intracellular pathogens from normal uninfected, cells because the pathogen's foreign proteins are processed and presented on the infected cells surface along with the host's endogenous proteins (see section **1.1.5.1**). In their anti-tumor role, CTLs have been shown to recognize and lyse tumors and to mediate lesional regression *in vivo*. Even though tumors express mainly "self" antigens, CTLs appear to preferentially target malignant cells (but spare normal neighboring cells) based on molecular changes have been acquired during

their transformation (130, 161). Tumor-associated antigens that have been shown to be recognized by CTLs include (130, 161): 1) **Viral antigens**: tumors induced by viruses may express viral antigens and thus present foreign peptides at their cell surface. These antigens are called tumor-specific antigens (TSA) since they are not present in non-tumor cells. For example, cervical carcinomas express the antigens E6 and E7 that are derived from the inducing human papilloma virus (HPV). 2) **Caner/testis (CT) antigens**: antigens expressed only in tumors with the exception of normal male germ cells in the testis. These antigens are a result of the reactivation of genes in tumors that are usually inactive in normal cells. The MAGE, GAGE, BAGE, and NY-ESO-1 families of proteins are good examples of CT antigens. 3) **Differentiation antigens**: represent antigens expressed in tumors from a unique cell type. Most of the defined differentiation antigens are melanocyte differentiation antigens, such as MART-1, tyrosinase, and gp100, expressed in nomal melanocytes and melanomas. 4) **Widely-expressed or overexpressed antigens**: the genes encoding these antigens are widely expressed in a variety of normal tissues and in many histologically unrelated tumors. Many of these antigens are expressed in normal tissues at very low levels while they are overexpressed in tumor cells. Examples of these antigens include wild type p53 and MUC1. 5) **Tumor-specific antigens**: antigens that arise in tumor cells due to mutations of normal genes. Some mutations occur only in a single tumor while other mutations occur in multiple tumors and may play a role in carcinogenesis. Examples of these antigens include mutated p53 and H- and K-ras. 6) **Fusion proteins** (161): antigens derived from proteins translated from the fusion of distant genes resulting from chromosomal translocation involved in tumorogenesis. The *bcr-abl* fusion gene in chronic myeloid leukemia (CML) is expressed as BCR-ABL fusion protein and is responsible for both the induction and progression of CML.

The capture and processing of the above described antigens by DCs is important in generation of epitopes that can be cross-presented and be recognized by tumor-specific CTLs (162). Effector CTLs generated in secondary lymphoid organs traffic to the tumor site and lyse tumors cells via direct cell-to-cell contact based on the recognition of epitopes expressed on tumors cells, which process endogenously expressed proteins through the MHC class I pathway (163, 164). Once they come in contact with their antigen-bearing target cells, CTLs can utilize at least two pathways to kill target cells by inducing them to undergo apoptosis: either the granule exocytosis pathway or the Fas-mediated pathway (reviewed in(165)). In the granule exocytosis pathway, the CTL releases lytic granules that contain perforin, granzymes and granulysin (only in humans) into the region of contact between the CTL and the target cell. These cytotoxic granules and their components are synthesized and stored in the cytoplasm by the CTL when it is activated to proliferate through TCR clustering due to specific-peptide binding. When the CTL recognizes the target cell in the periphery, it directs the secretion of the granules only at the antigen-bearing cell due to the reorientation of its cytoskeleton that causes it to become polarized to the point of contact with the target cell. Upon release from lytic granules, perforin monomers polymerize to form trans-membrane channels or pores on the target cell's plasma membrane. These pores allow the secreted granzymes, such as granzyme B, to enter the target cell's cytosol and initiate programmed cell death by cleaving and activating one or more members of the caspase family of proteases. These activated caspases in the target cells then cleave additional apoptotic substrates producing a proteolytic cascade that results in DNA fragmentation and apoptosis. Once apoptosis is induced in a target cell, a single CTL can be involved in the sequential killing of several other targets by detaching and reorienting its granules to another region of cell-cell contact. The importance of perforin in CTL mediated anti-

viral and anti-tumor immunity is highlighted by the absence of cytotoxic activity of CD8+ T cells from perforin-deficient mice against virus infected cells or fibrosarcoma tumor cells *in vitro* (166). *In vivo*, perforin-deficient mice were significantly more susceptible to the growth of fibrosarcoma as evidenced by the growth of tumors even when mice were inoculated with doses of tumor cells that are ten to a hundred times lower than that required to form progressive lesions in normal mice (166). In tumors that down-regulate MHC class I molecules as a means of evading immune surveillance, natural killer (NK) cells play a major role in controlling their growth by employing a similar perforin/granzyme pathway and a death receptor pathway (157). However, lytic granules in NK cells are pre-formed during their development and can be de-granulated immediately upon target cell contact (157).

Even though the granule exocytosis pathway plays a major role in the cytotoxicity of CD8+ effector cells, CTLs also employ the perforin-independent pathway mediated by Fas ligation to kill target cells (reviewed in (167)). Engagement of Fas expressed on a target cell with Fas ligand (Fas-L) expressed by CTLs results in the induction of apoptosis in the target cell since Fas contains what is known as "death domain" in its cytoplasmic tail. Fas-L induced trimerization of Fas leads to the recruitment of the adaptor protein FADD (Fas-associated protein with death domain) that binds via its own death domain to the death domain in Fas. Via its death-effector domain (DED), FADD then recruits the DED-containing procaspase-8. This results in the proteolytic cleavage and activation pro-caspase-8 and its release into the cytoplasm. The active caspase-8 then initiates a cascade of proteolytic cleavage and activation of other members of the caspase family that carry out the DNA fragmentation and apoptosis process.

While target cell apoptosis plays a major role in CTL effector function, CTLs further contribute to the immune response via the secretion of immunomodulatory cytokines, including

IFN-γ, TNF-α, and TNF-β, upon recognition of antigen on target cells (168). IFN-γ is known to up-regulate the cellular expression of MHC class I molecules and thus enhance antigen presentation to CD8+ T cells (168, 169). This increases the chance that CTLs will be able to recognize and attack pathogen infected or malignant cells as target cells during their surveillance mission (168, 169). IFN-γ also plays a direct role in inhibiting viral replication and in activating macrophages (168). The cytokines TNF-α and TNF-β act synergistically with IFN-γ to activate macrophages and kill target cells expressing TNFR-I (tumor necrosis factor receptor-I) (168).

1.4. Role of CD4+ T_H1/T_H2 Cells in Anti-Tumor Immune Responses

Most tumors express MHC class I molecules and have the capacity to present tumor-derived peptides to CTLs. However, tumors generally fail to express co-stimulatory molecules, such as B7.1 and B7.2, required for providing signal 2 for the activation of CTLs. Therefore, the activation of tumor antigen-specific CTLs and their differentiation into effector cells is largely dependent on DCs that indirectly present (i.e. cross-present) to them exogenous tumor-derived antigens in conjunction with MHC class I and co-stimulatory molecules (63, 162). This cross-priming of CD8+ T cells by DCs has been demonstrated to require the active participation of CD4+ T helper cells (99-101). Studies by several groups have shown that the CD4+ T cell "help" for the initiation of DC-mediated CTL activation is provided via the interaction of CD40 molecules on DCs with CD40 Ligand expressed by activated CD4+ T cells (99-101). Further, these studies demonstrated that in the absence of CD4+ T cells, the "help" required for CTL priming could be substituted by engaging CD40 molecules on DCs using anti-CD40 antibodies or soluble CD40L molecules (99-101). This interaction between DCs and T cells is a

bidirectional process and does not require the simultaneous presence of all three cells, i.e. DCs, CD4+ and CD8+ T cells (99). DCs initially stimulate the antigen-specific CD4+ T cells by presenting 13 to 23 amino acid long peptides through MHC class II molecules (99, 170). The activation of CD4+ T cells results in the up-regulation of CD40 ligand (CD40-L) expression on their cell surface and allows the CD4+ T cells to deliver activating signals back to the DCs (99, 170). The cross-linking of CD40 expressed on DCs by the interacting CD4+ T cell's CD40-L results in the "conditioning" of the DC to acquire powerful CTL stimulatory capability including the up-regulation of co-stimulatory and adhesion molecules, the maintenance of high levels of MHC-peptide complexes at the cell surface, and the production of significantly high levels of bioactive interleukin IL-12p70 (95, 96, 99-101).

Depending on their activation condition, precursor CD4+ T helper cells can polarize toward T helper 1 (T_H1) or T helper 2 (T_H2) cells that exhibit distinct profiles of cytokine production. T_H1 cells produce IL-2, IFN-γ and tumor necrosis factor-β ((TNF-β) and induce cell-mediated immunity by activating macrophages or CTLs (reviewed in (171). Activated macrophages more efficiently kill pathogens localized within their phagosomes while activated CTLs kill tumors or pathogen infected cells. In contrast, T_H2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and induce humoral immunity by activating B cells. Activated B cells proliferate and differentiate into plasma cells that secrete antibodies that are involved in mediating the immune response against extracellular pathogens and parasites as well as in the development of allergic responses. The exogenous cytokines that are present within the T cell priming microenvironment are critical in determining the differentiation pathway that a naïve precursor CD4+ T cell will follow (125, 126, 172). The cytokines IL-12p70 and IFN-γ are involved in inducing naïve T cell differentiation into T_H1 cells whereas IL-4 supports their development into T_H2 cells (125, 126,

172). Once naïve T cells have differentiated into T_H1 or T_H2 effector cells, they can negatively regulate the opposing helper T cell's development. That is, IFN-γ produced by T_H1 cells inhibits T_H2 cellular proliferation, while T_H1 development is blocked by IL-4 and IL-10 produced by T_H2 cells (173). Further, GATA-3—a transcription factor selectively expressed in T_H2 cells—regulates T_H2 cytokine expression and shuts down T_H1 development (174) while T-bet—a T_H1-specific transcription factor—controls the expression of IFN-γ in T_H1 cells and represses the opposing T_H2 cell development (175).

CD4+ T cells are important players in efficient and long-lasting CTL mediated anti-tumor immunity, even though most tumors do not express MHC class II molecules on their cell surfaces and thus cannot be directly recognized by effector CD4+ T cells. The lack of anti-tumor immunity in CD4+ T cell knockout or CD4+ T cell-depleted mice that are immunized with tumor-specific vaccines is strong evidence that CD4+ T cells play a critical role in mediating/supporting anti-tumor immunity. For example, Dranoff et al showed that immunization of a B16 melanoma tumor bearing mice with irradiated tumor cells expressing murine granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated potent, long-lasting, and specific anti-tumor immunity (176). However, the depletion of CD4+ T cells either before, or after, vaccination abrogated the development of CD8+ T cell-dependent anti-tumor systemic immunity (176). "Licensing" DCs that have taken up tumor-derived antigens to more efficiently activate tumor-specific CTLs is suggested to be the major mechanism by which CD4+ T cells evoke effective anti-tumor immunity (177, 178). Consistent with this "licensing" role of CD4+ T cells, Diehl et al. showed that systemic treatment with agonist anti-CD40 antibody (mimicking CD40-L expressed on CD4+ T cells) *in vivo* converted a tolerogenic CTL-epitope-based peptide vaccine into a strong CTL priming and eradication of established tumors (177).

Ossendorp et al. also reported that synergistic, long–term anti-tumor protective immune response was induced against a MHC class II-negative tumor when mice were simultaneously vaccinated with tumor-specific T helper and CTL epitopes (179). The involvement of CD4+ helper T cells in CTL-mediated responses does not appear to be at just during the CTL priming phase but also appears germane in the establishment of CTL memory (180). Once CD8+ T cells are primed to expand and differentiate into effector cells and traffic to sites of inflammation and clear the target antigen, many of them die via an apoptotic mechanism, while a few of them survive and become part of the host's antigen-specific memory pool (180). These memory T cells can rapidly re-acquire effector functions to kill tumors or pathogen infected cells as well as secrete cytokines that contribute to the overall effectiveness of the immune response (180). Consistent with the importance of CD4+ T cell help in generating a functional CD8+ T cell memory pool, Shedlock et al. demonstrated that memory CD8+ T cells generated in normal mice mounted a normal effective recall response when transferred into CD4-knockout mice (181). On the contrary, the recall response of memory CD8+ T cells generated in CD4-knockout mice and adoptively-transferred into normal mice was significantly defective (181). Similarly, Sun et al. found that even though mice lacking CD4+ T cells can generate CTL responses against *Listeria monocytogenes* (via direct activation of APCs and bypassing CD4+ T cell-mediated help) and clear the infection, the memory CTLs generated in the absence of CD4+ T cells were defective in their ability to respond to secondary challenge with *Listeria m.* (182). Mechanistically, Janssen et al. showed that T-cell help for secondary CTL expansion is "programmed" into CD8+ T cells during the priming phase and the presence of CD4+ T helper cells is not required after priming (183).

Subsequently, cytokines produced by effector CD4+ T cells play important roles in developing anti-tumor immune responses. IL-2 released by activated CD4+ T cells is an important cytokine for the recruitment and stimulation of CD8+ T cells. Fearon et al. have shown that a poorly-immunogenic murine colon cancer, that was transfected to produce IL-2, induced an anti-tumor response that protected mice from subsequent challenge with the parental tumor (184). This anti-tumor response occurred, even in the absence of CD4+ T cells, indicating that their helper role in the immune system can be bypassed by the administration of IL-2 (184). The IFN-γ produced by T_H1 CD4+ T cells is also critical for the development of anti-tumor immunity and contributes to enhanced antigen processing and presentation in DCs and tumors cells by up-regulating the expression of several molecules involved in the class I antigen processing pathway, such as MHC class I molecules, proteasome subunits and TAP (169, 177). Mumberg et al. have demonstrated that treatment of a MHC class II-negative tumor with anti-IFN-γ antibody abrogated the CD4+ T cell-mediated rejection of tumor cells *in vivo* (185). This anti-tumor response appears to occur through an indirect mechanism that depends on IFN-γ's effect on host but not tumor cells since tumors remain MHC class II negative even after stimulation with IFN-γ (185). In general, a predominantly T_H1-biased immune response by effector cells appears to be important in controlling tumor growth or mediating regression. Consistent with this paradigm, Tatsumi et al. reported that CD4+ T cell responses to peptides derived from the TAA MAGE-6 are highly-skewed toward T_H2-type responses without regard to stage of disease (186). On the contrary, the responses in normal donors and disease-free cancer patients were either mixed T_H1/T_H2 or strongly T_H1-type responses (186).

Yet, in a few tumors, CD4+ T cells can mediate their anti-tumor function via other effector pathways that do not involve CTLs (178, 187). Greenberg et al. demonstrated that the adoptive

transfer of activated CD4+ T cell clones specific for FBL-3 murine leukemia resulted in the induction of systemic anti-tumor immunity in adult thymectomized, irradiated, and T cell depleted mice (187). Pardoll and Topalian suggest that this CTL-independent CD4+ T cell mediated response may involve both tumor-specific T_H1 (activating macrophages to release reactive oxygen intermediates and nitric oxide) and T_H2 (recruiting and activating eosinophils to release toxic granule contents and free radicals) effector pathways that cooperate in tumor destruction (178). Indeed, activated macrophages and eosinophils have been found to infiltrate the site of tumor challenge in mice successfully treated with the adoptive transfer of CD4+ but not CD8+ T cells (178).

1.5. Role of CD4+ Regulatory T Cells (Tregs) in Anti-Tumor Immune Responses

1.5.1. Initial Characterization

Compared to the critical helper function of effector CD4+ T cells in conferring CD8+ T cell-mediated immunity, a small subset of CD4+ T cells known as regulatory T cells (Tregs) is involved in a paradoxical role of significantly inhibiting or suppressing host immune responses and inducing immune tolerance (188-190). These Tregs were first characterized as CD25+CD4+ T cells by Sakaguchi et al. based on the observation that the depletion of this subset of CD4+ T cells leads to a variety of organ-specific autoimmune diseases such as thyroiditis, gastritis, and insulitis (191). In Sakaguchi et al.'s experiment, when splenic or lymph node CD4+ T cells isolated from BALB/c nu/+ mice were adoptively transferred into recipient BALB/c athymic nude (nu/nu) mice, the mice did not develop autoimmune disease (192). In contrast, if the CD25+ population was first depleted of CD4+ T cells (by treatment with specific mAb and

complement before the adoptive transfer), all recipient athymic mice spontaneously developed histologically and serologically evident organ-specific autoimmune diseases similar to those seen in human organ-specific autoimmune diseases (191). However, the development of autoimmune disease in CD4+CD25− T cells recipient mice was inhibited when they were reconstituted with CD4+CD25+ cells suggesting that CD4+CD25+ T cells contribute to the maintenance of self-tolerance by down-regulating immune responses directed at self antigens (192). Asano et al. further confirmed the existence and function of Tregs when they reported that similar autoimmune diseases evident in thymectomized neonatal mice were the consequence of a developmental abnormality that reduces or eliminates the CD4+CD25+ T cell subpopulation from the periphery, leading to the activation of autoreactive T cells primed before the thymectomy (193). This thymectomy-induced autoimmune disease phenomenon was prevented when the mice were reconstituted with CD4+CD25+ splenic cells isolated from normal mice (193).

1.5.2. Subsets

Tregs recognize self-antigen peptides in the context of MHC class II molecules as evidenced by the inability of CD4+CD25+ T cells from MHC class II-deficient mice to suppress responder CD4+ T cells both *in vitro* or *in vivo* (194). CD4+ Tregs can be grouped into two general subpopulations: “naturally-occurring” or “constitutive” Tregs produced in the thymus and “inducible” or “adaptive” Tregs (i.e. Tr1 and Th3) induced from naïve CD4+ T cells in the periphery (188-190).

Naturally-occurring Tregs appear to arise in the thymus from the common double-positive (CD4+CD8+) precursor pool and require the expression of MHC class II-self-peptide complexes

on cortical thymic epithelium for their selection (194, 195). It has been suggested that since thymocytes that express a low affinity TCR are not selected to become CD25+ T cells and since high affinity self-reactive T cells are deleted by the thymus, the decision to commit to a Treg lineage seems to result from the moderate affinity of the expressed TCR for self-peptide-MHC class II molecules (195). That is, the TCR avidity that leads to Treg development appears to be in a range between positive and negative selection (195). In addition, the expression level of the self-antigen expression appears to be important in determining the level of CD4+CD25+ regulatory T cell formation (195, 196). Jordan et al. demonstrated that if transgenic mice expressing a TCR specific for hemagglutinin (HA) (TS1 mice) were mated with transgenic mice expressing HA under the control of a SV40 promoter (HA28 mice), TS1xHA28 mice contain the same numbers of S1-specific CD4+ T cells as found in TS1 mice (195, 196). However, when compared to the ~13% of HA-specific CD4+CD25+ Tregs present in TSA mice, ~50% of the HA-specific CD4+ T cells in TS1xHA28 mice were CD25+ regulatory T cells (195, 196). Treg development and expansion *in vivo* also appears to be dependent on the cytokine IL-2, since mice that are IL-2Rα− (197), IL-2Rβ− (198), or IL-2− (199) deficient develop lethal lymphoproliferation and autoimmune diseases and lack CD25+CD4+ Tregs (200). As expected, Almeida et al. showed that the adoptive transfer of normal mice CD25+CD4+ T cells into RAG-$2^{-/-}$ chimeras reconstituted with BM cells from IL-2Rα-deficient mice not only blocked the development of lethal autoimmune disease, but it also stabilized the total number of T cells to a level similar to that observed in control mice (200). In addition, B7:CD28 (201) and CD40:CD40L (202) interactions may play important roles in the homeostasis of CD4+CD25+ Tregs *in vivo*. Salomon et al. demonstrated that CD4+CD25+ Tregs are detected in markedly-reduced numbers in B7-1/B7-2-deficient and CD28-deficient NOD mice and their absence

results in the exacerbation of spontaneous diabetes (201). Kumanogoh et al reported that the CD25+CD45RB^{low}CD4+ subpopulation is markedly reduced in CD40-deficient mice and T cells from CD40-deficient mice triggered autoimmune diseases (202).

In addition to the naturally occurring CD4+CD25+ Tregs, IL-10 secreting Tregs, known as T regulator cell type 1 (Tr1), and TGF-β secreting Tregs, known as T helper 3 (T_H3), may be generated extrathymically and play an important role in the maintenance of peripheral tolerance (188-190). As previously indicated, these "adaptive" CD4+ Tregs are generally characterized by their unique cytokine production profile rather than by their expression of distinct cell surface markers and it is currently not known how these cells are developmentally related to naturally occurring Tregs (188-190). Tr1 cells were first generated *in vitro* by Groux et al. by chronic stimulation of naïve CD4+ T cells from ovalbumin (OVA) TCR transgenic mice with OVA peptide and irradiated splenic APCs in the presence IL-10 (203). These Tr1 cells exhibited low proliferative capacity, produced high levels of IL-10 (but low levels of IL-2 and no IL-4), suppressed the proliferation of antigen-specific CD4+ T cells, and protected SCID mice from colitis induced by pathogenic CD4+CD45RB^{high} T cells (203). Similarly, human Tr1 cells can be effectively generated from peripheral blood CD4+T cells after stimulation with allogeneic monocytes in the presence of exogenous IL-10 (203). T_H3 cells were first identified by Chen et al. who observed that the development of experimental autoimmune encephalomyelitis (EAE) was suppressed in SJL mice, which are susceptible to EAE after oral adminsitration of myelin basic protein (MBP) (204). T cell clones isolated from the mesenteric lymph nodes of these SJL mice produced high levels of TGF-β and variable levels of IL-4 and IL-10, but very low levels of IFN-γ and IL-2 (204). When transferred *in vivo*, these T_H3 clones cultured *in vitro* suppressed EAE induced with MBP in a TGF-β–dependent manner since injection of anti-TFG-β antibody

reversed their protective ability (204). Likewise, a significant increase in the frequencies of both MBP and proteolipid protein-specific, TGF-β-secreting T cell lines were found in multiple sclerosis patients treated with myelin (205). In a different series of experiments, Chen et al. demonstrated that naive peripheral CD4+CD25−Foxp3− T cells can be converted into anergic/suppressor cells that express Foxp3, CD25, $CD45RB^{-/low}$, intracellular CTLA-4, and membrane bound TGF-β by TCR stimulation in the presence of soluble TGF-β (206). When co-transferred with naïve responder CD4+ transgenic T cells, these TGF-β converted CD4+CD25− T cells were capable of inhibiting the antigen-specific expansion of the transgenic T cells *in vivo* (206).

1.5.3. Phenotype of Treg Cells

In the periphery, naturally occurring regulatory T cells constitute 5-10% of CD4+ T cells in both mice (191) and humans (207). Even though Tregs constitutively express the interleukin-2 receptor α (IL-2Rα) chain (i.e. CD25) on their cell surface, this does not represent an absolute and specific marker for Tregs, since recently activated non-regulatory T effector cells can also express CD25 (188-190). Likewise, other molecules that are constitutively expressed by Tregs and which may serve as potential markers for identifying these cells are also commonly associated with activated and/or memory cells (188-190). One of these molecules is CD45RB that is expressed at very low levels on Tregs (208). Indeed, Powrie et al. reported that the reconstitution of SCID mice with splenic $CD45RB^{high}$CD4+ T cells leads to the development of colitis and a lethal wasting disease, while reconstitution with reciprocal $CD45RB^{low}$ subset or unfractionated CD4+ T cells did not (208). Most importantly, if $CD45RB^{low}$CD4+ T cells were co-transferred with the $CD45RB^{high}$CD4+ T cells, the $CD45RB^{low}$ cells inhibited the wasting

disease and colitis (208). Another Treg-asscoiated molecule is cytotoxic T lymphocyte antigen-4 (CTLA-4 or CD152) that is reported to be expressed constitutively on Tregs and induced on other T cells upon activation (209). Takahashi et al. reported that CTLA-4 expression is limited to CD4+CD25+ T cells in normal mice and that *in vivo* blockade of CTLA-4 in normal mice breaks natural self-tolerance and elicits chronic organ-specific autoimmune diseases (209). Furthermore, in the presence of anti–CTLA-4 mAb in vitro, the ability of CD25+CD4+ T cells to suppress antigen-specific and polyclonal T cell activation and proliferation is abrogated (209). Glucocorticoid-induced TNF receptor family-related gene (GITR) is another marker that is detectable at very low levels on resting CD4+CD25− T cells, but is expressed at high levels on the cell surface of non-activated CD4+CD25+ and activated CD4+CD25− T cells (210, 211). Shimizu et al. demonstrated that when added to a culture of CD25+CD4+ T cells and CD25−CD4+ T cells in vitro, the agonist anti-GITR mAb (DTA-1) was able to abrogate the suppression mediated by splenic or thymocytes CD25+CD4+ T cells (210). The adoptive transfer of GITRhigh-depleted splenocytes (by treating with DTA-1 and complement) from BALB/c nu/+ mice to BALB/c nu/nu mice led to the development of autoimmune gastritis in recipient mice, with disease severity comparable to that observed in mice reconstituted with CD25-depleted splenocytes (210). Likewise, in vivo administration of DTA-1 mAb induced autoimmune gastritis in normal BALB/c mice without the in vivo depletion of CD4+CD25+ T cells that constitutively express the GITR (210).

Yet another marker that appears to be differentially expressed by Tregs and which might play an important role in regulating the suppressive capabilities of this cell population is the inducible costimulator (ICOS) moelcule, which is up-regulated on activated T cells and also found on memory T cells (212). Analysis of low, medium, or high expressors of ICOS from

secondary lymphoid organs of untreated mice indicates that ICOShigh T cells preferentially secrete IL-10, ICOSmedium T cells preferentially secrete IL-4, IL-5, and IL-13, and ICOSlow T cells preferentially secrete IL-2, IL-3, IL-6, and IFN-γ (213). In cases of autoimmunity, ICOS has been reported to be expressed on CD4+CD25+ T cells localized in the target organ and this seems essential for delivering signals to Tregs that sustain their function and create an anti-inflammatory environment (212). For instance, Herman et al. have reported that CD4+CD25+ cells in the pancreatic tissue of pre-insulitic mice express significantly higher levels of ICOS and IL-10 transcripts than the homologous cells in the draining lymph nodes (212). Indeed, they observed that the in vivo blockade of ICOS using anti-ICOS mAb treatment led to a rapid progression from early insulitis to diabetes by shifting the balance between effector and regulatory T cells toward effector T cells (212). Further, Greve et al. have demonstrated that diabetes-resistant Idd5.1 congenic mice developed more severe EAE when compared with NOD mice and this correlated with higher expression of ICOS and IL-10 production by activated NOD T cells compared with Idd5.1-derived T cells (214). Likewise, Dong et al. observed that ICOS-deficient mice are extremely sensitive to experimental allergic encephalomyelitis (EAE) when compared with wild-type mice, even on a genetically-resistant background (215). It has been suggested that the IL-10 stimulating role of ICOS appears to represent an important factor involved in regulating Treg function and the induction of T cell tolerance in autoimmunity (213). Consistent with this hypothesis, Akbari et al. showed that the induction of Treg development by mature pulmonary DCs isolated from the bronchial lymph nodes of mice exposed to respiratory allergen required T-cell costimulation via the ICOS−ICOSL pathway (115).

Furthermore, various investigators have reported the variable but constitutive expression of one or more of the following molecules on natural Tregs: CD62L (L-selectin), CD134 (OX-40),

CD103 (αε integrin), Neuropilin-1—a receptor involved in axon guidance, angiogenesis, and the activation of T cells—as well as, the chemokine receptors CCR2 (in mice), CCR4 and CCR8 (in humans) (211, 216-218).

While no cell surface marker has been identified that uniquely identifies the Treg subset, the recently-defined intracellular transcription factor Foxp3 appears crucial to Treg development and function (219-221). Foxp3 (fork-head box P3) is a member of the diverse winged helix-forkhead family of transcriptional regulators and has been shown to serve as a master switch in Treg differentiation and function (219-221). In humans, mutations in the gene encoding Foxp3 leads to a multi-sytem autoimmune disorder known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance) characterized by symptoms such as lymphadenopathy, thyroiditis, and early-onset insulin dependent diabetes mellitus (222). Similar lethal autoimmune disease is observed in scurfy mutant mice (X-linked frameshift mutation in the gene encoding Foxp3) and in Foxp3-defecient mice and was demonstrated to result from a deficiency of suppressive function in CD4+CD25+ Tregs, but not from a cell-intrinsic defect in CD4+CD25− T cells (219, 223). Consistently, the development of autoimmune diseases has been shown to be inhibited upon the adoptive transfer of CD4+CD25+ but not CD4+CD25− T cells isolated from normal mice into the Foxp3-deficient mice (219). Fontenot et al. further demonstrated the requirement of Foxp3 in Treg development by analyzing the origin of CD4+CD25+ Tregs in chimeric mice containing bone marrow (BM) from congenic Ly5.1+ mice mixed at 1:1 ratio with BM from either Foxp3̃ or Foxp3+ mice (219). They determined that the CD4+CD25+ Treg population in the Ly5.1+ and Foxp3− chimeras was solely of Ly5.1+ origin, both in the thymus and lymph nodes, indicating that Foxp3-defecient bone marrow cannot give rise to Tregs (219). In contrast, in Ly5.1+ and Foxp3+ chimeras, the CD4+CD25+ Tregs were

equally derived from both Ly5.1+ and Foxp3+ BMs indicating that Foxp3 expression is required for the development of Tregs (219).

High level expression of Foxp3 both at the mRNA and protein level is detected in CD4+CD25+ Treg, but not in naïve or newly-activated CD4+CD25− T cells (219-221). Recently, using mice expressing a GFP-Foxp3 fusion-protein reporter knockin allele (Foxp3gfp mice), Fontenot et al. demonstrated that Foxp3 expression in vivo is restricted to □□ T cells only and that almost all of Foxp3 expressing T cells are CD4+ T cells (~98.52%), with only very minor expression in a subpopulation of CD8+ (~1.7%), CD4+CD8+ (~0.92%) and CD4̃CD8̃ T cells (~0.74%) (224). Furthermore, consistent with the higher magnitude of autoimmune disorder associated with Foxp3 genetic deficiency compared to that caused by CD25+ T cell depletion, it was shown that regardless of the degree of CD25 expression on the surface of T cells, the expression of Foxp3 is correlated with suppressor cell activity (224). Consistent with its role as a Treg specification factor, ectopic expression of Foxp3 in vitro conferred suppressor function on peripheral CD4+CD25− T cells, permitting them to protect mice from autoimmune disease upon adoptive transfer (219, 220). Moreover, transgenic mice that overexpress Foxp3 have significantly increased percentages of CD4+CD25+ Tregs (7−10% in normal mice versus 15−20% in Foxp3 transgenic mice lymph nodes) and CD4+CD25− and CD4−CD8+ T cells from these mice were highly-capable of mediating suppressive activity (221).

1.5.4. Mechanisms of Treg-mediated immune suppression

CD4+ Tregs can exert their suppressive function by inhibiting the proliferation of, and cytokine production by, both naïve and effector CD4+ and CD8+ T cells (188-190). In order to

become suppressive, Tregs require antigen-specific activation or polyclonal TCR stimulation (225, 226). Interestingly, CD4+CD25+ Tregs exhibit an anergic phenotype *in vitro* in that they do not undergo cellular proliferation or produce IL-2 when they are cultured with stimuli, such as anti-CD3, concanavlin A (ConA), or allogeneic splenic APCs, that are known to provide powerful activation signals to conventional T cells even when a costimulatory signal is delivered by anti-CD28 (225, 226). It has been suggested that the basis for this anergic state is the lack of IL-2 gene transcription in CD4+CD25+ Tregs (227). Consistent with this view, Thronton et al. demonstrated that the presence of IL-2 or IL-4 is required in addition to a TCR stimulus in order to generate a strong Treg suppressor activity (228). In their experiments, CD4+CD25+ T cells exhibited poor survival and minimal induction of suppressor activity when stimulated with anti-CD3 antibody alone (228). However, stimulation of CD4+CD25+ T cells with anti-CD3 antibody in the presence of high-dose IL-2 or IL-4, but not IL-6, IL-7, IL-9, IL-10 or IL-15, resulted in the proliferation of the CD25+ cells and the induction of potent suppressor function (228). Even though the nature of the costimulatory signals required for CD4+CD25+ function remains unknown, Takahashi et al. reported that co-stimulation via CD28 does not appear to be required in this process, since CD28-deficient CD25+CD4+ T cells exhibit suppressive activity that is comparable to Tregs isolated from wild-type animals (209). Likewise, Thornton et al. have shown that anti-CD3 and IL-2 mediated activation of Tregs occurs even when the interaction of CD28 or CTLA-4 with CD80/CD86 is inhibited, suggesting that CD28/CTLA-4-mediated co-stimulation is not required for Treg activation (228). However, the role of CTLA-4 co-stimulation in the induction of Treg suppressor function remains controversial, since Takahashi et al. reported that the inhibition of proliferation seen in co-cultures of CD4+CD25+ and CD4+CD25− T cells is abrogated in the presence of intact anti-CTLA-4 mAb or its F_{ab}

fragment (209). On the contrary, CTLA4-deficient CD4+CD25+ Tregs have been shown to exhibit some degree of suppressive activity *in vitro* (209). In cases of antigen-specific activation of CD4+CD25+ Tregs, it appears that the concentration of antigen required for their induction of suppressive activity is significantly lower (10 to 100-fold) than that required for stimulating CD4+CD25− T cells (209). Once activated, Tregs suppress CD4+CD25− and CD8+ T cells in an antigen non-specific manner, that does not require additional stimulation via their TCR (225, 229)

The exact mechanism(s) by which Tregs mediate their suppressive function is still unknown. However, it appears that depending on the experimental model system and the subset of the CD4+ regulatory T cell being examined, Tregs may exert their suppression through cell-to-cell contact, the production of immunosuppressive cytokines, such as IL-10 and TGF-β, or a complex coordination of both (188-190). *In vitro*, CD4+CD25+ Treg suppression seems to be dependent on direct cell-to-cell contact, but not on immunosuppressive cytokine secretion (225, 226). Both Thornton et al. and Takahashi et al. demonstrated that the physical separation of a co-culture of CD4+CD25+ Tregs and CD4+CD25− responder T cells in a transwell system prevented Tregs from suppressing the proliferation of TCR-stimulated responder T cells, even when 10-fold higher CD4+CD25+ Tregs were used in the experiments (225, 226). The addition of neutralizing anti-IL-4, anti-IL-10, or anti-TGF-β mAb alone, or in combination, to co-cultures of CD4+CD25+ Tregs and CD4+CD25− responder T cells failed to abrogate the suppressive effects of Tregs (225, 226). Moreover, supernatants of anti-CD3 or ConA stimulated CD4+CD25+ Tregs did not exhibit any suppressive activity when added to cultures of anti-CD3 stimulated CD4+CD25− T cells (225). Cell contact-dependent suppression of T cell proliferation by Tregs appears to result from the inhibition of the production of IL-2 and the

upregulation of the IL-2 receptor in responder CD4+CD25− T cells (225, 226). Consistent with this view, the addition of high-doses of exogenous IL-2 or anti-CD28 mAb (to stimulate responder CD4+CD25−T cells to produce IL-2) to co-cultures of CD4+CD25+ Tregs and CD4+CD25− responder T cells abrogated Treg suppressive activity and terminated the Treg anergic state (225, 226). The cell surface molecules that are involved in such cell contact-dependent suppression have not been conclusively demonstrated to date. It has been suggested that in addition to CTLA-4, that GITR expressed by Tregs may also be involved in this mechanism of suppression (209-211, 230). Shimizu et al. and McHugh et al. showed that the addition of anti-GITR antibody (DTA-1) abrogated the suppression observed in co-cultures of CD4+CD25+ Tregs and CD4+CD25− responder T cells without breaking the anergic state of the CD25+CD4+ T cells (231). Accordingly, Shimizu et al. suggested that since the DTA-1 F_{ab} fragment was unable to abrogate suppression, and both soluble and plate-bound DTA-1 up-regulated NF-□B transcription in GITR expressing cells, that the DTA-1 mAb acts agonistically to actively transduce an anti-suppressive signal into CD25+CD4+ T cells (231). Conversely, Stephens et al. suggested that the mechanism by which GITR blockade abrogates suppression is by rendering activated CD25−GITR+ T cells resistant to CD25+CD4+ Treg-mediated suppression (232). This latter suggestion is based on the observation that upon the addition of anti-GITR antibody, an increase in T cell proliferation similar to that seen in the presence of CD4+CD25+ $GITR^{+/+}$ Tregs is evident, even when CD4+CD25–$GITR^{+/+}$ T cells were co-cultured with CD4+CD25+$GITR^{-/-}$ T cells (232). Similarly, no abrogation of suppression was observed in co-cultures of CD4+CD25–$GITR^{-/-}$ and CD4+CD25+$GITR^{+/+}$ T cells when anti-GITR antibody was added (232). Even though Thornton et al. had shown that CD4+CD25+ T cells do not directly regulate DC function (229), others have proposed that the contact-dependent

suppression by Tregs in vivo is mediated indirectly via modification of DCs (225, 233-236). Grohmann et al. reported that CTLA-4-Ig (CTLA4-Ig Fc fusion protein) regulates tryptophan catabolism in dendritic cells and requires B7 expression and autocrine IFN-γ production by the DCs (233) and Fallarino et al. showed that CD4+CD25+ cells induced tryptophan catabolism in DCs cells through a CTLA-4-dependent mechanism (234). Subsequently, Munn et al. demonstrated that the activation of indoleamine 2,3-dioxygenase (IDO) to its functional state requires ligation of B7-1/B7-2 molecules on the DCs by CTLA4/CD28 expressed on CD4+ T cells, but not CD8+ T cells (235). IDO is an enzyme that catabolizes the essential amino acid tryptophan to kynurenine and other metabolites and allows the DCs that express the active form of this enzyme to inhibit both CD4+ and CD8+ T cell proliferation via tryptophan depletion and/or apoptosis induction by tryptophan's pro-apoptotic metabolites (235). Cederbom et al. have suggested that CD4+CD25+ Tregs can further mediate their suppression via DCs by interfering with their antigen presentation capacity and, as a result, the activation of naïve T cells (236). This was based on the observation that co-culture of DCs with CD4+CD25+ T cells down-regulates surface expression of the co-stimulatory molecules CD80 and CD86 on the DCs, which occurs even in the presence of the DC activating anti-CD40 antibody, in a contact-dependent but soluble factor-independent manner (236). In addition to the above described active participation of DCs in Treg suppression, it appears that DCs may play only a passive role in some cases where Treg suppression mechanisms are evident, since CD4+CD25+ Tregs can suppress co-cultured CD4+C25− T cells even when the DCs are fixed with paraformaldehyde (i.e. they are metabolically inactive) (225, 236).

Despite what appears to involve a cell contact-dependent mechanism of suppression *in vitro*, Tregs seem to mainly employ cytokine-dependent mechanisms for exerting their

suppression *in vivo,* with IL-10 and TGF-β reported to be the two major cytokines that contribute to their suppressive actitivy (237-241). For example, Asseman et al. reported that the abrogation of the development of colitis in $CD45RB^{high}CD4+$ T cells reconstituted SCID mice by co-transfer of the $CD45RB^{low}CD4+$ T cells can be reversed by the *in vivo* administration of anti-TGF-β or anti-IL-10 receptor mAbs (237). Asseman et al. further showed that $CD45RB^{low}CD4+$ T cells isolated from IL-10-deficient mice were not able to protect mice from colitis induced by the pathogenic $CD45RB^{high}CD4+$ T cells, and when they were transferred alone, they themselves induced colitis in the SCID mice (237). Likewise, Kuhn et al. and Berg et al. reported that even though IL-10-deficient mice exhibit similar numbers of $CD45RB^{low}CD4+$ T cells as wild-type mice, they spontaneously develop enterocolitis and colon cancer that can be prevented in weanlings and ameliorated in adults by treatment with IL-10 (238, 239). *In vitro*, cross-linking of murine CTLA-4 has been reported to induce TGF-β production by CD4+ T cells and when CTLA-4 was cross-linked in T cells from TGF-β1-defecient mice, their ability to suppress the proliferation of responder cells is significantly reduced (242). Similar to results abotained for IL-10, the *in vivo* administration of anti- TGF-β1 mAb abrogated the colitis-protective effects of transferred $CD45RB^{low}CD4+$ T cells in $CD45RB^{high}CD4+$ T cell reconstituted SCID mice (240). In addition, even though CD4+CD25+ T cells from TGF-β1-deficient mice can suppress T cell proliferation *in vitro* to the same extent as those cells from wild-type mice, CD4+CD25+ T cells harvested from TGF-β1-deficient mice do not protect Rag-2 knockout recipients of $CD4+CD45RB^{high}$ T cells from developing colitis (241). Despite equivocal reports, TGF-β1 may also be involved in contact-dependent mechanism(s) of suppression mediated by Tregs (243). As described earlier, Takahashi et al. and Thornton et al. reported that anti-TGF-β antibody does not abrogate Treg-mediated suppression *in vitro* (225, 226). In contrast, Nakamura et al.

reported that CD4+CD25+ T cells activated by anti-CD3 Ab and APCs can mediate *in vitro* suppression that is abrogated by anti-TGF-β antibody or by physical separation of the Tregs and the responder cells in transwells (241). They showed that when stimulated by soluble anti-CD3 and APCs, Tregs produce low amounts of soluble TGF-β1, but mainly express surface-bound TGF-β1, whereas they also produce high-levels of soluble TGF-β1 after optimal stimulation with either plate-bound anti-CD3 and soluble anti-CD28 or plate-bound anti-CD3, IL-2 and anti–CTLA-4 (243). Therefore, it has been suggested that contact-dependent suppression mediated by Tregs occurs through surface bound latent TGF-β1 that becomes active when it binds to one or more activating proteins (such as thrombospondin-1/αvβ6 integrin) on the surface of responder CD4+CD25− T cells and requires high-dose, high-affinity anti-TGF-β1antibody to neutralize its effect (243). Yet, Barthlott et al. have proposed that competition for shared resources, independent of specificity, or effector function can serve as one possible mechanism underlying T cell regulation *in vivo* (244). This is based on the observation that monoclonal TCR transgenic T cells that do not possess Treg phenotype or function were able to abrogate wasting disease induced after transfer of a small number of naive CD45RB^{high}CD4+ T cells into RAG knockout mice (244). They demonstrated that the co-transferred transgenic T cells' ability to regulate the pathogenic CD45RB^{high}CD4+ T cells was directly correlated with high homeostatic expansion potential resulting from a higher avidity for self-peptide-MHC complexes (244). The regulation of the persistence of *Leishmania major* in the skin after healing in resistant C57BL/6 mice by CD4+CD25+ Tregs may represent one example of this mechanism (245). During the stage of lesion formation and immune clearance of parasites from the dermis, it has been observed that the ratio of Tregs-to-effector T cells decreases, not due to a decrease in the absolute number of Tregs, but due almost entirely to the expansion and recruitment of effector T cells into the

affected sites (245). In contrast, at the onset of the chronic phase of disease, the ratio of Tregs-to-effector T cells increases significantly resulting in the suppression of effector cells that leads to the establishment of chronic disease (245).

1.5.5. Role of Treg cells in tumor immunity

The role of Tregs in regulating the immune system is not limited to self-tolerance and autoimmune diseases. They have also been implicated in negative control of other immune responses *in vivo* including graft-versus-host disease (GVHD), viral and bacterial infections, allergy, and tumor immunity (231, 245-249). Before the characterization of Tregs as CD4+CD25+ T cells by Sakaguchi et al., the observation that immunogenic tumors grow progressively in their immunocompetent hosts led some investigators to propose that tumor growth favors the generation of suppressor cells that block the anti-tumor immune response initially generated by the host (250). This proposal appeared to be more relevant in cases of concomitant tumor immunity in which hosts bearing a progressive tumor completely inhibited the growth of the same tumor inoculated at a distant site (250). Once tumors grew progressively, reaching a certain critical size, however, even concomitant tumor immunity deteriorated rapidly (250). Consistent with this proposal, Berendt and North demonstrated that the adoptive transfer of sensitized T cells from immune hosts leads to the complete rejection of large established tumors only when the tumors were growing in thymectomized T cell-deficient recipient mice, but not in normal recipient mice (250). In addition, complete tumor rejection observed in the T cell deficient mice could be abrogated if splenic T cells isolated from donors with established tumors, but not from normal donors, were transferred before the tumor-specific T cells were administered, suggesting a T cell-mediated suppression of the ongoing anti-tumor immune

response (250). It was further shown that by eliminating tumor-induced suppressor T cells using cyclophosphamide treatment or sub-lethal whole-body gamma-irradiation of donors with established tumors—before collecting their splenic T cells for adoptive transfer—, complete restoration of anti-tumor immunity mediated by passively transferred immune T cells can occur (251, 252). Moreover, the suppressive splenic T cells were identified as CD4+ T cells based on anti-L3T4 (i.e. anti-CD4) antibody depletion or anti-mitotic drug (i.e. vinblastine) treatment studies that selectively eliminated cycling CD4+ T cells (but not non-cycling CD8+ T cells) and resulted in CD8+ T cell mediated regression of an advanced lymphoma (253, 254).

Once Tregs *in vivo* were identified as CD4+CD25+ T cells, Shimizu et al. demonstrated that athymic nude mice that had received syngeneic splenic cells depleted of CD25+ cells at the same time as transplantation with radiation leukemia cells, exhibited tumor growth for a short while, but these lesions ultimately regressed and the animals were protected against a secondary challenge with a higher dose of the same tumor (231). In contrast, all the athymic nude mice that received whole spleen cells or a 3- -to-1 mixture of CD25− cells and CD4+ T cells (of which 10% are CD25+) died as a result of tumor progression (231). In the *in vitro* setting, Shimizu et al. further showed that elimination of CD25+ cells from normal, tumor-unsensitized mice splenocytes resulted in the expansion of tumor-specific CD8+ T cells (after stimulated with leukemia cells) and tumor-nonspecific NK-like killer cells (when stimulated with or without leukemia cells), suggesting that the removal of Tregs enhances NK cell activity in addition to sensitizing tumor-specific CD8+ CTLs (231). Depending on the immunogenecity of the tumor, the *in vivo* depletion of CD25+ T cells by the administration of anti-CD25 antibody before, or no later than a day after, tumor inoculation has also been shown to result in the *in vivo* regression of immunogenic tumors (such as radiation leukemia cells, Meth A, myeloma) or in the significant

suppression of weakly/poorly immunogenic tumor (such as B16 melanoma or MCA205 fibrosarcoma) growth, thereby allowing longer survival periods without eradicating the tumors (231, 249, 255). Subsequently, Sutmuller et al. reported that blockade of CTLA-4 expressed by Tregs or depletion of CD25+ Tregs equally enhanced the therapeutic efficacy of a B16-GM-CSF tumor cell vaccine directed against the B16 melanoma (256). However, increased frequencies of CTLs specific for the melanocyte/melanoma differentiation antigen TRP-2 and maximal tumor rejection was observed when CTLA-4 blockade was combined with the depletion of CD25+ Treg cells, suggesting that CTLA-4 blockade and CD25+ T cell depletion affect alternative regulatory mechanisms (256).

Not surprisingly, increased numbers of CD4+CD25+ Tregs have been detected in the peripheral blood, tumor tissue, and/or tumor draining lymph nodes of patients with diverse forms pf cancer, including: patients with early-stage non-small cell lung cancer (257), late-stage ovarian cancer (257, 258), pancreas (259), breast adenocarcinoma (259), hepatocellular carcinoma (260), gastric (261) and esophageal (261) cancers. These CD4+CD25+ Tregs have been shown to constitutively co-expressed CTLA-4, CD45RO, and/or GITR markers and/or to secrete the immunosuppressive cytokines IL-10, TGF-β or both (259-262). Furthermore, CD4+CD25+ Tregs isolated from cancer patients significantly suppressed the proliferation of CD4+CD25− cells or IFN-γ secretion of antigen-specific CD8+ T cells in a dose-dependent manner (259-262). Wolf et al. additionally reported that pre-incubation of CD56+ NK cells with CD4+CD25+ T cells from cancer patients significantly reduced the ability of the NK cells to lyse target cells whereas pre-incubation with CD4+CD25− T cells resulted in only a slight decreased such activity, suggesting that Tregs from cancer patients can be effective inhibitors of NK cell-mediated anti-tumor responses (262).

Consistent with a possible negative contribution to anti-tumor immune responses, Curiel et al. reported higher frequencies of CD4+CD25+Foxp3+ Tregs in malignant ovarian carcinoma, that were correlated with a higher risk of death and significantly reduced survival, even after controlling for stage, surgical removal and other factors known to affect survival (258). For example, they found that patients with tumor Treg counts of 346 or more per ten high-powered microscopic field (HPF) experienced a 25.1-fold higher risk of death and a 4.2-fold reduction in survival compared to those patients with tumor Treg counts of 131 or less per ten HPF (258). For instance, CD4+CD25+ T cells were undetectable in normal ovarian tissues and only a very few CD4+CD25+ T cells were found in nonmalignant ascites/tumors, whereas CD4+CD25+ T cells were found to be enriched in the ascites/tumors of patients with stage III and stage IV tumors when compared with their blood (258). It appears that human Tregs in later ovarian cancer stages rarely enter the draining lymph nodes and are preferentially recruited to, and accumulate at, tumor sites in order to create an anti-inflammatory environment that affords protection from immune attack (258). The observation that malignant ascites induced significant migration and transmigration of Tregs *in vitro* lead Curiel et al. to investigate how these Tregs are preferentially recruited into the malignant tumor (258). They found out that tumor cells and tumor-infiltrating macrophages produced the chemokine CCL22 and anti-CCL22 antibody blocked ascites-induced or recombinant CCL22-mediated Treg chemotaxis *in vitro* and reduced *in vivo* trafficking of human Tregs into human ovarian tumors reconstituted in NOD/SCID mice (258). Consistently, all tumor Tregs were found to express CCR4, which is a chemokine receptor for CCL22 and CCL17 (258). As a result, Curiel et al. propose that human tumor-associated Tregs are recruited to the tumor site by the chemokine CCL22 produced by ovarian tumor cells and tumor-infiltrating macrophages through the CCR4 chemokine receptor (258).

1.6. DC-Based Immunotherapy for Cancer

Based on their potent antigen presentation and T cell stimulation capability, DCs have been widely targeted for use in immunotherapy to enhance the immune system's ability to recognize and eliminate tumors. The potential of DCs to present peptides in the context of both MHC class I and II molecules and activate both the CD4+ T helper cell and CD8+ CTL arm of the adaptive immune system has provided an additional reason for using these cells to optimize the therapeutic outcome of immunization against tumor antigens. Generally, the set ups for DC based therapies have involved generating DCs *in vitro*, loading them with tumor antigens, and administering them to the patient/animal, and monitoring the tumor-specific immune response and its effect on tumor growth (263). The ability to generate large numbers of DCs *in vitro* from peripheral blood mononuclear cells and bone marrow cells by culture in cytokines such as GM-CSF, IL-4, and TNF-α has helped significantly in allowing their manipulation and use in sufficient numbers. Several approaches have been used to pulse DCs with tumor antigens by incubating them with the following sources: 1) irradiated/apoptotic tumor cells, 2) tumor lysates, 3) whole proteins of tumor-associated antigens, 5) peptides eluted from tumor cells or synthesized based on algorithms for MHC binding motif, 6) tumor RNA, 7) tumor DNA, 8) tumor-derived exosomes, 9) viral vectors, such as adenovirus, containing cDNA encoding tumor-associated antigens (263). In addition, DCs can be transfected with cytokine genes, such as IL-12p70, or matured with activation stimuli, such as CpG motifs, to enhance their antigen presentation and T cell priming potential (59, 264, 265). Several animal studies have reported the successful use of *in vitro* generated and tumor-antigen pulsed/cytokine modified DCs to induce or enhance the anti-tumor response and/or cause complete regression of tumors (59, 60, 266-272). The DC induced anti-tumor immune response appears to be primarily cell-mediated

since sub-lethally irradiated naive mice can be efficiently protected against a subsequent tumor challenge by transferring spleen cells, but not sera, from immunized mice (266). Consistently, a marked upregulation of IFN-γ production has been detected in the draining lymph nodes and spleens of tumor-bearing mice immunized with tumor-antigen pulsed DCs compared with mice immunized with DCs alone or DCs pulsed with tumor-irrelevant antigens (59, 266). Moreover, the protective or therapeutic efficacy of tumor antigen pulsed DCs is significantly hampered by the depletion of either CD4+ or CD8+ T cells from tumor-bearing mice before the application of the therapy (59, 266, 268, 270). Immunization with tumor antigen pulsed DCs has also been shown to have the additional benefit of inducing a protective anti-tumor memory response, in that, those mice that have rejected their tumors were able to inhibit tumor growth when re-challenged with the same tumor (59, 266, 268, 270).

Even though tumor antigen pulsed DC-based vaccines have shown some degree of efficacy in animal models, the efficacy of these treatments in human clinical trials as cancer therapies has been very limited (273). Several studies have demonstrated that it is indeed possible to generate tumor-specific T cells in vaccinated patients *in vivo* as detected by sensitive techniques such as tetramer staining or ELISPOT (273). For example, patients treated with DCs loaded with a cocktail of peptides derived from melanoma antigens (such as MelanA/MART-1, tyrosinase, MAGE-3, and gp100) or tumor lysates have exhibited augmented levels of melanoma-specific CD4+ and CD8+ T in their peripheral blood T cell pool (274-276). Further, the success of DC-based vaccines has been clearly evident in a few patients that developed the autoimmune disease vitiligo as a side-effect of the activation of melanoma-specific CTLs that cross-reacted with antigens expressed by normal melanocytes (276). However, in many clinical trials, the achievement of clinical responses in terms of tumor regressions and prolonged disease free status

has been, and remains a significant challenge (273). When taking an aggregate look at several clinical trials, it appears that vaccination of melanoma patients with DCs pulsed with melanoma antigens has resulted in a better clinical response rate (9.5% regression) than immunization with other sources of vaccines such as peptide vaccines, viral vectors, and tumor cells (less than 4.6% regression) (277). Therefore, additional studies are warranted not only to better harness the great potential of DCs as antigen presenting cells, but also to maintain the anti-tumor responses generated by them.

Another DC-based approach that has been employed to generate enhanced anti-tumor immunity is the generation of DCs *in vivo* in tumor-bearing hosts by treatment with the cytokine Flt3 Ligand. Flt3-L has been shown to significantly expand both functionally mature CD8α+ and C8α− CD11c+ DCs in mice and both myeloid and plasmacytoid circulating DCs in humans (278-280). Flt3-L tretament induces transient DC expansion in both lymphoid and non-lymphoid tissues of mice including bone marrow, lymph nodes, spleen, Peyer's patches, thymus, liver, and lungs (278, 279). Lynch et al. first showed that daily injections of Flt3-L into mice bearing syngeneic, methylcholanthrene-induced fibrosarcoma leads to delayed tumor growth and, in some mice, complete tumor rejection (281). The anti-tumor immunity established in those mice that successfully rejected their tumors is protective in that the adoptive transfer of their splencoytes to naïve mice protects recipient animals from a subsequent tumor challenge (281). Lynch et al. further demonstrated that the enhanced therapeutic benefit by Flt3-L treatment appears to result mainly from the activation of tumor-specific CTLs since depletion of Thy1+ cells or CD8+ T cells (but not CD4+ T cells) from immune splenocytes before adoptive transfer abrogated their ability to mediate tumor rejection after injection (281). The observation that the treatment of tumor bearing SCID mice, which lack B and T cells but not NK cells, with

Flt3-L resulted in slowed tumor growth even though none of the mice rejected their tumors suggested that NK cells might also contribute to the anti-tumor immune response (281). However, it is worth noting that the therapeutic effects of Flt3-L treatment appeared to be limited by the dose of cytokine used for treatment, tumor size at the start of cytokine treatment, and the duration of the cytokine treatment (281). Barring these limitations, it is not known why treatment of many tumor-bearing mice with what appears to represent an optimal dose of Flt3-L results in very limited efficacy. Even though slowed tumor growth or metastasis has been observed in murine melanoma, lymphoma, colon adenocarcinoma, and MCA-102 and MCA-205 sarcoma (282-285) bearing mice treated with Flt3-L, many of these tumors are not rejected, and even in other tumors that are rejected, successfully-treated mice relapse once the therapy is discontinued, suggesting a failure in the development/maintenance of protective anti-tumor immune responses (286-288)

STATEMENT OF THE PROBLEM

Dendritic cells play critical roles in the development and maintenance of antigen-specific adaptive immune responses. The unique and potent capability of DCs to cross-present exogenous and cell-associated antigens and prime naïve CTLs makes them potential targets for use in immunotherapy to enhance the immune system's ability to recognize and eliminate tumors. For instance, the positive correlations of elevated numbers of tumor infiltrating DCs (TIDCs) with increases in tumor infiltrating lymphocytes (TILs) and improved patient prognosis or reduction in the establishment of metastatic disease (289-293) circumstantially supports the ability of constitutive cross-priming by TIDCs to regulate tumor progression. However, I observed that the treatment of tumor bearing mice with combinations of rFL and rGM promoted the critical immunological endpoints of enhanced numbers of TIDCs and CD8+ TILs but failed to show any discernible benefit in terms of inhibiting the progressive growth of tumors *in vivo*. Therefore, I closely examined these mice for the functional state of their TIDCs and splenic DCs, for the existence of activated systemic and tumor-infiltrating tumor-specific CD8+ T cells, and for the presence of regulatory T cells to assess for possible mechanism(s) to explain the absence of therapeutic outcome in rFL and rGM treated CMS4 bearing mice.

2. Combinational rFlt3-Ligand and rGM-CSF Treatment Promotes Enhanced Tumor Infiltration by Dendritic Cells and Anti-Tumor CD8+ T Cell Cross-priming, but is Ineffective as a Therapy

2.1. ABSTRACT

Dendritic cells (DC) play significant roles in the development and maintenance of anti-tumor immune responses. Therapeutic recruitment of DC into the tumor microenvironment has the potential to result in enhanced anti-tumor T cell cross-priming against a broad array of naturally-processed and -presented tumor-associated antigens. I have observed that the treatment of BALB/c mice bearing syngeneic CMS4 sarcomas with the combination of recombinant Flt3 ligand (rFL) and recombinant granulocyte-macrophage colony stimulating factor (rGM) for five sequential days is sufficient to optimize the number of tumor infiltrating DC (TIDC). However, despite the significant increase in the number of TIDC, the therapeutic benefit of rFL and rGM treatment is minimal. These TIDC do not exhibit a "suppressed" or "suppressor" phenotype *in vitro* and their enhanced numbers in cytokine-treated mice were associated with increased levels of peripheral anti-tumor CD8+ T effector cells and with an augmented population of CD8+ TIL. These data suggest that rFL + rGM therapy of murine tumors fails at a mechanistic point that is downstream of specific T cell priming by therapy-induced TIDC and the recruitment of these T cells into the tumor microenvironment.

2.2. INTRODUCTION

The induction and maintenance of an effective anti-tumor immune response is critically dependent on dendritic cells (DC). The ability of DC to capture and process tumor-derived antigens into peptide epitopes recognized by CD4+ and CD8+ T cells and to migrate to secondary lymphoid organs in order to present these antigens to naïve T cells is essential in alerting the immune system to combat the assault of pathogens or malignancy (3). Immunogenic DC are equipped to provide (at least) three signals needed for the activation of naïve T cells and their development and polarization into specific subsets of effector T cells: peptides complexed with major histocompatibility complex (MHC) molecules on the surface of DC (i.e. signal 1; ref. (294)); T cell co-stimulatory molecules, such as CD80 and CD86 (i.e. signal 2; ref. (295)); and secreted cytokines, such as IL-12 and IL-10, that play important roles in the functional polarization of activated T cells into Type-1, Type-2 or regulatory type effector cells (i.e. signal 3; refs. (128),(117)). The importance of DC in providing these three coordinate signals to responder T cells is underscored by defects in immunity when one or more signals are absent during priming events. The presentation of peptides by DC without accompanying co-stimulatory signals has been shown to result in specific T cell deletion and anergy (97). DC that express high MHC complexes and co-stimulatory molecules but do not produce IL-12p70 have been shown to be "tolerogenic" or to lack the capability of inducing T_H1-type immune responses that appear critical for the eradication of tumors and intracellular pathogens (113).

Given the central role dendritic cells play in initiating and maintaining antigen-specific immune responses, it is not surprising that in some diseases, such as cancer, that alterations in DC development and function are associated with tumor escape from immunosurveillance (140,

141, 154). Typically, tumor infiltrating dendritic cells (TIDC) have been reported to express low levels of MHC class II molecules and to lack expression of co-stimulatory molecules (152, 296). DC isolated from tumor-bearing mice and humans show significantly reduced abilities to activate peptide-specific CD8+ T cells and to stimulate allogeneic T cells when compared to normal control DC (131, 132). In contrast, T cells isolated from cancer patients or tumor-bearing mice and stimulated with DC from normal controls generate normal T cell responses (131, 132), suggesting that defects in DC-mediated cross-priming of tumor-reactive T cells *in situ* may be a major problem for immunotherapy-based approaches in the cancer setting.

In my studies, the treatment of mice bearing syngenic CMS4 (a methylcholanthrene-induced sarcoma) tumors with rFL + rGM results in the recruitment of large numbers of DC infiltrates into s.c. tumors *in vivo*, in the absence of discernable therapeutic benefit. TIDC in the treated mice do not exhibit a phenotype consistent with the induction of T cell anergy or deletion, and indeed, I noted elevated levels of tumor-specific CD8+ T cells in the spleens of mice and enhanced CD8+ T cell infiltration into treated tumors *in vivo*. These data suggest additional rate-limiting blockade of therapeutic T cell functionality post-priming that must be circumvented in order to allow for the effective immunotherapy of tumor-bearing animals.

2.3. MATERIALS AND METHOD

2.3.1. Mice

Six- to eight weeks old female BALB/cJ and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the pathogen-free animal facility of the Hillman Cancer Center at the University of Pittsburgh Cancer Institute. All animal work was performed in accordance with an IACUC-approved protocol.

2.3.2. Tumor Establishment

CMS4, a methylcholanthrene (MCA)-induced sarcoma of BALB/c origin, was cultured in complete medium (CM) (10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM Hepes buffer and 2mM L-glutamine in RPMI-1640 medium, all from Invitrogen Corporation (Carlsbad, CA). CMS4 expresses H-2K^d class I molecules but it exhibits a negative phenotype for the CD11c, CD8α, CD11b, B220, CD86, CD80, and I-A^d markers. *In vitro* cultured CMS4 cells were tested by IMPACT for known mouse pathogens, such as MPV and Mycoplasma, at the University of Missouri (St. Louis, MO) and were found to be negative. Cultured CMS4 cells were washed three times with PBS and 5 x 10^5 tumor cells were then resuspended in 100 μl of PBS and injected subcutaneously in the right flank of BALB/cJ mice. CT26, an H-2^d N-nitroso-N-methylurethane-(NNMU) induced colon carcinoma cell line, and RENCA, an H-2^d renal adenocarcinoma cell line, were cultured and evaluated in the same way as CMS4 cells. Tumor growth was monitored every 2-3 days by measuring the width and length of the tumors using a DigiMax slide caliper (Bel-art Products, Pequannock, NJ) and area calculated as the product of

these values. Treatment groups included 3-5 mice, as indicated. Data are shown as group mean ± standard deviation.

2.3.3. Combinational Cytokine Therapy

For in vivo DC expansion, mice were injected with 20 μg each of rFL and rGM (8) for three to seven consecutive days, as indicated in the text and figure legends. Injections were administered subcutaneously in the scruff of the neck, with cytokines diluted in a total volume of 100 μl PBS. Both cytokines were the kind gifts of Pharmacia Corporation (St. Louis, MO) or purified in the laboratory from reagents kindly provided by the same company.

2.3.4. Analysis of Single-cell Preparations from Tumor and Spleen

Spleens isolated from untreated or rFL + rGM treated mice were placed on nylon cell strainers, gently mashed using the flat end of a 3-ml syringe plunger, and washed with PBS. ACK buffer was used to osmotically lyse red blood cells from the cell suspensions prior to analyses. To prepare tumor cell suspensions, tumors were resected from mice and cut into small (1-10 mm3) pieces and incubated in complete medium containing 0.01% collagenase, 20 □g/ml DNase, and 30 U/ml Hyaluronidase (all from Sigma, St. Louis, MO) with constant shaking at RT for 45 minutes. The tumor pieces and the media were then passed through 70 □m cell strainers and the tumor tissue further mashed with a syringe plunger and washed with CM. The cell suspension was centrifuged at 1300 RPM for 5 minutes, prior to undergoing 2 additional washes using CM. To isolate total tumor-infiltrating leukocytes (TIL), the cells were resuspended in CM and underlaid with an equal volume of Lympholyte-M medium (ρ = 1.088 g/ml, Accurate Chemical and Scientific Corporation, Westbury, NY), then centrifuged at RT for 15 minutes.

Cells at the gradient interface were collected and washed twice with CM. For isolating CD11c+ DC, spleen or TIL cells were F_c receptor blocked with anti-CD16/32 antibody (BD Pharmingen, San Diego, CA), incubated with CD11c MicroBeads (Miltenyi Biotec Inc, Auburn, CA) and then positively selected on MiniMACS magnetic columns per the manufacturer's instructions.

2.3.5. Flow Cytometry

Cells are transferred to polypropylene tubes, pelleted by centrifugation and resuspended in FACS buffer (0.1% BSA and 0.05% sodium azide in PBS). The following antibodies and their corresponding isotype controls (all purchased from BD-Pharmingen) were used for staining: biotinylated CD11c, CD8α-PE, CD11b-PE, B220-FITC, CD86-PE, CD80-PE, and I-A^d-FITC. After adding the appropriate antibody, the cells are incubated in the dark at 4^{o}C for 30 minutes, then washed twice by centrifugation using FACS buffer. For the detection of apoptosis, FITC-conjugated pan caspase inhibitor, Z-VAD-FMK (Promega Corporation, Madison, WI), was used to stain cells using a protocol identical to that for antibodies above. Cells stained with primary antibodies were then incubated with Streptavidin-PerCP, washed twice, fixed in 1% paraformaldehyde and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Data were analyzed by using WinMDI (The Scripps Research Institute, La Jolla, CA) or EXPO32 (Beckman Coulter) software.

2.3.6. Confocal Immunofluorescence Staining and Metamorph Quantitation

Tumor tissue samples were embedded in OCT medium (Tissue-Tek; Sakura Finetechnical Co, Ltd), frozen and stored at -80^{o}C. Tissue sections 5-μm thick were then prepared using a cryostat microtome, mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, and

stored at -80 ^{o}C. For staining, the sections were fixed in 2% paraformaldehyde (Sigma) at room temperature (RT) for 40 minutes and blocked with normal goat serum for 40 minutes. The sections were then incubated with unconjugated (CD11c), biotinylated (CD11c, CD3, I-A^{d}), or PE-conjugated (CD11b, CD8α, B220) primary antibodies or matching isotype controls (all from Pharmingen) for 1hr. This was followed by incubation with goat anti-hamster Cy3 (Jackson ImmunoResearch, West Grove, PA) or streptavidin-Alexa488 (Molecular Probes, Eugene, OR) for 1 hour. Finally, Hoechst or Sytox Green (Molecular Probes) was applied to stain the nuclei. Images were acquired using Olympus Provis fluorescence or Olympus Fluoview 500 confocal microscopes (Olympus, Melville, NY). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings.

Numbers of CD11c+ DC in tumor sections were quantitated using Metamorph v.6.1 software (Universal Imaging Corp., Downingtown, PA). For each tissue section, both the CD11c-Cy3 and Sytox Green nuclear staining images were captured sequentially to avoid bleed through between the channels. For each tissue section, 10 or more images were captured from non-overlapping image field areas. The threshold settings for CD11c quantitation were set based on isotype control stained tissues and the count on them was always zero. The percentage of CD11c+ cells was calculated as follows: (CD11c+ staining count per field/total nuclei count per field) * 100. For each treatment group, tumor sections from 3-4 mice/cohort were analyzed.

2.3.7. In Vitro Activation of DC

CD11c MicroBead-selected DCs were resuspended in 200 μl CM containing 5 x 10^{5} cells. The following concentrations of dendritic cell activation stimuli were used: 10 μg/ml lipopolysaccharide (LPS from Sigma), 75 μg/ml *Staphylococcus aureus* Cowan strain I (SAC

from EMD Biosciences, San Diego, CA) or 100 μg/ml Polyriboinosinic polyribocytidylic acid (poly (I:C) from Sigma). Each stimulation condition was performed in duplicate wells in 96-well plates. The plates were placed in a humidified incubator at 37 ^{o}C and 5% CO_2 for 24 hours. At the end of the incubation period, the plates were centrifuged and the cell-free supernatants were collected in eppendorf tubes and stored frozen at -80 ^{o}C until used in ELISA. Data are shown as mean ± standard deviation of 2-3 independent experiments.

2.3.8. Analysis of DC stimulation of alloreactive T cells in vitro

H-2^{d} CD11c+ DC isolated from the spleens of untreated normal mice or rFL + rGM treated tumor bearing mice, or from single cell digests of resected CMS4 tumors were co-cultured with H-2^{b} CD4+ splenic T cells at DC to T cell ratios of 1:1, 1:10, or 1:100 in 96-well round bottom plates. The plates were then incubated at 37^{o}C and 5% CO_2 for three days. On the 4th day, CD4+ T cell proliferation was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) assay according to the manufacturer's procedure. In brief, MTT solution was added to each well and the plates were returned to the incubator for an additional 4 hours. After the incubation period, the MTT formazan crystals were dissolved in 0.1 N HCl in anhydrous isopropanol. The absorbance was measured at 570 nm (vs. 690 nm; background). Data are shown as mean ± standard deviation of 2-3 independent experiments.

2.3.9. DQ-Ovalbumin Uptake Assay

CD11c+ DC were resuspended at 50 x 10^{3} cells/100 μl of complete medium, with 100 μl of the cell suspension transferred to each of four polypropylene tubes. DQ-Ovalbumin (Molecular Probes) was added to two of the tubes at a concentration of 10 μg/ml. One set of tubes (one

containing and one lacking DQ-Ovalbumin) was placed on ice while a second set of tubes was incubated at 37 ^{o}C for 1 hour. At the end of the incubation period, the cells were washed with FACS buffer, stained with anti-CD11c antibody, washed, and evaluated immediately by flow cytometry.

2.3.10. In Vitro Stimulation (IVS) of C8+ T Cells

CD8+ T cells were isolated by MACS selection from the CD11c-CD4- fraction of splenocytes or tumor infiltrating leukocytes (TIL). Then, 2 x 10^5 CD8+ T cells were co-cultured with 5 x 10^4 irradiated (100Gy) CMS4 sarcoma cells or 4T1 (H-2^d) mammary carcinoma cells in 96-well round bottom plates in a humidified incubator at 37 ^{o}C and 5% CO_2 for 72 hours. At the end of the culture period, the plates were centrifuged to pellet the cells and the cell-free supernatants were collected and stored at -80 ^{o}C until IFN-γ ELISAs were performed. Since tumors from cytokine untreated mice contain only very few infiltrates of CD8+ T cells by day 14, it was not possible to isolate enough cells to perform comparative analysis with CD8+ T cells isolated from the tumors of rFL + rGM treated mice.

2.3.11. ELISA

ELISAs were performed using IL-12p70, IL-10, or IFN-γ specific OptEIA ELISA sets (both from BD-Pharmingen) according to the manufacturer's instructions. In brief, 96-well flat bottom plates (Corning Costar, Corning, NY) were coated with 50 μl/well of IL-12p70, IL-10, or IFN-γ capture antibodies, incubated overnight at 4 oC, washed, blocked with 3% BSA in PBS/0.05% Tween solution for 1 hour at RT, and washed again. Fifty microliters per well of recombinant IL-12p70, IL-10, or IFN-γ standards or culture supernatants were added to the

wells, incubated for 2 hours at RT, then washed. Fifty microliters of a mixture of biotinylated IL-12p70, IL-10, or IFN-γ detection antibody and avidin-HRP was then added and incubated for 1 hour at RT, and then plates were washed. Finally, 100 μl/well of substrate solution (1:1 mixture of H2O2 and tetramethylbenzidine, KPL, Gaithersburg, MD) was added to the plates and incubated at RT. Chromogen development was stopped by addition of 50 μl of 1M H2SO4 and the optical absorbance determined at 450 nm (vs. 570 nm; background control) using a microplate reader (Dynatech Laboratories, Chantilly, VA). Data are shown as mean ± standard deviation of 3 independent experiments.

2.3.12. Statistical Analysis

Statistical analyses were performed using an unpaired 2-tailed Student's T test. SPSS for windows (SPSS Inc., Chicago, IL) software was used to conduct the analysis. Comparative differences yielding P-values less than 0.05 were considered significant.

2.4. RESULTS

2.4.1. Combined treatment of mice with rFL + rGM leads to an increase in systemic DC numbers in vivo

DCs are rare event cell populations in both lymphoid and non-lymphoid tissues. One of my goals was to define and apply a cytokine treatment regimen capable of expanding optimal numbers of dendritic cells from their hematopoietic progenitors in the bone marrow and to subsequently mobilize these cells into tissues. In initial experiments, I treated BALB/cJ mice with rGM alone, rFL alone, or a combination of the two cytokines and assessed their impact on the percentage of DC in the spleen. As shown in **Fig. 1A**, the percentage of CD11c+ DC among total splenocytes is very low (~2.45%) in control mice that did not receive any cytokine treatment. This percentage increased to ~5.1% (**Fig. 1B**) and ~15.25% (**Fig. 1C**) when the mice were treated with 20 μg/day of rGM or rFL alone for seven days, respectively. As previous noted by others (278, 279, 297), rFL as a single agent was superior at inducing the expansion of DC when compared to rGM alone. However, the highest increase in the number of DC present in the spleen resulted from the treatment of mice with the combinations of both rFL + rGM (~24.44%, **Fig. 1D**). To achieve this level of increase, the mice were treated with 20 μg/ml of each cytokine for seven consecutive days, as reported by Bjorck (8). The increase in DC numbers paralleled the general increase in the size of spleens, with the spleens harvested from mice treated with rFL + rGM being larger than those harvested from mice treated with rFL or rGM alone (data not shown). When cell suspensions were prepared from the spleens of cytokine treated and nontreated mice and the total numbers of splenocytes compared, a similar trend for rank order of organ cellularity was also observed (data not shown). The fold increases in

cellularity compared to untreated mice were: ~1.3x, ~1.6x, and ~2.7x for spleens from mice treated with rGM only, rFL only or both cytokines, respectively. Overall, the absolute numbers of DC in the spleen were increased ~2.7x, ~10x and ~27.9x in mice treated with rGM, rFL or both cytokines, respectively. These data suggest that in order to maximally increase the number of DC in vivo, mice should be treated with the combination of rFL + rGM.

2.4.2. Significantly enhanced infiltration of tumor tissues by DC is observed in mice treated with both rFL + rGM

Since tumors have been reported to negatively affect DC development and function *in situ* (298, 299), I next wished to determine whether cytokine-induced expansion to peripheral compartments in treated mice would extend to the tumor microenvironment of CMS4-bearing animals. I treated BALB/cJ mice with established day 7 sarcomas with daily injections of rFL + rGM using a (initially empirical) 7 day course. On the day following the last cytokine administration, mice were sacrificed and their resected tumors examined by immunofluorescence microscopy for CD11c+ DC infiltration. No cellular staining was observed when isotype control antibody was used to stain tumor sections from both untreated and cytokine treated mice (**Fig. 2A**). As shown in **Fig. 2B**, there were very few CD11c+ DCs in tumor sections isolated from mice with untreated CMS4 tumors. On the contrary, those mice receiving cytokine treatment displayed high levels of CD11c+ DC infiltration (**Fig. 2C**). In such tumors, DCs were distributed throughout the lesion, although it was evident that some areas contained higher focal densities of DC infiltrates than others.

2.4.3. Optimal levels of DC infiltration into the tumor or spleen tissues occur with slightly different kinetics

The presence of optimal numbers of DC in the tumor microenvironment would be presumed important for the initiation and maintenance of tumor-reactive T cells in secondary lymphoid organs, as well as the tumor microenvironment itself (300, 301). Within tumor lesions, DCs would be envisioned to acquire tumor debris via direct (57, 59) or indirect (53, 54, 302) killing/death of tumor cells and to consequently cross-present processed tumor antigens to T cells in the spleen and lymph nodes. Given these considerations, I studied the kinetics of DC infiltration into the tumor in order to determine a treatment schedule that would allow for maximal infiltration of established tumors by cytokine-induced DC. Therefore, mice bearing day 7 CMS4 tumors were treated with rFL + rGM 3, 5, or 7 consecutive days and the number of CD11c+ DC infiltrating the tumors was quantitated using Metamorph software after stained tissue sections were imaged by confocal microscopy. In parallel, the frequencies of splenic CD11c+ DC were quantitated by single-cell flow cytometric analysis from these same animals. As shown in **Fig. 3A**, the average percentage of TIDC in control, non-cytokine treated mice was less than 1% of total cells imaged within the tumor microenvironment. In these mice, the highest level of TIDC infiltration observed per confocal image field was 1.7%. In contrast, the treatment of CMS4-bearing mice for even three days with rFL + rGM was sufficient to increase the average percentage of TIDC to 8.4% of total cells in the tumor lesion (range 4.8-11.4%; **Fig. 3B**). However, the maximal increase in TIDC numbers was observed when mice were treated with the combined cytokine regimen for five consecutive days (i.e. average of 17.6%, range 2.4-40.9%; **Fig. 3B**). Notably, tumor-bearing mice treated for 7 consecutive days did not exhibit TIDC frequencies that exceeded those observed for the 5-day treatment. Indeed, the overall

percentage of TIDC in animals treated for 7 days appeared somewhat lower than that of mice treated for 5 days (average 15.6% vs. 17.6%, respectively, p-value = 0.733), although this level of TIDC infiltration remained elevated when compared with the 3-day treatment results. These data suggest that five days of treatment with rFL + rGM is sufficient to optimize the number of TIDC. The kinetics of DC mobilization into the spleen was however, distinct from that of the tumor. Average CD11c+ DC frequencies in the spleens of treated animals showed continuous increases throughout the evaluation process; i.e. 7.9% (3-day treatment), 16.1% (5-day treatment) and 27% (7-day treatment). Indeed, cytokine treatment for a full seven days was necessary to achieve an optimal level of CD11c+ DC in the spleen.

2.4.4. The enhancement of CD8α+ dendritic cells in the spleen of rFL + rGM treated mice is not mirrored in the tumor

The lack of discernable therapy effect by cytokine co-administration, despite the effective recruitment of CD11c+ DC into the tumor suggested the performance of several corollary studies. First, it is abundantly clear that CD11c+ DCs contain several phenotypic and functionally distinct subpopulations that have been linked with contrasting states of immunity (84, 128). These include: CD8α+CD11c+ (previously referred to as "lymphoid"), CD8α-CD11c+ (previously referred to as "myeloid") and B220+CD11c^{lo} ("plasmacytoid") DC (8, 279, 303).

As shown in **Fig. 4A**, the spleens of untreated, non-tumor bearing mice contained ~23% CD8α+CD11c+, ~12% B220+CD11c^{lo} and ~71% CD11b+CD11c+ DC. Treatment of control mice with rFL + rGM principally impacted the mobilization/expansion of CD8α+ DC into/within the spleen (**Fig. 4B**), with the percentage of CD8a+CD11c+ cells doubling (i.e. ~48%

vs. ~23% in controls). On the contrary, the relative composition of the B220+ DC subset was lower in the treated mice (~5% vs. 12% in controls), but this cell population appeared to express a relatively higher level of the CD11c marker than control B220+ DC. Within the CD11c+ DC subpopulation, there were two largely distinct cohorts defined in the treated mice. One population (~35%) expressed very high levels of the CD11b marker, while the majority of the DC (~65%) expressed low-to-intermediate levels of CD11b. When compared to the spleen, the tumor of treated mice was deficient in CD8α+ CD11c+ TIDC. Indeed, the percentage of CD8α+ TIDC (~6%, **Fig. 4C**) was eight times lower than that seen in the spleen. The level of B220+CD11c+ TIDC was also reduced in the treated tumors when compared to the matched splenic samples (i.e. 3% vs. 12%). However, the percentage of TIDC that expressed high levels of CD11b on their surface was more than double that seen in the spleen. Approximately 83% of TIDC expressed high levels of CD11b and the fluorescence intensity of the expression was approximately one half log higher than that noted for DC in the spleen. These data suggests that combined cytokine therapy of CMS4 tumors results in the preferential and profound infiltration of tumors by CD11b+ but not CD8α+ or B220+ TIDC.

2.4.5. rFL + rGM induced TIDC exhibit apparently normal phenotype and function

While "myeloid" CD11b+CD11c+ DC can be strong stimulators of primary CTL responses (304, 305) and rFL + rGM treatment promoted significantly elevated levels of this type of TIDC, it remained unclear as to whether these DC exhibited an immunostimulatory phenotype. In this regard, "immature" or "mature" CD11b+CD11c+ DC exhibit very different immunostimulatory indices (91). Immature DC express low levels of co-stimulatory molecules on their surface whereas mature DC display upregulated levels of these markers, and tumors have been reported

to induce or restrict TIDC towards an immature phenotype (146, 296, 298). As a result, I assessed MHC II/I-A^d and co-stimulatory molecule expression levels on freshly-isolated TIDC from rFL + rGM treated mice. As shown in **Fig. 5A**, the majority of TIDC from CMS4 tumors consistently expressed the I-A^d, B7.1 (CD80), and B7.2 (CD86) molecules on their cell surface. The level of expression of these molecules on the TIDC was high, and consistent with what is considered to be a comparatively mature DC phenotype. Splenic CD11c+ DC from these same treated mice also expressed I-A^d and CD86 molecules (**Fig. 5B**), though surprisingly, at slightly lower levels than those noted for the matched TIDCs. A major difference was observed in the mean fluorescence intensity (MFI) associated with the CD80 marker, which showed a level four times higher in TIDC than splenic DC. The majority of splenic DC from untreated, non-tumor bearing mice expressed lower levels of I-A^d and CD86 molecules, consistent with their classification as immature DC (**Fig. 5C**). In terms of CD80 expression, however, the majority of these DC expressed levels similar to those seen in splenic DC from rFL + rGM treated mice, but lower than those seen for TIDC. These data suggest that the CMS4 microenvironment does not profoundly inhibit, and could counter-intuitively, support the maturation process of TIDCs *in situ*.

Some tumors are known to secrete factors, such as gangliosides, that can induce the apoptotic death of DC (150). Hence, I carefully assessed TIDC for their pro-apoptotic phenotype using a fluorescent, cell-permeable derivative of z-VAD-FMK (which irreversibly binds to activated caspases in cells undergoing apoptosis) as a probe. As shown in **Fig. 5**, no significant FITC-VAD-FMK staining was detected in TIDC or splenic DC isolated from the cytokine-treated mice.

Since the TIDC analyzed by flow cytometry were obtained from tumor tissues by collagenase, hyaluronidase, and DNase digestion, I also performed *in situ* staining analyses of tumor tissue sections resected from mice treated with rFL + rGM in order to confirm the observed phenotypes of the TIDC. As shown in **Figs. 6A and 6B**, TIDC were easily distinguished by CD11c+ staining and by their hair-like projections (dendrites; see inset). In **Fig. 6A**, tumor sections were double-stained with anti-CD11c and CD11b antibodies. Similar to the data obtained by flow cytometry (**Fig. 4C**), virtually all of the CD11c+ TIDC co-expressed CD11b on their surface, although a range of fluorescence intensities were observed for both the CD11c and CD11b markers. Staining with anti-CD8α or -B220 antibodies did not reveal co-staining with CD11c+ TIDC in tumor sections, but was readily detectable in spleen sections (data not shown). In **Fig. 6B**, TIDC were shown to co-express the CD11c and I-A^d molecules. I noted minimal cell staining with the FITC-VAD-FMK probe in tumor sections, and those cells reactive with this reagent were CD11c-negative (data not shown).

2.4.6. TIDC produce higher levels of IL-10 rather than IL-12p70 upon activation in vitro

Despite an apparently immunogenic phenotype expressed by cytokine-induced TIDC, the presence of such cells *in situ* did not lead to "clinical benefit". Given the reported importance of IL-12p70 and IL-10 as immunostimulatory vs. immunosuppressive DC-produced cytokines that may differentially affect the functional outcome of T cell cross-priming (306, 307), I analyzed the ability of TIDC to produce these cytokines after *in vitro* activation. TIDC and splenic DC isolated from untreated or rFL + rGM treated mice did not spontaneously secrete IL-12p70 when cultured in the absence of exogenous stimuli (**Fig. 7A**). However, the addition of the TLR4-

ligands LPS or *Staphylococcus aureus* (SAC) or the TLR7-ligand poly (I:C) resulted in IL-12 p70 production from all DC populations, with the sole exception of poly (I:C)-stimulated splenic DC isolated from mice treated with rFL + rGM. There was no significant difference in IL-12p70 production levels between LPS- and SAC-stimulated TIDC and untreated mice splenic DC. Despite what appears to be normal levels of IL-12p70 production from TIDC, these cells exhibited a tendency to produce higher than normal levels of IL-10 (**Fig. 7B**). When considered together, the average amount IL-10 detected in TIDC cultures stimulated with LPS, SAC, or Poly I:C was respectively 17.5, 10.8, or 8.8 times higher than that of IL-12p70.

2.4.7. TIDC mediate soluble antigen uptake and processing and effectively stimulate allogeneic T cell responses in vitro

The inability of increased TIDC frequencies translating into anti-tumor efficacy in rFL + rGM treated mice could relate to the inability of these DC to uptake and cross-present antigens (63) to anti-tumor T cells in vivo. In order to test whether TIDC maintain their antigen uptake and processing functions, I cultured freshly-isolated CD11c+ TIDC with DQ-ovalbumin for 1 hour. DQ-ovalbumin is well suited for this purpose since it is a self-quenched conjugate of ovalbumin that emits green fluorescence upon internalization (via mannose receptor mediated endocytosis) and subsequent proteolytic degradation. I observed a high level of green fluorescence in TIDC that were incubated at 37^{o}C when compared to TIDC that were kept at 4^{o}C (**Fig. 8A**). The degree of DQ-Ovalbumin uptake and degradation—reflected in the brightness of the green fluorescence emitted—was significantly higher in TIDC than in both splenic DC from rFL + rGM treated (**Fig. 8B**) or untreated control mice (**Fig. 8C**). In addition, there was only a small proportion of TIDC that failed to process DQ-Ovalbumin. These data suggest that the

CMS4 microenvironment does not profoundly interfere with the ability of cytokine-induced TIDC to uptake and process exogenous antigens

I next performed mixed leukocyte reactions to assess whether TIDC were functionally competent to activate alloantigen-reactive T cells. I stimulated B6 (H-2^b) derived CD4+ splenic T cells with BALB/cJ -derived (H-2^d) TIDC at T:DC ratios of 1:1, 10:1 and 100:1. As shown in **Fig. 8D**, TIDC were capable of inducing allogeneic T cell proliferation. Though not statistically significant, the level of T cell proliferation induced by TIDC was consistently higher than that observed for untreated H-2^d splenic DC. However, splenic DC isolated from tumor-bearing mice treated with rFL + rGM were comparatively better than TIDC in their ability to activate alloantigen-reactive T cells ($p < 0.05$).

2.4.8. rFL + rGM treatment results in the systemic cross-priming of anti-tumor T cells and enhanced T cell infiltration of tumor lesions in situ

Given the approximately normal functional characteristics noted for TIDC in the rFL + rGM treated mice (despite the lack of therapeutic benefit), I chose to directly assess whether combined cytokine treatment was associated with enhanced activation and recruitment of anti-tumor CD8+ T cells *in vivo*.

I initially evaluated splenic CD8+ T cells in these animals for evidence of specific tumor-reactivity by stimulating the CD8+ T cells with irradiated tumor cells *in vitro* and assessing their IFN-γ production. To address the CMS4 specificity of the splenic CD8+ T cells, I also stimulated the CD8+ T cells with an unrelated H-2^d mammary carcinoma cell line, 4T1. Consistent with the observation by Tatsumi et al.(59), no IFN-γ production was detected from splenic CD8+ T cells isolated from untreated, CMS4-bearing mice upon stimulation with CMS4

or 4T1 tumor cells (**Fig. 9A**). On the contrary, splenic CD8+ T cells from rFL + rGM treated mice produced high levels of IFN-γ when they were cultured with CMS4 cells. These IFN-γ producing CD8+ T cells appeared CMS4-specific, since no IFN-γ production was detected upon stimulation with 4T1 cells. This data suggest that TIDC that had ingested tumor-derived antigens, were able to traffic to secondary lymphoid organs/spleen and to effectively cross-present peptides to tumor-specific CD8+ T cells.

I also co-stained tumor sections from mice that were untreated or treated with rFL + rGM using anti-CD3 and anti-CD8 antibodies. As shown in **Fig. 9B**, very few CD8+ T cells could be imaged in tumor sections from tumor sections made from untreated mice. All the CD8+ cells detected in tumor sections co-expressed the CD3 molecule, confirming their identity as T cells. In marked contrast, tumor sections isolated from rFL + rGM treated mice were infiltrated by large numbers of CD3+CD8+ T cells. These T cells were located throughout the tumor lesion and were not confined to peripheral areas of the lesion by the tumor stroma (as is the case in some cancers (308)). I further assessed whether the tumor infiltrating CD8+ T cells were CMS4-specific after *in vitro* restimulation with irradiated CMS4 or 4T1 tumor cells. Similar to what I observed for splenic CD8+ T cells from rFL + rGM, the tumor-infiltrating CD8+ T cells responded to stimulation with CMS4 cells by producing high level of IFN-γ and failed to produce this cytokine when cultured with 4T1 cells. These data suggest that rFL + rGM treatment-induced CD8+ T cells that infiltrate CMS4 tumors *in vivo* are specifically primed to recognize antigen expressed by CMS4 cells. It appears that CMS4 specific T cells primed in secondary lymphoid organs/spleen are able to leave these organs and be recruited into, or expanded within, tumor sites.

2.4.9. The therapeutic impact of rFL and rGM treatment is minimal in controlling tumor growth in vivo

While my data clearly suggest a five day cytokine regimen to be effective for the promotion of maximal TIDC numbers and the cross-priming tumor-specific CD8+ T cells, treated CMS4 bearing mice did not display statistically significant therapeutic benefits from cytokine administration (**Fig. 10A**). The lack of therapeutic benefit by this regimen was not restricted to CMS4 tumors but was also observed in BALB/cJ mice bearing syngeneic CT26 (**Fig. 10B**) or RENCA (**Fig. 10C**) tumors.

2.5. DISCUSSION

Though most tumors express MHC class I molecules, they typically lack co-stimulatory molecules, such as B7.1 and B7.2 that are required for the priming of cytotoxic T lymphocytes (CTL). As the only antigen presenting cells that can activate naïve T cells (2), it is therefore imperative that functionally-appropriate DCs access the tumor microenvironment and consequently cross-prime tumor–specific T cells in the periphery that have the potential to regulate tumor growth or mediate its regression. The ability of *in vitro* generated dendritic cells to mediate anti-tumor efficacy when pulsed with tumor derived antigens (peptides or proteins, apoptotic tumor cells, or tumor cell lysates) and applied as a therapy in the tumor-bearing host (309), suggests that treatments that are capable of similarly promoting cross-priming events *in situ* may exhibit at least a comparable degree of effectiveness. The ability of constitutive cross-priming by TIDC to regulate tumor progression is circumstantially supported by correlations of elevated numbers of TIDC with improved patient prognosis (290) or reduction in the establishment of metastatic disease (292) as noted in other reports.

With the intent of promoting enhanced TIDC numbers and improved cross-priming of tumor-reactive T cells, I performed this preliminary assessment of combined rFL + rGM therapy in an established CMS4 sarcoma model. Previous studies have shown that the treatment of mice (278) or humans (310) with the hematopoietic cytokine FL results in significant expansion of DC in both lymphoid and non-lymphoid tissues *in vivo*. In some cases, the treatment of mice with rFL resulted in complete rejection or reduction in tumor growth rate, with this impact shown to be dependent on CD8+ T cells and/or NK cells (281, 282). In my studies, I treated CMS4 bearing mice with rFL + rGM since the combined treatment synergistically expanded more

dendritic cells than treatment with either cytokine alone. However, in contrast to reports of significant expansion of DCs as well as NK cells in mice treated with Flt3 ligand (311), I did not detect an increase in the number of DX5+ NK cells in the spleen of mice treated with rFL + rGM that paralleled the significant expansion of DCs. The absolute number of NK cells in the spleen of untreated, non-tumor bearing mice was $2.39 \times 10^6 \pm 3.92 \times 10^5$ (3% of total splenocytes) while in the spleen of rFL + rGM treated, non-tumor bearing mice, it was $2.25 \times 10^6 \pm 9.65 \times 10^4$ (1.3% of total splenocytes) and the difference was not statistically significant, i.e. p-value = 0.67. Despite the enhanced presence of systemic DC *in vivo*, combined treatment with rFL and rGM did not induce tumor regression or deceleration of growth. As a result, I focused my attention on the tumor microenvironment in order to determine whether CMS4 affects DC infiltration or their ability to function properly and prime CTL. This analysis is important in that several studies have shown that tumors can interfere with dendropoiesis (150, 298, 299) or with DC function (132, 296), allowing for tumor avoidance of recognition by the immune system. I have found out that the presence of CMS4 does not inhibit DC development from progenitors, and these APCs are not prevented from infiltrating into the tumor. Indeed, CMS4 tumors in animals treated systemically with rFL + rGM were highly infiltrated by DC that were not observed in control, tumor-bearing animals. The optimization of the number of DC within the tumor required only five days of treatment with the combined cytokines, where as the optimal level of DC in spleen required seven days of treatment. It is possible that the observed differences in the kinetics of DC mobilization into the tumor and spleen could be due to tissue-specific limitations on maximal capacity. However, it is also possible that the lower number of TIDC in 7-day treated compared to 5-day treated mice may also suggest that some of the recruited TIDC are leaving the tumor and trafficking to the draining lymph nodes between days 5 and 7, as there was

no evidence for increased apoptosis among any of the TIDC populations assessed. This hypothesis is consistent with my findings that treated mice bearing tumors have significantly higher levels of peripheral tumor-reactive T cells in their spleens and infiltrating CD8+ TIL than untreated mice.

Consistent with their ability to cross-prime CD8+ T cells *in situ*, rFL + rGM-mobilized TIDC uniformly expressed high levels of I-A^d, CD86, and CD80, and thus were not maintained in the tumor environment as grossly immature DC. In extended analyses, these TIDC were effective in taking up and processing the surrogate antigen DQ-BSA and were capable of stimulating the proliferation of allo-reactive splenic T cells in vitro. Furthermore, TIDC isolated from mice treated with rFL + rGM appeared to maintain their ability to produce normal levels of IL-12 p70 when stimulated with LPS, SAC or poly (I:C). Perhaps most importantly, *in situ* analyses demonstrated that anti-CMS4 CD8+ T cells were effectively primed to a greater extent in tumor-bearing mice treated with rFL + rGM and that these cells were present within TIL populations.

Disappointingly, despite my observation of these preferred immunologic endpoints, rFL + rGM administration failed to effectively treat established CMS4 tumors. A similar lack of therapeutic effect was also observed in mice bearing established CT26 or RENCA tumors. It remains unclear whether the uniform "myeloid" (CD11b+CD11c+) nature of the TIDC population in the cytokine treated animals limits the magnitude, type and functional capabilities of responding T cells, despite systemic mobilization of other stimulatory DC subsets including CD8α+CD11c+ DCs, which have been shown to be the predominant DC subset to produce IL-12 (84, 85) and also to cross-present exogenous cellular antigens to CD8+ CTLs (312). At the same time, the localization of a relatively low number of CD8α+ DCs within the tumor does not

preclude their participation in the activation of tumor-specific T cells since the rFL + rGM expanded lymphoid tissue resident CD8α+ DCs may be able to capture tumor-antigens from DCs that have trafficked from the tumor site. For instance, de Haan et al. reported that after in vivo priming with ovalbumin-loaded β2-microglobulin–deficient splenocytes, CD8α+ DCs are able to take up and present the cell-associated OVA to CD8+ T cells (312). Likewise, Allan et al. demonstrated that even though CD8α+ DCs are not detected in the epidermis, after an HSV infection confined to the epidermis layer of the skin, CD8α+ DCs isolated from skin draining lymph nodes were able to stimulate transgenic CTLs specific for an HSV-derived antigen (313). Alternatively, the capability of cytokine therapy-induced TIDC to produce significantly higher levels of IL-10 rather than IL-12 p70 when stimulated with microbial stimuli may be a relevant factor in the lack of clinical effect. Stimulation of naïve T cells with IL-10 producing immature DCs or IL-10 incubated DCs have been shown to lead to their differentiation into regulatory T cells (Tregs) that inhibit the proliferation and cytokine secretion of other antigen-specific T cells (314-316). However, given their high expression levels of I-A^d and costimulatory molecules, as well as their IL-10 production capabilities, my described TIDCs exhibit more phenotypic similarities with the semi-mature/mature DC described by Akbari et al. (117), McGuirk et al. (116), and Menges et al. (114) that appear capable of inducing T regulatory subset 1(Tr1)-like, CD4+IL-10+ T cells *in vivo*. Akbari et al. showed that after respiratory exposure to OVA, pulmonary DC found within the bronchial lymph nodes expressed IL-10 and when cultured with OVA-specific CD4+ T cells induced high levels of IL-10 production by responder T cells (117). Moreover, they observed OVA-specific T cell unresponsiveness when they adoptively transferred pulmonary DC from $IL10^{+/+}$ but not $IL\text{-}10^{-/-}$ mice suggesting that IL-10 secreted by the DCs plays an important role in the differentiation of Tregs. These"tolerogenic" pulmonary

DCs exhibit a typical "mature" phenotype: B7-1^{hi}B7-2^{hi}CD40^{+}MHC class II^{int}CD8α-CD205^{lo}CD11c+ and have a reduced capacity to endocytose FITC-dextran. McGuirk et al. reported that the interaction of filamentous hemagglutinin (FHA), a virulence factor from *Bordetella pertussis,* with DC induced IL-10 production but it also inhibited LPS-induced IL-12 and inflammatory chemokine production (116). When the DCs that interacted with FHA were co-cultured with naïve T cells, they selectively stimulated the induction of Tr1 cells. These Tr1 cells were characterized by their secretion of high levels of IL-10 and the *in vitro* and *in vivo* suppression of protective T_H1 responses against infection by *B. pertussis* or an unrelated pathogen. FHA-activated DC expressed similar levels of I-A^{d}, CD80, and CCR5 molecules as found on immature DC; however, they appeared to represent a distinct subtype of DC since their CD86 and CD40 expression levels were elevated when compared with immature DC. Menges et al. further demonstrated that in contrast to injections of immature DCs or DCs matured with LPS plus anti-CD40, injections of DCs matured with TNF-α and pulsed with an auto-antigenic peptide protected mice from experimental autoimmune encephalomyelitis (EAE) via the induction of peptide-specific predominantly IL-10 producing CD4+ T cells (114). These TNF-α matured DCs exhibited high levels of major histocompatibility complex class II and costimulatory molecules but they produced very low levels of pro-inflammatory cytokines and no IL-12 p70 and IL-10. TIDC in rFL + rGM treated mice appear to express combinations of "mature" DC surface phenotype and "immature" DC antigen uptake phenotype and the capacity for high amount of IL-10 production, hence, could play a role in activating and/or expanding Tr1-type T cells that are capable of impacting the efficacy of my applied therapy *in vivo*. Despite the high level priming of tumor-specific CTLs in lymphoid organs of rFL + rGM-CSF treated mice and the subsequent trafficking of these cells to the tumor site, the concomitant

induction and tumor infiltration by Tregs would be expected to hinder the effector function of these CTLs and create an environment conducive to continued tumor growth. I am currently analyzing this possibility in rFL + rGM treated CMS4 bearing mice as the model system.

3. Treatment-Enhanced CD4+Foxp3+GITR^{Hi} Regulatory Tumor Infiltrating T Cells Limit the Effectiveness of Cytokine-Based Immunotherapy

3.1. ABSTRACT

Regulatory T cells (Tregs) can suppress activated CD4+ and CD8+ T effector cells and modulate the functional outcome of cellular immunity. Recent studies in tumor-bearing hosts suggest that Treg may serve as an impediment to spontaneous or therapeutic anti-tumor CD8+ T cell-mediated immunity within tumor sites. In a previous report, I observed minimal therapeutic impact, but significantly enhanced T cell cross-priming and lesional infiltration of tumor-reactive CD8+ T cells, in CMS4 sarcoma bearing BALB/cJ mice after treatment with recombinant Flt3 (rFL) ligand and GM-CSF (rGM). Here, I show that this cytokine regimen also results in profound infiltration of the tumor by CD4+ T cells that express mRNA transcripts for *Foxp3*, IL-10 and TGF-β and that could produce IL-10 protein *in situ*. Approximately 50% of the CD4+ T cells in the tumor co-expressed CD25, while ~90% of the CD4+ T cells co-expressed high levels of the glucocorticoid-induced TNF receptor (GITR) on their cell surface. In addition, intracellular staining for Foxp3 protein revealed that rFL + rGM treatment results in a significant expansion of CD4+Foxp3+ T cells in the spleen of both non-tumor bearing and tumor bearing mice and the expression of this marker associated with Treg differentiation and function in nearly half of CD4+ TILs. These CD4+ T cells also exhibited an activated/memory ($ICOS^{high}CD62L^{low}CD45RB^{low}$) T cell phenotype and suppressed the proliferation of naïve alloreactive CD4+ T cells in mixed leukocyte reactions (MLR), which was partially antagonized by inclusion of anti-GITR antibody. Furthermore, they suppressed IFN-γ production from *in vivo* cross-primed anti-CMS4 CD8+ T cells when co-cultured *in vitro*. Most importantly, *in vivo*

depletion of CD4+ T cells resulted in the enhanced ability of therapy-induced CD8+ T cells to markedly regulate tumor progression.

3.2. INTRODUCTION

CD8+ cytotoxic T lymphocytes (CTLs) play a critical role in adaptive anti-tumor immunity by recognizing and attacking tumor cells in an antigen-specific manner (130, 317). While suppression within the tumor microenvironment can prevent effective CTL priming *in situ*, even when tumor-specific CTLs are activated during tumor progression, effector T cell function may be inhibited directly or indirectly by tumor-associated factors (318, 319). Recent studies have shown that in some cases, the suppression of immune responses to self or foreign antigens can be attributed to the presence of regulatory T cells (Tregs) that are reactive against self-antigens and capable of negatively regulating effector T cell function (190). Consistent with this observation, increased levels of Treg have been detected in the peripheral blood and tumor tissue of cancer patients (259, 262), with the increased frequency of such suppressive cells correlated well with reduced overall survival (258).

Regulatory T cells typically represent 5-10% of peripheral CD4+ T cells in both humans and mice and the *in vivo* depletion of this T cell subset results in increased predilection to develop a state of auto-reactivity culminating in the development of organ-specific autoimmune diseases, including thyroiditis and gastritis (192, 193). Similarly, the adoptive transfer of purified Tregs prevents the development of autoimmune diseases through enforcement of functional tolerance to self-antigens (208). While no single antigen has been accepted as a unique marker for Treg discrimination, these cells typically express high levels of CD25 (interleukin 2 receptor-α (IL-2Rα) chain) on their cell surface, in addition to the glucocorticoid-induced TNF receptor family-related gene (GITR), mostly intracellular cytotoxic T lymphocyte antigen 4 (CTLA-4; CD152) and OX-40 markers (191, 207, 210, 211). Treg also exhibit an

activated/memory cell phenotype and express low levels of the CD45RB ($CD45RB^{low}$) molecule and high levels of the inducible costimulator ($ICOS^{high}$) molecule (208, 212). Perhaps most notably, the forkhead/winged-helix family transcription factor *foxp3* seems to be expressed uniquely by the Treg subset of CD4+ T cells (219-221), although a recent report suggests that it may also be expressed by a minor population of CD8+, CD4+CD8+, and CD4–CD8– T cells as well (224). Interestingly, the ectopic expression of *foxp3* in naïve CD4+CD25– T cells has been shown to confer suppressive activity in those cells (219, 220). Tregs do not proliferate or produce IL-2 in response to T cell receptor (TCR) triggering although they are capable of inhibiting proliferate responses and cytokine production by effector cells in a non-antigen specific manner (226, 229). The mechanisms by which Tregs mediate their suppression *in vitro* and *in vivo* remain areas of intensive evaluation. *In vitro*, Treg-mediated suppression requires direct cell-to-cell contact and is abrogated by physical separation of Tregs and responder T cells, but not by neutralization of the cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (226, 320). However, *in vivo*, the cytokines IL-10 and TGF-β do appear to actively participate in Treg-mediated suppression of cellular immunity since anti-IL-10 receptor and anti-TGF-β antagonist antibodies inhibit Treg function (237, 241).

In my earlier study, I observed that the treatment of BALB/cJ mice bearing syngeneic CMS4 sarcomas with recombinant Flt3 ligand (rFL) and granulocyte-macrophage colony stimulating factor (rGM) resulted in the *in vivo* cross-priming of anti-CMS4 CD8+ T cells (Berhanu et al., manuscript submitted). However, there was only a minimal impact on tumor growth, despite the infiltration of significant numbers of these tumor-specific CD8+ T cells into the tumor lesion. I report here that the tumors in cytokine treated mice are infiltrated by high frequencies of CD4+T cells that exhibit a suppressive phenotype characteristic of Tregs, with

approximately half of these cells co-expressing CD25 and 90% co-expressing high level of GITR and almost 50% co-expressing the Foxp3 protein intracellularily. Since recent studies have demonstrated that the depletion of CD4+CD25+ T cells *in vivo* may not only prevent tumor growth but also enhance the immunogenecity of tumor vaccines (249, 321-323), I sought to explore the role Tregs might play in the unresponsiveness of the CMS4 bearing mice to treatment with rFL + rGM. *In vivo* depletion of CD4+ T cells in CMS4-bearing mice using anti-CD4 antibody administration resulted in CD8+ T cell-dependent inhibition of tumor growth and extended survival in mice treated with the combinational cytokine therapy.

3.3. MATERIALS AND METHOD

3.3.1. Mice

Six- to eight week old female BALB/cJ and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the pathogen-free animal facility of the Hillman Cancer Center at the University of Pittsburgh Cancer Institute. All animal work was performed in accordance with an IACUC-approved protocol.

3.3.2. Tumor Establishment

CMS4, a methylcholanthrene (MCA)-induced sarcoma of BALB/cJ origin, was cultured in complete medium (CM) (10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM Hepes buffer and 2mM L-glutamine in RPMI-1640 medium; all from Invitrogen Corporation (Carlsbad, CA). Cultured CMS4 cells were washed three times with PBS and 5×10^5 tumor cells were then resuspended in 100 μl of PBS and injected subcutaneously in the right flank of BALB/cJ mice. Tumor growth was monitored every 2-3 days by measuring the perpendicular width and length of the tumors using a DigiMax slide caliper (Bel-art Products, Pequannock, NJ) and area calculated as the product of these values and expressed as mm^2. To calculate the tumor volume, the diameter of the height of the tumors is also co-measured and used in the following formula: $V = (\prod/6) \times (\text{length}) \times (\text{width}) \times (\text{height})$ and expressed as mm^3. Treatment groups included 3-5 mice, as indicated.

3.3.3. Combinational Cytokine and Depletion Therapy

Mice were injected subcutaneously in the scruff of the neck with 20 μg each of rFL and rGM for five consecutive days in a total volume each of 100 μl PBS. Both cytokines were the kind gifts of Pharmacia Corporation (St. Louis, MO) or purified in the laboratory from reagents kindly provided by the same company. In order to deplete CD4+ and/or CD8+ T cells, mice were injected intraperitoneally with 200 μls of anti-CD4 (GK1.5 hybridoma; ATCC, Manassas, VA, USA) and/or anti-CD8 (53-6.72 hybridoma; ATCC) ascites fluid on day 8 (day at which rFL and rGM treatment started) and 100 μls on days 13 and 16 or on days 13, 16 and every three days thereafter. Mice injected with rat isotype control antibody (Sigma) were used as controls for the depletion experiments. Anti-CD4 or anti-CD8 antibody administration resulted in the depletion of >99% of CD4+ or CD8+ T cells, respectively, as assessed by flow cytometry (data not shown).

3.3.4. Preparation of Single-Cell Suspensions from Tumor and Spleen

Spleens were placed on nylon cell strainers, gently mashed using the flat end of a 3-ml syringe plunger, and washed with PBS. ACK lysis buffer was used to osmotically lyse and remove red blood cells from the cell suspensions. To prepare tumor cell suspensions, tumors were resected from mice and cut into small (1-10 mm^3) pieces and incubated in complete medium containing 0.01% collagenase, 20 μg/ml DNase, and 30 U/ml Hyaluronidase (all three from Sigma, St. Louis, MO) with constant shaking at RT for 45 minutes. The tumor pieces and the media were then passed through 70 μm cell strainers and the tumor fragments were further mashed with a syringe plunger and washed with CM. The tumor cell suspension was then centrifuged at 1300 RPM for 5 minutes, prior to undergoing 2 additional washes using CM. To

isolate total tumor-infiltrating leukocytes (TILs), the cells were resuspended in CM and underlaid with an equal volume of Lympholyte-M medium (ρ = 1.088 g/ml, Accurate Chemical and Scientific Corporation, Westbury, NY), then centrifuged at RT for 15 minutes. Cells at the gradient interface were collected and washed twice with CM.

3.3.5. CD4+ and CD8+ T Cell Isolation

Splenocytes or TILs were first F_c receptor blocked with anti-CD16/32 antibody (BD Pharmingen, San Diego, CA) for 15 minutes at 4 ^oC. To remove dendritic cells, anti-CD11c MicroBeads (Miltenyi Biotec Inc, Auburn, CA) were added to the cells, incubated, and positively-selected on MiniMACS magnetic columns per the manufacturer's instructions. CD4+ T cells are then isolated by positive selection from the CD11c negative flow through fraction after staining with anti-CD4 MicroBeads. Finally, CD8+ T cells are isolated by positive selection from the CD11c negative, CD4 negative flow through fraction after incubation with anti-CD8 MicroBeads. Greater than 90% purity is achieved via these positive selections as assessed by flow Cytometry. Since tumors from cytokine untreated mice contain only very few infiltrates of CD4+ or CD8+ T cells by day 14, it was not possible to isolate enough cells to perform comparative analysis with CD4+ or CD8+ T cells isolated from the tumors of rFL + rGM treated mice.

3.3.6. Flow Cytometry

Cells are transferred to polypropylene tubes, pelleted by centrifugation and resuspended in FACS buffer (0.1% BSA and 0.05% sodium azide in PBS). The following antibodies and their corresponding isotype controls were used for surface staining: CD4-PE, CD4-biotin, CD25-

FITC, CD62L-FITC, CD45RB-PE (all purchased from BD-Pharmingen), ICOS-FITC (eBioscience, San Diego, CA), and GITR-FITC (R&D Systems, Minneapolis, MN). After adding the appropriate primary antibody, the cells are incubated in the dark at 4^{o}C for 30 minutes, washed twice by centrifugation using FACS buffer. For CD4-biotin detection, the stained cells were further incubated with streptavidin-PerCP for 30 minutes in the dark at 4^{o}C and washed twice. Cells were then fixed in 1% paraformaldehyde and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Data were analyzed by using EXPO32 (Beckman Coulter) software. For Foxp3 intracellular staining, CD4+ T cells were surface stained as described above and further processed using PE anti-mouse/rat Foxp3 Staining kit (eBioscience). In short, pelleted cells were resuspended in eBioscience Fix/Perm buffer, incubated overnight at 4^{o}C, and washed once with FACS buffer. The cells were then washed twice with 1x eBioscience permeabilization buffer, blocked for 15 minutes at 4^{o}C with 2% normal rat serum in permeabilization buffer, and incubated for an additional 30 minutes at 4^{o}C with PE-conjugated anti-mouse/rat Foxp3 or rat IgG_{2a} isotype control antibody in permeabilization buffer. Finally, the cells were washed twice with 1x permeabilization buffer and resuspended in FACS buffer, prior to analysis by flow cytometry.

3.3.7. Immunofluorescence Staining and Imaging

Tumor tissue samples were embedded in OCT medium (Tissue-Tek; Sakura Finetechnical Co, Ltd), frozen and stored at -80^{o}C. Tissue sections 5-μm thick were then prepared using a cryostat microtome, mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, and stored at -80^{o}C. For staining, the sections were fixed in 2% paraformaldehyde (Sigma) at room temperature (RT) for 40 minutes and blocked with normal goat serum for 40 minutes. The

sections were then incubated with biotinylated CD11c or CD3 antibodies or PE-conjugated CD4 or CD8α antibodies or unconjugated IL-10 antibody or matching isotype controls (all from Pharmingen) for 1hr. This was followed by incubation with streptavidin-Alexa Fluor 488 or goat anti-rat Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 hour. Finally, Hoechst 33342 (Molecular Probes) was applied to stain the nuclei. The images were acquired using Olympus Provis fluorescence microscope (Olympus, Melville, NY). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings. *In situ* staining for TGF-β was not performed because antibody staining does not distinguish between the inactive and active forms of TGF-β since it is secreted as latent forms consisting of the cytokine and the TGF latency-associated peptide (TGF-LAP) and is activated in the extracellular environment (324).

3.3.8. Allogeneic Mixed Leukocyte Reaction (MLR)

$2x10^5$ BALB/cJ CD4+T cells from normal mice spleen were co-cultured in duplicate wells with 10 Gy irradiated $2x10^5$ total C57BL/6 splenocytes in 96-well round bottom plates. CD4+ TILs cells isolated from the tumors of rFL and rGM treated mice were then added to the MLR cultures in decreasing numbers at 1:1, 1:2, 1:5, and 1:10 ratios to assess for their suppressive activity. To determine whether GITR stimulation reverses suppression, 20 μg/ml of purified anti-GITR antibody (clone DTA-1; eBioscience) was added to the 1:2 ratio cultures. The plates were incubated in a humidified incubator at 37°C and 5% CO_2. After three days, the cultures were pulsed with 1μCi ^{3}H-thymidine/well (NEN, Boston, MA) for the last 16 hours of culture. ^{3}H-thymidine incorporation was then quantitated using a liquid β-scintillation counter (Wallac, Gaithersburg, MD). Data are shown as mean ± standard deviation from duplicate wells.

3.3.9. In Vitro Stimulation (IVS) of C8+ T Cells

2 x 10^5 CD8+ T cells from the spleen of rFL + rGM treated mice were co-cultured with 5 x 10^4 irradiated (100Gy) CMS4 sarcoma cells in duplicate wells of 96-well round bottom plates in a humidified incubator at 37^{o}C and 5% CO_2 for 72 hours. To asses for the suppression of IFN-γ production, 1x10^5 CD4+ T cells isolated from the tumors of rFL + rGM treated mice or from the spleen of non-tumor bearing cytokine treated mice were added to the CD8+ T cell cultures. At the end of the culture period, the plates were centrifuged to pellet the cells and the cell-free supernatants were collected and stored at -80^{o}C until IFN-γ ELISAs were performed.

3.3.10. ELISA

ELISAs were performed using IFN-γ specific OptEIA ELISA set (BD-Pharmingen) according to the manufacturer's instructions. In brief, 96-well flat bottom plates (Corning Costar, Corning, NY) were coated with 50 μl/well of IFN-γ capture antibody, incubated overnight at 4^{o}C, washed, blocked with 3% BSA in PBS/0.05% Tween solution for 1 hour at RT, and washed again. Fifty microliters per well of recombinant IFN-γ standards or culture supernatants were added to the wells, incubated for 2 hours at RT, then washed. Fifty microliters of a mixture of biotinylated IFN-γ detection antibody and avidin-HRP was then added and incubated for 1 hour at RT, and then plates were washed. Finally, 100 μl/well of substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine, KPL, Gaithersburg, MD) was added to the plates and incubated at RT. Chromogen development was stopped by addition of 50 μl of 1M H_2SO_4 and the optical absorbance determined at 450 nm (vs. 570 nm; background control) using a microplate reader (Dynatech Laboratories, Chantilly, VA). The lower limit for the

detection of IFN-γ by this assay was 31.25 pg/ml. Data are shown as mean ± standard deviation from duplicate wells.

3.3.11. RNA Isolation

Cells were washed twice with PBS and resuspended in TRIzol reagent (Invitrogen), then transferred to eppendorf tubes and incubated at room temperature (RT) for 5 minutes. Chloroform (Sigma) was then added to the tubes, shaken vigorously, incubated at RT for 3 minutes and centrifuged at 12,000 x g for 15 minutes at 4 ^{o}C. The clear aqueous phases were then transferred into eppendorf tubes and RNA precipitated by the addition of isopropanol, followed by incubation of specimens at RT for 10 minutes, and centrifugation at 12,000xg for 10 minutes at 4 ^{o}C. The RNA pellet was then washed by resuspending in 70% ethanol and centrifugation at 7,500xg for 5 minutes at 4 ^{o}C. Finally, the RNA was air-dried and resuspended in RNAse-free water, with yields determined by measuring OD at 260 nm and protein content at 280 nm.

3.3.12. Reverse Transcriptase PCR (RT-PCR)

One μg of RNA was used for reverse transcription and was performed in a 20 μl total reaction mixture containing 2 μl 10x PCR Buffer II, 2.5 mM dNTPs mixture, 5 mM $MgCl_2$, 2.5 μM random hexamer primer, 1U/μl RNAse inhibitor, and 5U/μl MuLV reverse transcriptase (All from Applied Biosciences, Foster City, CA). The reaction condition was 42^{o}C for 30 min, 95^{o}C for 5 min, and 4^{o}C for 5 min. Polymerase Chain Reaction (PCR) was performed using 2 μl of this cDNA in a 50 μl total reaction mixture consisting of 29.5 μl sterile water, 5 μl 10x PCR Buffer II, 1.6 mM dNTPs mixture, 2 mM $MgCl_2$, 0.05 U/μl Ampli-Taq DNA polymerase, and

0.5 μM each of forward and reverse primers. The specific primer sequences were obtained from published sources as follows: *foxp3* (T_A=63°C; ref. (325)), TGF-β (T_A=57°C), and IFN-γ (T_A=63°C; ref. (326)) and IL-10 (T_A=55°C; ref. (327)). The PCR reaction condition was 94°C for 3 min, 25 cycles of amplification (94°C for 45 sec, primer specific-annealing temperature for 45 sec, and 72°C for 1 min), and 72°C for 7 minutes. PCR products were confirmed by gel electrophoresis on standard 1.4% agarose gels stained with ethidium bromide and visualized by exposure to ultraviolet light. The images were captured using UVP gel camera (UVP, Inc., Upland, CA) utilizing Lab Works software (PerkinElmer, Boston, MA).

3.3.13. Statistical Analysis

Statistical analyses were performed using an unpaired 2-tailed Student's T test. SPSS for windows (SPSS Inc., Chicago, IL) software was used to conduct the analysis. Comparative differences yielding P-values less than 0.05 were considered significant.

3.4. RESULTS

3.4.1. Significantly enhanced infiltration of CMS4 tumors by CD4+T cells after treatment with rFlt3-L (rFL) + rGM-CSF (rGM)

The capture, processing, and presentation of tumor-derived antigens by dendritic cells (DC) and the subsequent activation of tumor-specific CD8+ CTLs appears crucial to the initiation of anti-tumor immune responses (328). CD4+ T cells can play a helper role in the priming of CD8+ CTLs, an effector role in mediating DTH-type inflammatory responses, or a suppressive role by inhibiting the priming/effector function of CTLs (329, 330). In my previous report, I observed that treatment of BALB/cJ mice bearing CMS4 sarcomas with rFL + rGM resulted in profound tumor infiltration by CD11c+ DC as well as T cell populations, which included CMS4-specific CD8+ CTLs, although the cumulative immunity failed to demonstrably impact tumor progression (Berhanu et al., manuscript submitted). Upon further inspection, I now show that while day 14 established CMS4 tumors in untreated mice are infiltrated by very few tumor infiltrating CD11c+ DC, CD4+ T cells or CD8+ T cells, the number of such cells in the tumors of animals treated with rFL + rGM was dramatically increased (**Fig. 11**). CD11c+ DC and both T cell subsets within the treated tumors exhibited a similar, diffuse distribution pattern throughout the lesions and were not anatomically restricted to the periphery of the tumor. Since I have previously shown that CD8+ T cells isolated from tumor digests were capable of responding specifically to stimulation with CMS4 cells *in vitro*, I next sought to determine whether the balance of functional capacity(ies) mediated by CD4+ TILs could, at least in part, explain the lack of efficacy of this combined cytokine therapy.

3.4.2. A high proportion of CD4+ TILs isolated from rFL + rGM treated mice display a regulatory T cell phenotype

In order to compare the surface phenotype of tumor infiltrating CD4+ T cells with that of splenic CD4+ T cells, CD4+ T cells were isolated from tumor cell digests of rFL + rGM treated mice and examined by flow cytometry. Based on prior reports ((191, 207)), I initially discriminated Treg by evaluating CD4+ T cells for constitutive, co-expression of the CD25 marker. As shown in **Fig. 12**, ~53% (49.25 ± 5.23) of CD4+ TILs (F/G-TIL) in cytokine-treated mice co-expressed CD25 on their cell surface, where as only ~11% (10.74 ± 1.97) of CD4+ T cells (F/G-Tu-SP) from the spleen of the same mice co-expressed CD25 (**Fig. 12**). Notably, rFL + rGM treatment does not appear to have such a dominant impact on splenic frequencies of CD4+CD25+ T cells. Indeed, I observed the percentages of splenic CD4+CD25+ cells in untreated/non-tumor bearing (U-NOR-SP), treated/non-tumor bearing (F/G-NOR-SP) and untreated/tumor-bearing (U-Tu-SP) mice to be ~7% (6.13 ± 2.00), ~10% (9.55 ± 2.62), and ~6% (5.76 ± 0.60), respectively. I also examined the TILs and spleen CD4+ T cells for co-expression of the GITR marker previously shown to be preferentially expressed by regulatory T cells at high levels (210, 211). Interestingly, ~90% (90.19 ± 1.05) of CD4+ TILs in treated mice co-expressed this molecule (**Fig. 12**), and the high fluorescence intensity level of expression was consistent with that typically reported for regulatory T cells (210, 211). $GITR^{HI+}$CD4+ T cell frequencies in TILs were dramatically greater than those observed for the spleens of cytokine-treated control, non-tumor-bearing (i.e. ~28% (26.70 ± 2.06) mice or tumor-bearing (i.e. ~29% (29.82 ± 5.48)) mice. Interestingly, and in contrast to the data generated in the CD25 marker analysis, rFL + rGM treatment appeared to result in increased percentages of splenic $GITR^{HI+}$CD4+ T cells when compared to untreated control, non-tumor-bearing (i.e.~7% (9.25 ±

4.42)) mice or tumor-bearing (i.e.~11% (11.84 ± 4.95)) mice receiving no treatment. These differences in the percentages of CD4+GITR^{Hi} T cells among cytokine treated and untreated mice were statistically significant: p = 0.0034 for U-NOR-SP vs. F/G-NOR-SP and p = 0.013 for U-Tu-SP vs. F/G-Tu-SP. These results suggest that combined cytokine treatment with rFL + rGM may peripherally expand and preferentially recruit CD4+ T cells exhibiting a regulatory T cell phenotype into CMS4 tumor lesions.

3.4.3. CD4+ TILs isolated from rFL + rGM treated mice express an activated/memory phenotype consistent with that of regulatory T cells

Since adoptively-transferred CD45RB^{low}CD4+ T cells isolated from the spleen of normal mice can act as regulatory T cells in preventing organ-specific autoimmune disease in T cell-deficient mice reconstituted with CD45RB^{High}CD4+ T cells (192, 208), I examined the level of CD45RB expression on CD4+ T cells isolated from the TILs and spleen of rFL + rGM treated mice. As shown in **Fig. 13**, the fluorescence intensity of the CD45RB molecule on the surface of CD4+ T cells isolated from tumor was significantly lower (~5 times) than that of CD4+ T cells isolated from the spleen of the same animals (~29 vs. ~153 MFI, respectively). This expression level was also ~15 times and ~19 times lower than that observed for splenic CD4+ T cells harvested from untreated/tumor-bearing (~29 vs. 426 MFI, respectively) or untreated/non-tumor bearing (~29 vs. 562.34 MFI, respectively) mice, respectively. Additionally, in parallel with increased GITR expression, it appeared that there were more CD4+ T cells exhibiting a lower expression level of the CD45RB marker in rFL + rGM treated mice when compared to untreated mice, whether the animals harbored tumors or not.

I also evaluated CD4+ TILs for co-expression of the inducible costimulator (ICOS) protein, since upregulated levels of this marker have been detected on regulatory T cells in pancreatic lesions and its blockade rapidly converts early insulitis to diabetes by disrupting the balance of Treg and T effector cells (212). Contrary to their reduced levels of CD45RB expression, CD4+ TILs from cytokine treated mice expressed higher levels of ICOS than any other splenic CD4+ T cells examined (**Fig. 13**). ICOS expression was virtually non-existent (MFI = 1.0) on splenic CD4+ T cells isolated from untreated, normal or tumor-bearing mice or from rFL + rGM treated normal controls. However, the MFI for ICOS expression on TILs and splenic CD4+ T cells in cytokine treated CMS4 bearing animals were both elevated compared to those controls (~39 and ~8, respectively). Furthermore, a population of IL-10 producing regulatory T cells that developed *in vivo* from naive CD4+CD25− T cells during a T helper type 1 (T_H1) polarized response has been shown to express high level of ICOS, but only low levels of CD62L (331). As shown in **Fig. 13**, CD4+ TILs from treated mice failed to express the CD62L marker (involved in the recirculation of naïve T cells from blood into peripheral lymphoid tissues, ref. (332)) on their surface, whereas the majority of splenic CD4+ T cells were CD62L+. However, it was also clearly evident that some of the splenic CD4+ T cells from cytokine-treated, tumor-bearing mice expressed low level of CD62L, while some expressed high levels of CD62L.

3.4.4. rFL + rGM treatment significantly expands CD4+Foxp3+ regulatory T cells in the spleen and results in high level infiltration of the tumor by CD4+Foxp3+ TILs

Foxp3 is important in the development and function of CD4+ regulatory T cells and is predominantly expressed in this subset of T cells (219, 220). Consistent with the regulatory T cell phenotype of tumor CD4+ T cells described so far, ~54% (49.56 ± 6.48) of CD4+ TILs from

rFL + rGM treated mice were found to express this intracellular transcription factor (**Fig. 14**). Interestingly, I detected double the percentages of CD4+Foxp3+ T cells in the spleens of rFL + rGM treated mice compared to what is normally found in untreated mice, in both tumor-bearing and non tumor-bearing mice. That is, ~29% (28.69 ± 1.42) and ~28% (27.98 ± 0.54) of splenic CD4+ T cells from rFL + rGM treated tumor bearing and non-tumor bearing mice, respectively, expressed the Foxp3 protein. In marked contrast, ~15% (14.13 ± 0.74) and ~13 % (11.89 ± 2.2) of splenic CD4+ T cells in untreated tumor bearing and non-tumor bearing mice, respectively, were Fox3 positive. These data suggest that combined treatment with rFL and rGM results in a coordinate expansion of DC and CD4+Foxp3+ T cells *in vivo*. Moreover, I co-stained CD4+ T cells with anti-Foxp3 and anti-CD25 or anti-Foxp3 and anti-GITR mAbs in order to examine the percentages of CD25+CD4+ or GITR+CD4+ T cells that co-express Foxp3. As shown in **Fig. 14**, ~62% of CD25 expressing CD4+ TILs were Foxp3+ while ~60% of GITR expressing CD4+ TILs were Foxp3+. In the spleens of cytokine-treated mice, the majority (>82%) of CD25– or GITR-expressing CD4+ T cells were Foxp3+, whereas in untreated mice, a slightly lower percentage (>73%) of CD25+ or GITR+ co-expressing CD4+ T cells were Foxp3+. In all of the CD4+ T cells analyzed, I observed that more of the Foxp3+CD4+ T cells were also GITR+ rather than CD25+, suggesting that GITR might be a better single marker than CD25 in identifying Tregs. The percentages of CD4+Foxp3+ T cells that also expressed CD25 were ~73%, ~36%, ~31%, ~28%, and ~30% for F/G-TIL, F/G-Tu-SP, F/G-NOR-SP, U-Tu-SP, and U-NOR-SP CD4+ T cells, respectively. In contrast, the percentages of CD4+Foxp3+ T cells that co-expressed GITR were ~97%, ~81%, ~83%, ~57%, and ~50% for F/G-TIL, F/G-Tu-SP, F/G-NOR-SP, U-Tu-SP, and U-NOR-SP CD4+ T cells, respectively. Even though it was not as high as the frequency of GITR+CD4+ TILs (i.e. ~97%), when compared to splenic CD4+ T cells, the

majority (~73%) of CD4+Foxp3+ TILs still co-expressed CD25. In addition to the intracellular staining of Foxp3 protein, I also performed RT-PCR using *foxp3* specific primers on total RNA isolated from CD4+ T cells to determine whether the increase in Foxp3 expression observed in rFL + rGM-CSF treated tumor bearing mice was reflected at the transcriptional level (**Fig. 17**). Consistent with my flow cytometry data, high levels of *foxp3* mRNA transcripts were detected in CD4+ TILs and splenic CD4+ T cells of tumor-bearing mice treated with rFL + rGM and this level of expression was higher than that observed for CD4+ T cells isolated from untreated, normal or tumor-bearing mice. As expected, *in vitro* cultured CMS4 cells did not express *foxp3* mRNA transcripts.

3.4.5. CD4+ TILs isolated from rFL + rGM treated mice suppress alloreactive CD4+ T cell proliferation and IFN-γ secretion by tumor-specific CD8+ T cells

The hallmark of CD4+ regulatory T cells is their functional ability to suppress responder/effector T cell proliferation and cytokine production (209, 226, 229). As a result, I wanted to determine whether CD4+ TILs suppressed the *in vitro* proliferation of responder CD4+ T cells in an allogeneic mixed leukocyte reaction (MLR). Naïve CD4+ T cells were isolated from normal BALB/cJ (H-2^d) mice and co-cultured with C57BL/6 (H-2^b) splenocytes as stimulators along with titrated numbers of unfractionated CD4+ TILs harvested from cytokine treated BALB/cJ mice. In accordance with their regulatory T cell phenotype, the CD4+ TILs effectively suppressed the proliferation of alloreactive responder T cells in a dose-dependent manner (**Fig. 15A**). Since the CD4+ TILs strongly expressed GITR, and anti-GITR antibodies have been shown to mitigate Treg function (210), I added anti-GITR antibody to the suppressed cultures and re-evaluated proliferative responses. As shown in **Fig. 15B**, the addition of a sub-

optimal concentration of anti-GITR antibody resulted in the partial normalization of allo-specific T cell proliferation. The proliferation observed in the presence of anti-GITR antibody was approximately 2.33 times that observed in the absence of the antibody (cpm = ~76 x 10^3 vs. ~33 x 10^3). This data suggests that CD4+ TILs may utilize GITR expressed on their surface to suppress the expansion of effector T cells at the tumor site in a contact-dependent manner.

I also evaluated whether CD4+ TILs from cytokine-treated mice could suppress IFN-γ production from *in vivo* primed anti-CMS4 CD8+ T cells. I have previously reported that rFL + rGM treatment of CMS4-bearing mice results in the enhanced cross-priming of tumor-specific CD8+ T cells *in situ* (Berhanu et al., manuscript submitted). For the current studies, I co-cultured splenic CD8+ T cells harvested from rFL + rGM treated CMS4-bearing mice with irradiated CMS4 tumor cells *in vitro* in the absence or presence of CD4+ TILs isolated from cytokine-treated animals. CD4+ T cells isolated from the spleens of rFL + rGM treated, non-tumor bearing mice were used as controls for TILs. As shown in **Fig. 16**, the addition of CD4+ TILs, but not control CD4+ splenocytes significantly reduced IFN-γ production from *in vivo* primed CD8+ T cells re-stimulated with CMS4 tumor cells *in vitro*. This data suggests that even though CD4+ Tregs present in the spleen may not affect the priming of tumor-specific CD8+ T cells, once these primed CD8+ T cells traffic into the tumor sites, their effector function may be significantly hindered by co-infiltrating CD4+ TILs.

3.4.6. CD4+ TILs from rFL + rGM treated mice express higher levels of TGF-β and IL-10 mRNA transcripts.

To characterize possible mechanism(s) by which the regulatory tumor infiltrating CD4+ T cells could mediate their suppression, I assessed the mRNA expression level of the two

cytokines, i.e. IL-10 and TGF-β, linked to Treg-mediated suppression in vivo (226, 320). Interestingly, the only CD4+ T cell population in which IL-10 mRNA transcripts were detected was CD4+ TILs (**Fig. 17A**). Regardless of the cohort evaluated, splenic CD4+ T cells failed to express detectable levels of IL-10 mRNA. Consistent with this data, immunofluorescence staining of tumor tissue sections from rFL + rGM treated mice revealed expansive staining with an anti-IL-10 antibody (**Fig. 17B**). Since IL-10 mRNA expression or IL-10 protein production was not detected from *in vitro* cultured CMS4 tumor cells by RT-PCR (**Fig. 17A)** or ELISA (data not shown), I presume that the tumor cells are not a prominent source of IL-10 *in situ*. In terms of TGF-β mRNA expression, CD4+ TILs expressed dramatically elevated levels of this transcript when compared to splenic CD4+ T cells in the same rFL + rGM treated mice (**Fig. 17A**). However, in contrast to IL-10, TGF-β mRNA expression was also detected in splenic CD4+ T cells isolated from untreated, normal or tumor-bearing mice and *in vitro* cultured CMS4 tumor cells. When taken together, the IL-10 and TGF-β data suggest that the CD4+ regulatory TILs may represent activated, effector cells, while splenic Treg may exist in a more resting state. I further noted that CD4+ TILs in cytokine-treated mice also expressed very low levels of gamma interferon (IFN-γ) mRNA transcripts, suggesting that the number of functional T helper type 1 (T_H1) cells among the CD4+ T cells is likely rather limited. It is also possible that the IFN-γ transcripts are expressed in the regulatory CD4+ TILs, since a recent study has demonstrated that alloantigen-reactive CD25+CD4+ T cells, but not CD25−CD4+ Tregs display a significant increase in IFN-γ mRNA expression when reencountering alloantigen *in vivo* and their functional activity was impaired dramatically in IFN-γ deficient mice (333). Also, a population of regulatory T cells that developed *in vivo* from naive CD4+CD25− T cells has been reported to produce both IL-10 and IFN-γ (331).

3.4.7. In vivo depletion of CD4+ T cells results in significantly improved anti-tumor efficacy of rFL + rGM therapy that is mediated by CD8+ T cells

Given my previous data supporting the ability of rFL + rGM treatment to promote the effective cross-priming and recruitment of anti-CMS4 CD8+ T cells into tumor lesions, I next sought to determine whether depletion of CD4+ T cells via injection of anti-CD4 mAb removed Treg-mediated restrictions on therapeutic CD8+ T cells *in vivo*. In my first experiment, I treated mice bearing day 8 established CMS4 tumors with depleting anti-CD4 or anti-CD8 antibodies. This time point coincided with the initiation of the treatment of CMS4 bearing mice with rFL + rGM. Antibodies were re-injected on days 13 and 16 post-tumor inoculation (i.e. 1 and 4 days after discontinuation of cytokine treatment). As shown in **Fig. 18**, the injection of anti-CD8 antibody alone or an isotype-matched control rat IgG failed to impact CMS4 tumor growth. Similarly, injection of anti-CD4 antibody failed to slow CMS4 tumor progression in animals that did not receive cytokine treatment. The only treatment group in which CMS4 growth was significantly inhibited was the cohort treated with rFL + rGM and co-injected with the depleting anti-CD4 mAb. The reduced rate of tumor growth in these mice occurred most acutely between days 17 and 23, as indicated by the plateau of the tumor growth curves of the mice in the group. This period of slowed growth appears to coincide with the window of time during which complete depletion of CD4+ T cells is expected to occur, However, after day 25 (9 days after the last treatment of anti-CD4 antibody), progressive tumor growth is re-established. These data suggest that CD8+ T effector cells promoted by therapy-induced cross-priming can control CMS4 tumor growth during the period of stable CD4+ (Treg) depletion. In this experiment, I further noticed that CD4 depletion in rFL + rGM treated mice not only slowed down tumor growth based on surface area, but also volume assessment. That is, the impact of CD4 depletion

in rFL + rGM treated mice is perhaps better represented by examining its impact on the three-dimensional rather than its two-dimensional growth. Unfortunately, since this additional impact on the volume of the tumor was observed only after CD4 depletion of rFL + rGM treated mice, I did not record height measurements at all timepoints. However, in my repeat experiment shown in **Fig. 19 and Fig. 20**, all three dimensions (length, width, and height) were recorded beginning on day 8 after tumor inoculation in order to compare both the areas and volumes of the tumors among the different treatment cohorts. Furthermore, since a plateau of tumor growth was observed in CD4-depleted, rFL + rGM treated mice during the depletion period in the **Fig. 18** experiment, I extended the antibody treatments beyond day 16 and continued to apply them every three days thereafter. In this experiment, I also determined whether CD8+ T cells were active participants in regulating tumor growth once CD4+ T cells were effectively depleted. Hence, anti-CD4 and anti-CD8 antibodies were co-administered in order to deplete both CD4+ and CD8+ T cells and to observe the impact of co-depletion on CMS4 growth. As shown in **Fig. 19 and Fig. 20** and consistent with the data in **Fig. 18**, significant inhibition of tumor growth was observed in the cohort of mice treated with rFL + rGM and co-injected with depleting anti-CD4 mAb but not rat control antibody. In contrast, injection of anti-CD4 antibody failed to slow CMS4 tumor progression in four out of five mice that did not receive rFL + rGM treatment. It was also clearly evident that the continued administration of anti-CD4 antibody resulted in an indefinitely prolonged period of tumor growth inhibition, as indicated by the long plateau in the growth curves of CD4-depleted, cytokine-treated mice. The impact of CD4 depletion was even more pronounced when tumor growth was assessed in terms of a clinically-relevant measure of volume (**Fig. 20**), rather than just surface area (**Fig. 19**). For example, on day 22 after tumor inoculation, the average tumor areas in the isotype treated (cytokine untreated), anti-CD4 treated

(cytokine untreated), anti-CD4 + anti-CD8 treated (cytokine untreated), isotype treated (cytokine treated), and anti-CD4 + anti-CD8 treated (cytokine treated) mice were 4.9, 4.2, 4.7, 4.1, and 5.5 times, respectively, that of anti-CD4 treated (cytokine treated) mice. The average tumor volumes on the same day for these mice were 10.8, 8.2, 9.5, 7.5, 12.5 times, respectively, that of anti-CD4 Ab-treated (cytokine treated) mice. The effect on CMS4 growth due to CD4 depletion in rFL + rGM treated mice was CD8+ T cell mediated since the depletion of CD8+ T cells along with CD4+ T cells resulted in progressive tumor growth without any period at which a plateau of growth arrest could be observed. Furthermore, the mice treated with cytokine and anti-CD4 antibody looked very healthy and active without any physically noticeable negative impact from such a prolonged period of CD4 depletion. As can be seen in **Fig. 21**, 80% of these cytokine and anti-CD4 antibody treated mice had small tumors and were alive upto the end of the observation period, i.e. 62 days after tumor inoculation, compared to the 28 days by which the majority of the mice in the other cohorts either died or had to be sacrificed due to sickness and high tumor burden. In summary, these *in vivo* data suggest that CD8+ T cells promoted by rFL + rGM therapy-induced cross-priming can control CMS4 tumor growth during the prolonged period of stable CD4+ (Treg) depletion.

3.5. DISCUSSION

In this study, I show that rFL + rGM treatment enhanced infiltration of CMS4 tumors by CD4+ T cells that exhibit regulatory T cell phenotype and function. CD4+ TILs not only expressed surface markers typical of Tregs, they also functionally suppressed the proliferation and IFN-γ production of responder naïve alloreactive CD4+ and tumor-specific CD8+ T cells, respectively. These CD4+ TILs from rFL + rGM treated mice were uniformly $CD45RB^{low}$ and $ICOS^{High}$ and nearly half of them co-expressed CD25, where as more than 90% of them expressed high levels of the GITR molecule on their cell surface. Unfortunately, both CD25 and GITR have been reported to be expressed by not only regulatory T cells, but also by activated non-regulatory T cells (191, 207, 210, 211). Unlike the complicated nature of using these surface markers to distinguish Tregs, Foxp3 appears to be a Treg specific factor since naïve or newly-activated, non-regulatory T cells do not express this marker and ectopic expression in deficient cells bestows suppressive function (219, 220, 224). Intracellular staining of CD4+ TILs for Foxp3 expression showed that approximately half of these T cells express high levels of this protein. As a result, I conclude that at least 50% of the CD4+ TILs after treatment with rFL and rGM are regulatory T cells. Approximately 97% of these CD4+Foxp3+ TILs expressed GITR, whereas only approximately 73% expressed the CD25 marker, suggesting that GITR expression may be an integral component of the Foxp3 expressing Treg functional phenotype. . The lack of CD25 expression by some of the Foxp3+CD4+ TILs does not appear to be an unusual finding, as it confirms data presented in a very recent report by Fontenot et al. who showed that in mice expressing a GFP-Foxp3 fusion-protein reporter knockin allele (*Foxp3*^{gfp} mice), only ~60% of Foxp3+CD4+ splenic T cells and ~47% of Foxp3+CD4+ lung T cells expressed CD25,

suggesting that not all Tregs can be identified based on constitutive high expression of CD25 on their cell surface (224).

However, given 1) the lack of BALB/cJ CD4+ TIL proliferation when co-cultured with allogeneic C57BL/6 splenocytes, 2) the high level suppression of naïve alloreactive CD4+ T cell proliferation and tumor-specific CD8+ T cell IFN-γ production by co-culture with unfractionated CD4+ TILs, and 3) the abrogating effect of anti-GITR antibody on Treg function, it is possible that the $GITR^{HI+}$CD4+ TILs (which may or may not express detectable levels of the CD25 molecule on their cell surface) that do not express Foxp3 may still be involved in suppressing the anti-tumor response within the tumor site, serving as regulatory T cells. In this vein, the idea of bystander suppression or "infectious tolerance" proposed by Qin et al. (334) in order to explain why no additional immunosuppression was necessary to maintain lifelong transplantation tolerance induced in adult mice after combined treatment with non-lytic mAbs to CD4 and CD8 T cells (on days 0, 2, and 4 after allogeneic skin grafting even though the thymus continues to make new T cells) and the reports of Jonuleit et al. (335) and Dieckmann et al (336), may be applicable in understanding how the significantly immunosuppressive tumor environment is mainitained by CD4+ TILs in rFL + rGM treated mice. Jonuleit et al. and Dieckmann et al. demonstrated "infective tolerance" by showing that human CD4+CD25+ Tregs, via cell-to-cell contact, convey suppressive capacity to non-regulatory CD4+CD25− T helper cells, that in turn, inhibit the activation of conventional, freshly isolated CD4+ T helper cells (335, 336). Contrary to the cell contact-dependent and fixation-resistant mechanism of suppression employed by the "suppression infecting" CD4+CD25+ Tregs, the newly "suppression infected" CD4+ helper T cells appear to mediate their suppressive activity via cell contact–independent mechanism(s), involving soluble TGF-β or IL-10, that may be abrogated

by fixation (335, 336). Interestingly, Dieckmann et al showed that CD4+CD25+ Tregs induced high level production of IL-10 in co-cultured CD4+CD25− T cells even when they were added to the cultures after activation and fixation, suggesting the involvement of cell surface molecule(s) in imparting suppression (336). Furthermore, Vieira et al have demonstrated that murine Tregs generated *in vitro* or *in vivo* after antigenic stimulation of CD4+CD25− naïve T cells expressed high levels of the GITR marker, but not Foxp3, and inhibited the *in vitro* proliferation of responder T cells as efficiently as Foxp3+CD4+CD25+ Tregs, suggesting that Foxp3 expression may not be a prerequisite for the suppressive capability of IL-10-producing "adaptive" Tregs (337). Interestingly, even though these Foxp3-negative Tregs produced IL-10, the inhibition of T cell proliferation was mediated by an IL-10-independent, but cell contact-dependent mechanism that is abrogated in the presence of anti-GITR antibody (337). Similarly, Bynoe et al. reported that CD4+CD2$\tilde{5}$ regulatory T cells induced in vivo by epi-cutaneous immunization with myelin basic protein peptide did not express Foxp3 but inhibited the development of EAE and suppressed T cell proliferation in a cytokine-independent, but cell contact-dependent manner (338). In my system, CD4+Foxp3+ T cells could represent naturally-occurring regulatory T cells that have been expanded from those cells that arose directly from the thymus whereas the CD4+Foxp3− T cells might represent "adaptive" Treg-like cells that have been peripherally induced within the tumor site by direct cell contact with the CD4+Foxp3+ T cells or indirectly via immunosuppressive cytokines and/or DCs presenting antigen in the presence of intratumoral immunosuppressive cytokines. The relevance of "infectious tolerance" in this system is further supported by the detection of IL-10 and TGF-β RNA transcripts in CD4+ TILs that have been freshly-isolated from tumors in rFL + rGM treated mice. The exclusive expression of IL-10 in CD4+ TILs (but not splenic CD4+ T cells from the same mice) and the high-level of expression

of TGF-β in CD4+ TILs (but not splenic CD4+ T cells from the same mice) suggest that these two cytokines might mediate contact independent suppression associated with the regulatory CD4+ TILs. The *in situ* detection of IL-10 protein in the tumors of rFL + rGM treated mice further suggests that this cytokine may play a significant role in the localized suppression of effector cells within the tumor microenvironment. Indeed, Akbari et al. have demonstrated that IL-10 producing adaptive regulatory T cells can develop in vivo from CD4+CD25− naïve T cells after respiratory exposure to antigen and these cells can block the development of allergen-induced airway hyper-reactivity (AHR) (115). Moreover, in this AHR model, both the development and the inhibitory function of regulatory cells were dependent on the presence of IL-10 and on ICOS−ICOS-ligand interactions (115). In my experimental system, these conditions clearly exist in the tumor microenvironment in that IL-10 is present, nearly 90% of the CD4+ TILs express high levels of the ICOS marker, and ~47% of the tumor infiltrating dendritic cells express high level ICOSL (also known as LICOS, B7-related protein 1 (B7RP-1), B7H, and GL50). Additionally, Treg-mediated suppressive function inside the tumor can be amplified even further in the presence of IL-10, since it has been demonstrated that IL-10 can serve as a positive autocrine factor that can act directly on T cells (even in an APC-free system) to enhance the development of IL-10 producing Tregs (339).

Since increased levels of its RNA transcripts have been detected in CD4+ TILs, TGF-β can further supplement the suppressive environment maintained within the tumor microenvironment by Tregs. TGF-β can actually play a powerful role in Treg-mediated suppression because it can mediate its effects in both a contact-dependent (i.e. membrane bound) and contact-independent (i.e. soluble cytokine) manners of suppression (241, 243). In addition to having a similar capacity as IL-10 in mediating "infectious tolerance" (206, 340), TGF-β may

also be involved in the stimulation and expansion of CD25+CD4+ Tregs within the tumor site, an effect that has been observed both *in vitro* {Yamagiwa, 2001 #809; }and *in vivo* (325, 341). For instance, Peng et al. showed that intra-islet Foxp3+CD25+CD4+ T cells were significantly expanded locally and that these cells inhibited diabetes onset upon a transient pulse of TGF-β in the pancreatic islets by using a tetracycline on/off system (325). Marie et al. further showed that in the absence TGF-β mediated signaling, the homeostatic expansion, the expression of Foxp3, and the suppressor activity of CD25+CD4+ Tregs were all significantly reduced (341). Yet another potential impact of Treg cell-produced TGF-β could involve inhibition of the effector function of the tumor-specific CD8+ T cells within the tumor site. Indeed, Green et al. demonstrated that CD4+CD25+ Tregs inhibited the *in situ* differentiation of islet-reactive CD8+ T cells into CTLs via TGF-β–mediated signaling as evidenced by the inability of CD4+CD25+TGF-β+ Tregs to suppress autoreactive anti-islet CD8+ T cells bearing a dominant negative TGF-β receptor type II transgene (342). Likewise, Chen et al. reported that CD25+CD4+ Tregs block CD8+ T cell-mediated tumor rejection by suppressing the cytotoxicity of expanded CD8+ T cells (343). Interestingly, they also found that the presence of Tregs *in vitro* or *in vivo* did not interfere with the expansion, IFN-γ, and TNF-α production of antigen-specific CD8+ T cells (343). The mechanism of Treg suppression in their experiment was TGF-β signaling dependent since Treg-mediated suppression is abrogated and associated with tumor rejection and unimpaired CTL cytotoxicity if the tumor-specific CD8+ T cells expressed a dominant-negative TGF-β receptor (343). It has further been reported in the literature that antigen presentation by APCs in the presence of TGF-β may lead to the development of regulatory CD8+ T cells that suppress effector cells (344, 345)}. For instance, in models of anterior chamber-associated immune deviation (ACAID)—in which systemic and antigen-

specific impairment in the delayed-type hypersensitivity (DTH) response is induced by the injection of antigen into the anterior chamber of the eye—although CD4+ Tregs are required for the development of tolerance induced by TGF-β-treated APCs, CD8+ Tregs appear to be the mediators of DTH impairment (345). However, I do not think that regulatory CD8+ T cells play a significant role in my model system since CD8+ TILs isolated from the rFL + rGM treated mice were not inhibited in their capacity to respond to *in vitro* stimulation with CMS4 cells by producing IFN-γ production. However, I cannot rule out the potential involvement of tumor-infiltrating DCs (TIDCs) in amplifying the immunosuppression at the tumor site and/or draining lymph node as increased levels of Tregs infiltrate the tumor and start to interact with DCs and to change the cytokine profile of the tumor microenvironment. In addition to providing TCR and co-stimulatory signaling via peptide-MHC class II molecules and CD80 and CD86 molecules, respectively, to enhance the activation/expansion of Tregs, the interaction of ICOS ligand (B7RP-1) expressed on TIDCs (**Appendix A**) with ICOS expressed by Tregs may induce/enhance IL-10 production by Tregs. Indeed, Lohning et al have demonstrated that the activation of CD4+ T cells with the highest expression of ICOS results in the predominant production of the anti-inflammatory cytokine IL-10 (213). Moreover, the interaction of Tregs with TIDCs may induce indoleamine 2,3-dioxygenase (IDO) functional activity in TIDCs via the ligation of their CD80/CD86 molecules and initiate the immunosuppressive pathway of tryptophan catabolism. IDO is an enzyme that degrades the essential amino acid tryptophan into kynurenine and other downstream pro-apoptotic metabolites, such as such as quinolinic acid and 3-hydroxy-anthranilic acid, and its expression is highly inducible by IFN-γ (346). Additionally, soluble CTLA-4-Ig or cloned CTLA-4 expressing Tr1 cells have been shown to induce functional IDO in DCs suggesting that Tregs may utilize the IDO mechanism to suppress T-cell

responses and promote tolerance (233, 346). It appears that this suppression is mediated not by inhibiting T cell activation but by preventing clonal expansion as a result of rapid death of the activated T cells via the depletion of tryptophan from the local microenvironment and/or apotosis induced by tryptophan metabolites (346-348). To test the possible involvement of IDO in my model system, I performed IDO-specific western blots on cell lysates prepared from CD11c+ and CD11c– fractions of TILs and splenocytes of rFL + rGM treated CMS4 bearing mice (**Appendix B**). Interestingly, I detected IDO expression in both CD11c+ and CD11c− cells isolated from both the tumor and the spleen. To assess whether the IDO were functionally active, I performed HPLC analysis on cell-free supernatants of L- tryptophan incubated, freshly-isolated CD11c+ TIDCs and splenic DCs to detect its downstream metabolite, kynurenine. It appears that the IDO expressed by CD11c+ TIDC is functionally active, since I were able to detect 12 ± 2 μM kynurenine in their culture without any additional stimuli while I did not detect any kynurenine in the CD11c+ splenic DCs (data not shown). As IL-10 has been reported to upregulate the expression of IDO in DCs (349, 350), the IL-10 detected within the tumor may be involved in maintaining the enzymatic activity of IDO expressed in TIDCs. This will be consistent with data by von Bubnoff et al. showing that clinical unresponsiveness (in which Tregs are thought to play a role) in aeroallergen-sensitized atopic individuals correlates well with significantly higher systemic activity of IDO and increased levels of IL-10 during allergen exposure (351). Even though I did not detect significant leukocytic apoptosis in tumors sections obtained a day after rFL + rGM treatment, once additional Tregs accumulate/expand, it is possible that local depletion of tryptophan and apoptosis induction by tryptophan metabolites could contribute to the hindrance of the effector function of tumor infiltrating CD8+ T cells. Further, it is possible that TIDCs trafficking to the draining lymph nodes after having been in contact with Tregs in

the tumor microenvironment, and having upregulated their IDO activity, might dampen the active and ongoing priming of tumor-specific CD8+ T cells induced by rFL + rGM treatment.

Taken together, it appears that GITR, IL-10, TGF-β, and other cell surface or soluble factors associated with the prevalent "natural" and/or "adaptive" Tregs infiltrating/induced at the tumor site due to rFL + rGM treatment have the potential to create a modified tumor microenvironment that is hostile to effector CD8+ T cells. Therefore, the concurrent infiltration of effector CD8+ T cells and regulatory CD4+ T cells into tumors treated with rFL + rGM may explain why this combinational cytokine treatment fails to ultimately negatively impact tumor growth, despite its ability to promote functional DC infiltration of tumor lesions and consequent CD8+ T cell cross-priming. In different experimental systems, CD4+ Tregs have been reported to suppress CD8+ T cell priming (330, 352-354), IFN-γ production (355-357) and/or cytolytic function (342, 343, 358). In my rFL + rGM treatment tumor model, Tregs present within the CD4+ TILs population likely inhibit the effector function of the CD8+ T cells that have specifically trafficked into the tumor site, thereby limiting their anti-tumor efficacy. The production of high levels of IFN-γ by *in vitro* stimulated CD8+ T cells isolated from the spleen of rFL + rGM treated mice indicates that despite their concomitant expansion, splenic Foxp3+CD4+ Tregs do not inhibit the priming and differentiation of these tumor-specific CD8+ T cells. This conclusion is consistent with my finding that tumors from cytokine-treated mice were significantly infiltrated by CMS4-specific CD8+ T cells and that these CD8+ TILs produced high levels of IFN-γ when stimulated *in vitro* with CMS4 but not an unrelated syngenic tumor, 4T1. The ability of CD4+ TILs to significantly hamper IFN-γ production from co-cultured tumor-specific CD8+ T cells upon *in vitro* stimulation with CMS4 cells suggests that they have been activated *in vivo* and can participate in a similar scenario that possibly occurs *in*

vivo in the tumor microenvironment. Since IFN-γ plays a critical role in CD8+ T cell mediated anti-tumor immune response (including preventing rapid tumor burden by direct or indirect induction of angiostasis or direct destruction of existing tumor blood vessels (359, 360), the inhibition of its production by CD4+ Tregs will have major negative consequences in the host's ability to control tumor growth. For example, it has been clearly demonstrated that 1) tumor rejection is impaired in IFN-γ (361) or IFN-γ receptor (362) knockout mice, 2) tumor rejection can be abrogated by IFN-γ neutralizing antibodies (363), and 3) IFN-γ production correlates positively with the ability of adoptively-transferred, tumor-specific CD8+ T cells to mediate tumor regression (364). Even though I did not assess the impact of CD4+ TILs on CD8+ T cell cytotoxicity, I believe that this function would also be hampered, like IFN-γ production. The combined suppression of IFN-γ production and cytolytic function of tumor infiltrating CD8+ T cells by CD4+ TILs would optimize the chances that a tumor would escape immune regulation. Indeed, there was no difference in tumor growth between cytokine-treated and untreated mice suggesting that tumor-infiltrating CD8+ T cells in cytokine treated mice failed to effectively control tumor growth. However, this cytokine treatment-induced Treg mediated suppression of an active anti-tumor response appears to be reversible. The high level *in vitro* production of IFN-γ by CD8+ TILs suggested to us that as long as Tregs are not present at the effector site, CD8+ T cells can regain/carry out their effector function. Consistent with this suggestion, the *in vivo* depletion of CD4+ T cells for a short period of time significantly inhibited tumor growth in rFL + rGM treated animals during the depletion period. Although the tumor continued its fast growth rate during the CD4+ T cell recovery and normalization periods, it was evident that CD4+ T cell depletion exerts a positive impact on the ability of therapy-induced effector CD8+ T cells to (at least) transiently control tumor growth. As a result, in the repeat experiment, I

maintained the depleted state for a longer period of time by the administration of anti-CD4 mAb every three days. As I expected, the extension of the depletion period resulted in continued inhibition of tumor growth in cytokine treated mice. On the contrary, CD4+ T cell depletion in cytokine untreated mice did not result in such a pronounced suppression of tumor growth. The anti-CD4 Ab treatment induced impact on tumor growth in rFL + rGM treated mice was clearly dependent on the ability of CD8+ T cells to arrest tumor growth since the co-depletion of CD8+ T cells along with CD4+ T cells, abrogated the observed therapeutic effects associated with anti-CD4 mAb administration. These data clearly demonstrate that rFL + rGM treatment can yield a CD8+ T cell-dependent therapeutic benefit, but only in the absence of treatment-expanded Foxp3+CD4+ Tregs and possibly "infectious tolerance" generated Foxp3−CD4+ Tregs in the immunosuppressive tumor microenvironment. It was interesting to observe such an extended period of tumor growth arrest without any major decrease or increase in tumor area or volume. It appears that anti-tumor CD8+ T cells support a period of active symbiosis, allowing the tumor to co-exist without overtly hindering the activities/health of the host. I did not observe any physical impediments in rFL + rGM treated, tumor-bearing mice that were additionally treated with the CD4 depleting mAb for up to 62 days after tumor inoculation. On the contrary, anti-CD4 and anti-CD8 mAb treated mice did not survive beyond 28 days after tumor inoculation.

It is possible that in the CMS4 tumor model, CD4+ T helper cells may not be required for the continued priming of tumor-specific CD8+ T cells (as seems to be the case in CD4 depleted, cytokine treated mice) but that their effector function at the tumor site might still be important for the complete resolution of the anti-tumor immune response. For instance, this CD4+ T helper cell function may involve the secretion of cytokines that can recruit and activate macrophages and/or eosinophils within the tumor site to produce both superoxide and nitric

oxide and cause tumor destruction (178, 365). Hung et al, for example, showed that despite no loss in anti-tumor CTL activity, protection against B16 melanoma tumor challenge was significantly diminished in vaccinated, IL-5 (cytokine critical for differentiation of bone marrow progenitors into eosinophils) knockout mice and that this was associated with the absence of eosinophils at the tumor challenge site (365). They also showed that NO produced by tumor infiltrating macrophages exerts tumoricidal activity and that there was a substantial decrease in protection against tumor challenge in vaccinated, iNOS knockout mice (365). These studies suggest that maximal anti-tumor immunity may require the participation of additional non-T effector cells, other than CD8+ T cells.

Since intraperitoneal anti-CD4 antibody administration depletes both helper and regulatory CD4+ T cells, it will be interesting to observe in future experiments whether complete tumor regression can be achieved by selective targeting of Tregs or their immunosuppressive cytokines. While one choice would be for the use of ant-CD25 antibody in this application, there is also a possible disadvantage to using this antibody since cytokine treatment-activated effector CD4+ and CD8+ T cells may also be targeted for depletion, since they are also likely to express CD25. Another alternative treatment strategy would be to use anti-GITR Abs to mitigate Treg function, rather than necessarily depleting this cell population While it does not appear to deplete Treg cells, the anti-mouse GITR mAb (DTA-1) has been shown to neutralize the suppressive activity of CD25+CD4+ Treg *in vitro* (**Fig. 5** and ref. (210)) and to induce organ-specific autoimmune disease in normal mice (210). Even though it is not clear whether GITR stimulation acts by stimulating a suppression blocking signal to CD25+CD4+ Treg (210) or rendering activated GITR+ T cells resistant to CD25+CD4+ Treg-mediated suppression (232), the net result could enforce specific CD8+ T cells to mediate their tumoricidal functions *in situ*.

Yet a third alternative to total CD4 T cell depletion is to block the effects of IL-10 and TGF-β as these suppressive/anti-inflammatory cytokines may mediate the inhibitory activity of CD4+ TILs. Even though Treg may employ additional (as yet unknown) mechanism of suppression *in vivo*, the administration of neutralizing anti-IL-10/IL-10 receptor and/or anti-TGF-β antibodies or TGF-β soluble receptors (TGF-β-SR) could potentiate the therapeutic impact of rFL + rGM treatment in tumor bearing animals. Indeed, very recently, Yu et al have demonstrated that the regression of a tumor tumor cell line transfected with a gene encoding an immunogenic antigen can be achieved either by intratumoral/intraperitoneal depletion of CD4+ cells or by intraperitoneal treatment with anti–IL-10 receptor or anti-TGF-β (323). However, as Fuss et al. showed that anti-TGF-β mAb administration prevents TGF-β secretion, but leaves IL-10 secretion intact, while anti-IL-10 mAb administration prevents both TGF-β and IL-10 production (366), any therapeutic benefit that results from the use of these antibodies would need to be closely examined in order to determine the role each cytokine plays in Treg-mediated suppression.

In addition to attempting to directly target Tregs and their cytokines, treatments that potently activate DCs could also be envisioned to bypass Treg mediated suppression by creating a predominant pro-inflammatory tumor microenvironment. For instance, imiquimod (Aldera) is a synthetic compound that activates immune cells via the TLR-7 MyD88-dependent signaling pathway and has been shown to strongly induce the maturation and proinflammatory cytokine—such as IL-12, IFN-α, and TNF-α—production by macrophages (367) and dendritic cells (368). Palamara et al. reported that topical imiquimod treatment of intradermally-induced melanomas resulted in the accumulation of plasmacytoid DCs in both the spleen and the tumor and lead to either complete regression or a significant reduction of tumor burden (369). Likewise, Craft et

al. showed that the protective anti-tumor effects of a live, recombinant listeria vaccine against murine melanoma is significantly enhanced by imiquimod application and is clearly evident by the development of a localized vitiligo (370). In my model system, I have combined rFL + rGM treatment with topical imiquimod to examine whether this strong adjuvant can help reverse Treg mediated suppression of the anti-tumor immune response (**Appendix C**). However, imiquimod treatment did not show any additional influence on CMS4 growth in rFL + rGM treated mice over that seen in cytokine untreated mice, and the tumors continued their progressive growth within a week of treatment cessation. Hence, it appears that imiquimod treatment is insufficient to disrupt the immunosuppressive tumor microenvironment maintained by cytokine treatment-induced Tregs. Recently, Pasare et al. have shown that CD4+CD25+ T cell-mediated suppression can be overcome by LPS or CpG induced ligation of TLR-4 or TLR-9, respectively, in DCs by making responder T cells refractory to suppression (371). This effect is mediated by IL-6 acting synergistically with as yet unidentified other soluble factor(s) and is almost completely abrogated by neutralization of IL-6 (371). In TIDCs or splenic DCs from rFL + rGM treated mice, stimulation with LPS results in elevated levels of TNF-α, IL-1β, IL12 p40 (**Appendix D**), IL-12 p70, and IL-10 (**Fig.7**) and extremely elevated levels of IL-6. Therefore, LPS/CpG motifs administration might synergize with rFL + rGM treatment, yielding a better therapeutic outcome via the mitigation of Treg mediated suppression by IL-6 and other factors. Repeated treatments with LPS/CpG motifs might be necessary in order to maintain a pro-inflammatory environment, however, since single LPS treatments have been reportedly unable to abrogate Treg mediated suppression of anti-tumor immunity in vivo (323). However, Yang et al. have demonstrated that withdrawal of LPS results in a rapid reduction (>90%) in DC production of pro-inflammatory cytokines in vitro and daily administration of LPS for 4

consecutive days led to a significant increase in protection of mice from A20-HA lymphoma in the presence of CD25+CD4+ Tregs cells. Therefore, like what I observe in my group of CD4 depleted, cytokine treated mice, even in treatments involving strong inducers of DC activation, it appears that there is a strong tendency for Tregs to quickly re-assert their dominance over effector cells once the signals from those treatments start to wane.

4. SUMMARY AND CONCLUSIONS

The goal of immunotherapy for cancer is to restore the ability of the host's immune system to control the progressive growth of tumors. In order for the adaptive immune system to recognize and attack tumors, tumor-derived antigens need to be presented to the tumor-specific T cells residing in the secondary lymphoid organs. Once activated, the tumor-specific T cells proliferate, differentiate into effector cells and traffic to the target site, i.e. the tumor. In most cases, both CD4+ helper T cells and CD8+ T cells are required for optimal anti-tumor immunity. Because of the lack of co-stimulatory (CD80/CD86) molecule expression on their cell surface, most tumors cannot deliver the co-stimulatory signaling necessary for the activation of naïve tumor-specific T cells. In addition, until their metastasis stages of differentiation, tumors are localized in the periphery and cannot directly interact with the T cells present in the lymphoid organs. As a result, the presence of an antigen presenting cell that can acquire antigen from tumors in the periphery, migrate to the lymphoid organs, and present tumor antigen-derived peptides in the context of the appropriate MHC, co-stimulatory, and cytokine molecules is of critical importance for the initiation and amplification of the immune response against tumors. As APCs that meet all these requirements, DCs have been shown to play a central role in the priming and activation of both CD4+ and CD8+ T cells. As such, the administration of *ex vivo* generated and tumor-derived antigen (irradiated/apoptotic tumor cells, tumor lysates, whole proteins, peptides, RNA, DNA, exosomes, viral vectors encoded TAAs) exposed DCs have been reported to enhance anti-tumor immunity in both murine and human hosts albeit at varying degrees (60, 266-272, 274-276). For example, in the CMS4 tumor model, Mayordomo et al. have demonstrated that the treatment of CMS4 bearing mice with DCs pulsed with wild-type p53 peptide induced peptide-specific CD8+ CTLs and caused tumor rejection in 60% of the mice

(267). Several reports have also shown that the recruitment of DCs directly into the tumor microenvironment by engineering tumors to express cytokines (such as GM-CSF or IL-4) or chemokines (such as SLC or ELC/MIP-3β) or by the intratumoral administration of recombinant cytokines/chemokines bypasses the need for *ex vivo* manipulation of DCs and correlates with enhanced anti-tumor responses (301, 372-375). In our laboratory, Tatsumi and colleagues demonstrated that DCs engineered to secrete both IL-12 and IL-18 and administered intratumorally induced complete rejection of CMS4 tumors without the need to pulse the injected DCs with tumor antigen (59). Interestingly, splenic CD8+ T cells isolated from these treated mice reacted *in vitro* against a wide range of naturally-presented, tumor peptide epitopes suggesting that the treatment resulted in the activation of a broad, diversified repertoire of anti-tumor CD8+ T cells (59). Most of all, the findings in human cancers, such as oral and lung squamous cell carcinomas and gastric carcinomas, that patient prognosis and reduction in the establishment of metastatic disease correlates positively with the level of infiltration of the tumors by DCs suggests that not only the absence/presence but also the magnitude of their presence may impact immune response against tumor growth (289-293). Indeed, significant increases in tumor infiltrating lymphocytes are observed in tumors with higher densities of DCs (289, 290).

All of the above observations prompted us to hypothesize that since progressive tumors can act as a reservoir of antigens that can be processed and presented by APCs to activate a broad repertoire of tumor-specific T cells, that by optimizing the presence of functionally active DCs in the tumor microenvironment, there would be the potential to promote cross-priming events *in situ* and to enhance the therapeutic anti-tumor immune response. I observed that the treatment of tumor-bearing mice with combinations of rFL + rGM-CSF was significantly better

than either treatment alone in increasing the number of DC *in vivo*. As a result, combinational treatment with rFL + rGM-CSF was used to determine a schedule resulting in the maximal number of DCs infiltrating CMS4 tumors. I found that seven days of cytokine treatment were required for optimal numbers of CD11c+ DCs to be achieved in the spleen, while only five days of treatment was required for optimal numbers of DCs to infiltrate tumors. However, the treatment of mice bearing CMS4 sarcoma (or RENCA or CT26) tumors for five days with rFL + rGM failed to provide any therapeutic benefit(s) as evidenced by the lack of inhibition of progressive tumor growth. These findings led us to examine whether there was a lack of tumor-specific T cell cross-priming, despite the presence of high levels of DCs not only in the tumor but also in the lymphoid organs of cytokine-treated animals. Consistent with the scarcity of DCs and CD4+ and CD8+ T cells within the tumors of untreated mice, I failed to detect any IFN-γ production from their splenic CD8+ T cells when stimulated with irradiated CMS4 cells *in vitro*. Interestingly, despite the lack of therapeutic effect, the tumors of cytokine treated mice were highly-infiltrated with CD4+ and CD8+ T cells in addition to DCs. The TIDCs expressed elevated levels of MHC class II molecules and CD80/CD86 molecules, stimulated allogenic CD4+ T cells, and were very efficient at antigen uptake and processing, strongly suggesting that they possessed the functional competency to prime naïve T cells. Consistent with activation of tumor-specific T cells due to rFL + rGM treatment, CD8+ T cells isolated from both the spleen and the tumors of cytokine treated hosts produced high levels of IFN-γ when stimulated *in vitro* with irradiated CMS4 cells, but not the unrelated tumor cells, 4T1. Based on the observation of 1) progressive CMS4 growth in the presence of activated, tumor-specific T cells, 2) the CD4+ Tr1-like cell inducing phenotype (114, 116, 117) of the TIDCs and SPDCs, and 3) the infiltration of the tumors with similar levels of CD4+ T cells as CD8+ T cells, I hypothesized that rFL +

rGM treatment may also lead to the induction of regulatory T cells that can actively suppress the effector function of CD8+ TILs and hence limit the effectiveness of the therapeutic anti-tumor immune response. Consistent with my hypothesis, I found that cytokine treatment doubled the percentage of Foxp3—the regulatory T cell specification factor—expressing CD4+ T cells in the spleens of both none-tumor-bearing, normal controls and CMS4 bearing mice. Within the tumor site, almost half of the CD4+ T cells infiltrating the tumor expressed intracellular Foxp3 protein of which 97% were $GITR^{High}$ and 73% were CD25+. The CD4+ TILs also expressed high level of ICOS but very low levels of CD45RB and CD62L molecules. In co-culture assays *in vitro*, these CD4+ TILs almost completely abrogated the proliferative responses of naïve CD4+ T cells and significantly reduced cytokine production from tumor-specific CD8+ T cells. I believe that these CD4+ TILs may utilize both cell contact-dependent and -independent mechanism to suppress CD8+ T cells *in vivo*. Since GITR is highly-expressed in almost 90% of the CD4+ TILs and anti-GITR antibody partially abrogates the functional suppressive activity of these cells in vitro, this molecule may be involvedin cell contact-dependent mechanism(s) of active suppression. The increased levels of IL-10 and TGF-β RNA transcripts detected in CD4+ TILs and the IL-10 protein detected within the tumor strongly suggest that these two cytokines may participate in Tregs-mediated contact-independent mechanism(s) of suppression. Of course, TGF-β may also be involved in contact-dependent suppression, as it has been reported that CD4+CD25+ Tregs can express membrane bound TGF-β and employ this in inhibiting CD4+CD25− T cell proliferation (243). When taken together, 1) the anergic state of the CD4+ TILs, 2) the extreme magnitude of suppressed naïve T cell proliferation in the presence of unfractionated CD4+ TILs, 3) the detection of IL-10 transcripts in CD4+ TILs but not splenic CD4+ T cells, and 4) the similar level of GITR expression between Foxp3+ and Foxp3− CD4+

TILs, these findings suggest that these Foxp3+CD4+ TILs are likely active *in situ* and may have imparted their suppressive capacity to Foxp3–CD4+ TILs via "infectious" tolerance. The conversion of Foxp3–CD4+ TILs into regulatory T cells can be expected to have an additive suppressive effect in addition to that mediated by Foxp3+CD4+TILs and may explain the lack of any discernible impact on tumor growth from rFL + rGM treatment. Indeed, remarkable tumor growth arrest was observed in cytokine-treated mice that were also treated with CD4 depleting mAb. This impact on tumor growth was reversible if CD4+ and CD8+ T cells were co-depleted, strongly suggesting that in the absence of CD4+ TILs, tumor infiltrating CD8+ T cells can effectively control tumor growth. However, it appears that the continuous administration of anti-CD4 antibody is necessary in order to maintain this state of tumor growth arrest in cytokine-treated mice, since tumor progression is rapidly re-established once anti-CD4 mAb treatment is discontinued. It would appear that CD4+ Tregs rapidly recover after cessation of Ab-depletion, again limiting the beneficial impact of rFL + rGM administartion.

The percentage of Foxp3+CD4+ T cells is ~13% of all CD4+ T cells in untreated normal mice while it represents ~28% in rFL + rGM treated normal mice. This increase in the percentage of Foxp3+CD4+ T cells also results in a ~2.6 fold increase in the number of these cells in cytokine-treated mice ($5.71 \times 10^6 \pm 1.81 \times 10^5$) when compared to untreated mice ($2.14 \times 10^6 \pm 1.87 \times 10^4$). These findings suggest that rFL + rGM treatment increases the number of Foxp3+CD4+ T cells by stimulating their expansion and not by inducing Foxp3 expression in Foxp3–CD4+ T cells. But, how does rFL and rGM treatment lead to the expansion of CD4+Foxp3+ T cells in the spleen of normal mice? I speculate that the markedly increased presence of DCs exhibiting a mixed populational phenotype, i.e. immature ($CD40^{lo}$ (**Appendix A**), antigen $uptake^{high}$) and mature (MHC class II^{Hi}, $CD86^{Hi}$, $CD80^{Hi}$) DCs, that express ICOS

ligand on their surface, and have the propensity to secrete significantly higher levels of IL-10 rather than IL-12 p70 upon activation, plays a role in Treg expansion. Salomon et al. demonstrated that CD4+CD25+ Tregs are detected in markedly-reduced numbers in B7-1/B7-2-deficient and CD28-deficient NOD mice and their absence results in the exacerbation of spontaneous diabetes (201). Their data suggests that the co-stimulation of murine CD4+CD25+ Tregs via CD28/B7 interaction is essential for their development and homeostasis *in vivo* (201). Studies by Jordan et al. suggest that the expression level of self-antigens determines the level of CD4+CD25+ Treg formation (195, 196). Therefore, it appears that both TCR and CD28 signals are necessary for optimal Treg development *in vivo*. In my experimental system, the high levels of expression of MHC class II and CD80 and CD86 molecules on rFL + rGM expanded DCs meet both of these conditions necessary for the induction of Treg proliferation. It appears that Tregs have a moderate affinity for self-peptide:MHC class II molecules (195). Interestingly, I found that TIDCs in rFL + rGM treated animals were efficient at mediating endocytosis as evidenced by their ability to take up and process DQ-Ovalbumin (presumably via mannose receptors). As exogenous self antigens in extracellular milieu are acquired by DCs by macropinocytosis, phagocytosis, and receptor-mediated endocytosis, they are efficiently processed and presented in MHC class II complexes. As a result, in the absence of inflammatory stimuli (as is the case in rFL + rGM treated normal mice) the increase in the number of highly-endocytic splenic DCs would be expected to increase the repertoire and density of self-antigens presented on those DCs. The increase in the number of DCs in the spleen would also be expected to enhance the likelihood of the interaction between DCs and Tregs resident in the spleen. Foxp3+CD4+ Tregs that recognize self peptides presented on splenic DCs would be capable of expanding efficiently because these DCs provide the requisite co-stimulatory signals

for Treg cell activation. It is also possible that Treg TCR and CD28 ligation by DCs could result in the upregulation of ICOS by the Tregs, allowing these cells to receive additional proliferative signals via the ICOS ligand expressed on the DCs.

Even though the specific factors/events necessary for the recruitment of murine Tregs *in vivo* is an understudied area, recent studies suggest that the chemokines MCP-1/CCL2 (CCR2 ligand), MIP-1α (CCR1, CCR5 ligand), MIP-1β (CCR1,CCR5, CCR7, CCR8 ligand), and SDF-1α (CXCR4 ligand) may play important roles in Treg chemotaxis (325, 376-379). Of these chemokines, Tregs appear to respond most strongly to CCL2. Bruhl et al. have reported that the CCL2 receptor, CCR2, is expressed on the $CD44^{high}CD45RB^{low}$CD25+CD103+ subset of CD4+ regulatory T cells (377). To determine whether there are any differences in the chemokine secretion between TIDCs and splenic DCs in relations to Tregs, I performed multi-chemokine Luminex assays on TIDCs and splenic DCs from rFL + rGM-CSF treated mice cultured overnight in the absence or presence of LPS. Interestingly, unstimulated TIDCs, but not splenic DCs, produced high levels of the chemokines MCP-1 and IP-10 (**Appendix E**). On the contrary, unstimulated splenic DCs, but not TIDCs, produced high levels of MIP-1α. CD4+ T_H1 and CD8+ T cells predominatly express CCR1, CXCR3 and CCR5 and respond to IP-10 via CXCR3 (380). As stated earlier, tumor-specific CD8+ T cell priming occurs in the spleen, even in the presence of cytokine treatment expanded Tregs, but the effector function of CD8+ T cells at the tumor site is blocked by the presence of Tregs. I hypothesize that the production of both CCR2 (Treg chemoattractant) and IP-10 (CD4+ T_H1 and CD8+ T cell chemoattractant) by TIDCs may play a critical role in bringing the CD4+ Tregs and CD8+ TILs into close proximity, allowing Treg-mediated immune suppression to occur.. Since DCs express both MHC class I and II molecules, both Tregs and CD8+ T cells can interact with a TIDC that have attracted both

populations of T cells. I believe that this three cell interaction could amplify Treg mediated suppression in various ways. First, Tregs may be able to directly suppress neighboring CD8+ T cells by direct cell-to-cell contact. Second, Foxp3+CD4+ T cells can confer suppressive capacity on neighboring CD4+ T_H1 cells, which in turn can suppress neighboring or distant CD8+ T cells via cell-to-cell contact or induced secretion of immunosuppressive cytokines. Third, Tregs can transmit immunosuppressive signals to TIDCs (such as the activation of the enzymatic activity of IDO), which in turn can become immunosuppressive to the CD8+ T cells. Fourth, TIDCs can provide positive signals (such as induction of IL-10 production via ICOS:ICOS Ligand interaction) that will allow the Tregs to activate their cell contact independent mechanism of suppression so that Treg suppression can reach more than just the neighboring effector T cells. When CD4+ TILs are depleted, I would expect the interaction of the CD8+ TILs and TIDCs to result in the development of TIDCs capable of providing improved stimulatory rather than inhibitory signals and the cytokine profile in the area of the interaction to shift from a more anti-inflammatory milieu to a pro-inflammatory one. Likewise, I expect that splenic CD8+ T cells can be activated by splenic DCs in rFL and rGM treated mice, since these DCs do not produce CCR2 that may be involved in enforcing the three cell interaction. Indeed, I find that splenic DCs secrete MIP-1α, which strongly attracts T_H1 and CD8+ T cells but is a weak chemoattractant towards Tregs. Interestingly, DePaolo et al. reported that the induction of oral tolerance is mediated by Tregs and is abrogated in $CCL2^{-/-}$ and $CCR2^{-/-}$ mice (376). Blease et al. further reported that pulmonary allergic responses to an allergen were enhanced in $CCR2^{-/-}$ mice, suggesting the lack of recruitment of Tregs that normally control airway hyperreactivity (AHR) (381). Similarly, I think that in the absence of IP-10 or its inducing factor (i.e. IFN-γ) in the presesnce of CCL2 may also result in the abrogation of Treg mediated suppression due to the

inability to position the effector T cells in close proximity to CCR2+ Tregs. Thus, it is tempting for us to speculate that the unexpected occurrence of increased susceptibility to EAE (experimental autoimmune encephalomyelitis) in IFN-γ knockout mice (382) is due to the complete absence of IP-10, which is highly expressed in the CNS (central nervous system) of mice with an intact IFN-γ gene. Even though MCP-1 is upregulated in the CNS of IFN-γ knockout mice with EAE (382) and may result in an enhanced recruitment of Tregs into the CNS, the pathogenic T cells responsible for EAE may not be suppressed because they are not brought in close proximity to Tregs due to the absence of IP-10 in the same microenvironment that contains CCL2. Therfore, when needed, it might be possible to enforce Treg mediated tolerance at specific tissue location *in vivo* by the simultaneous administration of IP-10 and CCL2.

Overall, my findings demonstrate that suppression mediated by regulatory T cells can present a major roadblock for the successful implementation of immunotherapeutic approaches to treat cancers. The results of my study reinforce the need to consider not only resident Treg in tumor-bearing hosts at the time of therapy, but also the potential for therapy-induced augmentation of Treg function *in situ* that may limit treatment efficacy. Thus, the induction of high levels of tumor-specific CTLs by the immunotherapy is not sufficient to guarantee clinical efficacy, if the same therapy is also conducive to the expansion of Tregs *in situ*. As a result, if an immunotherapy for a cancer results in an enhanced activation of tumor specific CD8+ T cells but lacks any impact on tumor growth, the assessment of active suppression by Tregs is absolutely required before a decision should be made to abandon the methodology. Ultimately, even in the absence of direct inhibition of CTL effector function by tumor cells or tumor-secreted products,

the outcome of a purported therapeutic treatment may be decided by the functional balance between Tregs and tumor-specific CTLs within the tumor microenvironment.

5. FIGURES

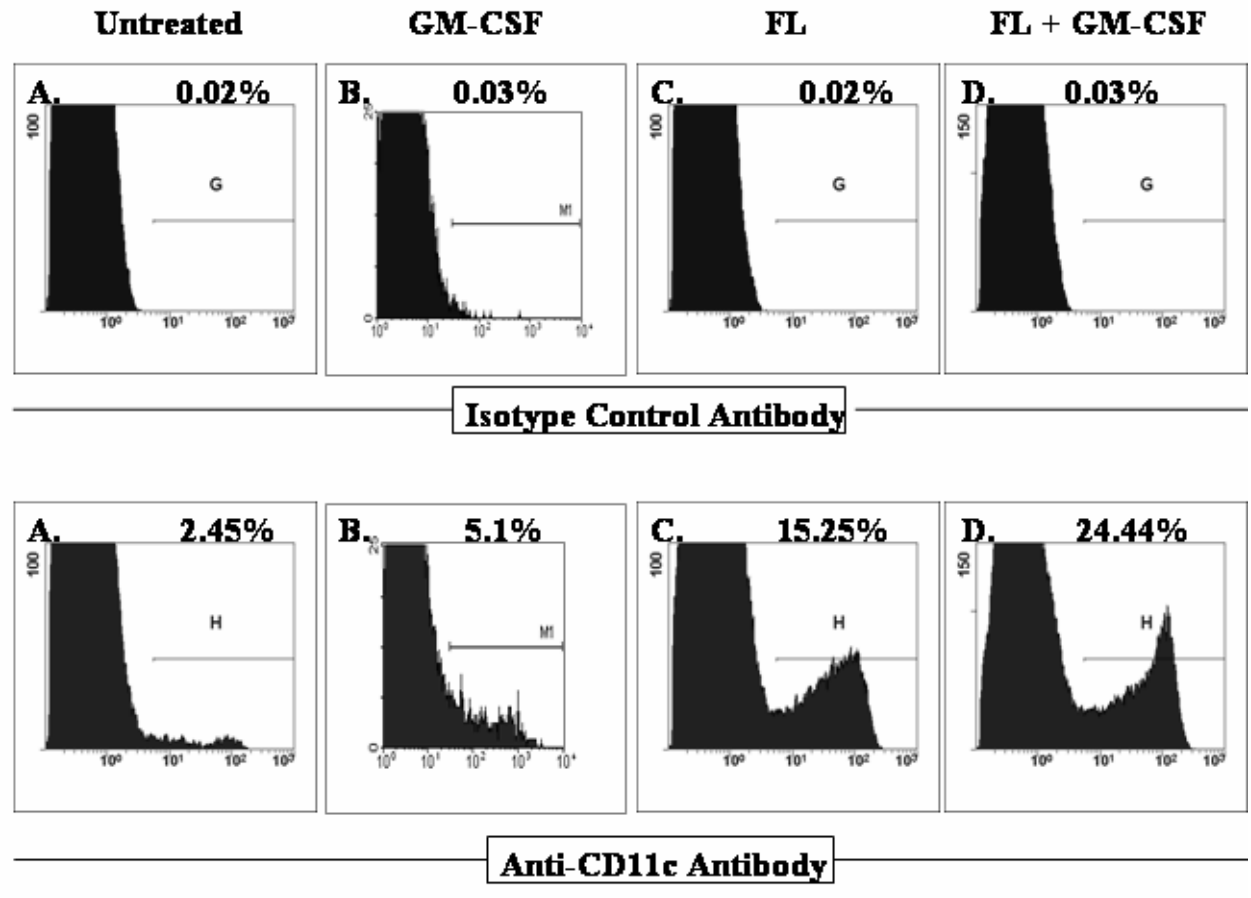


Figure 1. Combined treatment with rFlt3 Ligand (FL) and rGM-CSF (rGM) expands more splenic CD11c+ dendritic cells (DC) than either treatment alone.

Normal BALB/cJ mice were either untreated (Panel A) or treated with 20 μg/day of rGM alone (Panel B), FL alone (Panel C), or rFL + rGM (Panel D) for seven days. The mice were sacrificed on the eighth day, splenic cell suspensions were F_c receptor blocked and stained with isotype control or anti-CD11c antibody and analyzed by flow cytometry. The data shown is based on gating live splenocytes.

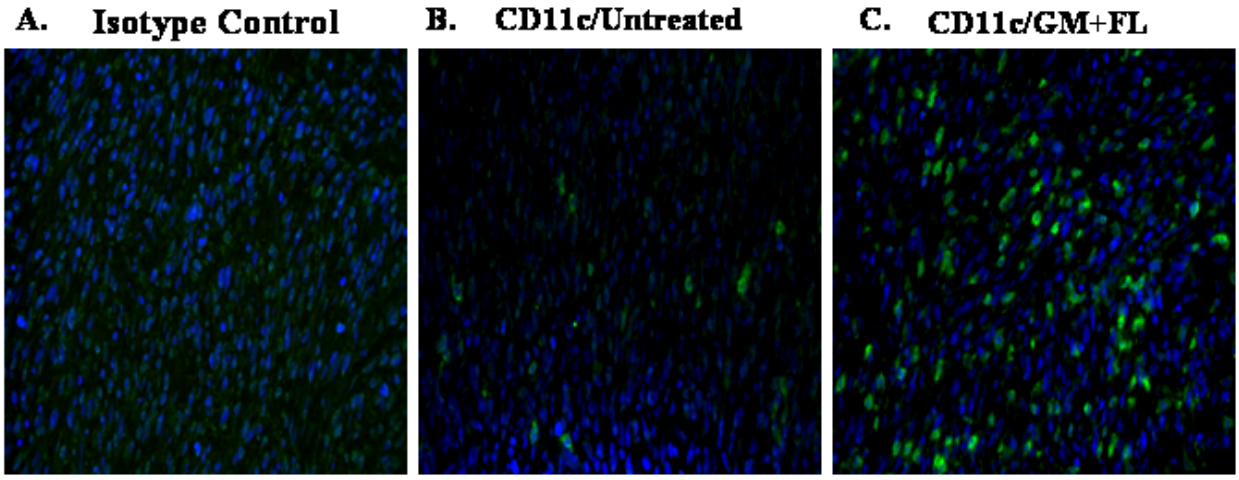


Figure 2. Treatment of mice with combinations of rFL and rGM also increases tumor infiltration by CD11c+ dendritic cells (TIDCs).

BALB/cJ mice bearing day 7 established CMS4 tumors were treated with 20 μg/day of each cytokine for seven days and then sacrificed on day 14 post-tumor inoculation. Five mm sections of the tumors were stained and analyzed by immunofluorescence microscopy. Nuclei are depicted in blue and CD11c+ cells are in green.

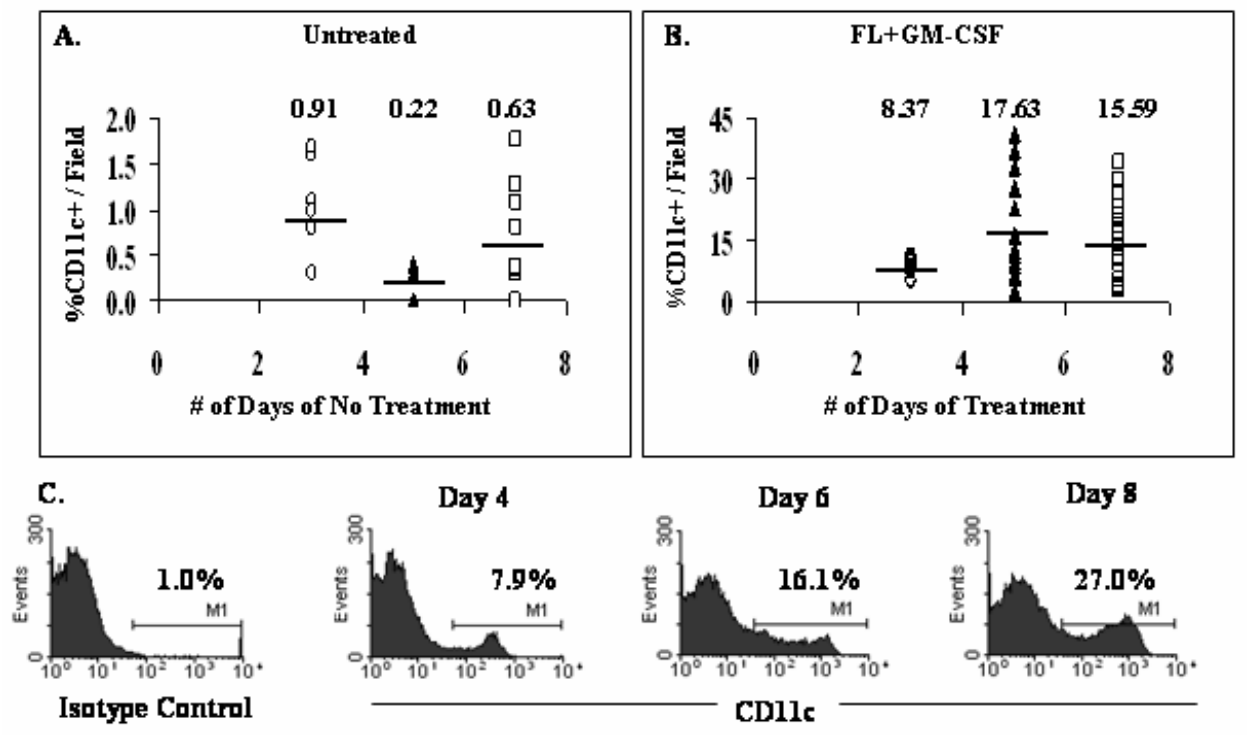


Figure 3. Five days of treatment with rFL + rGM results in optimal TIDC numbers.

BALB/cJ mice bearing day 7 established CMS4 tumor were either untreated or treated with 20 μg/day of each cytokine for 3, 5, or 7 days and sacrificed the following day. Panels A and B). The percentage of CD11c+ DC in the tumors was quantitated by Metamorph v.6.1 software after images were collected by confocal microscopy. The percentage composition of CD11c+ DC within a representative tumor is shown for each captured confocal image field based on the total number of nuclei. The numbers at the top and the bars in the graphs indicate the average % value. Panel C) The percentage of CD11c+ cells in the spleen was quantitated by flow cytometry performed on total splenocyte cell suspensions.

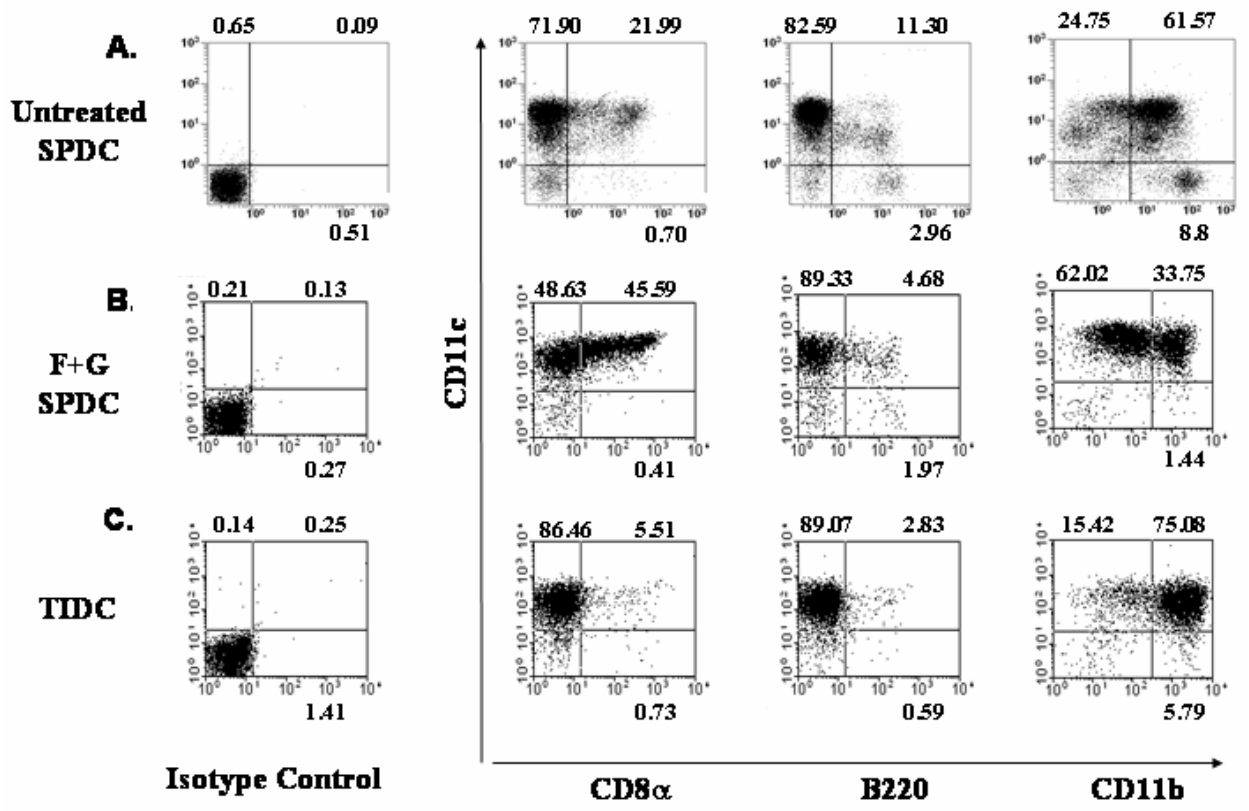


Figure 4. The vast majority of CD11c+ TIDC co-express the CD11b, but not the CD8α or B220 markers.

BALB/cJ mice bearing day 8 or 9 established CMS4 tumor were treated with 20 μg/day of each cytokine for 5 days and sacrificed the next day. Tumors were digested and processed on a Lympholyte-M gradient in order to obtain total tumor-infiltrating leukocytes (TIL). TIDC are isolated from the TIL by CD11c MACS bead selection. Splenic DCs were also isolated from the same mouse (F+G_SPDC) or from untreated non-tumor bearing mice (Untreated_SPDC) by CD11c MACS bead selection. The numbers in the panel insets represent the percentage of cells in the specific quadrant.

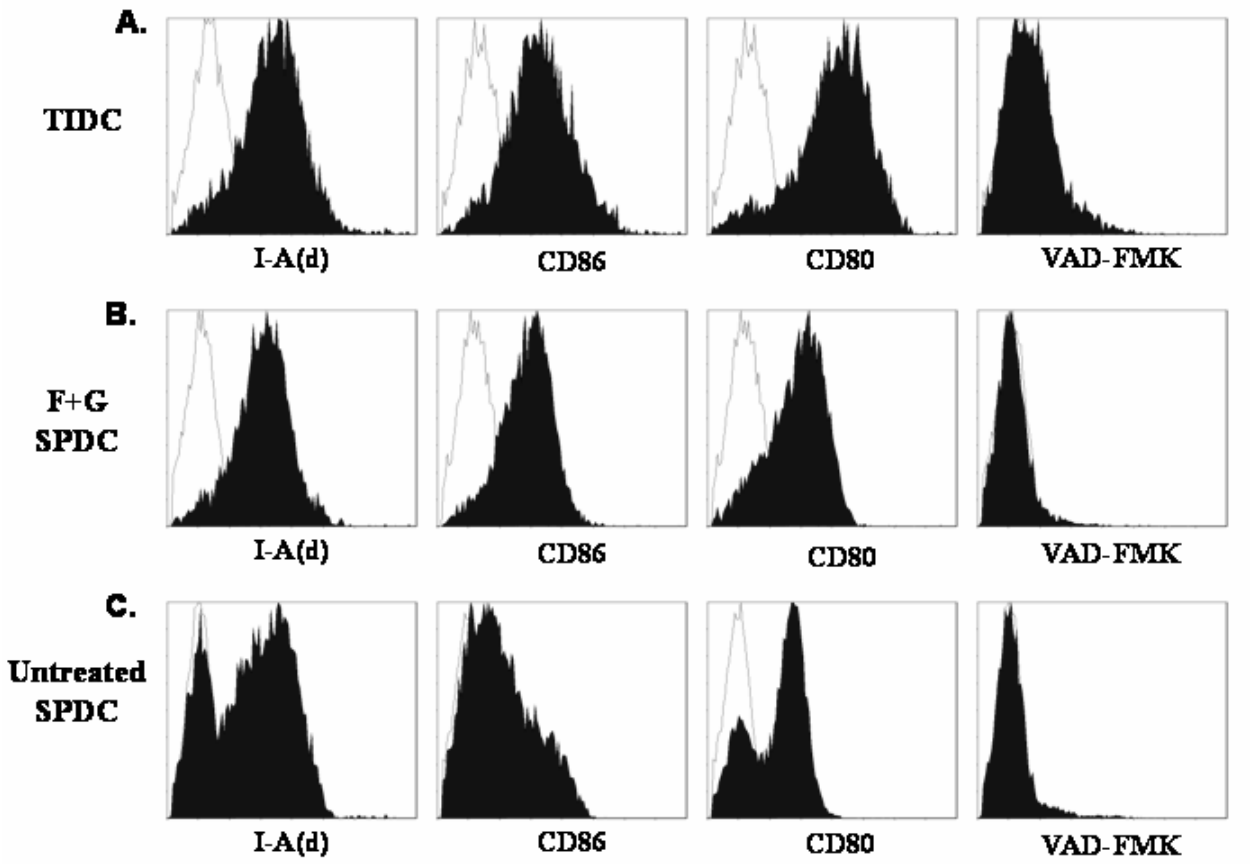


Figure 5. CD11c+ TIDCs exhibit the surface phenotype of mature dendritic cells and do not exhibit a "suppressed" or highly apoptotic phenotype.

The TIDC and SPDC described in **Fig. 4** were stained with antibodies specific for MHC class II and co-stimulatory molecules and analyzed by flow cytometry. The data shown is gated on CD11c+ dendritic cells. The first histogram represents isotype control staining whereas the second represents specific antibody staining.

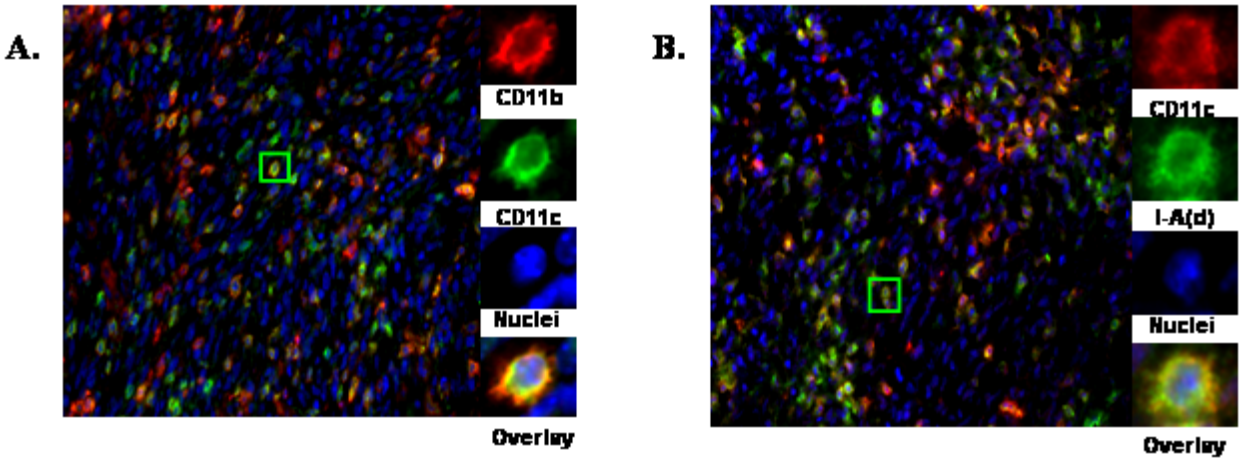


Figure 6. Immunofluorescence staining of tumor sections confirms the CD11b+CD11c+ (Figure A) and I-A^{d}+CD11c+ (Figure B) phenotype of TIDCs.

Tumor sections described in **Fig. 2** were stained with the indicated antibodies and analyzed by immunofluorescence microscopy.

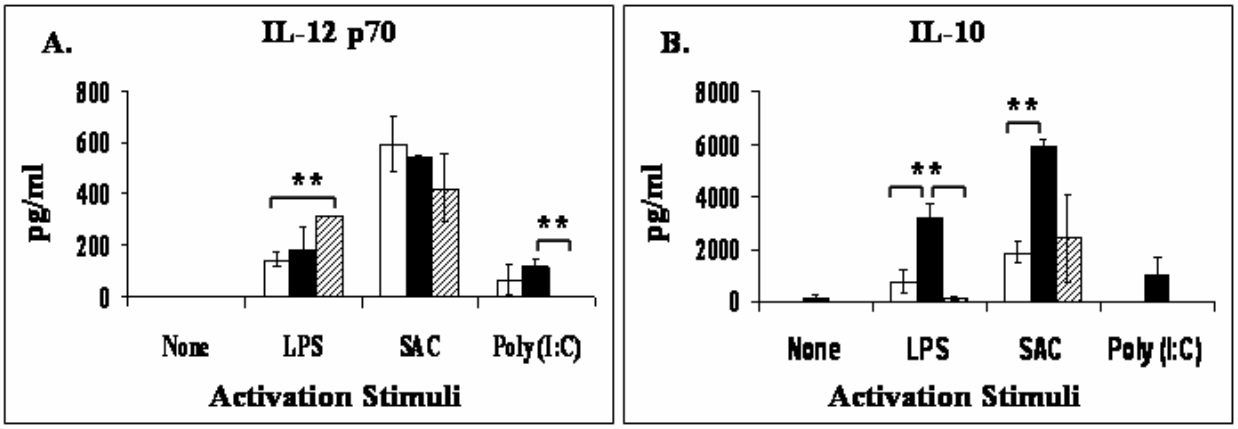


Figure 7. Both TIDCs and SPDCs isolated from rFL + rGM treated mice produce higher levels of IL-10 rather than IL-12 p70 when cultured with maturation stimuli.

TIDC and splenic DC described in **Fig. 4** were cultured for 24 hrs with or without activation stimuli and the supernatants assayed by cytokine-specific ELISA. ** Indicate p-value < 0.05. The open bars and the filled bars represent splenic DCs and TIDCs from rFL + rGM treated mice, respectively. The hatched bars represent splenic DCs from untreated normal mice.

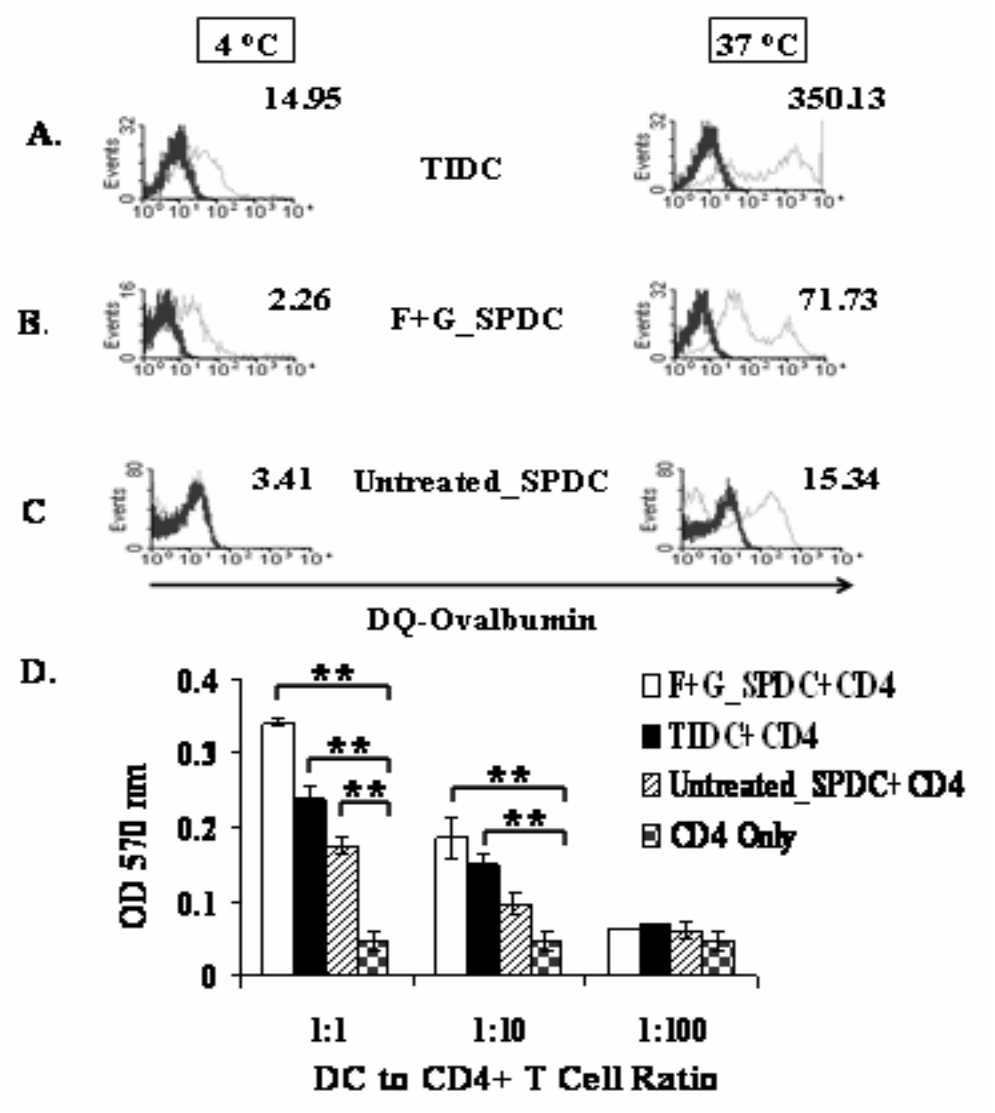


Figure 8. TIDCs are more efficient than SPDCs in mediating soluble antigen uptake and processing and are capable of inducing alloreactive T cell proliferation.

The TIDCs and SPDCs described in **Fig. 4** were assayed using DQ-Ovalbumin as a soluble antigen. Tubes to which DQ-Ovalbumin was added or not added were incubated at 4^{o}C and 37^{o}C for 1 hour, washed, and analyzed by flow cytometry. In panels A-C, the first histogram represents DC incubated without DQ-Ovalbumin and the second histogram represents DC incubated with DQ-Ovalbumin. The numbers above each histogram represent the mean fluorescence intensity of the DQ-Ovalbumin added cultures. In addition, the TIDC and SPDC were co-cultured with CD4+ splenic T cells from C57BL/6 mice for three days and assayed for MLR responses by 4 hour MTT assay (Panel D). ** Indicate p-value<0.05.

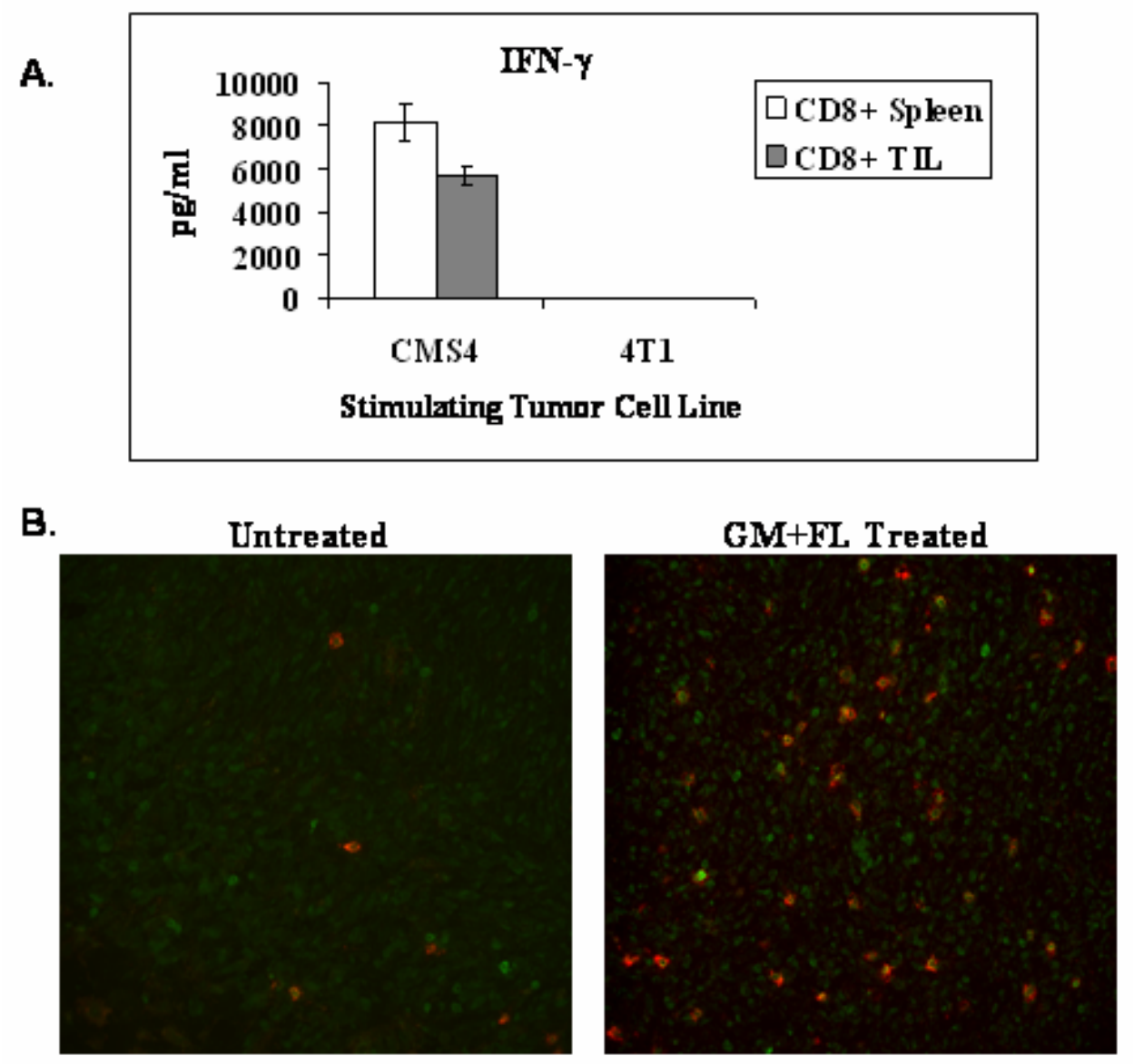


Figure 9. rFL + rGM treatment results in enhanced infiltration of specific CD8+ T lymphocytes within CMS4 tumors.

Panel A) CD8+ T cells were isolated by MACS selection from the CD11c−CD4− fraction of splenocytes or TIL of rFL + rGM treated mice on day 14. IFN-γ production was measured by ELISA after co-culture of the CD8+ T cells with irradiated CMS4 or control 4T1 tumor cells for 72 hrs. <115 pg/ml of IFN-γ was detected in CD8+ T cell only or CD8+ T cell and 4T1 tumor cell cultures. When splenic CD8+ T cells isolated from untreated mice were stimulated with irradiated CMS4 or control 4T1 cells, < 50 pg/ml of IFN-γ was detected. Panel B) Five mm sections of established day 14 CMS4 tumors described in **Fig. 2** were stained and analyzed by confocal microscopy. CD3+ T cells are depicted in green and CD8+ T cells are depicted in red.

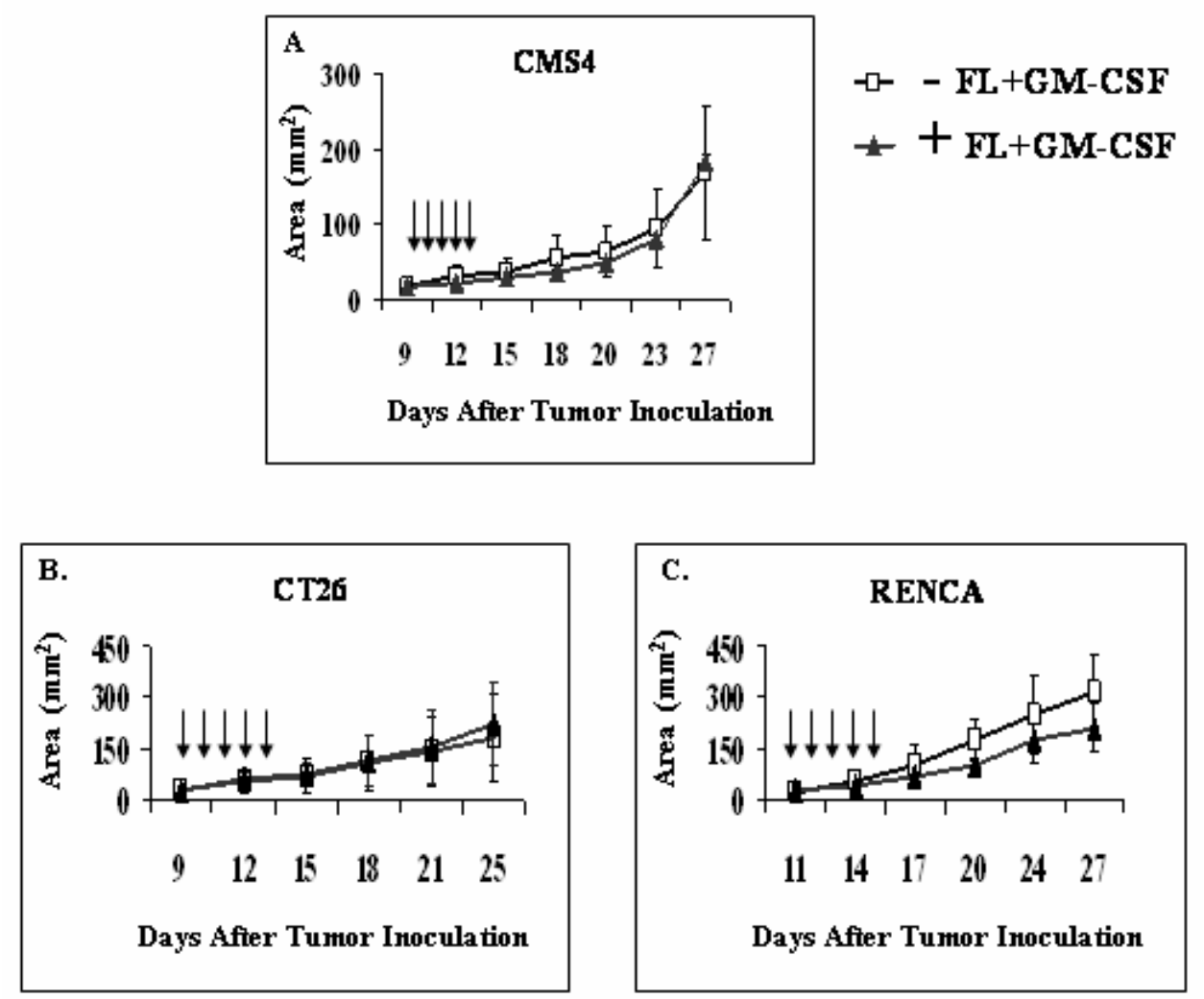


Figure 10. The impact of rFL+ rGM-CSF treatment on the growth of tumors *in vivo* is minimal.

BALB/cJ mice bearing established CMS4 (A), CT26 (B), or RENCA (C) tumors were treated with 20 μg/day of each cytokine for five consecutive days and tumor measurements were taken every 2-3 days. The arrows indicate the days in which the rFL + rGM treatment was administered.

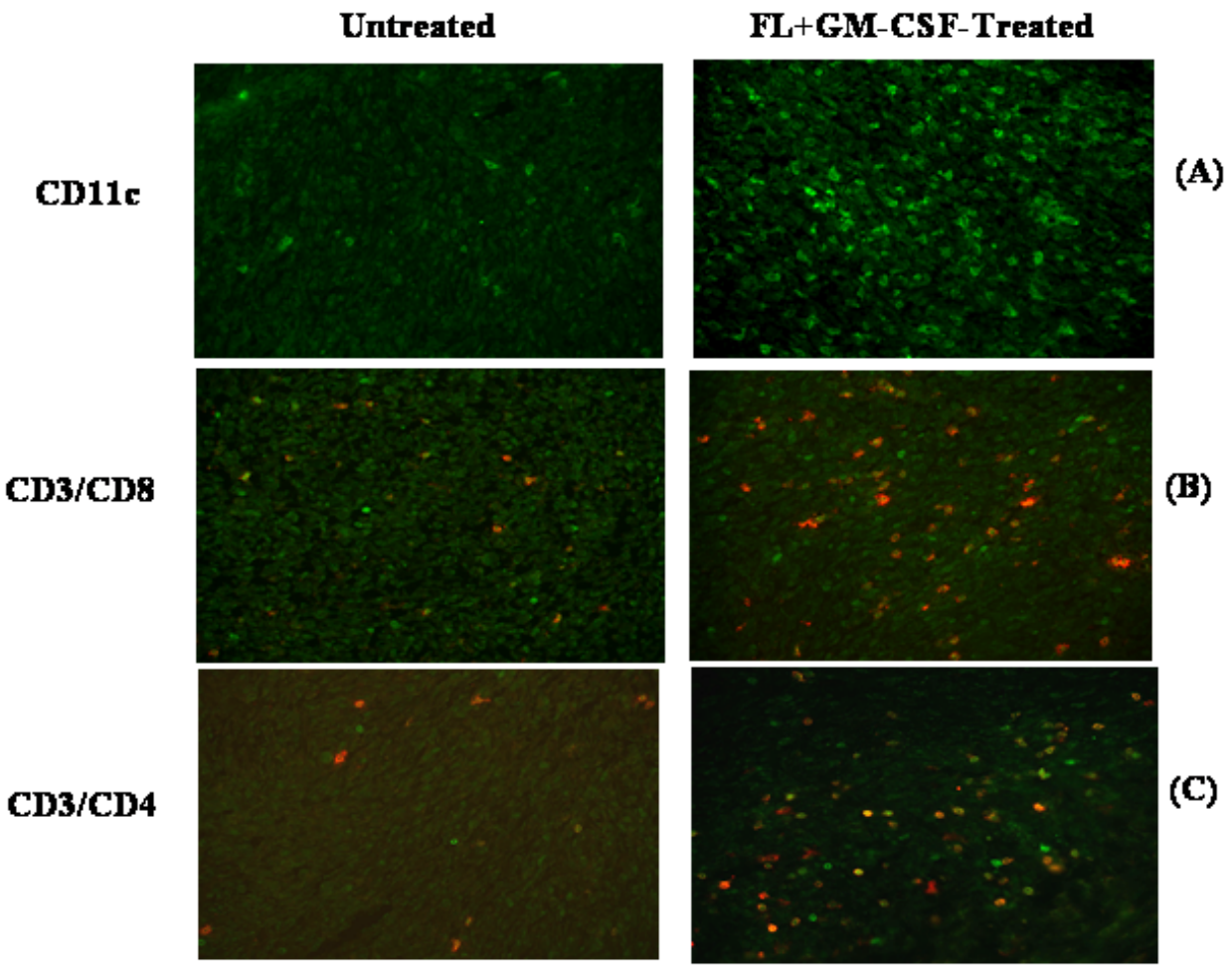


Figure 11. Tumors in mice treated with rFL + rGM display increased infiltration by CD11c+ dendritic cells, CD8+ and CD4+ T cells.

BALB/cJ mice bearing day 7 established CMS4 tumors were treated with 20 μg/day of each cytokine for seven days and sacrificed at day 14. Five mm sections of the tumors were stained and analyzed by confocal microscopy. CD11c+ dendritic cells (panel A) or CD3+ T cells are depicted as green-stained cells; CD8+ (panel B) or CD4+ (panel C) T cells are depicted as red-stained cells, with CD3+CD8+ or CD3+CD4+ T cells appearing yellowish due to co-staining. Data are representative of 3 independent experiments performed.

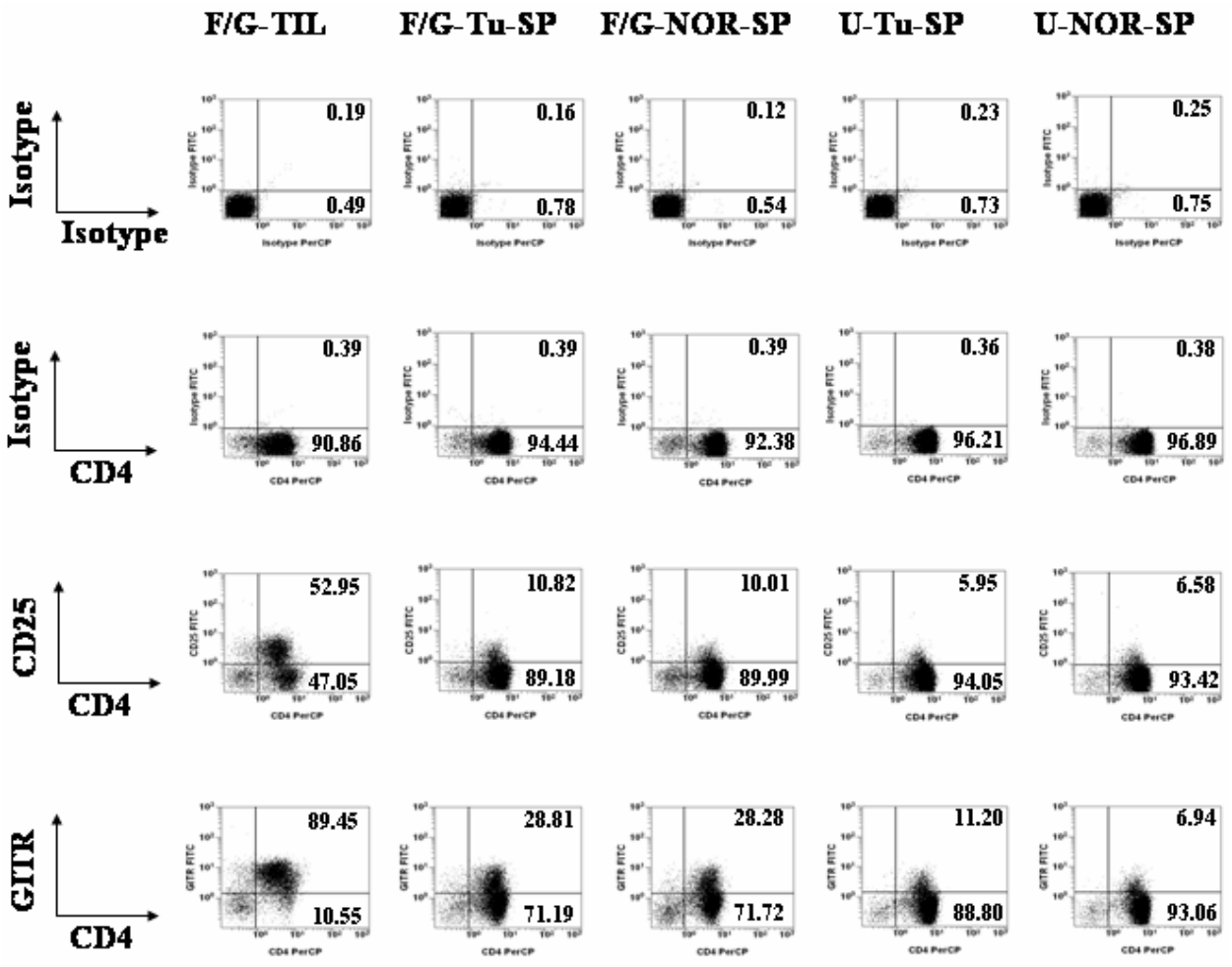


Figure 12. The majority of CD4+ TIL in rFL + rGM treated mice express a GITR^{HI+}CD4^{+} regulatory T cell phenotype and half of these cells co-express CD25.

CD4+ T cells were isolated by MACS selection from the CD11c- fraction of tumor-infiltrating leukocytes (F/G-TIL) or splenocytes (F/G-Tu-SP) of cytokine-treated, tumor-bearing mice, or splenocytes of untreated normal mice (U-NOR-SP) or cytokine-treated normal mice (F/G-NOR-SP) or untreated tumor-bearing mice (U-Tu-SP) and were stained and analyzed for CD25 and GITR co-expression by flow cytometry. Data shown is gated on total live selected cells. The numbers shown in the quadrants for Isotype/Isotype and CD4/Isotype represents the percentage of cells among all cells while the numbers shown in the quadrants for CD4/CD25 and CD4/GITR represents the percentage of CD4+ cells negative/positive for the specific marker. Data are representative of 4 independent experiments performed.

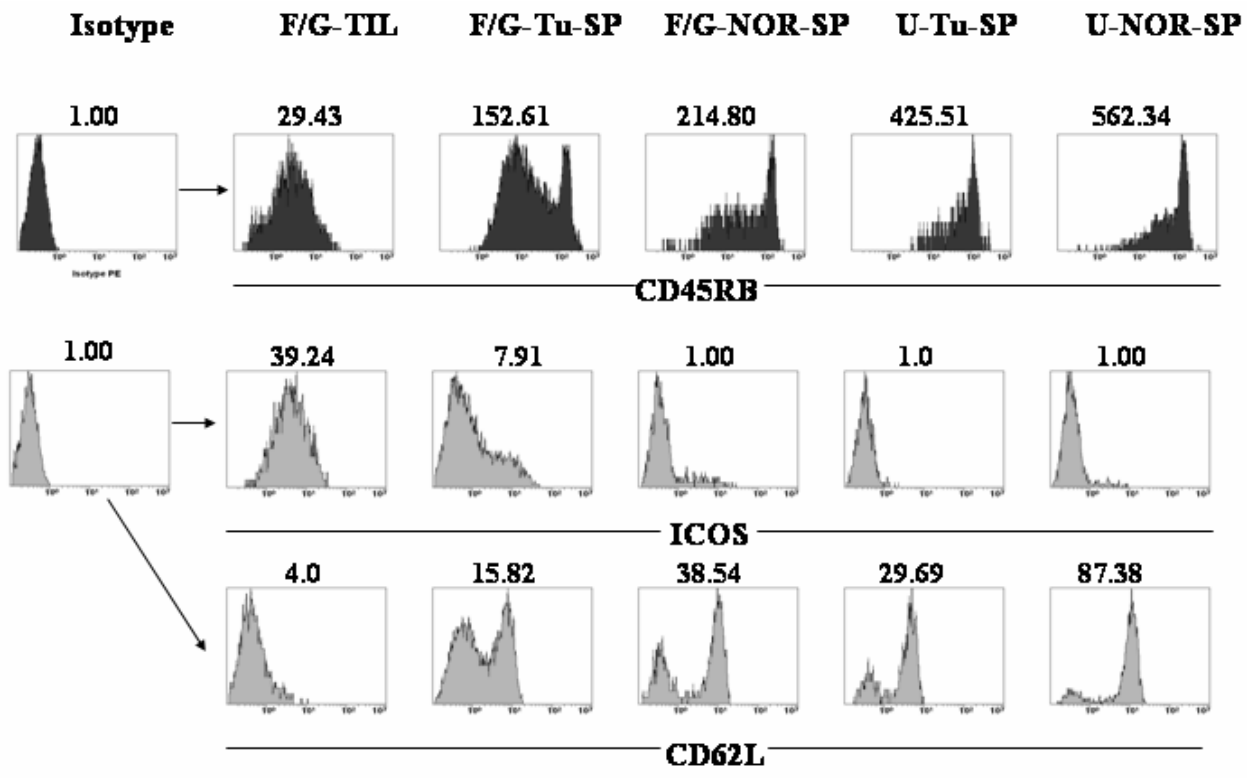


Figure 13. Most CD4+ TIL in rFL + rGM treated mice exhibit an activated/memory phenotype consistent with Treg cells.

The CD4+ T cells described in Figure 12 were stained with antibodies for the indicated activation/memory markers and analyzed by flow cytometry. The data shown is gated for CD4+ live cells. Since the median fluorescence intensities of the isotype controls were similar in all five cases, the control data for the F/G-TIL cohort is shown as a representative plot. The numbers above the figures represent median fluorescence intensity values for the indicated marker. Data are representative of 3 independent experiments performed.

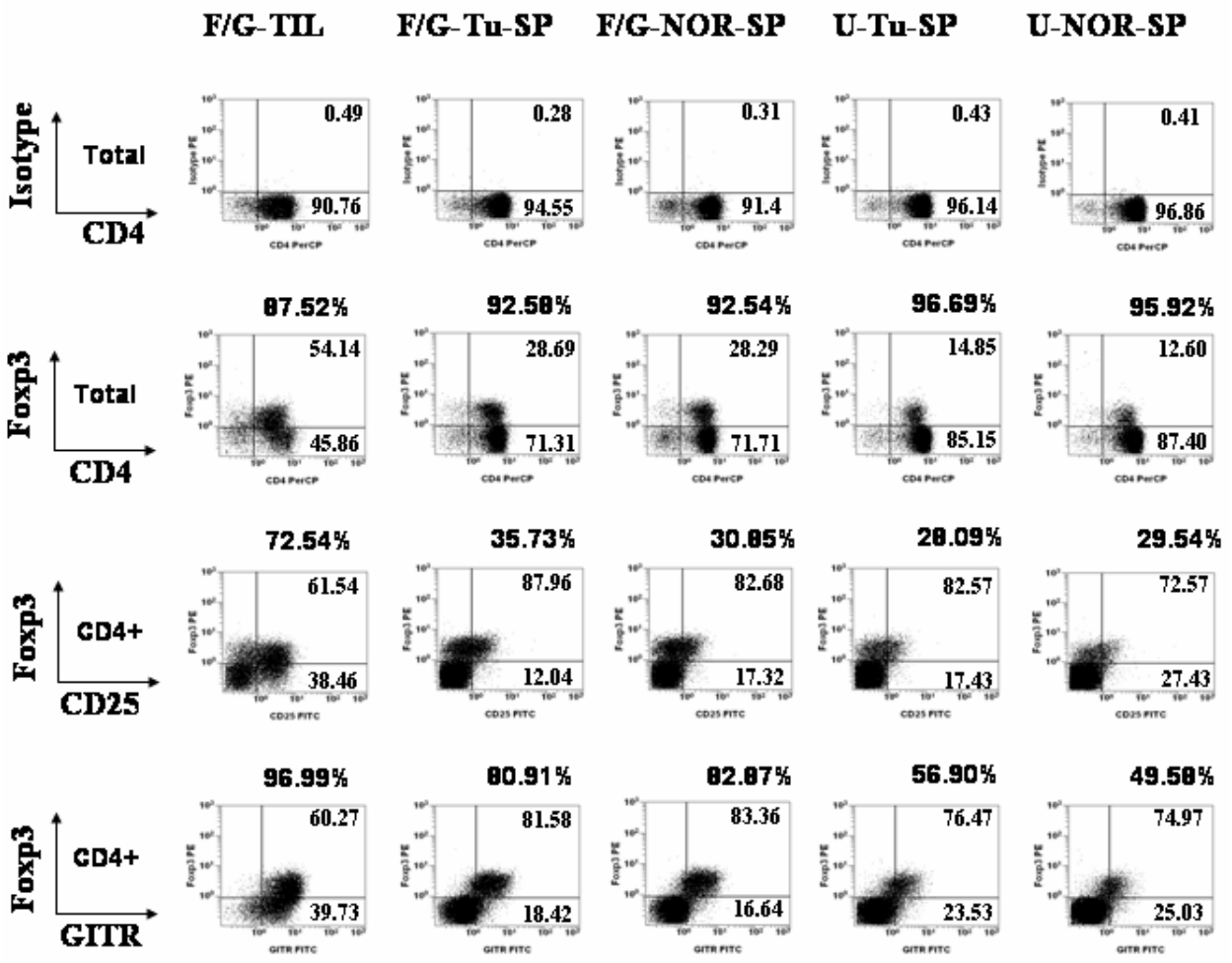


Figure 14. rFL + rGM treatment significantly expands CD4+Foxp3+ regulatory T cells in the spleen and the infiltration of the tumor by CD4+ TILs of which more than 50% are Foxp3+.

The CD4+ T cells described in Figure 12 were surface stained with isotype control, CD25, or GITR antibodies and intracellularily stained with isotype control or Foxp3 antibodies and analyzed by flow cytometry. The data shown for CD4/Isotype and CD4/Foxp3 is gated on total live selected cells while the data shown for CD25/Foxp3 and GITR/Foxp3 is gated on live CD4+ T cells. The numbers in the quadrants of the panels represent the percentage of CD4+ T cells or CD25+ T cells or GITR+ T cells that are negative/positive for Foxp3. The % numbers above the plots represent the percentage of Foxp3+ cells that are CD4+ or CD25+ or GITR+, respectively. Data are representative of 2 independent experiments performed.

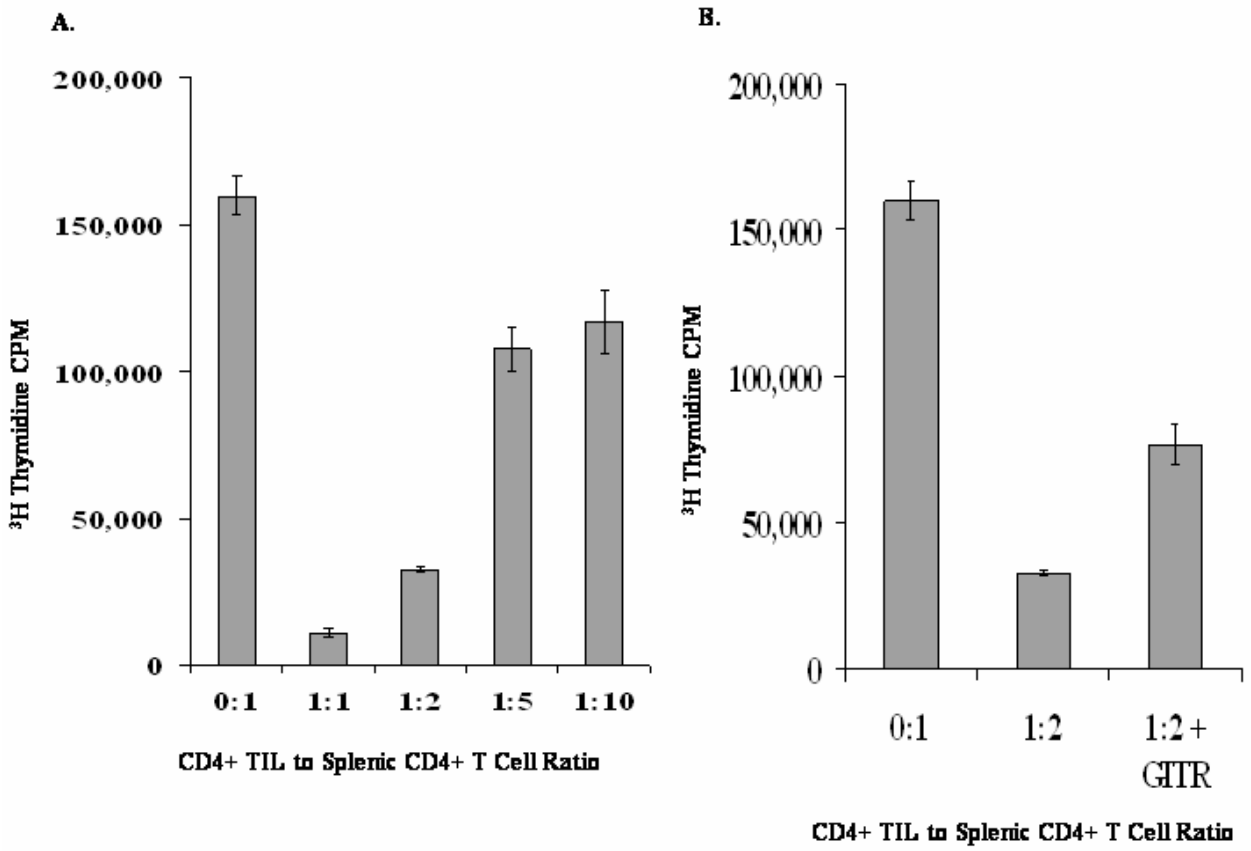


Figure 15. CD4+ TILs from cytokine-treated mice suppress alloantigen-specific T cell proliferation and are functionally antagonized by anti-GITR mAb.

(A) CD4+T cells (BALB/cJ) from normal mice were co-cultured with total C57BL/6 splenocytes at a ratio of 1:1. CD4+ TIL from rFL + rGM treated CMS4 bearing mice were added to these cultures at the indicated ratios. After three days, 1 μCi ^{3}H-thymidine was added for the last 16h of culture, and its incorporation consequently measured by β-scintillation counting. The background in BALB/cJ CD4+T cells only or CD4+TILs only cultures was <3200 cpm. Further, the proliferative response of CD4+TILs when co-cultured with allogeneic C57BL/6 splenocytes was <1700 cpm. **(B)** The experiment reported in panel A was repeated in the absence or presence of 20 μg/ml of anti-GITR (DTA-1) antibody. Data are representative of 3 independent experiments performed in each case.

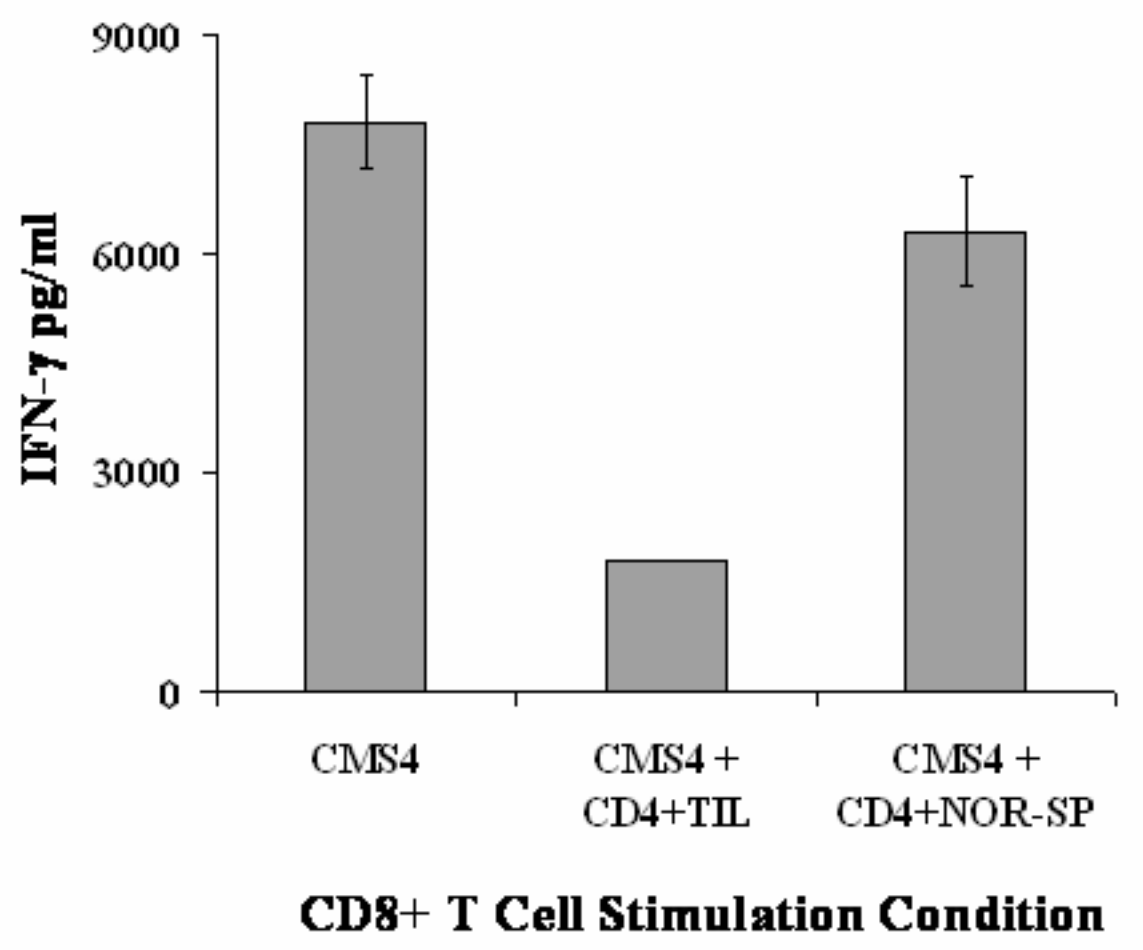


Figure 16. CD4+ TIL from cytokine-treated mice suppress IFN-γ secretion by anti-CMS4 CD8+ T cells isolated from rFL + rGM treated, tumor-bearing mice.

CD8+ T cells from rFL + rGM-CSF treated tumor-bearing mice spleen were co-cultured with CMS4 cells. CD4+ T cells from the spleens of rFL + rGM treated normal mice or CD4+ TIL were then added to tumor and CD8+ T cell cultures at a 1:2 (CD4+ T cell : CD8+ T cell) ratio. After 72 hrs of culture, supernatants were collected and analyzed by IFN-γ ELISA. Data are representative of 2 independent experiments performed.

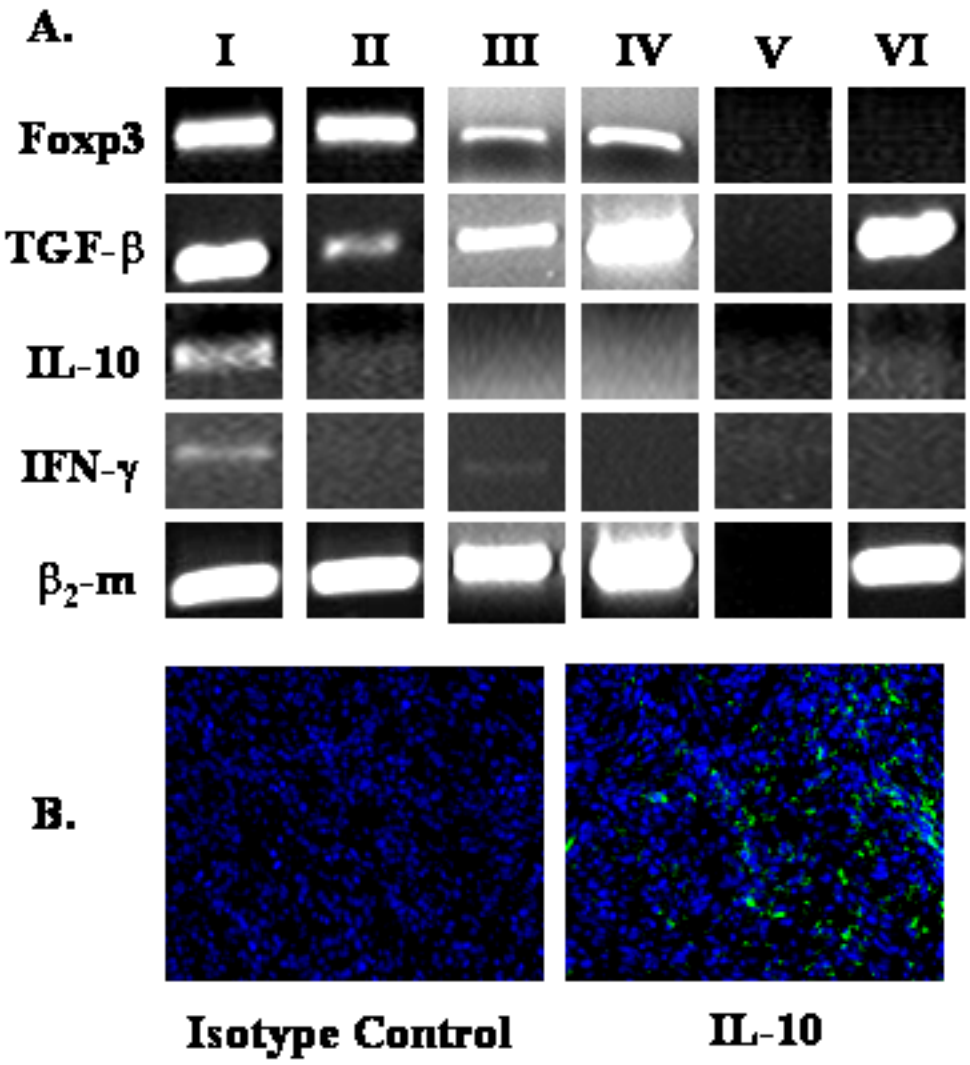


Figure 17. CD4+ TIL in cytokine-treated mice express transcription factor *foxp3*, IL-10 and TGF-β mRNA and *in situ*, IL-10 protein is detected in tumor sections.

(Panel A) Total RNA was isolated from tumor-infiltrating CD4+ T cells. One μg of RNA was reverse transcribed and PCR amplification using specific primers was performed. Beta-2-microglobulin (β_2m) was used as a reaction and loading control. PCR products were visualized in 1.4% agarose gels containing ethidium bromide. The numbers above the pictures represent the following cell populations: I- (CD4+ TIL), II-(CD4+ Tu FL+GM SP), III-(CD4+ Tu Untreated SP), IV-(CD4+ Normal SP), V-(- cDNA), VI-(In Vitro CMS4). (Panel B) Tumor sections described in Figure 11 were stained with isotype control or anti-IL-10 antibody and analyzed by immunofluorescence microscopy. Data are representative of 2 independent experiments performed.

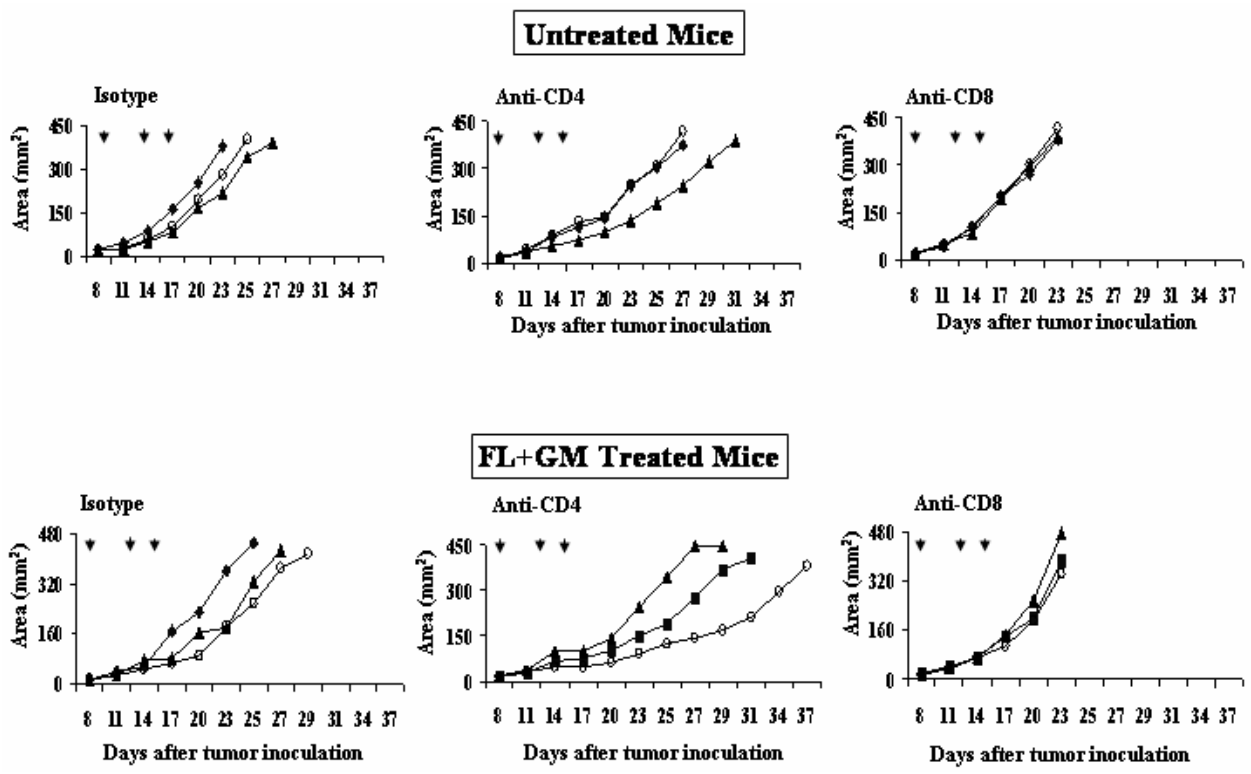


Figure 18. *In vivo* depletion of CD4+ T cells results in slowed tumor growth in rFL + rGM treated mice (but not untreated mice) only during the depletion period.

BALB/cJ mice bearing day 8 established CMS4 tumors were untreated or treated with rFL + rGM for five consecutive days. On days 8, 13 and 16, the mice received either rat isotype, anti-CD4 or anti-CD8 antibody ascites injections intraperitoneally (indicated by the three arrows in the figures). Tumor measurements were taken every 2-3 days. Data represent the tumor growth curves (area in mm^2) for each individual mouse of three in a given treatment cohort.

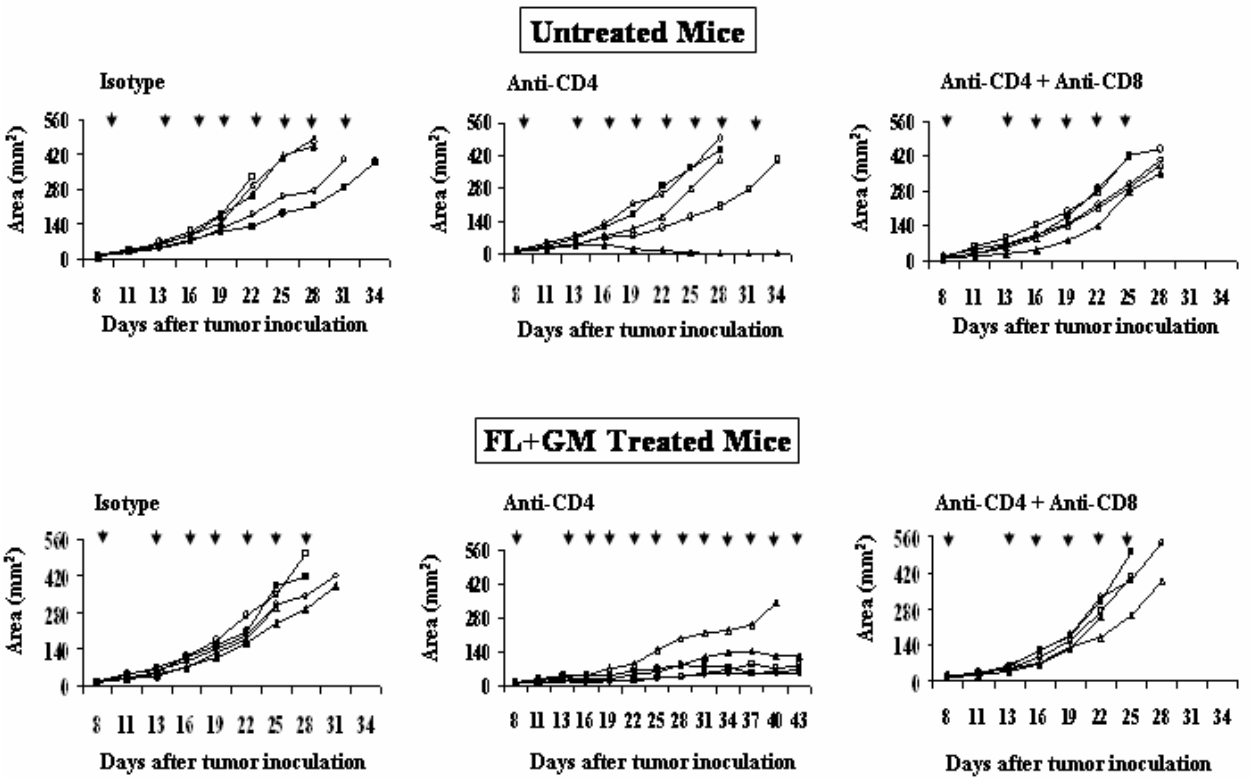


Figure 19. The maintenance of the *in vivo* depletion of CD4+ T cells results in a prolonged, significant inhibition of tumor growth in rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent.

The *in vivo* experiment described in Figure 18 was repeated with an extended period of depletion. BALB/cJ mice bearing day 8 established CMS4 tumors were treated with rFL + rGM for five consecutive days. On days 8, 13, 16, and every three days thereafter, the mice received rat isotype, anti-CD4, or combinations of anti-CD4 and anti-CD8 antibody ascites injections intraperitoneally (indicated by the arrows in the figures). Tumor measurements were taken every 2-3 days. Data represent the tumor growth curves (area in mm^2) for each individual mouse of five in a given treatment cohort.

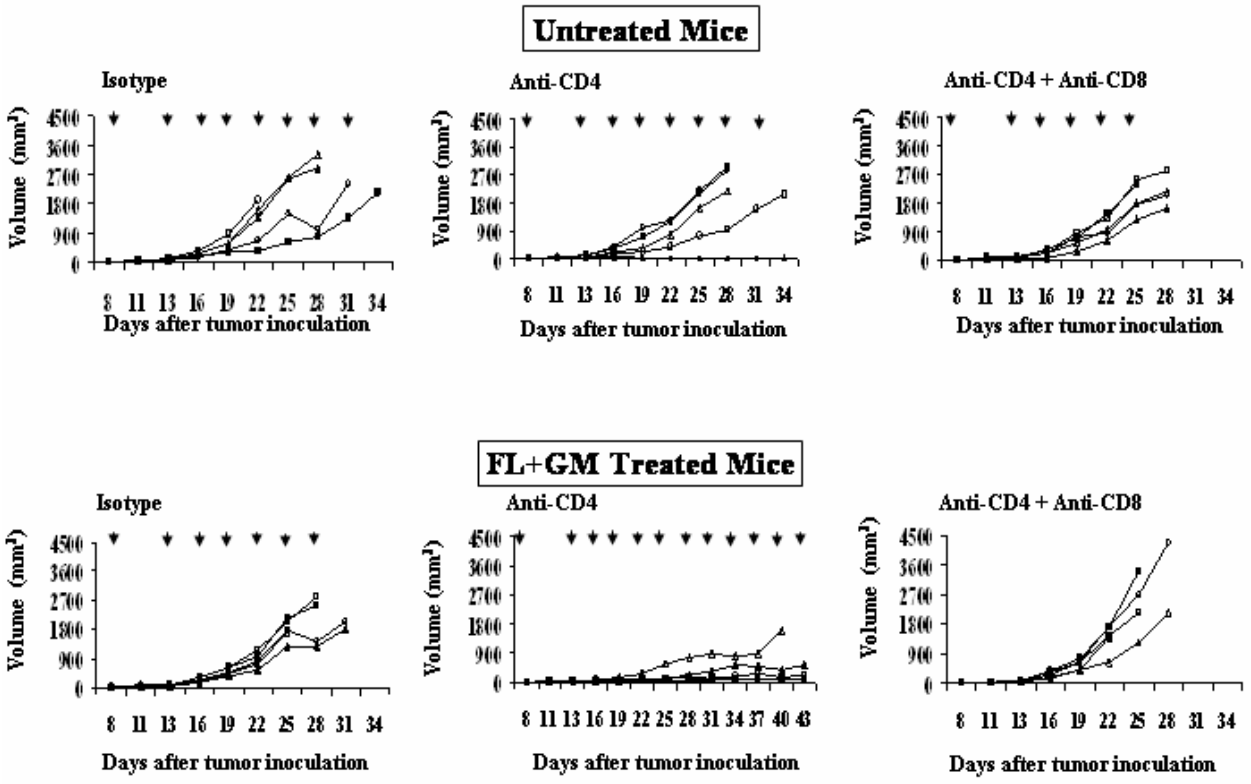


Figure 20. The maintenance of the *in vivo* depletion of CD4+ T cells results in a prolonged, significant inhibition of tumor growth in rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent.

The tumor growth curve shown in Figure 19A as tumor area (mm^2) is shown here in terms of tumor volume (mm^3). Tumor area is calculated by multiplying the largest perpendicular diameters, i.e. length (*l*) x width (*w*). To calculate the tumor volume, the diameter of the height of the tumors is also co-measured and used in the following formula: V = (∏/6) x (length) x (width) x (height). Data represent the tumor growth curves (volume in mm^3) for each individual mouse of five in a given treatment cohort.

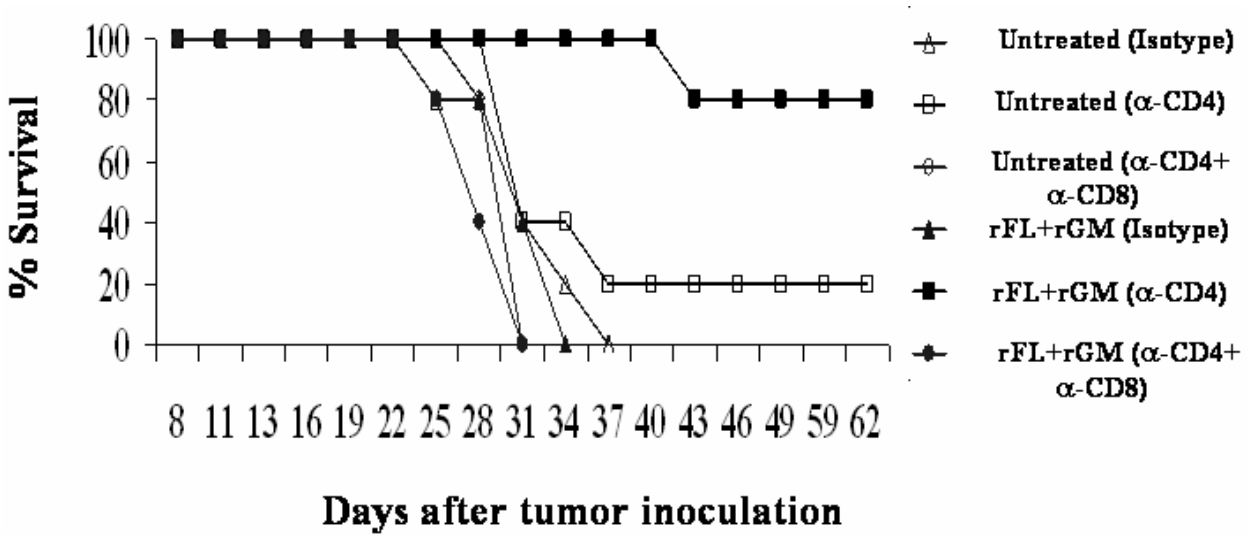
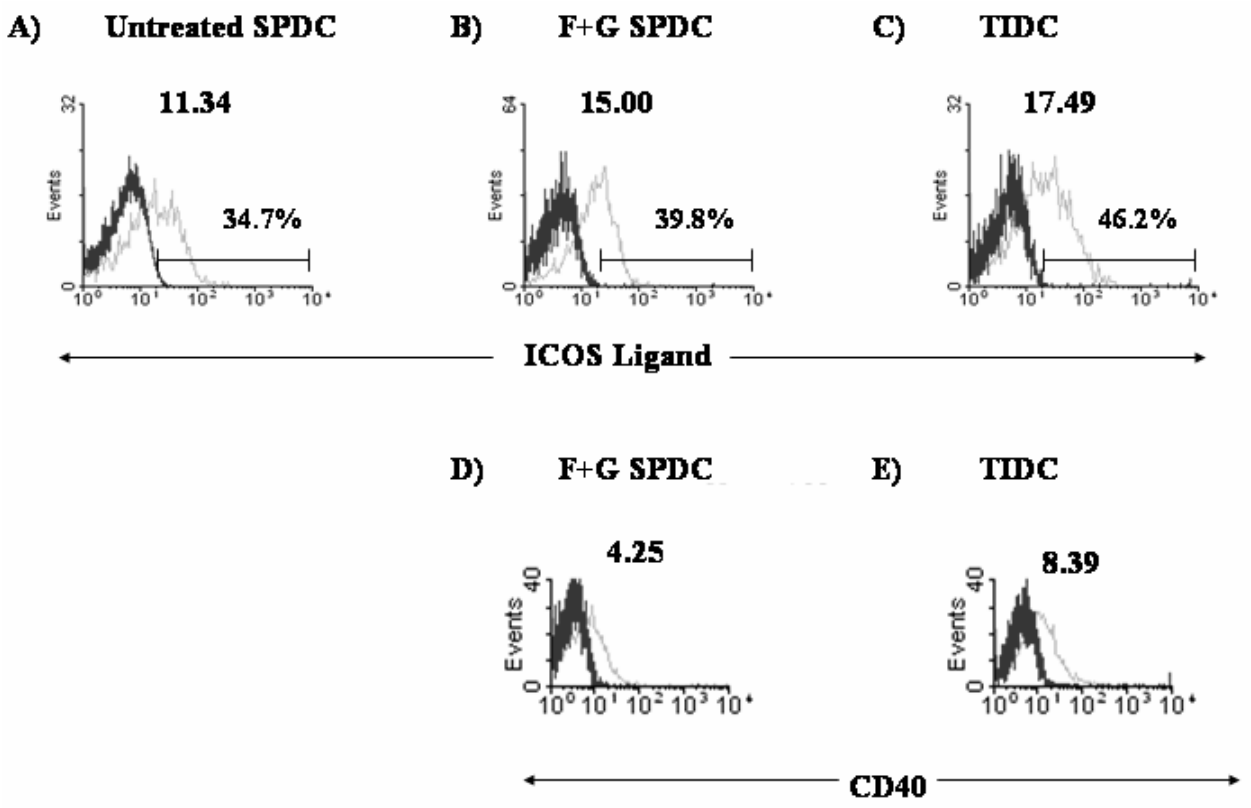


Figure 21. The maintenance of the *in vivo* depletion of CD4+ T cells results in prolonged survival of rFL + rGM treated mice (but not untreated mice), which is CD8+ T cell-dependent.

For the experiment described in Figure 19A, the percentage of survival of a treatment group is calculated by dividing the number of mice alive at a specific date after tumor inoculation by the number of mice in the group (i.e. 5).

APPENDIX A

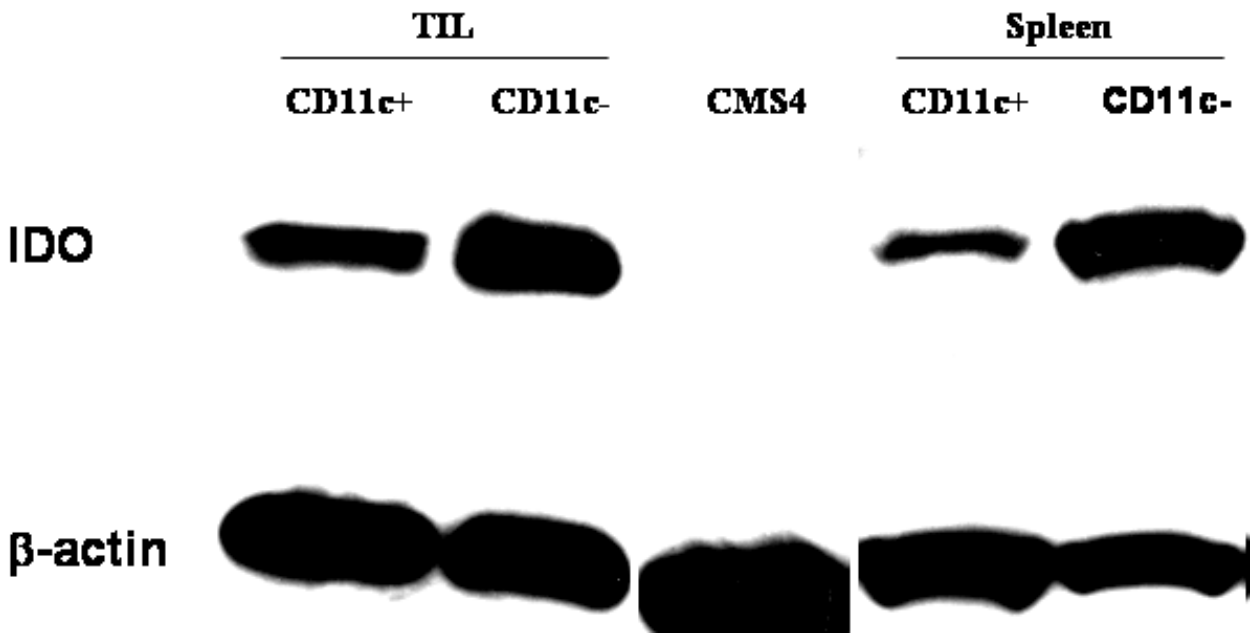
ICOS ligand (B7RP-1) and CD40 expression on dendritic cells



BALB/cJ mice bearing day 8 or 9 established CMS4 tumor were untreated or treated with 20 μg/day of each cytokine for 5 days and sacrificed the next day. Tumors were digested and processed on a Lympholyte-M gradient in order to obtain total tumor-infiltrating leukocytes (TIL). TIDC are isolated from the TIL by CD11c MACS bead selection. Splenic DC were also isolated from the same mouse (F+G_SPDC) or from untreated non-tumor bearing mice (Untreated_SPDC) by CD11c MACS bead selection. The numbers in panel A represent the mean channel fluorescence for ICOS ligand expression on CD11c+ DCs. The darker curve represents isotype control staining while the lighter curve represents specific antibody staining.

APPENDIX B

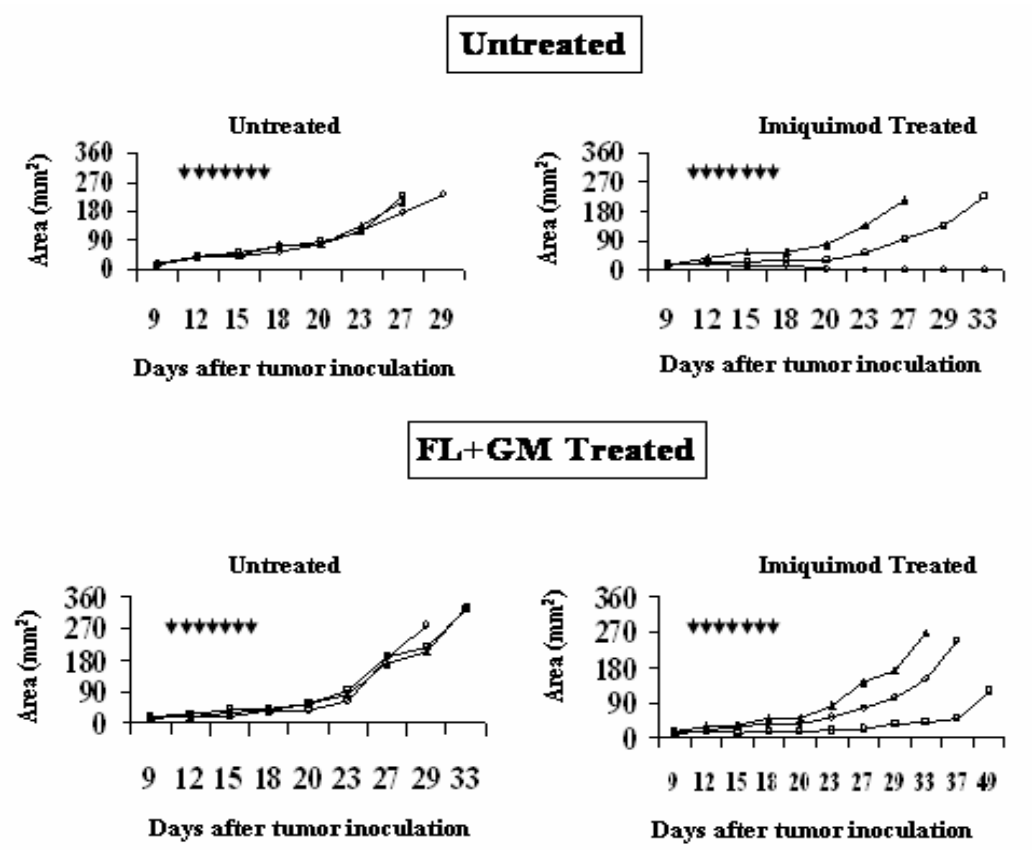
Indoleamine 2,3-dioxygenase (IDO) expression by TIDCs



Cell lysates were prepared from CD11c+ TIDC or SPDC and CD11c− TIL or splenocytes from rFL + rGM treated mice or from in vitro cultured CMS4 cells. The lysates were run on SDS-PAGE and western blotting was performed using anti-IDO antibody.

APPENDIX C

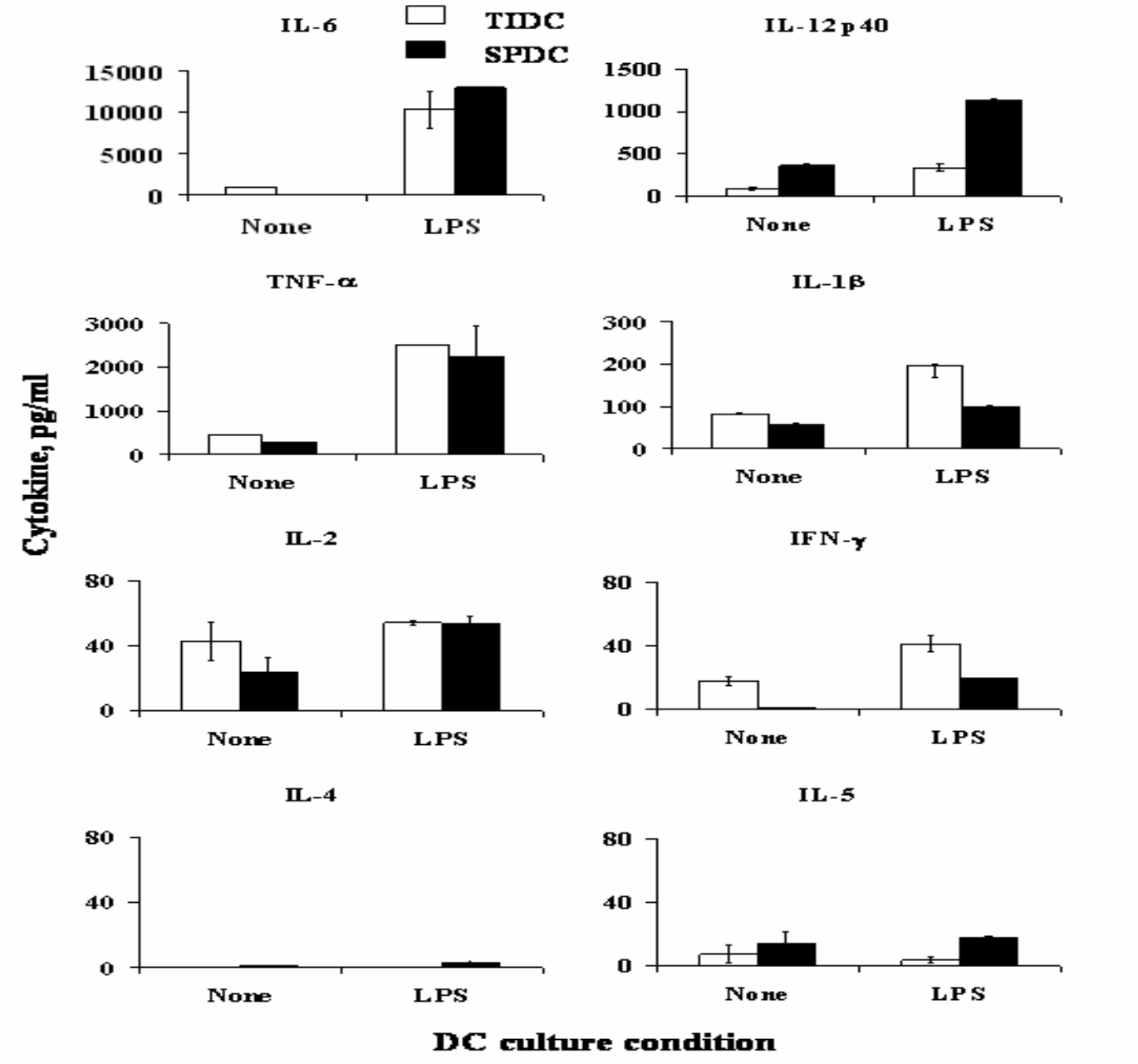
The effect of TLR7 agonist imiquimod on tumor growth



BALB/cJ mice bearing day 9 established CMS4 tumors were untreated or treated with rFL + rGM for five consecutive days. On days 11-17, imiquimod (Aldara) 5% cream (3M, Minnesota) was applied directly onto the tumors of some mice (indicated by the three arrows in the figures). Tumor measurements were taken every 2-3 days. Data represent the tumor growth curves (area in mm^2) for each individual mouse of three in a given treatment cohort.

APPENDIX D

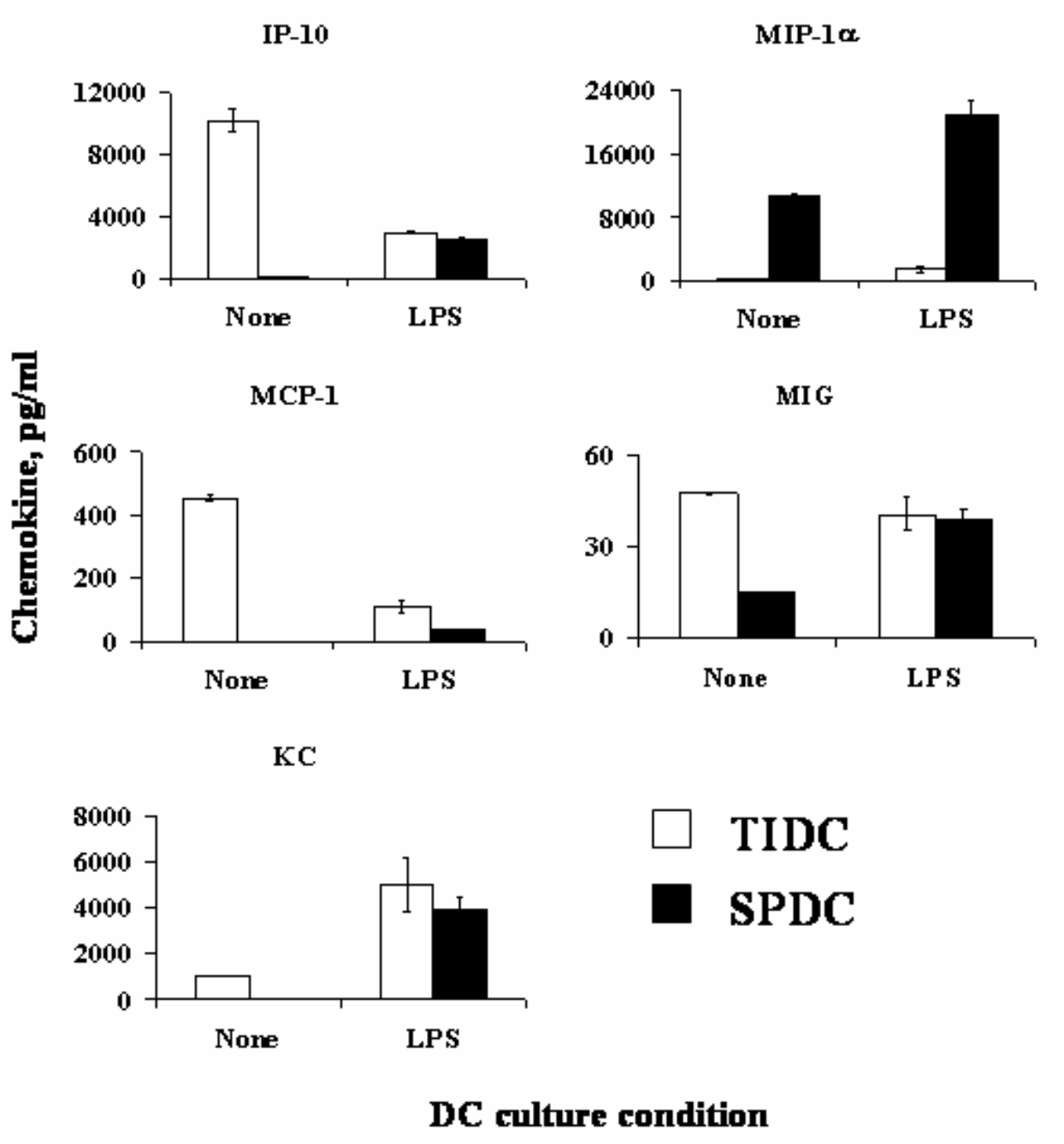
Cytokine secretion profile of TIDCs and SPDCs



TIDCs and splenic DCs described in **Fig. 4** were cultured for 24 hrs with or without LPS (10 μg/ml) and the supernatants assayed by multi-cytokine Luminex.

APPENDIX E

Chemokine secretion profile of TIDCs and SPDCs



TIDCs and splenic DCs described in **Fig. 4** were cultured for 24 hrs with or without LPS (10 μg/ml) and the supernatants assayed by multi-chemokine Luminex.

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