THE ROLE OF MHC-II DEPENDENT EVENTS IN THE SUPPRESSION MEDIATED BY $CD4^+FOXP3^+$ REGULATORY T CELLS

By

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This dissertation is submitted by Faith Daima Mmanywa and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

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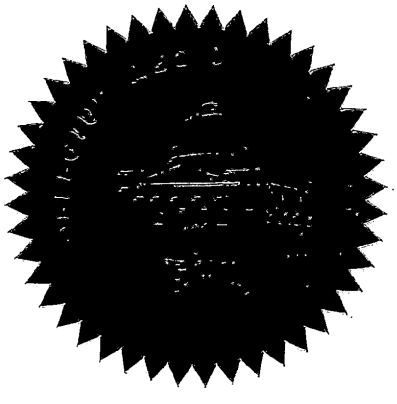

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FAITH DAIMA MMANYWA
The Role of MHC-II Dependent Events in the Suppression Mediated by $CD4^+Foxp3^+$ Regulatory T cells
(Under the direction of PANDELAKIS A. KONI, Ph.D.)

$CD4^+Foxp3^+$ regulatory T cells (Tregs) and antigen presenting cells (APCs) play an important role in maintaining peripheral tolerance but are otherwise exploited by tumors to create a state of unresponsiveness towards tumor antigens. The mechanisms of Treg mediated suppression are still not well understood. This work seeks to elucidate the role of major histocompatibility complex class II (MHC-II) dependent events in $CD4^+Foxp3^+$ Treg mediated suppression. The studies described here take advantage of novel conditional MHC-II deficient mice, which lack expression of MHC-II on peripheral APCs but still maintain their own naïve CD4 T cells and Tregs. In an *in vitro* system antigen-specific Tregs suppress CD8 T cell proliferation and effector molecule production in an antigen-specific and MHC-II dependent manner. *In vivo*, MHC-II deficiency resulted in a delay in tumor progression that was CD8 T cell dependent. We further describe two *in vivo* models in which the role of MHC-II dependent events in Treg mediated suppression can be tested. Therefore, a better understanding of Treg mediated suppression in the context of tumor-induced tolerance could provide potential strategies that could be utilized for anti-tumor immunotherapy.

INDEX WORDS: MHC-II, Regulatory T cells, Antigen presenting cells, Tumor-induced tolerance

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LIST OF ABBREVIATIONS

APC	-	Antigen presenting cell
BMDC	-	Bone marrow-derived dendritic cell
CD	-	Cluster of differentiation
CD11c	-	Cell marker on certain dendritic cells
CD25	-	The IL-2 receptor α chain constitutively expressed on regulatory T cells and activated T cells
CD28	-	T cell receptor for co-stimulation molecules CD80 and CD86 expressed on T cells
CD3	-	Associated with the T cell receptor. Required for cell surface expression and signal transduction of the T cell receptor
CD4	-	MHC class II restricted T cell co-receptor
CD44	-	A membrane glycoprotein expressed on mature T cells and with greater expression on activated and memory T cells than naïve T cells
CD8	-	MHC class I restricted T cell co-receptor
CFSE	-	Carboxy-fluorescein diacetate succinimidyl ester
CTLA-4	-	Cytotoxic T lymphocyte associated antigen-4 also known as CD152

DC	-	Dendritic cell
ELISA	-	Enzyme-linked immunosorbent assay
FACS	-	Fluorescence activated cell sorting
FITC	-	Fluorescein isothiocyanate
Foxp3	-	Forkhead winged helix transcription factor gene 3
GFP	-	Green fluorescent protein
GITR	-	Glucocorticoid-induced tumor necrosis factor receptor-related protein
HEL	-	Hen egg lysosome
iaβ	-	MHC-II beta chain gene
$ia\beta^{neo}$	-	MHC-II beta chain null allele
$ia\beta^{neo/\Delta}Tie2Cre^{+}$	-	Conditional MHC-II knockout mice
IFN-γ	-	Interferon gamma
IL-2	-	Interleukin 2, T cell growth factor
IL-2Rα	-	Interleukin 2 receptor α chain
IL-10	-	Interleukin 10
KO	-	Knockout
LAG-3	-	Lymphocyte activation gene-3
LN	-	Lymph node
MHC	-	Major Histocompatibility Complex
MHC class I	-	Major Histocompatibility Complex class I

MHC class II	-	Major Histocompatibility Complex class II
MIIC	-	MHC class II compartment
nTregs	-	Natural regulatory T cells
Neo	-	Neomycin-resistance gene
OVA	-	Ovalbumin
PE	-	Phycoerythrin
PerCP	-	Peridinin chlorophyll protein
pDC	-	Plasmacytoid dendritic cell
pLN	-	Popliteal lymph node
TCR	-	T cell receptor
TDLN	-	Tumor-draining lymph node
TGF-β	-	Transforming growth factor-β
Tie2	-	Kinase promoter specifically expressed in endothelial cell lineage
Tregs	-	Regulatory T cells
Tr1	-	Induced or adaptive regulatory T cells that produce interleukin 10
Tr3	-	Induced or adaptive regulatory T cells that produce transforming growth factor-β
Vα2	-	T cell receptor marker, used to identify OT-II and OT-I T cell receptor transgenic T cells
WT	-	Wild type

I. Introduction

I.A. Statement of Problem

Immunological self-tolerance is a property of the immune system that ensures that individuals do not mount a response to their own antigens. Therefore it is a state of unresponsiveness to a particular antigen that is induced by previous exposure to that antigen (1). 'Unresponsiveness' however is now understood to not simply be a result of passive processes but a phenomenon that requires ongoing active processes. Tumors also create a state of immunological tolerance towards tumor antigens that allows them to escape immune surveillance and grow unchecked in an immunocompetent host. The mechanisms of tumor-induced tolerance are not clearly understood but evidence suggests that tumors exploit many of the same mechanisms responsible for maintaining peripheral tolerance that are otherwise involved in preventing autoimmune diseases. Antigen presenting cells (APCs) and $CD4^+CD25^+$ regulatory T cells (Tregs) are key players in maintaining peripheral tolerance but at the same time are centrally involved in the mechanisms of tumor-induced tolerance. The relationship between APCs and Tregs and how they contribute to peripheral tolerance or tumor-induced tolerance has slowly begun to unfold. An area of intense research in recent years has been the mechanisms of Treg mediated suppression. This work seeks to elucidate the role of the major histocompatibility complex class II (MHC-II) dependent events in Treg mediated suppression. We first describe the generation of an *in vitro* system to test the hypothesis

that Tregs suppress CD8 T cell proliferation and effector molecule production in an MHC-II dependent manner. We employ novel conditional MHC-II knock out mice, containing their own naïve CD4 T cells and Tregs but lacking MHC-II expression on peripheral APCs. We then use these mice to investigate the role of MHC-II dependent events in tumor progression. Finally, we describe *in vivo* models, which would allow further investigation of the role of MHC-II dependent events in Treg mediated suppression of CD8 T cell effector function. We specifically look at the ability of Tregs to suppress CD8 T cell mediated delay of tumor progression and CD8 T cell *in vivo* cytotoxic killing.

I.B. Peripheral Tolerance

The immune system has mechanisms in place to discriminate against self and non-self antigens and also eliminates self-reactive T cells in order to establish and maintain self-tolerance. The majority of self-reactive T cells are eliminated during development in the thymus at which thymocytes bearing a T cell receptor (TCR) with high affinity for self-antigens are deleted by negative selection in a process of 'central tolerance' (2). This process, however, does not eliminate all self-reactive T cells, and some escape to the periphery where they continue to pose a pathogenic threat. Peripheral tolerance, therefore is required to control self-reactive T cells in the periphery (3, 4). The proposed mechanisms for peripheral tolerance include anergy, ignorance and mechanisms that evoke additional subsets of cells including dendritic cells (DCs) and Tregs (5). T cell anergy is a state in which T cells become unresponsive following antigen encounter but remain alive for an extended period of time in this hyporesponsive state (6). Anergy was first proposed to be a result of TCR stimulation in the absence of co-stimulation (signal 1

without signal 2) but it was later realized that it was an active process that involves inhibitory receptors such as the cytotoxic T lymphocyte associated antigen-4 (CTLA-4) (6). T cell ignorance to self-antigens could be simply due to antigens being sequestered in sites not easily accessible to immune cells or due to an inability to reach the antigenic threshold required to mount a T cell response (5). Along with these intrinsic factors, extrinsic mechanisms involving Tregs and dendritic cells are key players in maintaining peripheral tolerance.

Regulatory T cells

$CD4^+CD25^+$ Tregs derived from the thymus, known as natural Tregs (nTregs), represent about 5-10% of the CD4 T cell compartment (7, 8) and are in contrast to those that develop in the peripheral lymphoid tissues which are termed induced or adaptive Tregs [reviewed in (9, 10)]. Depletion of $CD4^+CD25^+$ leads to a variety of multi-organ autoimmune diseases that are prevented when Tregs from normal mice are reconstituted (7, 8). nTregs have been characterized by the expression of the interleukin-2 receptor α chain (IL-2Rα) known as CD25, glucocorticoid induced tumor-necrosis factor receptor related protein (GITR), lymphocyte activation gene-3 (LAG-3) and CTLA-4 but these markers are insufficient in characterizing this T cell subset as they are also expressed on activated T cells (7). It was not until the discovery that natural Tregs specifically express the forkhead winged helix transcription factor gene 3 (Foxp3) that a more specific and reliable marker of nTreg was established, especially in the murine system (11-14). Foxp3 is both required and sufficient for the development and function of nTreg, and genetic defects in this gene are associated with severe and lethal autoimmune disease (11-13).

Induced or adaptive Tregs include interleukin-10 (IL-10) producing cells (Tr1) and T helper type 3 (Tr3) cells that produce transforming growth factor-β (TGF-β), with the latter expressing Foxp3 (9, 10). Evidence of TGF-β-induced Tregs was demonstrated in experiments where TGF-β was shown to mediate the conversion of $CD4^+CD25^-$ to $CD4^+CD25^+Foxp3^+$ Tregs (15, 16). This conversion was more clearly demonstrated by Wan and Flavell who used Foxp3-mRFP knock-in mice. In these mice Foxp3 expressing cells are marked by mRFP expression allowing the removal of contaminating pre-existing $CD25^-$ cells (17). TGF-β-induced de novo expression of Foxp3 in $CD4^+CD25^-$ T cells that conferred suppressive function (17).

Mechanisms of Treg mediated suppression

The mechanisms of Treg mediated suppression have been widely studied but are still not clearly understood, and the mechanisms used *in vivo* are not exactly mirrored *in vitro*. *In vitro*, Tregs are hyporesponsive to stimulation with anti-CD3 or antigen but require TCR stimulation to exert their suppressive function (19-22). Upon TCR stimulation, this suppressive function occurs in an antigen non-specific manner (21). The suppression *in vitro* is thought to be cell to cell contact-dependent and cytokine independent (19, 20). Experiments in which $CD4^+CD25^+$ Tregs were separated from $CD4^+CD25^-$ responder T cells with a permeable membrane revealed the inability of $CD4^+CD25^+$ Tregs to suppress $CD4^+CD25^-$ responder T cell proliferation, suggesting a contact-dependent manner of suppression (19, 20). In these studies it was also suggested that Tregs inhibit IL-2 production of $CD4^+CD25^-$ responder T cells at least in part at the mRNA transcription level (19, 20). However, Tregs deficient in IL-2 or IL-2R from $Foxp3^{gfp}IL\text{-}2^{-/-}$ or $Foxp3^{gfp}IL\text{-}2R^{-/-}$ mice suppressed T cell proliferation of $CD4^+Foxp3^-$

responder T cells to a similar extent as Tregs from wild type Foxp3^{gfp} mice, suggesting that IL-2 is not necessary for Treg mediated suppression in vitro (23).

Among the cytokines that have been implicated in the *in vitro* suppressive mechanism of Tregs is TGF-β but with contradictory results. Tregs express membrane bound TGF-β1, and studies that employed neutralizing antibodies against TGF-β1 demonstrated that these Tregs failed to mediate suppression *in vitro* (24-26). On the contrary, a study using Tregs from TGF-β1$^{-/-}$ neonatal mice showed that TGF-β1$^{-/-}$ Tregs had similar suppressive capacity to Tregs from wild-type mice, suggesting that Treg mediated suppression is TGF-β independent *in vitro* (27). Despite these contradictory data with TGF-β, the role of cytokines in Treg mediated suppression *in vitro* is still being explored, and more recently a newly discovered member of the interleukin-12 (IL-12) cytokine family, interleukin-35 (IL-35) has been described as having a novel inhibitory role in Treg suppressive function *in vitro* and *in vivo* (28, 29). IL-35 is made of the Epstein-Barr virus-induced gene 3 (Ebi3) and p35 subunits, suggested to be secreted by Tregs and required for maximal Treg suppressive function (29). Therefore, the mechanisms of Treg mediated suppression *in vitro* merits further investigation, as a wide range of molecules could potentially be involved in their suppression, as reviewed in (30).

In vivo, Tregs have been shown to expand in an antigen-specific manner and do not display the anergic characteristic observed *in vitro* (31-33). Cytokines such as IL-10 and TGF-β have also been shown to be important in Treg suppression (22). Both IL-10 and TGF-β produced by Tregs have been implicated in the suppression of colitis in inflammatory bowel disease (IBD) models (22, 34). Another *in vivo* mechanism which

Tregs could be utilizing involves dendritic cells (DCs) (35). Mature bone marrow-derived dendritic cells (BMDCs) are able to break Treg anergy *in vitro* and allow for the proliferation and expansion of this T cell population (32). The expanded Tregs when transferred *in vivo* suppress autoimmune diabetes (36, 37). Tregs can also condition DCs to be suppressive (35). For example Tregs constitutively express CTLA-4, which is upregulated upon TCR stimulation (38). CTLA-4 upregulation in Tregs led to the induction of indoleamine 2, 3 dioxygenase (IDO) in DCs, which led to the suppression of effector T cells (39, 40). Two elegant studies using two-photon laser scanning microscopy revealed a possible *in vivo* mechanism that Tregs could be utilizing, which involved interactions between Tregs and DCs as well as CD4 effector T cells (41, 42). It was observed that contacts between autoantigen loaded DCs and effector T cells were shorter in the presence of Tregs than in their absence, suggesting that Treg could be preventing priming of autoreactive T cells (41). Moreover, Treg direct interaction with autoantigen loaded DCs inhibited DC interactions with effector T cells, thus preventing effective proliferation and differentiation of the effector T cells (42). However, it was not clear in these studies whether the interactions between Tregs and DCs produced any suppressive factors that could have affected the effector T cells. Therefore, Tregs could utilize multiple suppressive mechanisms *in vivo*.

Antigen Presentation Pathways

Before discussing the role of APCs in peripheral tolerance, a brief overview of the antigen presentation pathways is provided as the induction of tolerance or immunity begins with APCs capturing, processing and presenting antigens to T cells.

Professional APCs such as DCs, B cells and macrophages are responsible for displaying peptide-MHC complexes on their surface for the purpose of activating the immune system or inducing tolerance. There are two classical pathways for antigen presentation namely, the MHC class I pathway which presents endogenous antigens to CD8 T cells and the MHC class II pathway which presents exogenously derived antigens to CD4 T cells (43). Along with these two classical antigen presentation pathways, a third pathway called cross presentation exists. Cross presentation involves the presentation of exogenous peptides to the MHC class I pathway rather than to the MHC class II pathway to which they are normally presented (44). Subsets of DCs such as the murine $CD8\alpha^+$ DC have been shown to play a role in cross presentation (45, 46). This pathway is said to be important in immune surveillance of viral and tumor antigens (47). Extensive work has been done in this area and reviewed in (44, 47-49).

MHC class I antigen presentation pathway

Peptides to be presented in the MHC class I pathway are derived from endogenous proteins and generated by the action of proteolytic degradation by the proteasome and peptidases in the cytosol (50, 51). Proteins are targeted for proteasomal degradation by ubiquitination, which involves covalently linking the protein with ubiquitin (51). After ubiquitination the proteins are unfolded, the ubiquitin removed and proteins are passed through the proteasome (51). Proteasome digested products are further trimmed by the action of cytosolic peptidases (51). The antigenic peptides are then transported from the cytosol to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) composed of TAP1 and TAP2 (50, 51). In the ER, further trimming of the peptides takes place via ER aminopeptidases (ERAAP or

ERAP1) to yield 8-10 amino-acids peptides (50, 51). Meanwhile the synthesis of the MHC class I molecule takes place in the ER with the help of ER chaperone proteins. The MHC-I heavy chain first binds the chaperone protein calnexin which mediates folding and intra-chain disulfide bond formation (50). The heavy chain then dissociates from calnexin and assembles with β_2-microglobulin, and the MHC-I complex is attached to TAP by a protein called tapasin to become part of the peptide-loading complex (50). The other components of the MHC-I peptide-loading complex include the chaperone protein calreticulin and the soluble thiol oxidoreductase ERp57 (50). Antigenic peptides enter the ER through TAP and those with the appropriate sequence bind to the cleft of the MHC class I molecule (50). The peptide-MHC-I complex are then released from tapasin, exit the ER and are transported to the cell surface where they may be recognized by CD8 T cells. An illustration of the MHC class I pathway is shown in Figure 1.

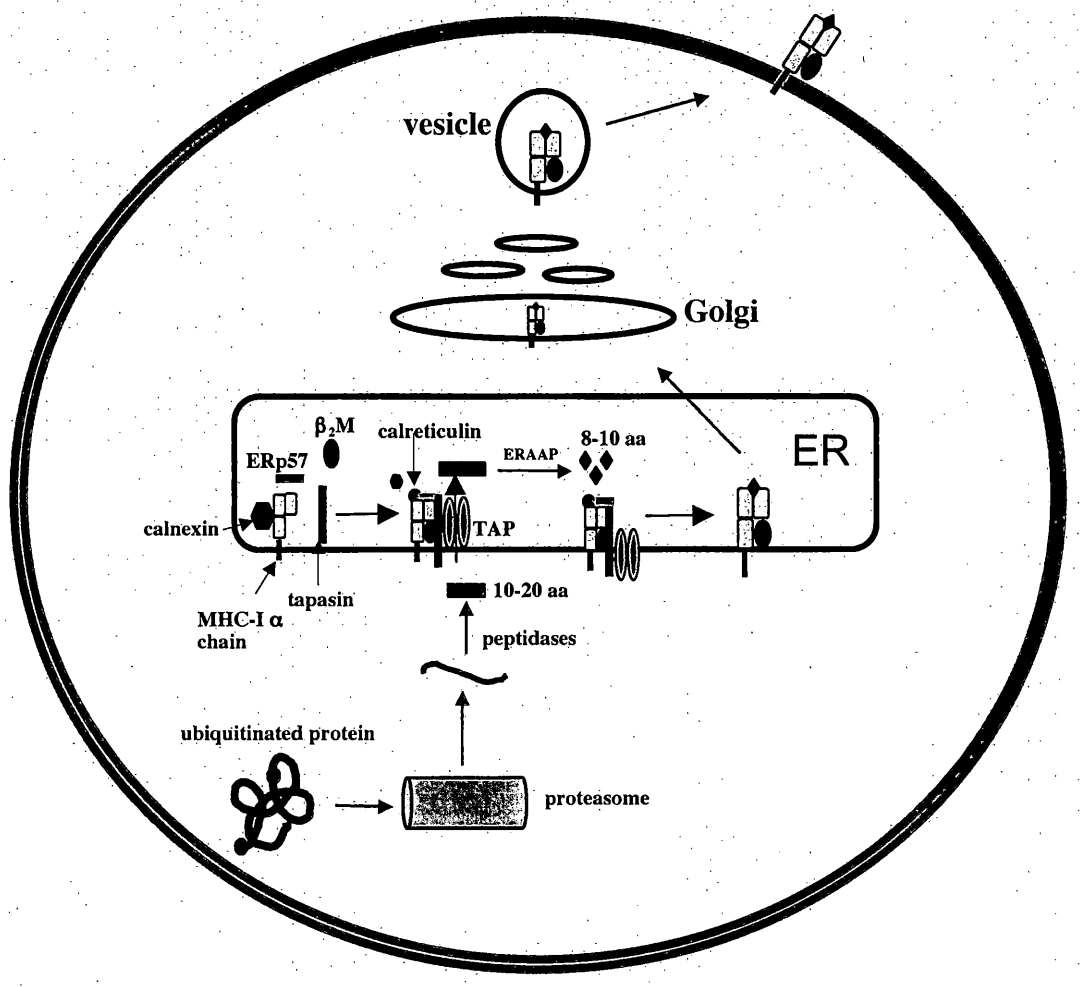


Figure 1: MHC class I antigen presentation pathway. Endogenous proteins are ubiquitinated and digested by the proteasome and cytosolic peptidases. Antigenic peptides are transported into the ER via TAP. Further trimming of the peptides can occur in the ER by such enzymes as ERAAP to peptides of 8-10 amino acids in length. Antigen peptides are loaded on the MHC-I peptide-loading complex composed of the MHC-I complex, calreticulin and ERp57 and attached to TAP by tapasin. The loaded peptide-MHC-I complex is then released from TAP and transported to the surface where it is displayed for CD8 T cell recognition.

MHC class II antigen presentation pathway

The generation of antigenic peptides for MHC class II presentation involves proteolytic degradation of exogenous proteins in the endocytic pathway (51). Proteins are degraded in endocytic vesicles by proteases such as cathepsins at low pH (52). The α and β components of the MHC class II molecules are assembled in the ER and a protein called the invariant chain (Ii) occupies the peptide binding clefts of the newly synthesized class II molecule (51). The assembled MHC class II complexes (MHC α/β-Ii) are transported from the ER to the Golgi in exocytic vesicles (53). The vesicles fuse with endosomes carrying internalized and processed antigens and these fused endosomes are called the MHC class II compartment (MIIC) (53). The Ii chain is removed in the MIIC by proteolytic enzymes such as cathepsin S and leaves a 24 amino acid remnant called the class II associated invariant chain peptide (CLIP) (54). CLIP is then removed from the peptide-binding cleft making it accessible to antigenic peptides. This process is facilitated by the chaperone protein HLA-DM (or HLA-DO in B cells) that exchange CLIP for antigenic peptides (54). The peptide-MHC-II complexes are then transported to the cell surface where they may be recognized by CD4 T cells. An illustration of the MHC class II pathway is shown in Figure 2.

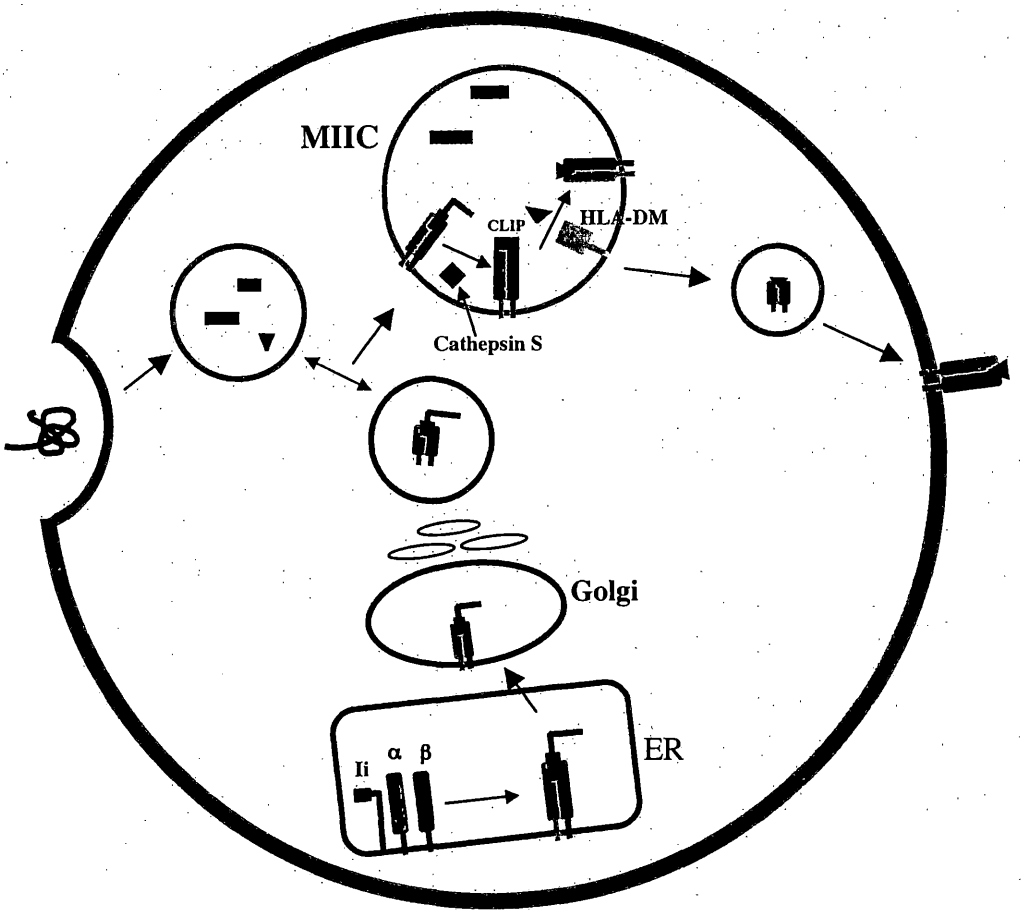


Figure 2: MHC class II antigen presentation pathway. Exogenous proteins are degraded in endocytic vesicles by proteases. In the ER the MHC class II complex (MHC α/β-Ii) is assembled and then transported to the Golgi in exocytic vesicles. The vesicles carrying the MHC α/β-Ii complex fuses with the endosomes containing the processed antigens to form MIIC. The removal of the Ii chain and replacement with antigenic peptides take place in the MIIC facilitated by HLA-DM. The peptide-MHC-II complexes are then transported to the cell surface where they may be recognized by CD4 T cells.

The role of APCs in Peripheral Tolerance

Here we discuss the role of APCs in peripheral tolerance using more specifically DCs as an example as they are considered to be the most potent APCs. A great deal of heterogeneity exists in the DC population as DCs can be distinguished by their surface markers, tissue distribution and on the basis of their progenitors (51). The maturation state of DCs has also been used to distinguish DCs and is considered to be important in the induction of either immunity or tolerance (55). Immature or semi-mature DCs (expressing low MHC and co-stimulatory molecules on their surface) have been shown to induce tolerance rather than immunity in the steady state by induction of anergy, deletion or generation of regulatory T cells (55). For example, experiments where peptide or whole protein was delivered to immature DCs through the DEC-205 (also called CD205) endocytosis receptor resulted in T cell deletion, and the mice became tolerant to subsequent immunization with peptide or whole protein in complete Freund's adjuvant (56, 57). By using this targeting technique to immature DCs through DEC-205, others have observed the induction $CD4^{+}CD25^{+}$ Tregs (58). However, this idea of maturation of DCs in the context of inducing immunity or tolerance has been challenged and evidence suggests that even mature DCs (expressing high MHC and co-stimulatory molecules on their surface) are also able to induce tolerance (59). Thus, this nomenclature of immature vs. mature DCs has been suggested to be somewhat misleading (59). Some have proposed defining DCs in terms of function instead of their maturational state and thus prefer the terms tolerogenic DCs for those DCs that induce tolerance and immunogenic DCs for those DCs that activate the immune system (59, 60). Some mature DCs are thus able to exert tolerogenic properties by their ability to express immunosuppressive

molecules such as IDO and induce the generation of regulatory T cells (61). Examples of such mature DCs involved in tolerance induction are discussed below in the context of tumor-induced tolerance, with the idea that the same mechanism could apply in peripheral tolerance.

I.C. Tumor-induced Tolerance

The Role of Tregs and APCs in Tumor -induced Tolerance

Tumors create an immunosuppressive environment that promotes tumor growth and hinders effective immunotherapy. Both CD4 and CD8 T cells have been shown to be rendered tolerant in the tumor microenvironment (62). Hemagglutinin (HA) specific CD4 T cells adoptively transferred into A20 B cell lymphoma tumor bearing mice were rendered anergic as measured by their poor proliferative response to cognate antigen *in vitro* and their lack of ability to be primed following vaccination *in vivo* (63). Also Pmel-1 antigen-specific CD8 T cells specific for the tumor/self-antigen gp100 were tolerized and unable to confer protection against B16 melanoma tumor expressing the self-antigen (64). Among the mechanisms employed by tumors to induce tolerance, APCs and Tregs play a crucial part (62). APCs can capture, process and present tumor associated antigens to activate tumor specific T cell responses. Even with this T cell activation, tumors are not eliminated and a state of tolerance to the tumor antigen is created. The role of APCs in the induction of tumor induced tolerance was demonstrated when bone marrow derived APCs were shown to be responsible for the induction of antigen specific CD4 T cell tolerance in mice challenged with A20 B cell lymphoma expressing HA antigen and in solid tumors (65, 66). The particular subset of APCs in these experiments was not explored but several lines of evidence point to DCs in particular as having a key role in

the induction of tumor tolerance (62). Plasmacytoid DCs (pDCs) for example have been shown to infiltrate ovarian carcinoma, a recruitment dependent on the stromal-derived factor 1 (SDF-1) secreted by tumor cells (67). pDCs also infiltrated primary melanoma lesions, head and neck squamous cell carcinoma (HNSCC) and cutaneous melanoma where they impair T cell responses (68-70). A subset of pDCs has also been found in tumor draining lymph nodes (TDLNs) of mice that constitutively express IDO that potently inhibits T cell responses (71, 72). Immature DCs expressing low levels of co-stimulatory molecules such as CD80, CD86 and CD40 are able to take up and process antigen but are unable to induce anti-tumor immune response but instead induce tolerance (62). In renal cell carcinomas, melanoma metastases and basal cell carcinoma an accumulation of immature DCs was observed (62). The tumor microenvironment could also modify DCs to be regulatory instead of activators of immune responses. These regulatory DCs secrete IL-10 and TGF-β and possibily induce naïve CD4 T cells to become Tregs in the tumor microenvironment (62).

Treg have also been shown to accumulate in human tumor models and hamper tumor specific effector T cell responses (73-77). In ovarian epithelial cancer, Treg were found in malignant ascites and tumor tissues where they appear to be in close proximity to CD8 T cells, suggesting a cell contact dependent suppression mechanism (74). Tregs have also been found in human tumor draining lymph nodes; however, a discrepancy exists as to the numbers of Treg. Some investigators observed that the number of Tregs found in TDLNs of ovarian epithelial cancer was decreased (74); whereas others noted an increased number of Tregs in metastatic melanoma lymph nodes (73). Tregs in both of these human tumor models express Foxp3 and suppress proliferation of effector T cells *in*

vitro (73, 74). The discrepancy in these results, however, does not undermine the TDLN as an important anatomical location in the study of Tregs in tumors. An excellent review by Munn and Mellor describes the TDLN as an immune privilege site, where active suppression takes place, as well as a site of accumulation and generation of Tregs (78).

Experiments in mouse tumor models in which Tregs were depleted resulted in tumor rejection, suggesting that this T cell population hinders effective anti-tumor immune responses (79-81). Others have shown that Tregs suppress tumor-specific $CD8^+$ T cells resulting in a lack of tumor immunity (82-84). There is considerable evidence suggesting that Tregs are involved in suppression of anti-tumor immune responses. However, the mechanism by which Tregs hamper anti-tumor immunity is not well understood. By using mice that lack the expression of MHC-II in the periphery (85), we will be able to elucidate the role MHC-II dependent events in Treg suppressive functions.

I.D. Regulatory T cells and MHC class II

MHC-II dependent events have been suggested in the generation of nTregs. The majority of thymic derived Tregs require high-affinity TCR/MHC-II interactions for their generation (86). Therefore, the expression of MHC-II on cortical epithelial cells was necessary and sufficient for Treg development in the thymus (87). However, there have been reports of Treg subsets that developed in the periphery in a MHC-II independent manner in MHC-$II^{-/-}$ mice (88-90). The role of MHC-II in Treg mediated suppression has also been illustrated by two recent studies. Andersson et al. showed that MHC-II dependent activation of Tregs *in vivo* was required for Tregs to exert their suppressive function *in vitro* after they were first cultured with IL-2 (91). Parallel studies by Liang and colleagues revealed that the binding of LAG-3 a marker expressed on Tregs (92), to

MHC-II on DCs resulted in the inhibition of DC activation, providing a potential mechanism by which Tregs could modulate DCs (93). MHC-II dependent events have also been suggested in the peripheral homeostasis of Tregs. Transfer of Tregs to IA$\beta^{-/-}$ RAG$^{-/-}$ mice revealed that homeostatic proliferation of Tregs is MHC-II dependent (94). Additionally, TCR/MHC interactions are required for the induction of Foxp3 expression (18). Studies in our laboratory with conditional MHC-II knockout mice suggest an indirect role for MHC-II in Treg peripheral homeostasis such that Treg maintenance is dependent on the availability of IL-2 from CD25^{low} self-reactive T cells, which are themselves partially MHC-II dependent (85). These mice are described below.

I.E. Characterization of Conditional MHC-II deficient mice

The Cre recombinase-*loxP* system is a powerful tool that allows conditional, i.e. tissue specific, ablation of targeted genes. Cre is a 38-kD protein derived from the *Escherichia coli* P1 bacteriophage that mediates DNA recombination between two 34 base-pair specific sites called *loxP,* resulting in the deletion of DNA sequences between these two sites (95). Mice bearing *loxP* sites of recombination in the MHC-II β chain gene (*ia*β^{neo}) were generated by Hashimoto et al., which when crossed with the appropriate Cre recombinase mice allow the deletion of MHC-II on specific cells (96). To achieve conditional deletion of MHC-II on APCs, Tie2Cre transgenic mice were employed (in which the Tie2 promoter drives Cre expression), which cause deletion in early hematopoietic progenitor and endothelial cells (97). *ia*$\beta^{neo/\Delta}$Tie2Cre^{+} conditional MHC-II deficient mice (85) were, therefore, generated by crossing *ia*β^{neo} allele mice (96) with Tie2Cre transgenic mice (97). Since the Tie2Cre transgene causes deletion of *lox*-P flanked targets in early hematopoietic progenitor cells and endothelial cells but not in

thymic epithelial cells (97), $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice did not lose the expression of MHC-II in thymic epithelial cells (85). The cortical and medullary epithelial cells were thus MHC-II sufficient, but CD11c^{+} DCs were MHC-II deficient as determined by immunohistochemistry (85). Further analysis with flow cytometry revealed that thymic CD11c^{high} DC from $ia\beta^{neo/\Delta}$Tie2Cre^{+} lacked MHC-II while the same population from $ia\beta^{neo/\Delta}$Tie2Cre^{-} littermates were MHC-II sufficient (85).

In the peripheral organs, a high degree of loss of MHC-II was seen in splenic B cells, CD11c^{+}B220^{-}CD8α^{+} and CD11c^{+}B220^{-}CD8α^{-} DCs (85). Moreover, this study reported the existence of a few low-deletion mice possessing about 20% MHC-II expression on their APC, but these mice could be excluded before experimentation by genotyping and peripheral blood cell analysis (85). $ia\beta^{neo/\Delta}$TIE2Cre^{+} mice showed increased total CD4^{+}CD8^{-} cells in the thymus compared to their age-matched wild-type counterparts (85). CD4^{+}CD25^{+}CD8^{-} Tregs that were also Foxp3^{+} were also found in the thymus but not significantly increased compared to wild-type mice (85). Naïve CD4 T cells and CD4^{+}CD25^{+} Tregs that were Foxp3^{+} were also present in the peripheral organs, with Treg numbers in these mice similar to those in their wild type littermates (85). And of interest, CD4^{+}CD25^{+} cells from $ia\beta^{neo/\Delta}$TIE2Cre^{+} mice suppressed CD4^{+}CD25^{-}CD8^{-} T cell proliferation *in vitro* with the same potency as wild-type mice. Therefore, these novel conditional MHC-II deficient mice provide a model in which the role of MHC-II dependent events in Treg mediated suppression and in tumor progression can be studied.

II. Materials and Methods

Mice

Mice used in experiments were 6-10 weeks of age. Foxp3^{gfp} knockin mice (18) were from Dr. Rudensky (University of Washington, Seattle Washington). C57BL/6J (B6) and B6.PL-*Thy1*^{a}/CyJ mice were from The Jackson Laboratory (Bar Harbor, ME). *ia*$\beta^{neo/\Delta}$Tie2Cre^{+} mice on a C57BL/6J background and their wild-type littermates (85) were generated by crossing Tie2Cre transgenic mice (97) with *loxP*-flanked IAβ (*ia*β^{neo}) allele mice (96). OT-I, MHC-I restricted recognizing $OVA_{257-264}$ peptide (SIINFEKL) (98) and OT-II, MHC-II restricted recognizing $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR) (99) TCR transgenic mice were backcrossed onto a B6.PL-*Thy1*^{a}/CyJ background. To generate OT-IIFoxp3^{gfp} mice, OT-II (99) mice were first backcrossed onto a B6.PL-*Thy1*^{a}/CyJ background and then crossed with Foxp3^{gfp} mice (18). Act-mOVA mice that express ovalbumin protein on the surface of all cells in the body (100) were crossed with OT-II mice to produce double transgenic mice OT-II x Act-mOVA.

Culture medium

Complete medium (10%FCS) was made from RPMI 1640 supplemented with 10% fetal calf serum (FCS) from Hyclone, 25 mM HEPES, 2mM L-glutamine, 50 μM β-mercaptoethanol, 100 IU/ml penicillin and 0.1mg/ml streptomycin. 1%FCS medium was made from RPMI 1640, 1% FCS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 IU/ml penicillin and 0.1mg/ml streptomycin.

FACS buffer was made from 1xPBS supplemented with 1% FCS. Medium for making liquid nitrogen stocks was made from10% DMSO (Sigma) in FCS. Reagents were from Cellgro.

Tumors

B16-OVA (MO4) (101) expressing full-length ovalbumin protein and the parental B16F10 (American Tissue Culture Collection, ATCC) were provided by Dr. Munn (Immunotherapy Center, Medical College of Georgia). B16F10 cells were grown in complete medium whereas the B16-OVA cells were grown in complete medium supplemented with 1 mg/ml of G418 (Cellgro). Cells were trypsinized using 1xTrypsin EDTA (Cellgro) for 2 minutes at 37°C then washed 3 times and resuspended in plain RPMI 1640 medium before injecting into mice. $5x10^5$ cells unless otherwise indicated in the experiment were subcutaneously injected into the abdominal flank area of $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice and wild-type counterparts. For CD8 T cell depletion studies, $ia\beta^{neo/\Delta}$Tie2Cre$^+$ and wild-type mice (3 mice per group) were first intravenously injected via the tail vein with 200 μg/mouse anti-CD8 antibody (Clone 53-6.7) on the same day as the tumor injections. Mice were then further injected with depletion antibody on day 3

and day 7. Tumor sizes were measured by taking the average of the perpendicular and vertical distance using a digital caliper (GENERAL TOOLS).

Antibodies

Antibodies from BD Pharmingen™ (unless otherwise specified) were used for extracellular cell surface staining and cell sorting and included antibodies against CD4 (RM4-5, FITC and APC), CD11c (HL3, FITC and PE), CD8b (H35-17.2, PE from eBioscience), CD8α (53-6.7, PerCP and APC) CD25 (PC61, PE and Biotin), Vα2 (B20.1, PE and Biotin), CD43 (1B11 Biotin), CD44 (IM7 in Cychrome), CD122 (TM-β1 Biotin), 120G8Bio (102) and Foxp3 (FJK-16s in PE and APC from eBioscience), with the clone and flourochromes indicated in the brackets. Streptavidin-PE, Streptavidin-PercP and Streptavidin-APC were used in secondary staining where biotinylated antibodies were used in the primary staining.

Anti-CD3 (145-2C11) and anti-CD28 (37.51) were used to make immobilized plates. Recombinant IL-2 was from R&D Systems. Depletion antibodies used were CD49b/Pan NK cells (DX5), Thy-1 (G7), Thy1.2 (30-H12), anti-NK1.1 (PK136) and anti-CD8 (53-6.7, provided by Dr. Lei Huang).

Cell Preparation

Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation in accordance with Laboratory Animal Services (LAS) guidelines. Single cell suspensions were made from spleens and pooled peripheral lymph nodes (inguinal, brachial and axillary lymph nodes) by crushing the organs between 0.1 mm pore diameter nylon filter mesh (Millipore) in 5 ml of PBS in a Petri dish using the plunger of a 5 ml syringe. Cells were filtered through the 0.1 mm pore diameter nylon filters, collected in 15 ml tubes and

centrifuged for 5 minutes at 1400 rpm. Lymph nodes were resuspended in appropriate medium. Spleen cells were depleted of erythroid cells by resuspending in 2 ml/spleen ACK lysis (BioWhittaker™) and incubated at room temperature for 30 seconds before diluting with PBS, and were then centrifuged for 5 minutes at 1400 rpm. Spleen cells were then resuspended in appropriate medium.

Tumor Digestion

The tumor mass from Foxp3^{gfp} mice was isolated and placed in Petri dishes (Falcon dishes, tissue culture treated, 60 x 15 mm from Becton Dickson). The tumors were carefully teased from any surrounding tissue and chopped into small pieces using forceps. The digestion medium was made with 50 ml of RPMI 1640 supplemented with 50 mg of collagenase, 5 mg of DNase and 5 mg of Hyalurodinase (Reagents from Sigma). 5 ml of this medium was dispensed into the cells. Cells were incubated at 37°C for 1 hour. After the incubation, cells were passed through a 40 μM Falcon cell strainer and centrifuged for 5 minutes. Cell were further washed twice with plain RPMI 1640 medium before resuspending in FACS buffer and staining with CD4APC (1:500). Cells were analyzed on a FACS calibur.

Flow cytometry cell sorting

Pooled erythroid cell-depleted spleens and peripheral lymph nodes prepared as described above were resuspended in 5 ml 1%FCS medium and placed at 4°C. Fc-Block (2.4G2; anti-CD16/CD32 from BD Bioscience) was added at a concentration of 2 μg/ml for 5 minutes at 4°C. Appropriate antibodies were added at a concentration of 0.5-2 μg/ml for 30 minutes at 4°C. Depending on the experiment, secondary antibodies were added after cells were first washed with 1% FCS medium and resuspended in the same

volume of 1% FCS medium. Secondary antibodies were added at the same concentration as primary antibodies of 0.5-2 μg/ml and incubated for an additional 30 minutes at 4°C. Cells were washed and resuspended in 2 ml of 1%FCS medium with 0.001 g of DNase I, to prevent cells from forming aggregates, and filtered. Cells were sorted on a MoFlo cell sorter (DakoCytomation) or a BD FACS Aria and collected in 0.5 ml of complete medium.

Tregs from OT-II and OT-II x Act-mOVA mice were sorted on the basis of their being $CD4^+CD25^+V\alpha2^+CD8\alpha^-$. Tregs from OT-IIFoxP3^{gfp} were first sorted based on $V\alpha2^+CD25^{hi}GFP^+CD8\alpha^-$, then modified to $V\alpha2^+CD25^{all}GFP^+CD8\alpha^-$ and finally modified to exclude both CD11c and CD8 T cells as $V\alpha2^+CD44^{hi}GFP^+CD11c^-CD8b^-$ or $CD4^+CD44^{hi}GFP^+CD11c^-CD8b^-$. The purity of Tregs from these mice was greater than 94%. OT-I CD8 T cells were first sorted on the basis of $V\alpha2^+CD8\alpha^+CD4^-CD11c^-$ and modified to only include naïve CD8 T cells as $CD8\alpha^+CD43^{lo}CD122^{lo}CD4^-CD11c^-$. The purity of OT-I CD8 T cells was greater than 92%.

Flow cytometry staining and analysis

Erythroid cell-depleted spleens or lymph nodes were prepared as described in the cell preparation section and resuspended in 2 ml FACS buffer. Fc-Block was added at a concentration of 2 μg/ml for 5 minutes at 4°C. Cells were stained with the appropriate antibodies at a concentration of 0.5-2 μg/ml for 30 minutes. Depending on the experiment, secondary antibodies were added after cells were first washed and resuspended in the same volume of FACS buffer. Secondary antibodies were added at a concentration of 0.5-2 μg/ml and incubated for an additional 30 minutes 4°C. Single color compensation tubes were prepared to calibrate the appropriate channels for each

staining. Cells were finally washed with 1ml of FACS buffer, centrifuged at 1400 rpm for 5 minutes and resuspended in FACs buffer. Cells were collected on a FACS calibur (Becton Dickson) and the data was analyzed using CellQuest software (BD).

Intracellular Foxp3 Staining

Foxp3 intracellular staining was performed for Tregs that did not incorporate the GFP allele using a kit from eBioscience as per the manufacturer's instructions. Cells to be stained were collected and resuspended in FACS buffer at a concentration of $1x10^6$ cells/ml. Cells were first stained with appropriate antibodies at a concentration of 0.5-2 μg/ml and incubated at 4°C for 30 minutes. Cells were washed three times, resuspended in 1 ml of freshly made Fix/Perm concentrate and incubated at 4°C overnight in the dark. After the overnight incubation cells were washed once with FACS buffer and then washed twice with 2 ml of permeabilization buffer. Foxp3 antibody was added at a concentration of 1 μl per 100 μl and incubated at 4°C in the dark for 30 minutes. Cells were washed twice with 2 ml of permeabilization buffer and resuspended in the appropriate volume of FACS buffer before analysis on a FACS calibur.

Antigen Presenting Cell (APCs) Preparation

Erythroid cell-depleted spleens from B6 or $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice were resuspended in 2 ml of 1% FCS medium. T cell and NK cell depletion antibodies (anti-Thy1.2 or anti-Thy-1 and anti-NK1.1 or anti-CD49) were added at a final concentration of 10 μg/ml. The cells were incubated at 4°C for 30 minutes. Cells were gently mixed every 5 minutes. After the incubation, cells were washed 1x with 1% FCS medium and resuspended in 2 ml of 1% FCS medium. Rabbit complement (Cedarlane) was added at a 10% (v/v) concentration and incubated at 37°C for 30 minutes. Cells were washed three

times with 1% FCS medium and resuspended in complete medium. Cells were then filtered through a 0.1 mm pore diameter nylon mesh filter and counted. Finally, cells were irradiated for 30 minutes (3000 Rads) before use in experiments.

Bone Marrow - Derived Dendritic Cell (BMDC) preparation

The femur and tibia bones from B6 mice were isolated. All muscles and tissues were removed and bones were sprayed with 70% ethanol. After careful cleaning, bone marrow cells were isolated from the bones by flushing with RPM1 1640 medium. The cells were collected in 15 ml tubes, which were filled completely with plain RPMI 1640 medium. Cells were centrifuged for 5 minutes at 1400 rpm. The cells were depleted of erythroid cells by adding 1 ml of ACK lysis and incubated at room temperature for less than 30 seconds. 1x PBS was added to stop the reaction and cells again collected by centrifugation for 5 minutes at 1400 rpm. Cells were resuspended in complete medium and filtered through a 0.1 mm pore diameter nylon mesh filter. Cells were cultured in complete medium supplemented with 5% GM-CSF. Every 2 days non-adherent cells were removed and fresh complete medium supplemented with 5% GM-CSF was added. On day 8 of culture the remaining loosely attached and non-adherent cells were collected and washed once in complete medium and resuspended in complete medium. The cells were matured overnight with 1 μg/ml LPS and in some cultures ovalbumin protein or OVA peptide were added. The next day the matured cells were washed, resuspended in complete medium and counted. The cells were irradiated for 30 minutes (3000 Rad) before use as APCs.

Treg activation

Sorted Tregs from OT-IIFoxp3^{gfp} mice were activated per published protocols (103) with some modifications. Briefly, sorted Tregs were incubated in plates with immobilized anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) in complete medium supplemented with IL-2 at 100 U/ml for 3-4 days prior to resting for an additional day in complete media with 10 U/ml of IL-2. A diagrammatic representation of the Treg activation protocol is summarized in Figure 3.

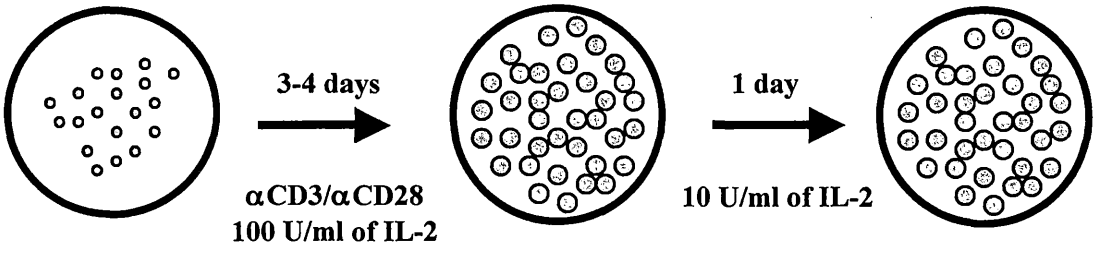


Figure 3: Treg activation protocol. Note at the end of the culture period cells must be viable and larger in size than at the start of the culture.

T cell proliferation and suppression assay

Sorted naïve T cells and Tregs were co-cultured or cultured alone in triplicate in various quantities as described in the results. $5x10^4$ irradiated T cell depleted APCs were then added in a 96-well U-bottom plate. For antigen-specific stimulation the indicated amounts of $OVA_{323-339}$, $OVA_{257-264}$, HEL (Hen egg lysosome) peptides or ovalbumin protein was added as described in each experiment in the results. The final volume in each well was 0.2 or 0.25 ml. Cells were incubated at 37°C in a 5% CO_2 humidified incubator for 72 hours unless otherwise specified. The last 8 hours of this incubation was with 1 μCi/well [^{3}H] thymidine (Amersham). Cells were harvested on a TOMTEC cell harvester and proliferation was determined using tritiated thymidine incorporation into DNA using a 1205 BetaPlate™ reader (Wallac).

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ ELISA was performed using the BD OptEIA™ kit (BD Bioscience) per the manufacturer's instructions. Briefly, capture antibody was diluted 1:250 in coating buffer (PBS/pH 8.5). A 96 well flat-bottom Maxisorp plate (NUNC™) was coated with 50 μl of the diluted capture antibody and incubated overnight at 4°C. After the overnight incubation, the wells were aspirated and washed 5x with 250 μl wash buffer (0.05% Tween-20 in 1xPBS) on a Wellwash 4 Mk 2 (Fisher Scientific) well wash. The plate was blocked with 200 μl assay diluent (10% FCS in PBS) and incubated at 37°C for 1-2 hours. The plate was washed 5x with wash buffer. 50μl of titrated amounts of standards and samples were added into the appropriate wells. The plate was sealed with a plastic wrap and incubated for 1 hour at 37°C. Wells were aspirated and washed 5x. 50 μl of working detector (detection antibody diluted in assay diluent 1:250 + avidin-HRP reagent

added at a 1:250 dilution) was added to each well. The plate was sealed and incubated at room temperature for 1 hour. Wells were aspirated and washed 10x (final wash). 50 µl of substrate solution (tetramethylbenzidine and hydrogen Peroxide) were added to each well and incubated at room temperature for 30 minutes or until the desired blue color was observed. 50 µl of stop solution (1M H_3PO_4 or 2N H_2SO_4) were then added and the absorbance was read immediately at 450 nm on a SpectraMax spectrometer (Molecular Devices). The data were processed using SoftMax Pro V5 software.

Activation of OT-I CD8 T cells for Adoptive Transfer

Activation of OT-I cells was adapted as described by Schuler and Blankenstein (104). Spleens from OT-I TCR transgenic mice were isolated and prepared as described in the cell preparation sections, resuspended in completed medium and counted. The volume was then adjusted to $2x10^6$ cells/ml, and the cells were cultured for 3 days with 1 µg/ml of OT-I peptide ($OVA_{257-264}$, SIINFEKL). After the three-day culture, cells were collected, washed three times, counted and resuspended in 1x PBS before injection intravenously (100 µl) into mice.

Osmotic shock loading of ovalbumin protein in B10.BR splenocytes

Ovalbumin protein was loaded into B10.BR splenocytes as previously described (105). Briefly, $15x10^7$ erythrocyte lysed B10.BR splenocytes were washed twice in plain RPMI and resuspended in 1 ml of hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 100 in RPMI medium with a pH of 7.2) containing 10 mg/ml of ovalbumin protein and incubated at 37°C for 10 minutes. Pre-warmed hypotonic medium (40% H_2O, 60% plain RPMI 1640) was added and the cell incubated at 37°C for an additional 2 minutes. After the incubation cells were immediately centrifuged, washed

twice with ice-cold 1x PBS and counted and 50 μl were injection into the footpads of mice. These dead cells served as the antigen presented by host APCs to activate adoptively transferred T cells.

CFSE labeling of Cells

Cells were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) labeled (106) for the *in vivo* cytotoxic killing assay described below. Spleen cells were prepared as described in the cell preparation section and resuspended in 1x PBS at a concentration of 10^7 cells/ml. CFSE was added at a final concentration of 0.2 μM or 2 μM and incubated at 37°C in a 5% CO_2 humidified incubator for 10 minutes. Cells were then diluted with 1x PBS, washed twice in 1x PBS, counted and resuspended in 1x PBS, and 0.2 ml was injected intravenously via the tail vein.

***In vivo* cytokoxic killing assay**

This assay was adapted as described by Mempel and colleagues (107). Briefly, naïve sorted $1x10^5$ OT-I $CD8^+$ T cells were adoptively transferred into B6 mice with or without $5x10^5$ sorted and activated OT-IIGFP$^+$ Tregs. One day later mice intravenously received $6x10^6$ irradiated T cell depleted splenocytes from Act-mOVA mice. Six days later mice were injected intravenously with a 1:1 mixture of B6 splenocytes, one half of which were labeled with 0.2 μM CFSE ($CFSE^{lo}$) and pulsed with 1 μg/ml of SIINFEKL peptide (targeting peptide) and the other half of which were labeled with 2 μM CFSE ($CFSE^{hi}$) and left untreated. A 1:1 mixture of these CFSE-labeled cells were injected into B6 mice that did not receive any T cells and served as controls. Spleen and lymph nodes were harvested 8 hours later, processed, stained with B220APC (1:500) and analyzed on

a FACS calibur. The percentage of specific lysis was determined using the formula: specific lysis (%) = $(1\text{-}CFSE^{low(exp)}/CFSE^{high(exp)} / CFSE^{low(control)}/CFSE^{high(control)}) \times 100$.

Statistical analysis

All statistical analysis were performed using the two-paired Student T-test. P-values of less than 0.05 were considered significant.

III. The generation of an *in vitro* system to test the role of MHC-II dependent events in Treg mediated suppression

A. Hypothesis

$CD4^+Foxp3^+$ Tregs suppress CD8 T cell proliferation and effector molecule production in a MHC-II dependent manner.

Although it is well established that Tregs suppress both CD4 and CD8 T cells, previous studies were performed with polyclonal Tregs in which the antigen specificity was not known (108, 109). However, in studies where antigen specificity was known the percent suppression of proliferation was less than 65% (110). Also in the quest to elucidate the mechanism of Treg suppression *in vitro*, Tregs were shown to suppress the proliferation and effector molecule production of CD8 T cells even in an APC free system (109). This result illustrates the idea that Tregs must be stimulated through their TCR before exerting their suppressive function; it does not rule out the prospect that Tregs could use APCs as a platform for interacting with other T cells and for their activation. Furthermore, *in vivo* the mechanism of Tregs mediated suppression has been shown to involve APCs (22, 41, 42). Moreover, the role of MHC-II dependent events in Treg mediated suppression is still not well defined. Here we describe the generation of

an *in vitro* system to test the hypothesis that $CD4^+Foxp3^+$ Tregs suppress CD8 T cell proliferation and effector molecule production in a MHC-II dependent manner

In order to generate an *in vitro* model in which MHC-II dependent events in the suppression mediated by $CD4^+Foxp3^+$ Tregs could be tested, antigen-specific Tregs and responders are particularly crucial so that the antigen-specific activation of both $CD4^+Foxp3^+$ Tregs, through the MHC-II pathway, and CD8 T cells, through the MHC-I pathway, can be assessed in the same culture. In this system the MHC-I pathway remains intact but presentation through the MHC-II pathway is rendered defective by the ablation of the MHC-II molecule on the surface of peripheral APCs. OT-I CD8 T cells from OT-I TCR transgenic mice were used as a source of responder cells. Also, a series of experiments led us to use OT-II$Foxp3^{gfp}$ TCR transgenic mice as a source of antigen-specific Tregs. Therefore, we first described this quest for the antigen-specific Tregs that were used in our model.

III.A.1. The generation of OT-II x Act-mOVA double transgenic mice

OT-II TCR transgenic mice possess very low frequencies of antigen-specific Tregs (111). Sorting Tregs from seven OT-II mice on the basis of posi tivity for CD4, CD25, and Vα2, for example, produced only $5x10^5$ cells (data not shown). In order to isolate significant numbers of Tregs for use in experiments, this limitation had to be overcome. In studies where double transgenic mice were generated by crossing TCR transgenic mice with mice expressing their cognate antigens, as in the cases of DO11 x RIP-mOVA, TCR-HA x *pgk*HA, Ld-nOVA x DO11 and TCR-HA x IgHA, the frequency of antigen-specific Tregs was shown to increase (31, 33, 112, 113). Thus, we also

generated double transgenic mice OT-II x Act-mOVA by crossing Act-mOVA mice that express membrane bound ovalbumin protein on the surface of all cells with conventional OT-II mice. Spleens from OT-II x Act-mOVA and OT-II mice were isolated and analyzed for Tregs. We observed about a 6-fold increase in the relative frequency of $CD4^{+}V\alpha2^{+}Foxp3^{+}$ Tregs from OT-II x Act-mOVA mice compared to OT-II mice (7.7% vs. 1.2%)(Figure 4). However, the total number of CD4 T cells in the double transgenic mice was dramatically decreased compared to OT-II mice (4% vs. 31%) suggesting CD4 T cells had undergone thymic deletion due to the presence of cognate antigen in the thymus (Figure 4).

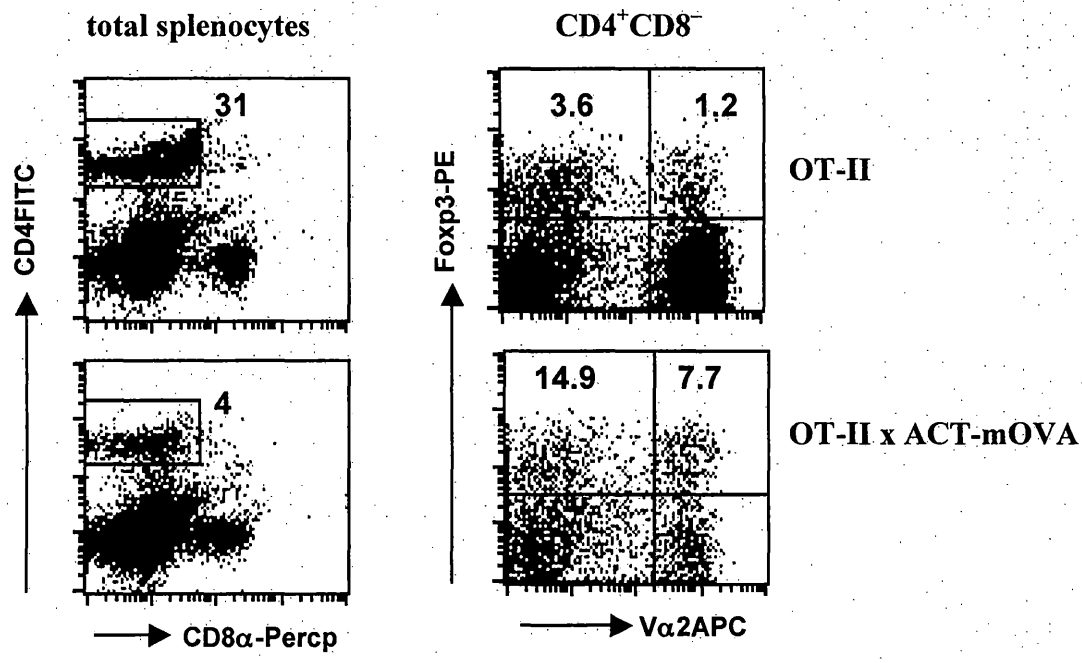


Figure 4: Increased percentage of Tregs from OT-II x Act-mOVA mice compared to OT-II mice. $1x10^6$ spleen cells from OT-II and OT-II x Act-mOVA mice were intracellular stained for Foxp3 using the PE-staining kit from eBioscience. Shown in the quadrants are the percentage of total $CD4^+$ T cells and Foxp3 expressing Tregs in OT-II and OT-II x Act-mOVA mice.

Since Tregs constitutively express the IL-2Rα chain (CD25) (8), standard Treg isolation protocols employ the CD25 marker. Tregs from OT-II x Act-mOVA mice were sorted for use in the experiment described in Figure 25. Spleens and peripheral lymph nodes from OT-II x Act-mOVA mice were isolated and prepared as described in the Materials and Methods section. Cells were stained with Vα2Biotin (1:1000)/StreptavidinAPC (1:500), CD4FITC (1:500), CD25PE (1:250) and CD8αPerCP (1:250). Tregs were sorted as $CD4^+CD25^+V\alpha2^+CD8\alpha^-$ on a MoFlo cell sorter. The profile of the sorted cells is shown in Figure 5. From eight OT-II x Act-mOVA mice we only obtained $4.18x10^5$ cells, similar to the numbers obtained from sorting Tregs from 7-8 OT-II mice (data not shown). The percentage of Tregs in the double transgenic mice was also comparable to conventional OT-II mice (Figure 5). Although there was an increase in the percentage of Tregs from OT-II x Act-mOVA mice (Figure 4), this did not translate to an increase in sorted Treg numbers. These results prompted us to seek alternative ways to increase Treg numbers for experimentation.

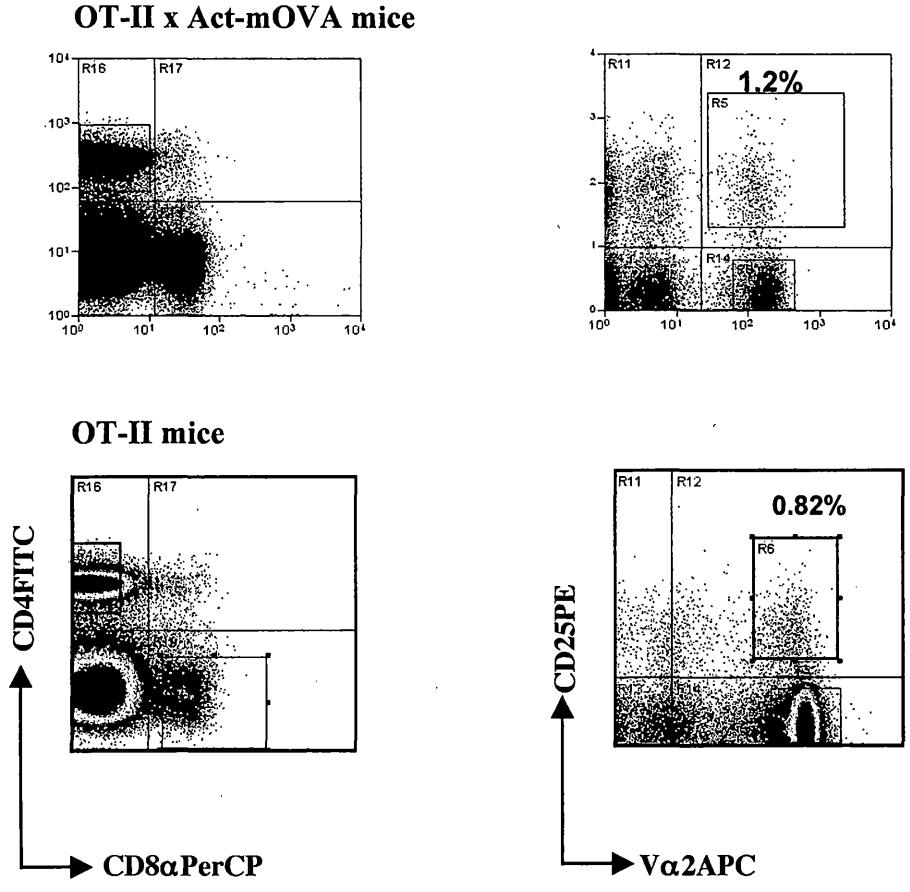


Figure 5: Sort profiles of Tregs from OT-II x Act-mOVA and OT-II mice. Spleens and pooled peripheral lymph nodes were isolated and prepared as described in the Materials and Methods section. Tregs were sorted on the basis of $CD4^+CD25^+V\alpha2^+CD8\alpha^-$. The percentage in the dot plots represents the total percentage of Tregs among $CD4^+$ T cells.

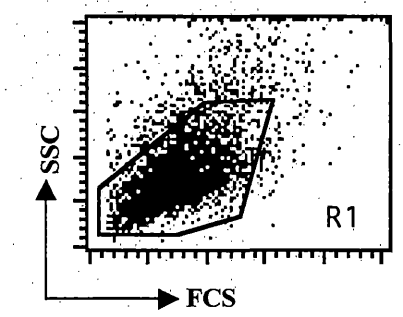
III.A.2. Increasing Treg numbers by *in vitro* expansion

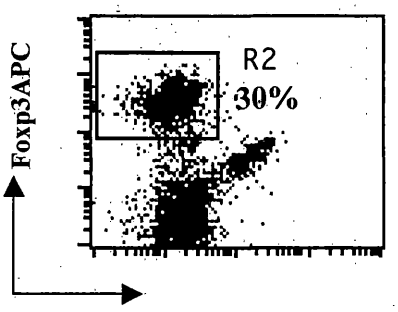
During this time period, Tang et al. described an *in vitro* method for expanding antigen-specific Treg resulting in about a 200-fold increase in Treg numbers in a period of two weeks (103). We therefore employed this *in vitro* expansion protocol to expand Tregs from OT-II mice. In one experiment, $3.64x10^5$ sorted OT-II Tregs ($CD4^+CD25^+V\alpha2^+CD8\alpha^-$, purity of about 94%) were seeded on immobilized anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) plates supplemented with 1000 U/ml of IL-2 and cultured for 7 days. Tregs were split on day 7 of culture and rested in complete medium supplemented with 100 U/ml of IL-2 for 3 more days. At the end of the 10-day culture, we obtained $10x10^6$ cells, about a 27-fold increase in the total cells (data not shown). However, only 30% of the total cells were $Foxp3^+$ Tregs with about 99% $V\alpha2^+$ (Figure 6a). About 50% of the cells in the culture were $V\alpha2^+Foxp3^-$ (Figure 6b). High amounts of exogenous IL-2 with even a small number of contaminating cells that were $V\alpha2^+CD25^-$ at the start resulted in increased expansion of the $V\alpha2^+CD25^-$ T cell population instead of $V\alpha2^+CD25^+$ Tregs. This result also suggests that sorting using CD25 does not produce a sufficiently pure population of Tregs that are $Foxp3^+$, as this marker is also expressed on activated T cells. These expanded Tregs failed to suppress OT-I CD8 T cell proliferation *in vitro* (Figure 7).

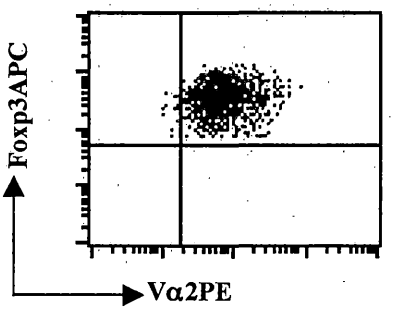
Of note, when $Foxp3^{gfp}$ reporter mice (described below) became available we were able to isolate OT-II Tregs using GFP instead of CD25 and modified the expansion protocol to using 100 U/ml of IL-2, resting the cells in 10 U/ml IL-2 and culturing the cells for a total of 3-4 days. This protocol is described in the Materials and Methods section and only gives a 2-fold increase in total cell numbers. However, the OT-II Tregs

remained viable (about 100%), were GFP^+ and suppressed OT-I CD8 proliferation and IFN-γ production in a dose-dependent manner even in the absence of OT-II peptide (Figure 8a-b and data not shown). Suppression of CD8 T cells by activated Tregs in the absence of cognate peptide is consistent with what others have observed (109). For this reason, the expanded Tregs could not be used to test the role of MHC-II dependent events in Treg mediated suppression *in vitro*.

A



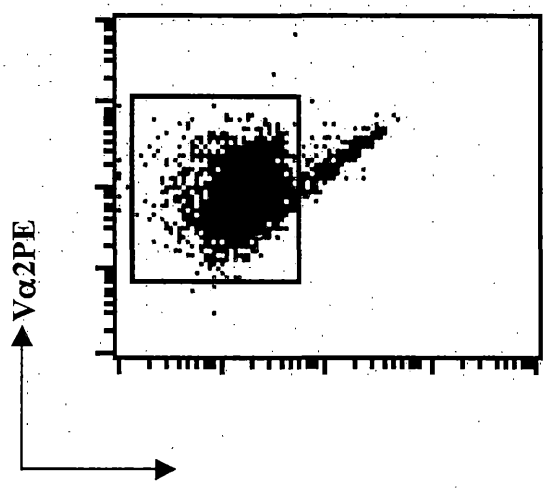


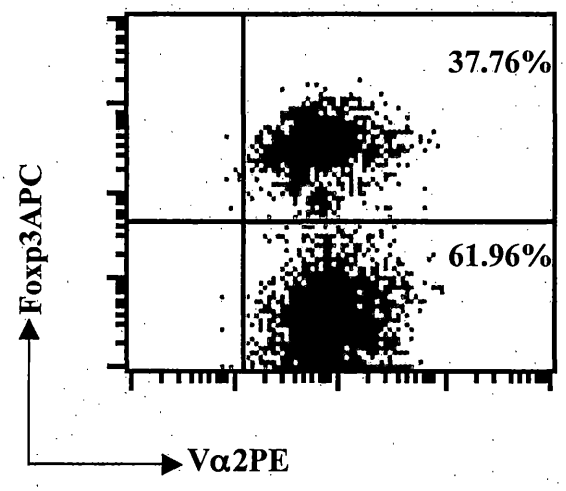


Quad	% Gated	% Total
UL	1.36	0.40
UR	98.64	28.78
LL	0.00	0.00
LR	0.00	0.00

Figure 6a: Intracellular Foxp3 staining of expanded Tregs from OT-II mice. OT-II Tregs were sorted and seeded on anti-CD3 and anti-CD28 immobilized plates in complete medium supplemented with 1000 U/ml IL-2. On day 7 of culture Tregs were rested with 100 U/ml of IL-2 in complete medium for an additional 3 days. Cells were stained with Vα2PE (1:1000) and fixed. The next day, cells were intracellularly stained with Foxp3APC (1:100). We observed a 27-fold increase in cell numbers (data not shown) but only 30% of the total cells were Foxp3^{+}.

B





Quad	% Gated	% Total
UL	0.09	0.07
UR	37.76	30.11
LL	0.19	0.15
LR	61.96	49.40

Figure 6b: An alternative analysis of expanded Tregs from Figure 6a. Second dot plot displays cells gated from R1 and R3. Expanded Tregs contained 50% Vα2$^+$ cells that were Foxp3$^-$.

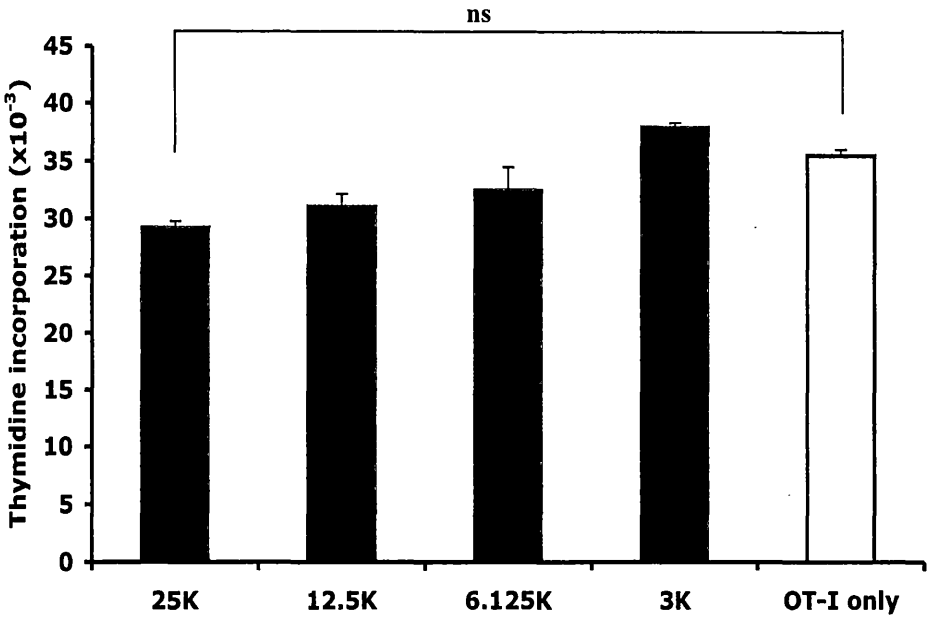


Figure 7: Expanded OT-II Tregs failed to suppress OT-I CD8 T cell proliferation. Indicated numbers of day 10 expanded OT-II Tregs were co-cultured with sorted $5x10^4$ OT-I CD8 T cells ($CD8\alpha^+V\alpha2^+CD4^-CD11c^-$) and $5x10^4$ irradiated T cell depleted APC, 0.03 μg/ml SIINFEKL peptide and 1 μg/ml $OVA_{323-339}$ for 72 hour. 1 μCi/well tritiated thymidine was added the last 8 hour of the incubation and proliferation expressed as the average ± standard deviation of triplicate samples. ns = not statistical significant.

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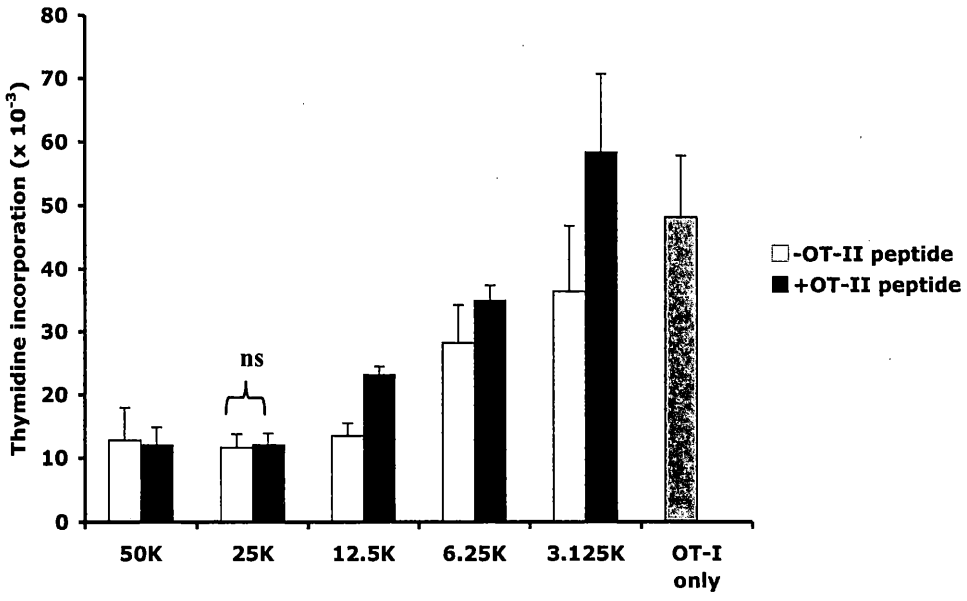


Figure 8a: Activated OT-IIGFP$^+$ Tregs suppress naïve OT-I CD8 T cell proliferation even in the absence of their cognate peptide. Sorted OT-IIGFP$^+$ Tregs (CD4$^+$GFP$^+$CD44^{hi}CD8b$^-$CD11c$^-$) were activated on immobilized anti-CD3/anti-CD28 plates with 100 U/ml of IL-2 for 3 days and rested in complete medium supplemented with 10 U/ml of IL-2 for one additional day. Indicated amounts of activated OT-IIGFP$^+$ Tregs were co-cultured with 2.5x10^4 sorted naïve OT-I CD8 (CD8α$^+$CD122^{lo}CD43^{lo}CD4$^-$ CD11c$^-$), 5x10^4 irradiated T cell depleted APCs, 100 pg/ml of SIINFEKL peptide and 1 μg/ml OVA$_{323-339}$ peptide in total volume of 0.2 ml in triplicates in a 96 well U-bottom plate for 72 hour. 1 μCi/well tritiated thymidine was added the last 8 hours of the incubation and proliferation expressed as an average ± standard deviation of triplicate samples. ns = not statistical significant.

B

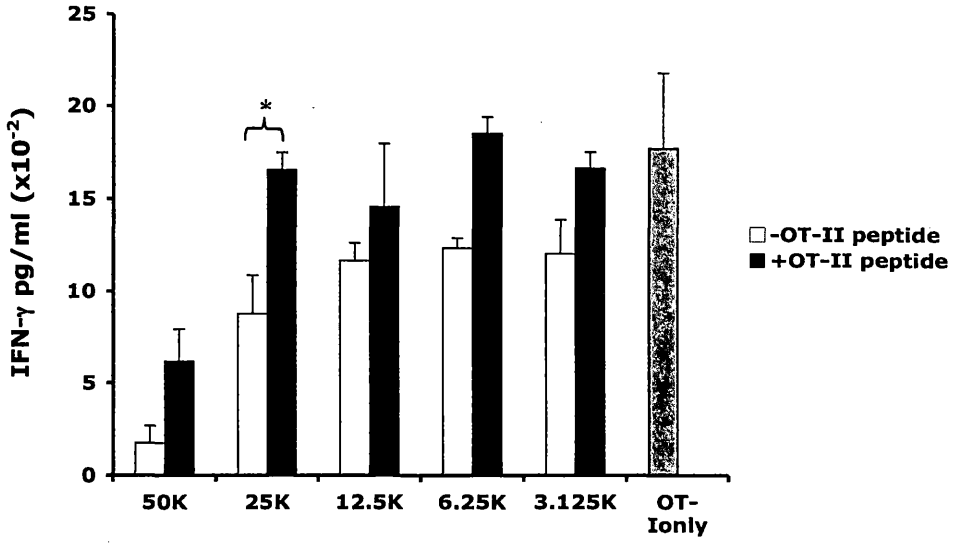


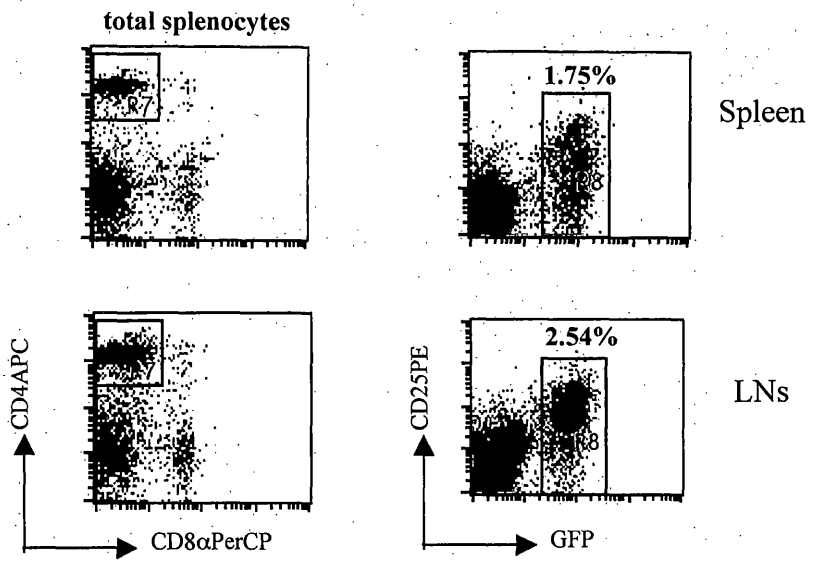
*Figure 8b. Activated OT-IIGFP^{+} Tregs suppress naïve OT-I CD8 T cell IFN-γ production even in the absence of their cognate peptide. Endpoint ELISA was performed per the manufacturer's instructions using the BD OptEIA^{TM} kit (BD Bioscience) with 50 μl of supernatant obtained in Figure 8a and the concentration expressed as an average ± standard deviation of triplicate samples. *$p<0.05$*

III.A.3. Generation of OT-IIFoxp3^{gfp} mice

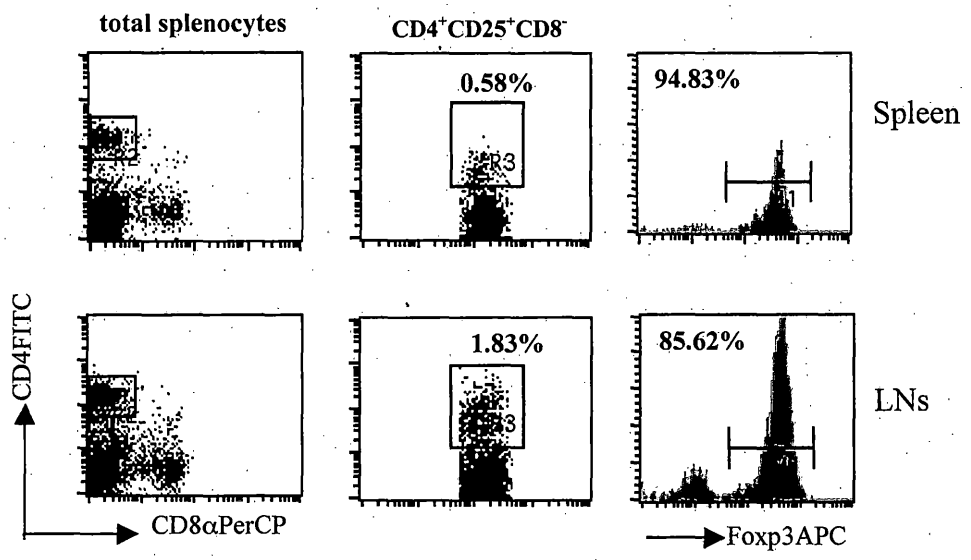
As mentioned, Tregs constitutively express CD25, which is also expressed by activated T cells. CD25 in our experiments was a poor surface marker for isolating OT-II Tregs by cell sorting due to low purity and low yields in numbers of sorted cells. The sacrifice of large numbers of OT-II mice was also required to obtain sufficient cells for experimentation. A more reliable and specific marker for identifying Tregs arose from with the determination that Foxp3 is the transcription factor for Treg development; this discovery paved the way for the development of reporter mice that enable the identification and isolation of Tregs with relatively high purity (18). Fontenot et al. described the generation of Foxp3^{gfp} mice harboring a Foxp3-GFP fusion protein reporter knock-in allele, thus marking Foxp3 expressing cells with GFP (18). Foxp3^{gfp} mice were therefore crossed with OT-II mice to generate OT-IIFoxp3^{gfp} mice that allowed the isolation and analysis of Tregs using GFP instead of CD25 expression. The frequency of Tregs in the spleen and lymph nodes of OT-IIFoxp3^{gfp} mice was analyzed by FACS and compared to conventional OT-II mice. OT-IIFoxp3^{gfp} mice showed an increased percentage of Tregs ($V\alpha2^{+}GFP^{+}CD25^{all}$) in both the spleen and lymph nodes compared to OT-II mice Tregs ($CD4^{+}V\alpha2^{+}CD25^{+}$) (Figure 9).

Figure 9: Increase in the percent of Tregs from OT-IIFoxp3^{gfp} mice compared to OT-II mice. Spleen and lymph nodes from 8 week-old OT-IIFoxp3^{gfp} and OT-II mice were isolated and prepared as described in Materials and Methods. OT-IIFoxp3^{gfp} cells were stained with CD4APC (1:500), CD25PE (1:250) and CD8αPercP (1:250). OT-II cells were stained with CD4FITC (1:500), CD25PE (1:250) and CD8αPercP (1:250). Cells were fixed overnight and the next day intracellularly stained with Foxp3APC (1:100). Cells were analyzed on a FACS Calibur. Shown on the gates and histograms are the percentage of OT-II Tregs among total CD4 T cells.

A. OT-IIFoxp3^{gfp} mice:



B. OT-II mice:



The use of GFP to identify Tregs provided a better strategy for sorting of OT-II Tregs. Spleens and peripheral lymph nodes from OT-IIFoxp3^{gfp} mice were isolated and prepared as described in the Materials and Methods section. Cells were stained with Vα2Biotin (1:1000)/StreptavidinAPC (1:500), CD25PE (1:250) and CD8αPerCP (1:250) and Tregs sorted on the basis of Vα2^{+}CD25^{all}GFP^{+}CD8α^{-} (both CD25^{+} and CD25^{-} cells were used as both of these populations were GFP^{+}). The purity of Tregs was greater than 95%. The sorting protocol for obtaining Tregs from OT-IIFoxp3^{gfp} mice was later modified to exclude CD8b and CD11c and allow simultaneous isolation of CD44^{lo} naïve CD4 T cells by staining cells with Vα2Biotin (1:2000)/StreptavidinAPC (1:1000) or CD4APC (1:1000), CD8bPE (1:1000), CD11cPE (1:500) and CD44cychrome (1:1000). Tregs were defined as Vα2^{+}GFP^{+}CD44^{hi}CD8b^{-}CD11c^{-} or CD4^{+}GFP^{+}CD44^{hi}CD8b^{-} CD11c^{-} and sorted on a FACS Aria. The sort profiles are shown in Figure 10 with a Treg purity of >95%. The number of Tregs obtained from these experiments was greatly increased. For example, in one experiment 2.8x10^{6} OT-IIGFP^{+} Tregs were obtained from cells sorted from eight OT-IIFoxp3^{gfp} mice compared to about 5x10^{5} Tregs from eight conventional OT-II mice (data not shown). Thus, by crossing OT-II mice with Foxp3^{gfp} mice, Tregs could be isolated using GFP, resulting in increased Treg numbers for use in experiments. Therefore, OT-IIFoxp3^{gfp} mice were the source of antigen-specific Tregs in all further experiments.

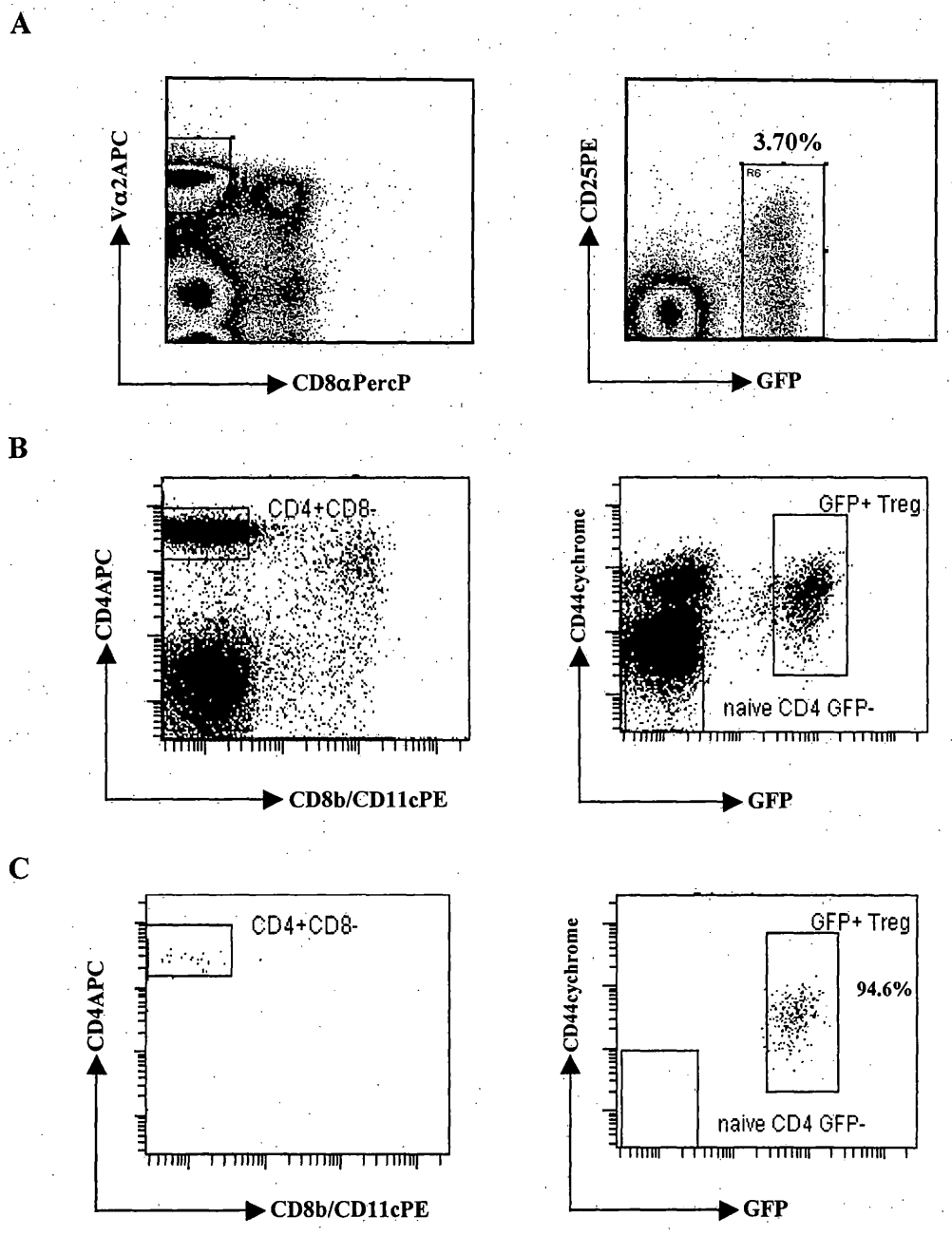
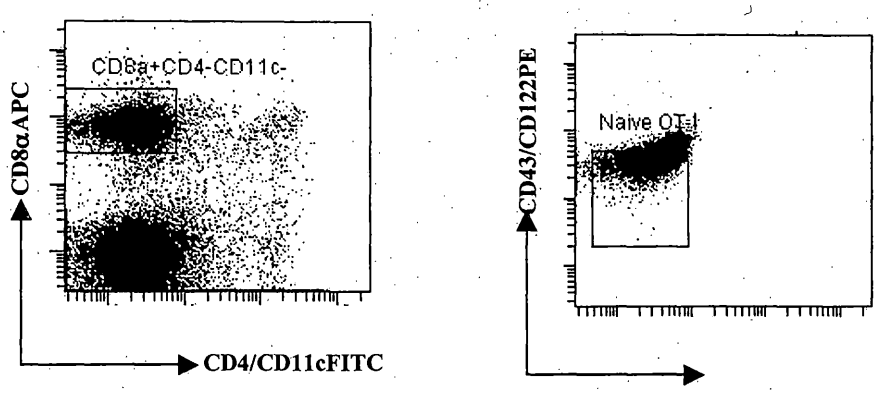


Figure 10: Profile of sorted Tregs from OT-IIFoxp3^{gfp} mice. Tregs were sorted on the basis of being (A) Vα2^{+}CD25^{all}GFP^{+}CD8α^{-} and then modified to (B) CD4^{+}GFP^{+}CD44^{hi}CD8b^{-}CD11c^{-} and in some experiments as Vα2^{+}GFP^{+}CD44^{hi}CD8b^{-} CD11c^{-}. (C) The purity of Tregs in this experiment was 94.6%.

III.A.4. FACS sorting of OT-I CD8 T cells

Antigen-specific CD8 T cells were derived from OT-I TCR transgenic mice and sorted as follows. Spleen and pooled peripheral lymph nodes from OT-I TCR transgenic mice were isolated and prepared as described in the Materials and Methods section. Cells were stained with Vα2Biotin (1:1000)/StreptavidinPE (1:500), CD4FITC (1:500), CD11cFITC (1:250) and CD8αAPC (1:500) and sorted on the basis of being $CD8\alpha^{+}V\alpha2^{+}CD4^{-}CD11c^{-}$ (data not shown). The sorting scheme was later modified to exclude activated and memory CD8 T cells. To do so, cells were therefore stained with CD122/CD43-Biotin (1:2000)/StreptavidinPE (1:1000), CD4FITC (1:1000), CD11cFITC (1:500) and CD8αAPC (1:1000). Naïve OT-I CD8 T cells were defined as $CD8\alpha^{+}CD4^{-}CD122^{lo}CD43^{lo}CD11c^{-}$ (Figure 11). The purity of naïve CD8 T cells was greater than 92%. All studies shown henceforth were performed using this latter method.

A



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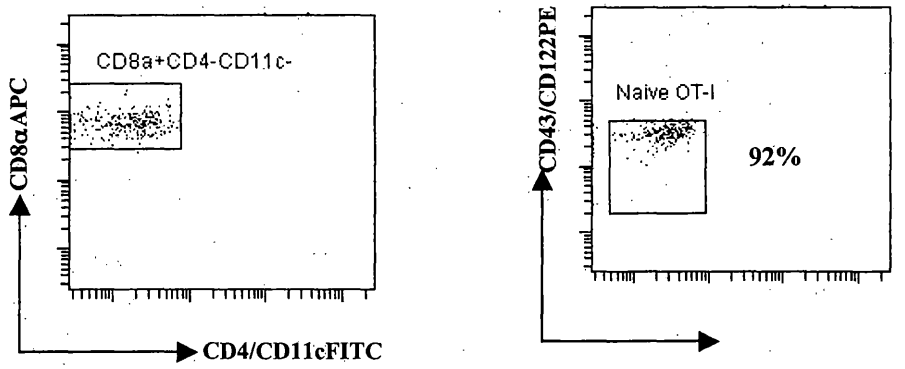

Figure 11: Profile of sorted naïve OT-I CD8 T cells. OT-I CD8 T cells were defined as $CD8\alpha^{+}V\alpha2^{+}CD4^{-}CD11c^{-}$ and later modified to $CD8\alpha^{+}CD4^{-}CD122^{-}CD43^{-}CD11c^{-}$ as shown in (A) and the purity of cells was approximately 92% (B).

III.A.5. $CD4^+Foxp3^+$ Tregs from OT-II$Foxp3^{gfp}$ mice respond to their cognate antigen

To study the origin and antigen specificity of Tregs, Tregs from TCR transgenic mice has been extensively used, but little is still known about their origin (111, 114). DO11.10 mice for example harbor a small number of $CD25^+$ Tregs that are known to require endogenous TCRα for their development as crossing these mice to a $RAG^{-/-}$ background abrogated their development (33, 115). Antigen-specific Tregs arising in DO11.10 mice exhibit suppressive activity upon stimulation with their cognate peptide suggesting that the Tregs may be expressing dual TCRs (33, 115). One of these TCRs is composed of the transgenic α/β chains and the other one composed of an endogenous α-chain and transgenic β-chain which responds to cognate peptide (115). We expect that these findings from DO11.10 mice will also hold true in OT-II transgenic mice.

OT-II$Foxp3^{gfp}$ Tregs were therefore tested for their response to their cognate peptide. Sorted $2.5x10^4$ Tregs ($CD4^+GFP^+CD44^{hi}CD11c^-CD8b^-$) from OT-II$Foxp3^{gfp}$ mice were cultured with $5x10^4$ irradiated T cell depleted B6 APCs in the presence of 1 μg/ml of OT-II peptide ($OVA_{323-339}$) or HEL peptide as a negative control, and with or without 10 ng/ml (100 U/ml) IL-2, in triplicate. As controls, $2.5x10^4$ naïve CD4 T cells ($CD4^+GFP^-CD44^{lo}CD11c^-CD8b^-$) were also cultured with 1 μg/ml $OVA_{323-339}$ or HEL peptide in the presence of $5x10^4$ irradiated T cell depleted B6 APCs (data not shown). Proliferation was determined by thymidine incorporation into DNA. Proliferation of OT-IIGFP^+ Tregs was only observed in co-cultures with $OVA_{323-339}$ peptide and IL-2 but not with peptides alone (either $OVA_{323-339}$ or HEL), HEL and IL-2 or IL-2 alone (Figure 12). Robust proliferation was observed in the control cultures of naïve CD4 T cells with

$OVA_{323-339}$ peptide but not with HEL peptide (data not shown). This is consistent with the fact that Tregs display anergic characteristics when stimulated *in vitro*, but TCR stimulation combined with exogenous IL-2 breaks this unresponsiveness and cause proliferation (19).

Mature BMDCs have been shown to break Treg anergy in the absence of exogenous IL-2 (32). The response of OT-II Tregs to $OVA_{323-339}$ peptide and ovalbumin protein was therefore tested using LPS-matured BMDCs in the absence of exogenous IL-2. $1.25x10^4$ Tregs ($V\alpha2^+GFP^+CD44^{hi}CD11c^-CD8b^-$) were co-cultured with $5x10^4$ LPS-matured BMDCs in the presence of 1 μg/ml $OVA_{323-339}$ or 1 mg/ml ovalbumin protein in triplicate. $1.25x10^4$ naïve CD4 T cells were used as controls and cultured the same way as the OT-IIGFP$^+$ Tregs (data not shown). Proliferation was determined by thymidine incorporation. Mature BMDCs caused robust proliferation of OT-IIGFP$^+$ Tregs in the presence of both $OVA_{323-339}$ peptide and ovalbumin protein (Figure 13).

A

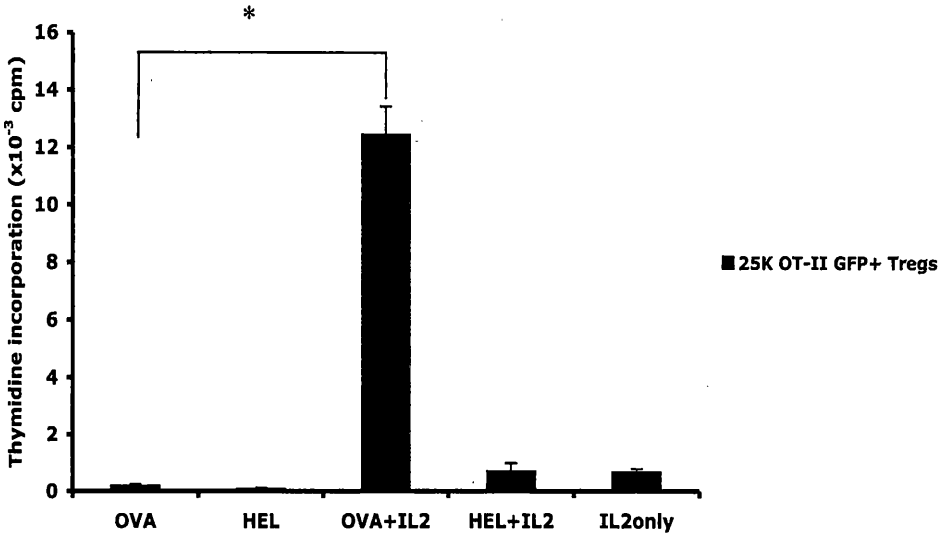

*Figure 12: $CD4^+Foxp3^+$ Tregs from OT-II$Foxp3^{gfp}$ mice respond to their cognate antigen. $2.5x10^4$ OT-IIGFP^+ Tregs defined as $V\alpha2^+GFP^+CD44^{hi}CD8b^-CD11c^-$ were stimulated in triplicates in the presence of $5x10^4$ irradiated T cell depleted APCs, 1 μg/ml of $OVA_{323-339}$ or HEL peptides with or without 10 ng/ml (100 U/mL) murine IL-2 for 72 hours at 37°C with the last 8 hours of the incubation with 1 μCi/well tritiated thymidine. Proliferation data expressed as an average ± standard deviation of triplicate samples. *$p<0.05$*

B

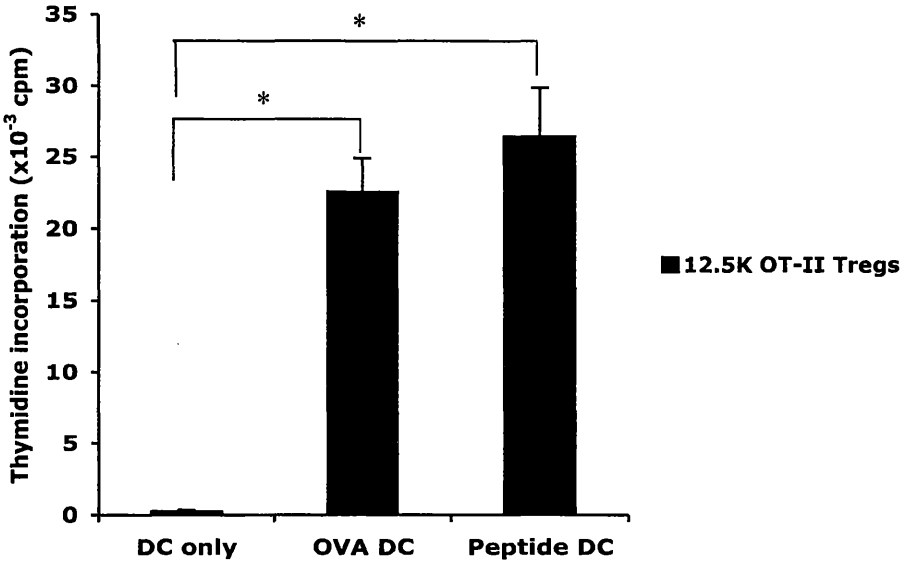

*Figure 13: BMDCs reverse CD4$^+$Foxp3$^+$ Treg from OT-IIFoxp3^{gfp} mice anergy in vitro. 1.25x10^4 OT-IIGFP$^+$ Tregs were cultured with 5x10^4 LPS matured BMDCs pulsed with ovalbumin protein or OVA$_{323-339}$ peptide and unpulsed BMDCs as controls. Proliferation was determined by thymidine incorporation after 72 hours of incubation and expressed as average ± standard deviation of triplicate samples. *$p<0.05$*

III.A.6. Determination of an optimal concentration of SIINFEKL peptide in the suppression of naïve CD8 T cells mediated by OT-IIGFP$^+$ Tregs

CD8 T cells from OT-I TCR transgenic mice respond vigorously to their cognate peptide SIINFEKL (116). The optimal concentration of SIINFEKL for this *in vitro* system was therefore determined. $2.5x10^4$ sorted naïve OT-I CD8 T cells ($CD8\alpha^+CD122^{lo}CD43^{lo}CD4^-CD11c^-$) were cultured with the same number of sorted OT-II Tregs ($V\alpha2^+GFP^+CD44^{hi}CD8b^-CD11c^-$) in the presence of $5x10^4$ irradiated T cell depleted B6 APC, 1 μg/ml $OVA_{323-339}$ peptide and various concentrations of SIINFEKL peptide in a total volume of 0.2 ml in triplicate. Proliferation was determined by thymidine incorporation. Naïve OT-I CD8 T cells showed robust proliferation to SIINFEKL peptide at concentrations of 100 pg/ml to 100 ng/ml while no proliferation was observed at 1 or 10 pg/ml (Figure 14). OT-II Tregs suppressed naïve OT-I CD8 T cells proliferation in response to SIINFEKL peptide showing greater than 80 percent suppression at all concentrations tested with the exception of 1 and 10 pg/ml (Figure 14). Therefore, 100 pg/ml or 1 ng/ml of SIINFEKL peptide was used in all subsequent experiments.

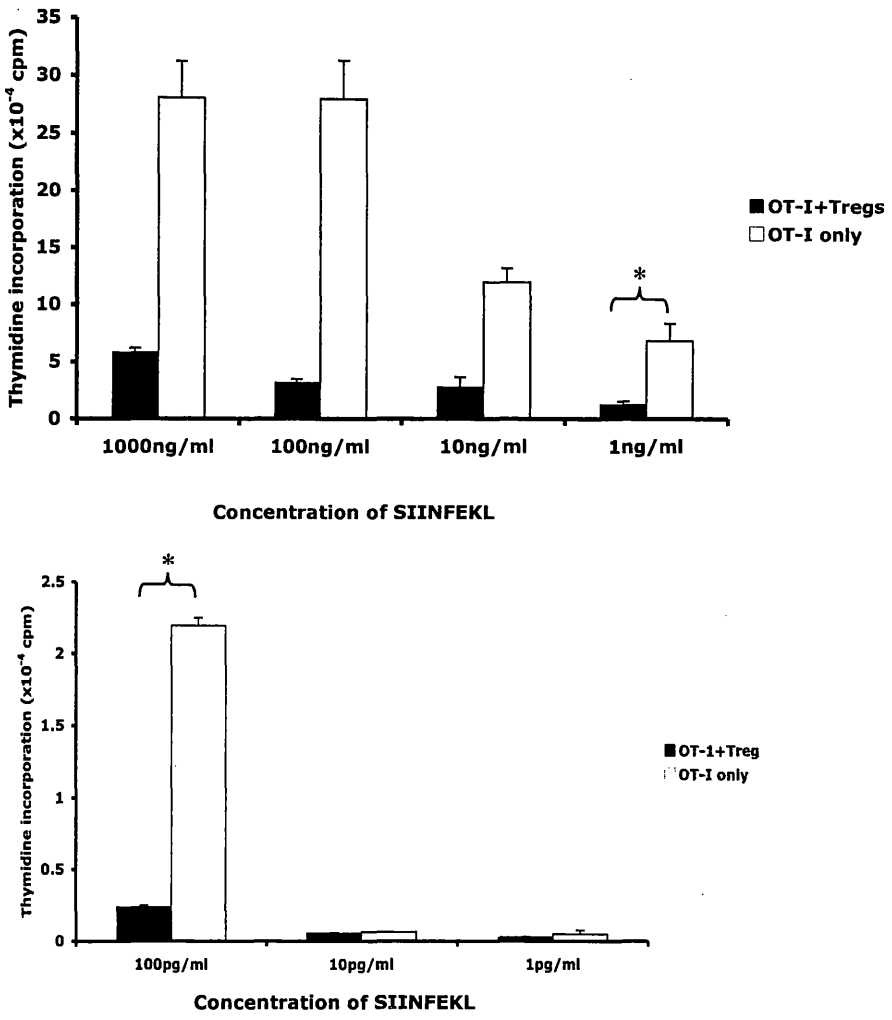

*Figure 14: Determination of an optimal concentration of SIINFEKL peptide in OT-IIGFP$^+$ Tregs mediated suppression of naïve OT-I CD8 T cells proliferation. 2.5x10^4 sorted naïve OT-I CD8 T cells (CD8α^+CD122^{lo}CD43^{lo}CD4$^-$CD11c$^-$) were cultured with the same number of sorted OT-IIGFP$^+$ Tregs (Vα2$^+$CD25$^+$GFP$^+$CD8α^-), 5x10^4 irradiated T cell depleted APCs, 1 μg/ml OVA$_{323-339}$ peptide and various concentrations of SIINFEKL peptide (x-axis) in a total volume of 0.2ml in a 72 hours thymidine incorporation assay with the last 8 hours with tritiated thymidine. Proliferation was determined as the average ± standard deviation of triplicate samples. *$p<0.05$*

III.A.7. Determination of an optimal concentration of $OVA_{323-339}$ peptide in the suppression of naïve CD8 T cell proliferation and IFN-γ production mediated by OT-IIGFP$^+$ Tregs

The concentration of $OVA_{323-339}$, the OT-II peptide, was also determined for use in this *in vitro* system. $2.5x10^4$ sorted OT-IIGFP$^+$ Tregs ($V\alpha2^+CD25^+GFP^+CD8\alpha^-$) and $2.5x10^4$ naïve OT-I CD8 T cells ($CD8\alpha^+V\alpha2^+CD122^-CD43^-CD4^-CD11c^-$) were cultured with $5x10^4$ mitomycin C-treated T cell depleted APCs, 100 pg/ml of SIINFEKL peptide and various concentrations of $OVA_{323-339}$ peptide in a final volume of 0.2 ml in triplicates. Cells were incubated at 37°C for 72 hours. Before adding 1 μCi/well of tritiated thymidine for the final 8 hours of incubation, 50 μl of the supernatant was removed to perform IFN-γ ELISA. At 10 μg/ml and 1 μg/ml concentrations of $OVA_{323-339}$ peptide, OT-IIGFP$^+$ Tregs showed 77 and 73 percent suppression of naïve OT-I CD8 T cell proliferation, respectively (Figure 15a). OT-IIGFP$^+$ Tregs at these concentrations also suppressed the production of IFN-γ by naïve OT-I CD8 T cells (Figure 15b). Therefore, 1 μg/ml $OVA_{323-339}$ peptide was used in subsequent experiments.

A

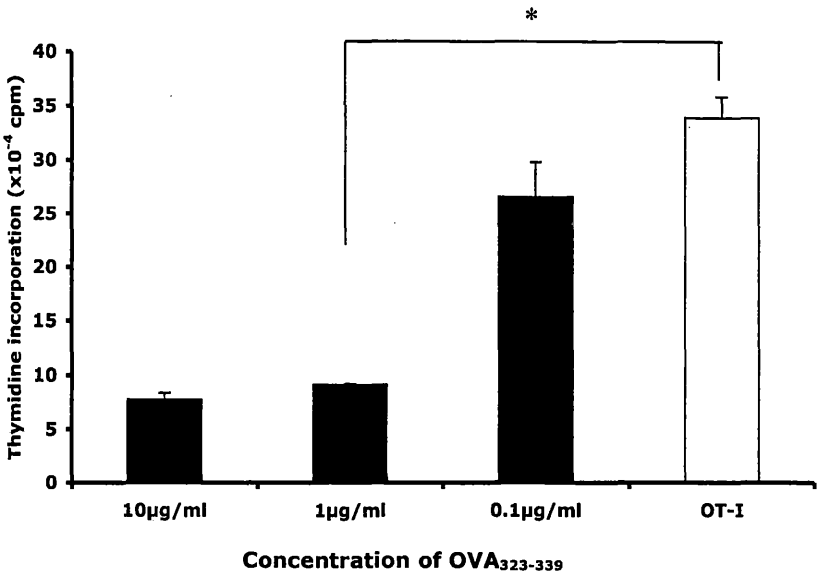

Figure 15a: Determination of an optimal concentration of $OVA_{323-339}$ peptide in the suppression of naïve OT-I CD8 T cell proliferation mediated by OT-IIGFP$^+$ Tregs. 2.5x10^4 sorted OT-IIGFP$^+$ Tregs (Vα2$^+$CD25$^+$GFP$^+$CD8α^-) and naïve OT-I CD8 T cells (CD8α^+CD122^{lo}CD43^{lo}CD4$^-$CD11c$^-$) were co-cultured with 5x10^4 mitomycin C-treated T cell depleted APCs, 100 pg/ml of SIINFEKL peptide and various concentrations of $OVA_{323-339}$ peptide (x-axis) in a final volume of 0.2 ml. Cells were incubated at 37°C for 72 hours. Before adding 1 µCi/well of tritiated thymidine for the final 8 hours of incubation, 50 µl of the supernatant was removed to perform IFN-γ ELISA. Suppression of proliferation was expressed as an average ± standard deviation of triplicate samples.

** $p<0.05$*

B

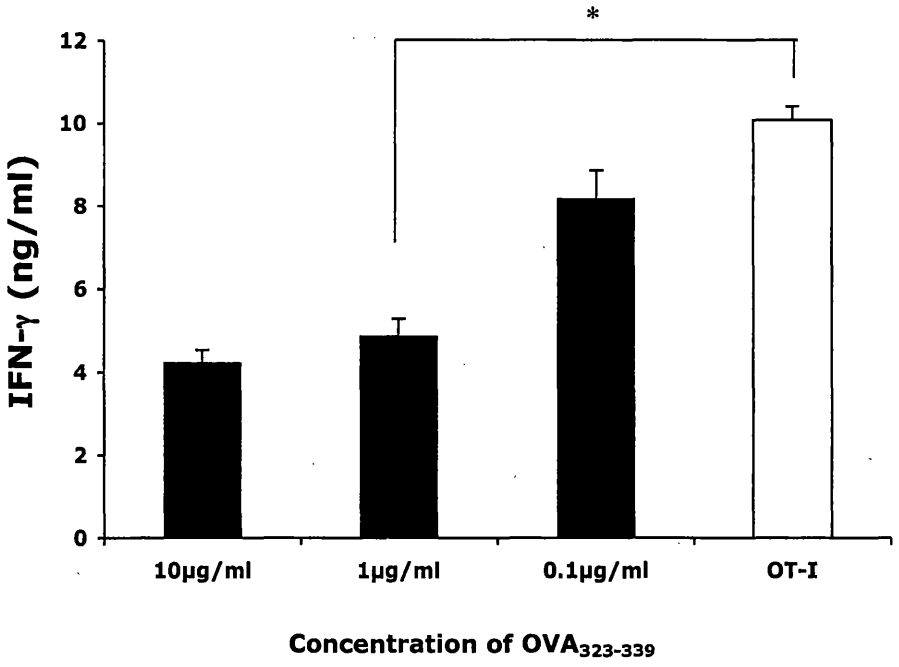

*Figure 15b: Determination of an optimal concentration of $OVA_{323-339}$ peptide in the suppression of IFN-γ production by naïve OT-I CD8 T cells mediated by OT-IIGFP^{+} Tregs. Endpoint ELISA was performed per the manufacturer's instructions using the BD OptEIA^{TM} kit (BD Bioscience) (see Materials and Methods) with the supernatant obtained in Figure 15a. OT-IIGFP^{+} Tregs suppressed IFN- γ production from naïve OT-I CD8 T cells; the concentrations are expressed as average ± standard deviation of triplicate samples. * $p<0.05$*

III.A.8. OT-IIGFP$^+$ Treg suppress naïve CD8 T cell proliferation and IFN-γ production in a dose dependent manner

Suppression of OT-I CD8 T cell proliferation and IFN-γ production was tested using the optimal concentrations of $OVA_{323-339}$ and SIINFEKL peptides with varying amounts of OT-IIGFP$^+$ Tregs. $2.5x10^4$ sorted naïve OT-I CD8 T cells ($CD8\alpha^+CD122^{lo}CD43^{lo}CD4^-CD11c^-$) were cultured with various numbers of OT-IIGFP$^+$ Tregs ($V\alpha2^+GFP^+CD44^{hi}CD8b^-CD11c^-$), $5x10^4$ irradiated T cell depleted APCs, 100 pg/ml of SIINFEKL peptide and 1 μg/ml $OVA_{323-339}$ peptide in a total volume of 0.2 ml in triplicate for 72 hours. Before adding 1 μCi/well tritiated thymidine for the last 8 hours of the incubation, 50 μl of the supernatant was removed to perform IFN-γ ELISA. Naïve OT-I CD8 T cells in the absence of OT-IIGFP$^+$ Tregs showed robust proliferation, which was potently suppressed by the addition of OT-IIGFP$^+$ Tregs in a dose dependent manner (Figure 16a). Addition of twice as many OT-IIGFP$^+$ Tregs revealed a greater suppression of naïve OT-I CD8 T cell proliferation by the OT-IIGFP$^+$ Tregs, with about 95 percent suppression observed (Figure 16a). OT-IIGFP$^+$ Tregs exhibited approximately 50% suppression of naïve OT-I CD8 T cell IFN-γ production at the various OT-IIGFP$^+$ Tregs ratios used (Figure 16b). Therefore, OT-IIGFP$^+$ Tregs potently suppress naïve CD8 T cell proliferation and effector molecule production in a dose dependent manner.

A

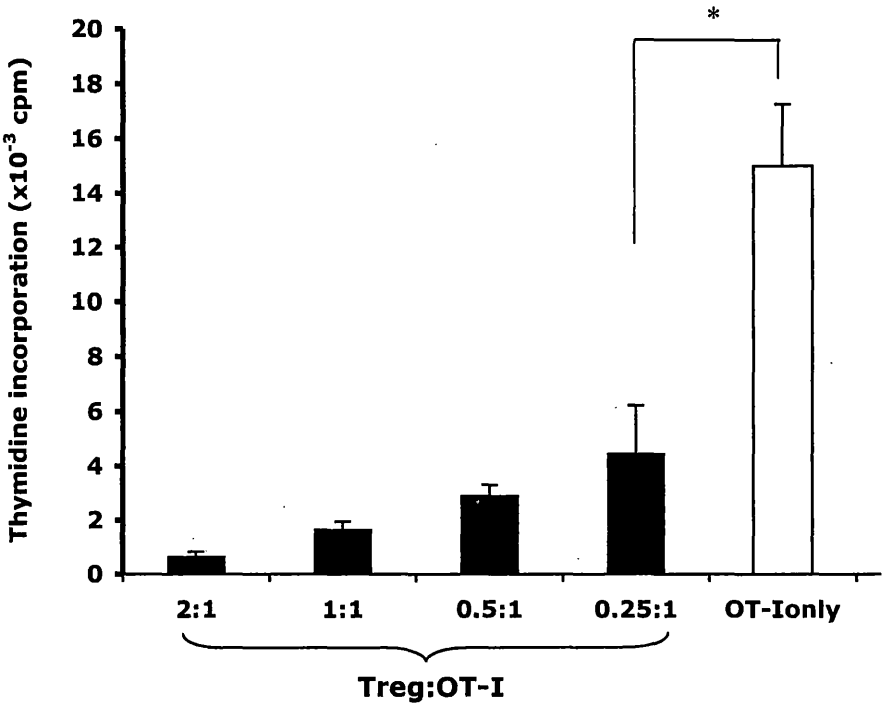

*Figure 16a: OT-IIGFP$^+$ Tregs suppress naïve OT-I CD8 T cell proliferation in a dose dependent manner. 2.5x10^4 sorted naïve OT-I CD8 T cells (CD8α$^+$CD122^{lo}CD43^{lo}CD4$^-$ CD11c$^-$) were cultured with various numbers of OT-IIGFP$^+$ Tregs (Vα2$^+$GFP$^+$CD44^{hi}CD8b$^-$CD11c$^-$), 5x10^4 irradiated T cell depleted APCs, 100 pg/ml of SIINFEKL peptide and 1 μg/ml OVA$_{323-339}$ peptide in a total volume of 0.25ml in triplicate in a 96-well U-bottom plate for 72 hours. 1 μCi/well tritiated thymidine was added for the last 8 hours of the incubation after removing 50 μl of the supernatant to perform IFN-γ ELISA. Proliferation data was expressed as an average ± standard deviation of triplicate samples. *p<0.05*

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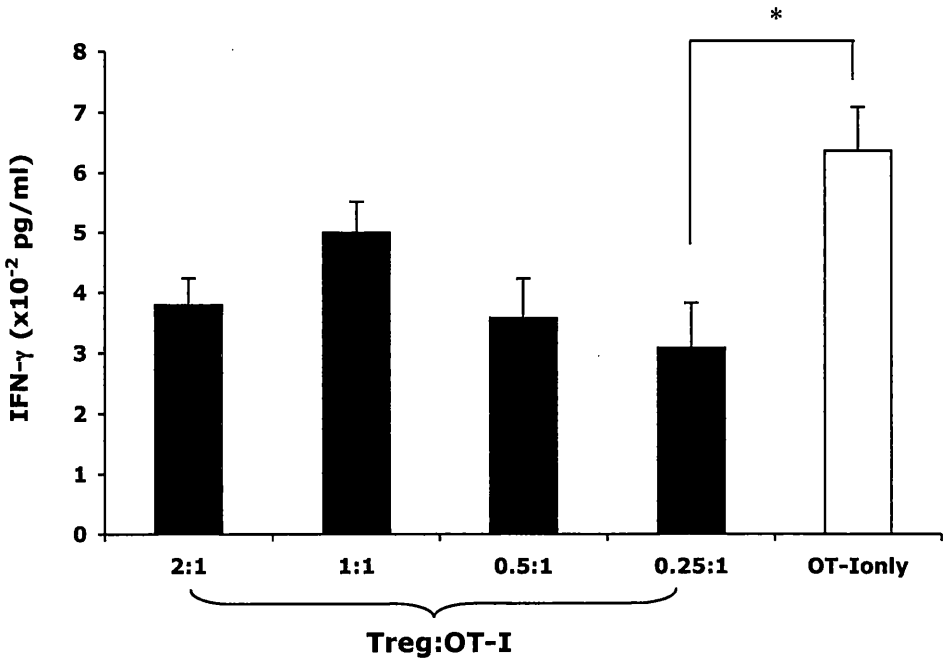

*Figure 16b: OT-IIGFP^{+} Tregs suppress naïve OT-I CD8 T cell IFN-γ production in a dose dependent manner. Endpoint ELISA was performed per the manufacturer's instructions using the BD OptEIA^{TM} kit (BD Bioscience) (see Materials and Methods) with the supernatants obtained in Figure 16a and the concentrations expressed as an average ± standard deviation of triplicate samples. *$p<0.05$*

III.A.9. OT-IIGFP$^+$ Tregs suppress naïve CD8 T cell proliferation and IFN-γ production in the presence of ovalbumin protein

After observing a OT-IIGFP$^+$ Tregs response to LPS-matured BMDCs in the presence of $OVA_{323-339}$ peptide and ovalbumin protein (Figure 13), the ability of OT-IIGFP$^+$ Tregs to suppress naïve OT-I CD8 T cell proliferation and IFN-γ production was tested using ovalbumin protein. $2.5x10^4$ sorted OT-IIGFP$^+$ Tregs ($V\alpha2^+GFP^+CD44^{hi}CD8b^-CD11c^-$) and naïve OT-I CD8 T cells ($CD8\alpha^+CD122^{lo}CD43^{lo}CD4^-CD11c^-$) were cultured in the presence of various amounts of ovalbumin protein with $5x10^4$ irradiated T cell depleted APCs in a total volume of 0.2 ml in triplicate in a 72-hour thymidine incorporation assay. Before addition of 1 μCi/well tritiated thymidine for the last 8 hours of incubation, 50 μl of supernatant was removed to perform IFN-γ ELISA. OT-IIGFP$^+$ Tregs potently suppressed naïve OT-I CD8 T cell proliferation at all ovalbumin concentrations tested (Figure 17a), with 80 percent suppression of naïve OT-I CD8 T cell IFN-γ production at 1 mg/ml ovalbumin protein (Figure 17b). Suppression of IFN-γ production was also seen at 0.3 mg/ml but not at 0.1 mg/ml concentration of ovalbumin protein (Figure 17b). Therefore, OT-IIGFP$^+$ Tregs suppress the proliferation and effector molecule production of naïve CD8 T cells in the presence of ovalbumin protein.

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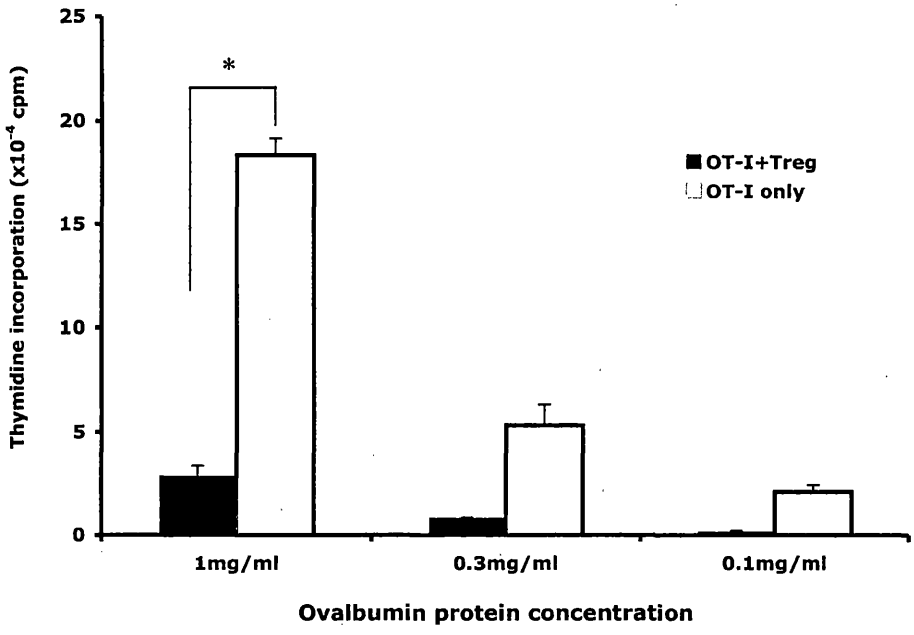

*Figure 17a: OT-IIGFP$^+$ Tregs suppress naïve OT-I CD8 T cell proliferation in the presence of ovalbumin protein. 2.5x10^4 sorted OT-IIGFP$^+$ Tregs (Vα2$^+$CD25$^+$GFP$^+$CD8α$^-$) and naïve OT-I CD8 T cells (CD8α$^+$CD122^{lo}CD43^{lo}CD4$^-$ CD11c$^-$) were cultured in the presence of various amounts of ovalbumin protein (x-axis) with 5x10^4 irradiated T cell depleted APCs in a total volume of 0.2 ml in a 72 hours thymidine incorporation assay. 50 μl of supernatant was removed from the culture before adding 1 μCi/well for the final 8 hours incubation prior to harvesting of the cells. Proliferation was determined as the average ± standard deviation of triplicate samples. *p<0.05*

B

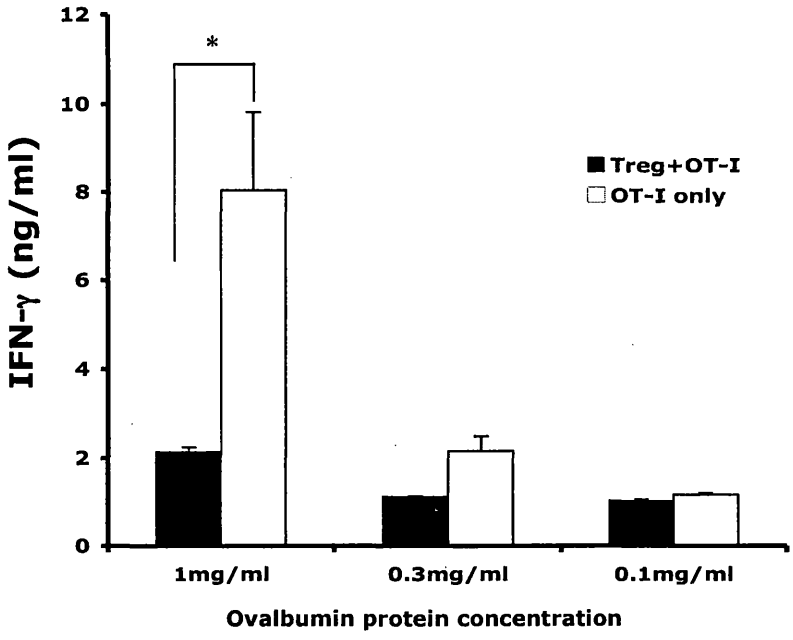

*Figure 17b: OT-IIGFP^{+} Tregs suppress naïve OT-I CD8 T cell IFN-γ production in the presence of ovalbumin protein. Endpoint ELISA was performed per the manufacturer's instructions using the BD OptEIA^{TM} kit (BD Bioscience) (see Materials and Methods) with the supernatants obtained in Figure 17a. OT-IIGFP^{+} Tregs suppressed IFN- γ production by naïve OT-I CD8 T cells with the concentrations expressed as average ± standard deviation of triplicate samples. *$p<0.05$*

III.A.10. OT-IIGFP$^+$ Tregs suppress naïve CD8 T cell proliferation and IFN-γ production in a MHC-II dependent manner

In vitro, Tregs require TCR stimulation to exert their suppressive function but have been shown to suppress in an antigen non-specific manner (22). This suppression *in vitro* has been suggested to be contact dependent and APC independent (22). However, the mechanism of this suppression is still not clearly understood, especially the involvement of MHC-II. We sought to elucidate the role of MHC-II dependent events in Treg mediated suppression. $ia\beta^{neo/\Delta}$Tie2Cre$^+$ conditional MHC-II knockout mice were generated and characterized in the lab (85). These mice lack the expression of MHC-II on all peripheral APCs (Figure 18 and reference 85). These mice and their littermates were used as a source of APCs in this assay. $2.5x10^4$ sorted naïve OT-I CD8 T cells (CD8α$^+$CD122^{lo}CD43^{lo}CD4$^-$CD11c$^-$) were cultured with various quantities of OT-IIGFP$^+$ Tregs (CD4$^+$GFP$^+$CD44^{hi}CD8b$^-$CD11c$^-$), $5x10^4$ irradiated T cell depleted MHC-II$^{-/-}$ APCs from $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice or MHC-II$^{+/+}$ APCs from wild type mice, 1 ng/ml of SIINFEKL peptide and 1 μg/ml $OVA_{323-339}$ peptide in a total volume of 0.2 ml in triplicate in a 96-well U-bottom plate for 72 hours. 1 μCi/well tritiated thymidine was added for the last 8 hours of the incubation. A duplicate plate was set up and supernatants taken 72 hours later for IFN-γ ELISA. OT-IIGFP$^+$ Tregs suppressed naïve OT-I CD8 T cell proliferation in a dose dependent manner in the presence of MHC-II replete APCs, suggesting faithful presentation of OT-II and OT-I peptides to their respective T cells (Figure 19a). In the presence of MHC-II deficient APCs from $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice, suppression of naïve CD8 T cells was not observed but instead cells showed robust proliferation at all Treg:CD8 T cell ratios used (Figure 19a). OT-

IIGFP$^+$ Tregs also suppressed IFN-γ production in the presence of MHC-II sufficient APCs even at 1:8 Treg: OT-I ratio. However, even in the presence of MHC-II deficient APCs some suppression was observed at high Treg ratio and was statistically significant at 1:1 Treg: OT-I ratio (Figure 19b). This result suggests that MHC-II independent mechanisms could also be involved at the high Treg ratio of 1:1 used in suppressing naïve CD8 T cell IFN-γ production. Another possible explanation could be that the OT-IIGFP$^+$ Tregs were consuming the IFN-γ produced by naïve CD8 T cells as the number of Treg increased, thus a decrease of IFN-γ was observed in the culture supernatant at the 1:1 ratio used. Another alternative explanation is that there was a small fraction of Treg that were actually recently activated *in vivo* before sorting from the mice, and this only became evident when the number of Tregs in each well was increased to the 1:1 Treg: OT-I ratio. These 'pre-activated' Tregs might be anticipated to suppress even without MHC-II dependent events. These different possibilities need to be further investigated. Nonetheless, MHC-II dependent events mediated by OT-IIGFP$^+$ Tregs are involved in the suppression of naïve CD8 T cell proliferation and IFN-γ production at low Treg ratio at least.

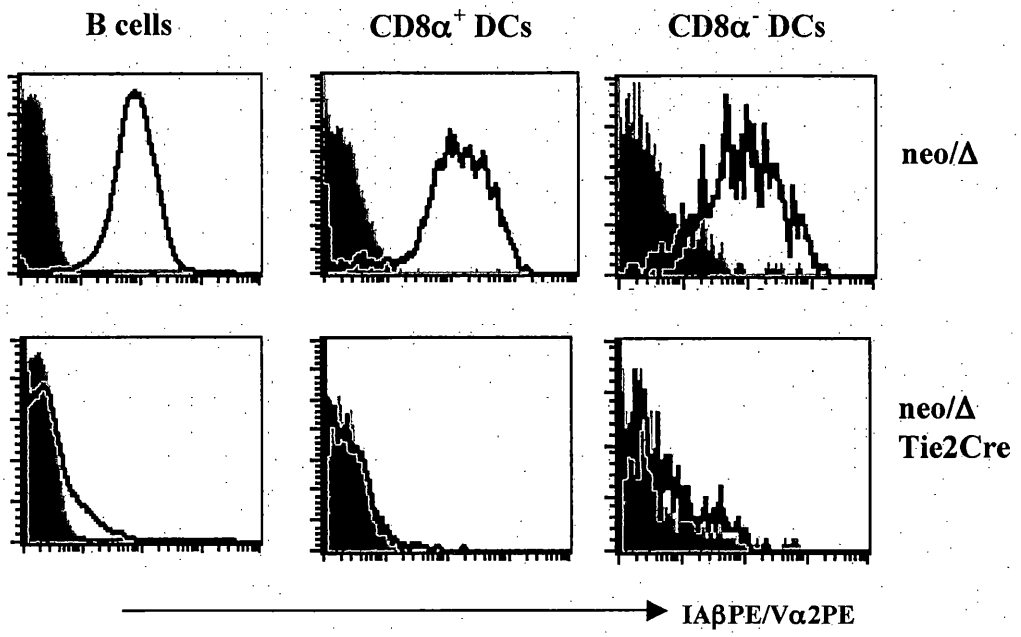

Figure 18: Loss of MHC-II on APCs from ia$\beta^{neo/\Delta}$ TIE2Cre^{+} mice. Spleens from 8 week-old ia$\beta^{neo/\Delta}$ TIE2Cre^{+} mice and their wild type littermates were isolated and prepared as described in Materials and Methods section. Cells where stained for CD11cFITC (1:250), IAβPE (1:1000), CD8αPercP (1:250), B220APC (1:500) and Vα2PE as an isotype control. Cells were analyzed on a FACS calibur. Histogram shows MHC-II expression (open plots) with isotype control (green plots) on gated B cells (B220^{+}CD11^{-}, first row) and CD11c^{+} DCs (CD11c^{+}B220^{-}CD8α^{+}, middle row or CD11c^{+}B220^{-}CD8α^{-}, last row).

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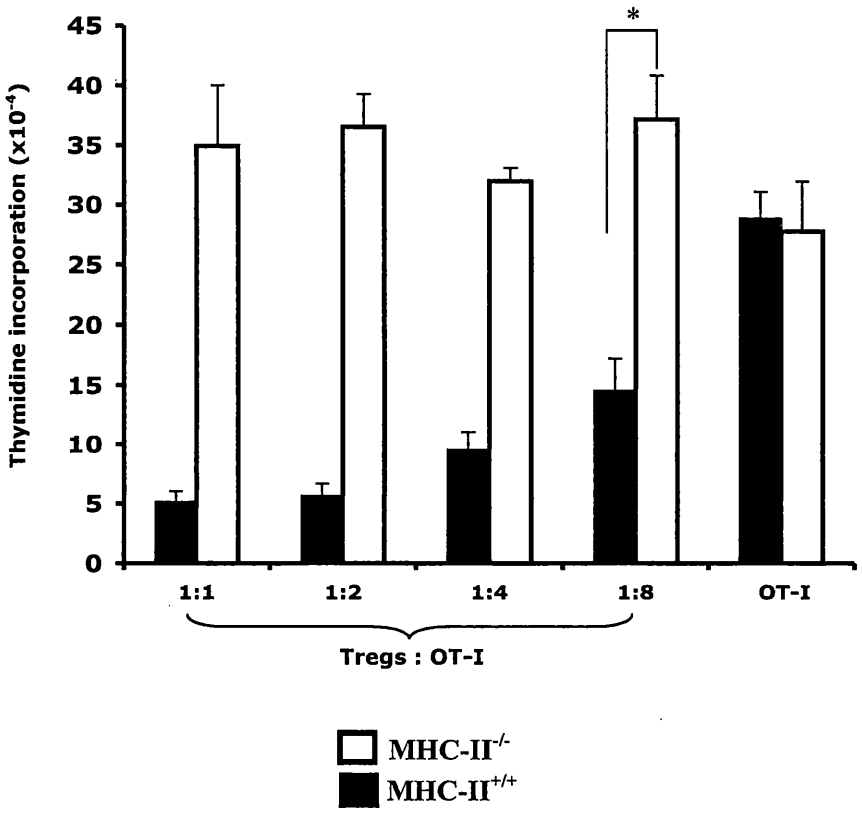

*Figure 19a: OT-IIGFP$^+$ Tregs suppress naïve OT-I CD8 T cell proliferation in a MHC-II dependent manner. 2.5x10^4 sorted naïve OT-I CD8 T cells (CD8α^+CD122^{lo}CD43^{lo}CD4$^-$CD11c$^-$) were cultured with various numbers of OT-IIGFP$^+$ Tregs (CD4$^+$GFP$^+$CD44^{hi}CD8b$^-$CD11c$^-$), 5x10^4 irradiated T cell depleted MHC-II$^{-/-}$ APCs or MHC-II$^{+/+}$ APCs, 1000 pg/ml of SIINFEKL peptide and 1 μg/ml OVA$_{323-339}$ peptide in a total volume of 0.2 ml in triplicate in a 96-well U-bottom plate for 72 hours. 1 μCi/well tritiated thymidine was added for the last 8 hours of the incubation and proliferation expressed as average ± standard deviation of triplicate samples. *$p<0.05$*

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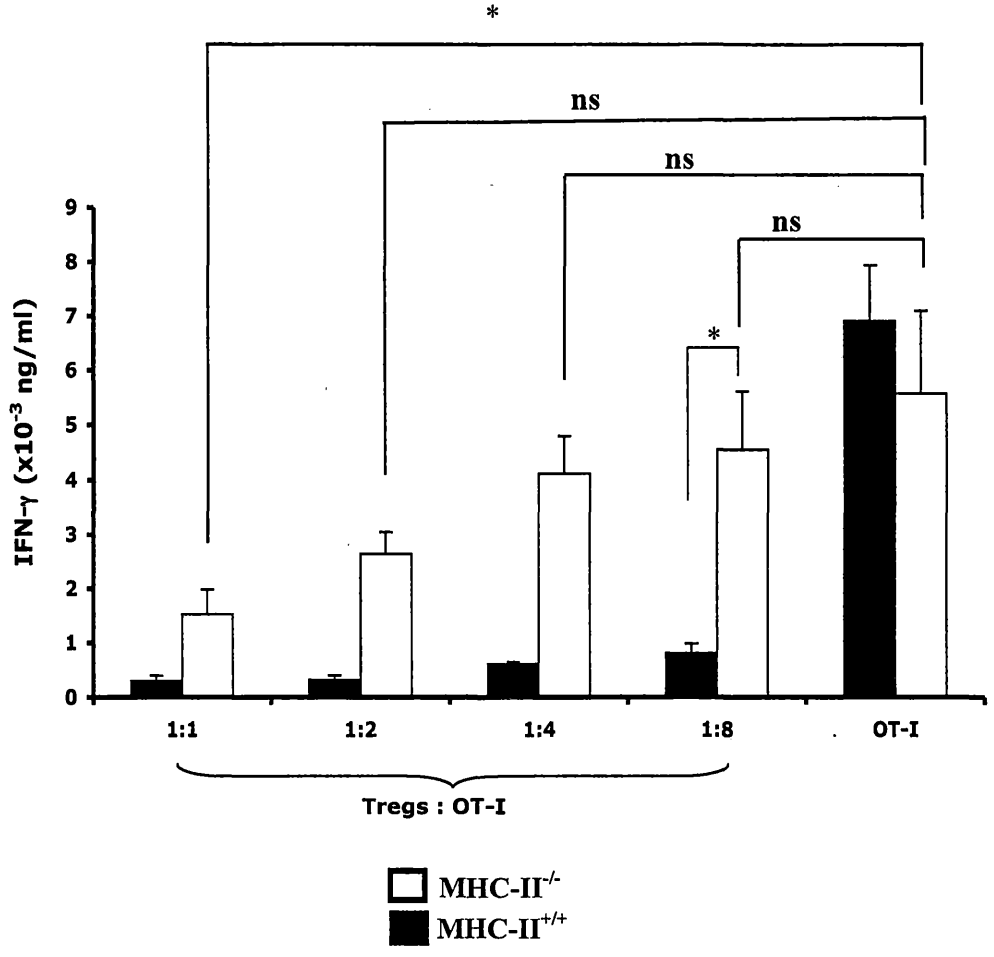


*Figure 19b: OT-IIGFP^{+} Tregs suppress naïve OT-I CD8 T cell IFN-γ production at low Treg ratio in a MHC-II dependent manner. Endpoint ELISA was performed per the manufacturer's instructions using the BD OptEIA^{TM} kit (BD Bioscience) with supernatants obtained from a duplicate plate set up as in Figure 19a. Concentrations were expressed as average ± standard deviation of triplicate samples. *$p<0.05$, ns = not statistical significant.*

IV. Discussion

APCs display peptide-MHC complexes on their cell surface generated through the MHC-I and MHC-II antigen presentation pathway leading to T cell activation or tolerance. The MHC-I pathway presents endogenously derived antigens to CD8 T cells but exogenous antigens can also be presented to CD8 T cells by cross presentation (43). Exogenous antigens are usually presented to CD4 T cells through the MHC-II pathway. Here we described the generation of an *in vitro* system in which the role of MHC-II dependent events in Treg mediated suppression was tested. Conditional MHC-II knock out mice were the source of APCs, while ovalbumin reactive Tregs from OT-IIFoxp3^{gfp} mice were the source of Tregs and naïve CD8 T cells from OT-I TCR transgenic mice were the source of responders. We show that CD4^{+}Foxp3^{+} Treg suppress CD8 T cell proliferation in a MHC-II dependent manner even at low Treg cell levels. MHC-II dependent suppression of CD8 T cell effector molecule production mediated by CD4^{+}Foxp3^{+} Treg required slightly higher Treg: OT-I ratios but still was very effective.

The use of Tregs from OT-IIFoxp3^{gfp} mice was a result of experiments trying to increase Treg numbers for use in experiments, as the frequency of these cells in conventional OT-II mice is very low. We first generated OT-II x Act-mOVA transgenic mice that showed an increased percentage of CD4^{+}Vα2^{+}Foxp3^{+} Tregs among total CD4 T cells. However, the overall percentage of CD4 T cells was drastically decreased in

these mice and did not translate to increased cell numbers when Tregs were sorted from these mice. Consistent with these observations, studies have shown the selection of a $CD4^+CD25^+$ Treg population in other models of double transgenic mice with a decrease in the overall CD4 T cell population possibly due to thymic deletion (111). Antigen expression by thymic epithelial cells has been implicated in the generation of antigen-specific Tregs in these double transgenic mouse models (111). Analysis in the DO11 x RIP-mOVA model, for example, showed that the total number of clonotypic T cells (KJ-126^+) was reduced, but there was an enrichment of the $CD4^+CD25^+$KJ-126^+ T cell population (33). In our model therefore, the total numbers obtained after cell sorting was similar to that obtained after sorting from OT-II mice. Due to these low numbers, we sought other ways to increase the frequency of Tregs.

We, therefore, turned to increasing Treg numbers by the *in vitro* expansion method described by Tang and colleagues (103). This method utilizes immobilized anti-CD3 and anti-CD28 with high amounts of exogenous IL-2. The limitation of expanding OT-II Tregs with this method was that although there was about a 27-fold increase in the total cell numbers, only 30% of the total cells expressed Foxp3, i.e. were Tregs. Another limitation was that the expanded Tregs were not able to suppress the proliferation of OT-I CD8 T cells. A possible explanation could be that the $V\alpha2^+CD25^-$ T cell population contaminated the original culture, and these cells proliferated and expanded more vigorously than the $V\alpha2^+CD25^+$ Tregs. This result suggests that even a small number of $CD25^-$ T cells in the presence of high amounts of exogenous IL-2 could result in a substantial decrease in Tregs purity in the final culture. To solve this problem of contaminating $CD25^-$ effector cells in the culture, treatment with 1-butanol (117) or

rapamycin (118) might allow the selective expansion of Tregs while blocking the proliferation of effector cells. Others have employed different surface markers to sort Tregs such as CD62L (103) and CD127 (IL-7 receptor alpha chain) (119). With the availability of Foxp3^{gfp} mice (18), we generated OT-IIFoxp3^{gfp} mice where Treg could be isolated using GFP. Using these mice, the percentage and total cell numbers of sorted Tregs was increased, and we were able to obtain Tregs with high purity and in greater numbers compared to conventional OT-II mice. OT-IIGFP^{+} Tregs when activated on immobilized anti-CD3 and anti-CD28 plates in the presence of low concentrations of IL-2 for 4 days suppressed the proliferation and IFN-γ production of naïve CD8 T cells even in the absence of $OVA_{323-339}$ the OT-II peptide. We therefore were not able to use these Tregs in this *in vitro* system because the cells were already activated. This further stressed the point that after Tregs are stimulated through their TCR *in vitro* they are able to exert suppressive functions. However, this does eliminate the possibility that activated OT-IIGFP^{+} Tregs might still need to be activated in an antigen-specific manner *in vivo* to exert suppression, as evidence suggests that this might be the case. Expanded autoantigen specific Tregs, for example, were shown to drain to the lymph node expressing the autoantigen where they were activated and divided, whereas Tregs that were not specific for the autoantigen showed no antigen-specific homing (103). These Tregs were able to prevent diabetes more potently than expanded polyclonal Tregs or antigen-specific Tregs that did not respond to the autoantigen (103). These results suggest that expanded antigen-specific Tregs still need antigenic stimulation *in vivo*. Homing experiments of activated OT-IIGFP^{+} Tregs to lymph node targeted with OVA_{323-}

$_{339}$ peptide will address the question of whether or not these Tregs accumulate in draining lymph nodes in an antigen-specific manner.

OT-IIGFP$^+$ Tregs responded to $OVA_{323-339}$ peptide in the presence of APCs and exogenous IL-2 or with mature BMDCs even in the absence of IL-2. This is consistent with the characteristics of Tregs, which are anergic when stimulated with anti-CD3 or cognate peptide but exhibit responsiveness when exogenous IL-2 or mature BMDCs added (19, 32). OT-IIGFP$^+$ Tregs also showed dose dependent suppression of naïve CD8 T cell proliferation and IFN-γ production. OT-II Tregs response and suppression upon $OVA_{323-339}$ stimulation suggests that they may be expressing dual TCRs as in other TCR transgenic mice, such as the DO11.10 mice (33, 115). One of these TCRs expresses an endogenous α-chain and a transgenic β-chain which confer specificity to cognate antigen (115). Evidence that DO11.10 Tregs utilize endogenous α-chain for their development was provided by crossing these mice with RAG$^{-/-}$ mice, which resulted in the disappearance of this T cell population in the DO11.10 x RAG$^{-/-}$ mice (33, 115). The expression of dual TCR by Tregs has also been shown in human peripheral blood CD4$^+$CD25$^+$ Tregs (120).

In our system, OT-IIGFP$^+$ Tregs potently suppressed naïve OT-I CD8 T cell proliferation by about 90% suppression at a 1:1 ratio compared to a study by Chai et al. in which they used the same responder cells and DO11.10 Tregs (110). The discrepancy in percent suppression observed could be due to the different sorting techniques used to isolate the Tregs and OT-I CD8 T cells. Since we used mice that incorporated the GFP allele, we were able to sort Tregs using GFP instead of CD25 thus obtaining a higher purity of Tregs. Sorting Tregs using CD25 could introduce activated effector cells and

thus decrease the purity of Tregs; some have suggested that the $CD25^{hi}$ cells are the most potent suppressor subset of Tregs (18). Fontenot et al. showed that Foxp3 expression and not CD25 expression correlates with Treg suppressive function as suppression of CD4 effector cells was observed with $CD4^+CD25^{hi}GFP^+$ or $CD4^+CD25^{lo}GFP^+$ but not with $CD4^+CD25^+GFP^-$ or $CD4^+CD25^-GFP^-$ cells from $Foxp3^{gfp}$ mice (18). Also while sorting OT-I CD8 T cells we excluded memory and activated CD8 T cells and isolated only naïve CD8 T cells. In the previously mentioned study, the responders used were OT-I $CD8^+CD25^-$ T cells and might contain memory CD8 T cells which might be harder to suppress. We also showed that OT-IIGFP^+ Tregs were able to suppress naïve CD8 T cell proliferation and IFN-γ production in the presence of ovalbumin protein. This suggests that APCs in this system were able to process whole protein and present antigen to both OT-IIGFP^+ Tregs and naive OT-I CD8 T cells.

Studies have suggested that antigen-specific Tregs are more potent in controlling autoimmune disease, such as type 1 diabetes, than polyclonal Tregs (37, 103). Most *in vitro* systems have utilized polyclonal Tregs in which the antigen specificity was not known. Using Tregs with known antigen specificity allowed us to show that $CD4^+Foxp3^+$ Tregs suppress CD8 T cell proliferation and effector molecule production in a MHC-II dependent manner *in vitro*. To our knowledge this is the first system to address the role of MHC-II dependent events in Treg mediated suppression using APCs that are deficient in MHC-II expression. APCs from the conditional MHC-II knockout mice were able to activate OT-I CD8 T cells to proliferate and produce IFN-γ. However, they failed to activate OT-IIGFP^+ Tregs to exert their suppressive function in co-culture with naïve OT-I CD8 T cells. Therefore, they were able to faithfully present antigen to

CD8 T cells but not to Tregs as a result of their lack of MHC-II. However, in co-culture experiments where antigen-specific Tregs and CD4 responders were from mice of different genetic backgrounds but activated in a antigen-specific manner by their respective APCs, suppression of proliferation did not seem to require that the target antigens be presented on the same APCs (21). We, therefore, generated an *in vitro* system in which the role of MHC-II dependent events in Treg mediated suppression could be tested. A model of MHC-II dependent events in Treg mediated suppression is shown diagrammatically in Figure 20.

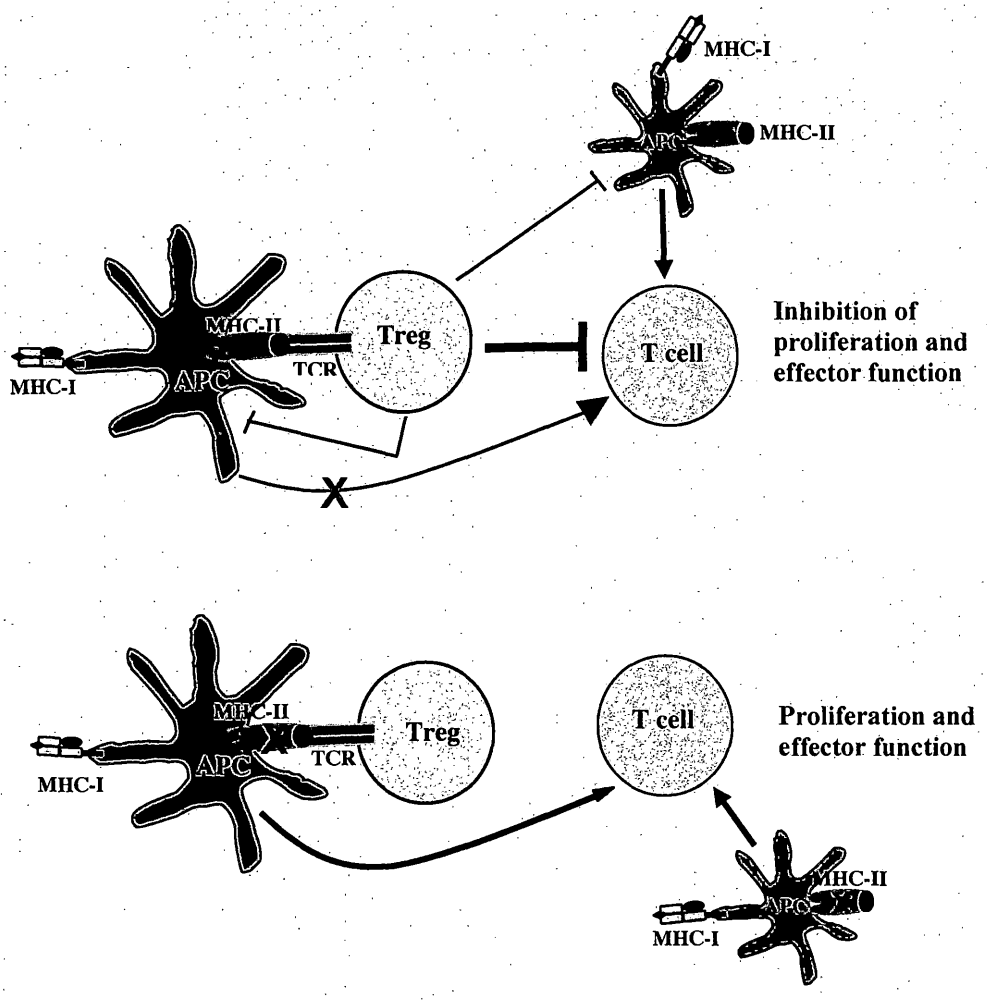


Figure 20: Model of MHC-II dependent events in Treg mediated suppression. In the presence of MHC-II, Tregs are activated and able to suppress CD8 T cell proliferation and effector molecule production. In the absence of MHC-II, Tregs are not activated and therefore are unable to exert their suppressive function, allowing CD8 T cell to proliferate and produce effector molecules.

V. The role of MHC-II in tumor progression

A. Hypothesis

MHC-II deficiency will cause a delay in tumor progression due to lack of Treg mediated suppression

Tumors induce a state of tolerance to their own antigens by employing some of the same mechanisms that are needed to maintain peripheral tolerance. The presence of such cells as DCs and Tregs in the tumor microenvironment has been suggested to be involved in the creation of such a tolerant state, allowing the tumor to grow unchecked in an immunocompetent host (62). Bone marrow derived APCs were shown to be involved in the induction of CD4 T cell tolerance in a B cell lymphoma model and solid tumors (65, 66). This population of tolerized CD4 T cells was shown to include naïve CD4 T cells, anergic T cells and tumor specific Tregs suggesting that Tregs could be generated *de novo* in the tumor microenvironment (121). Tregs in tumors have been suggested to inhibit CD8 T cell effector functions resulting in a lack of anti-tumor immune responses (82-84). Depletion of Tregs that suppress the effector function of tumor infiltrating CD8 T cells at tumor sites leads to reversed cytotoxic T lymphocyte (CTL) tolerization and rapid rejection of established tumors (81). Cao et al. demonstrated, using three different tumor cell lines, that these tumors induce granzyme B expression in Tregs (122).

Granzyme B expressing Tregs were shown to suppress tumor rejection mediated by NK and CD8 T cells (122). Furthermore, granzyme B-deficient mice more efficiently clear these tumors than did wild type mice (122). The mechanism of Treg mediated suppression of CD8 T cells in the tumor microenvironment are not clearly understood. What is also not well defined, despite the presence of APCs in the tumor microenvironment, is the involvement of MHC-II in Treg mediated suppression and in tumor progression. Using conditional MHC-II knock out mice, we therefore tested the role of MHC-II dependent events in tumor progression.

V.A.1. MHC-II deficiency causes delay of tumor progression

To understand the role of MHC-II dependent events in tumor progression, the growth kinetics of B16-OVA and B16F10 tumors in $ia\beta^{neo/\Delta}$Tie2Cre^{+} conditional MHC-II knockout mice were analyzed. $5x10^{5}$ B16-OVA or B16F10 tumor cells were subcutaneously injected into the abdominal flank area of 8 week-old $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice or wild type littermates. Mice were monitored for 30 days with tumor measurements starting on day 10 after tumor challenge. $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice that received a B16-OVA tumor challenge on average showed a delay in tumor growth compared to wild type mice, which exhibited rapid tumor growth (Figure 21a). Tumors in $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice appeared at approximately day 13 while in the wild type mice tumors were seen as early as day 7 (Figure 21a). Figure 21b showing tumor growth in individual $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice illustrate that the size of the tumors in two of the mice were smaller, in a third mouse tumor growth was not observed and in the fourth tumors grew to a similar extent as in the wild type mice. However, all of the $ia\beta^{neo/\Delta}$Tie2Cre^{+}

mice had a greater survival time compared to the wild type mice and were sacrificed at about day 30 of tumor growth (Figure 21b). Wild type mice were sacrificed when tumor diameters were about 13mm by day 16 and 20. $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice that received B16F10 tumors showed a similar trend with the exception of one mouse that showed rapid tumor growth similar to that observed in wild type mice (Figure 22a). However, on average B16F10 tumor growth in $ia\beta^{neo/\Delta}$Tie2Cre^{+} mice was delayed even in the absence of the neo-antigen (Figure 22b). These results tend to support the hypothesis that MHC-II deficiency results in a delay in tumor progression.

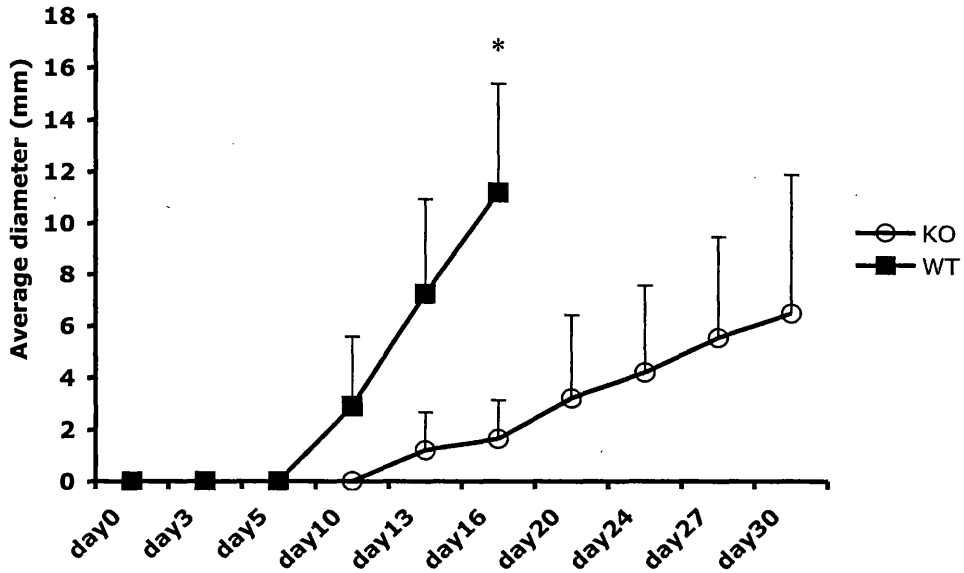


*Figure 21a: MHC-II deficiency results in a delay in tumor progression in mice bearing B16-OVA tumors. 5x10^5 B16-OVA tumor cells were subcutaneously injected into the abdominal flank area of 8 week-old ia$\beta^{neo/\Delta}$Tie2Cre$^+$ or wild type litter mates (4 mice per group). Tumors were monitored for 30 days with tumor sizes taken as the average of the perpendicular and vertical diameters. *$p<0.05$*

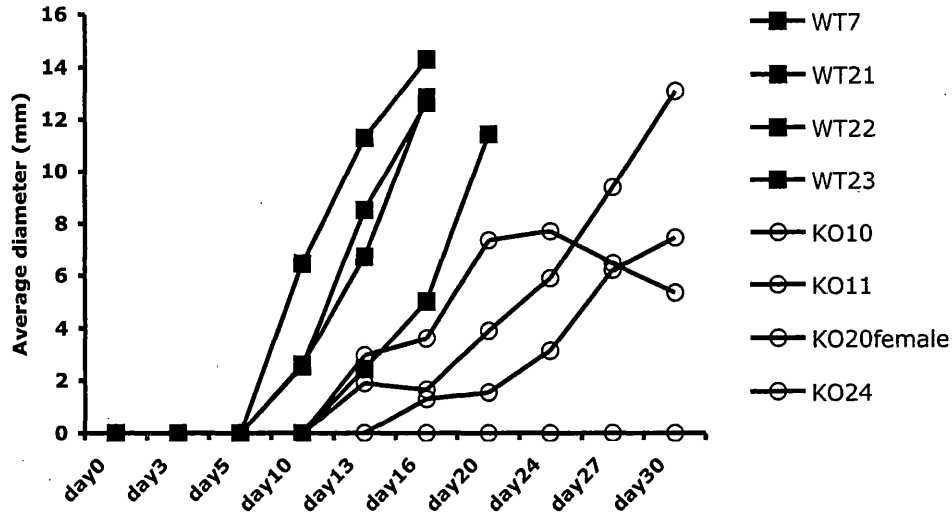


Figure 21b: MHC-II deficiency resulted in a delay in tumor progression in mice bearing B16-OVA tumors. The graph depicts tumor growth of mice for individual in the experiment described in Figure 21a.

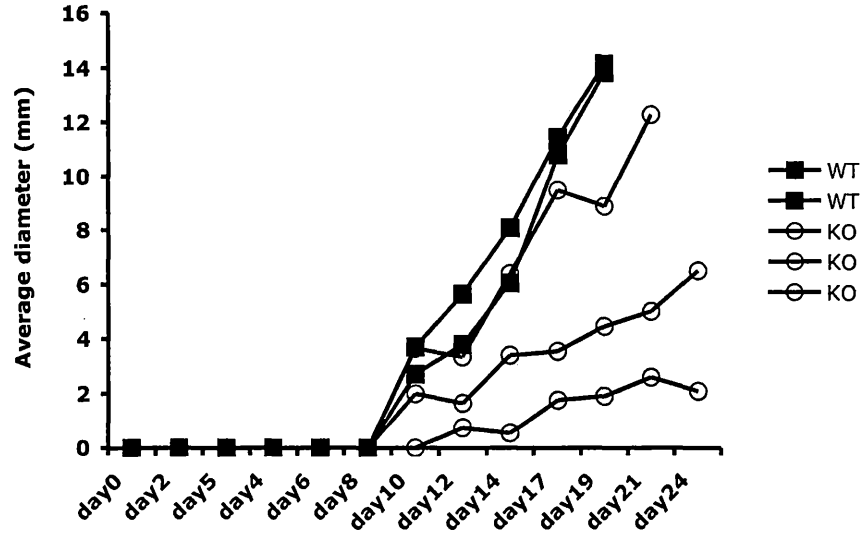


Figure 22a: MHC-II deficiency results in a delay in tumor progression in mice bearing B16F10 tumors. $5x10^5$ B16F10 tumor cells were subcutaneously injected into the abdominal flank area of three ia$\beta^{neo/\Delta}$Tie2Cre$^+$ mice (KO) and two wild type litter mates (WT). Tumors were monitored for 24 days with tumor size taken as the average of the perpendicular and vertical diameters for each mouse.

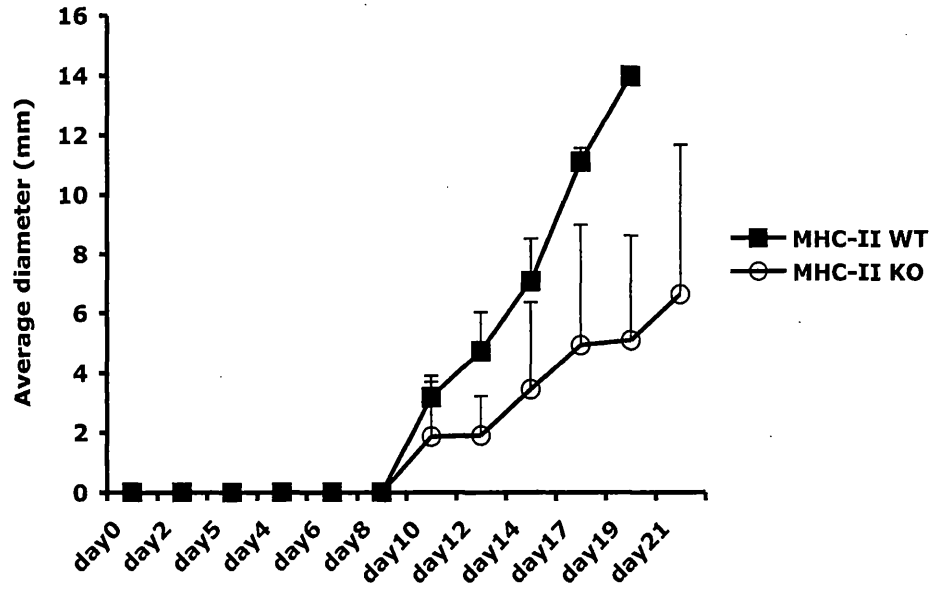


Figure 22b: MHC-II deficiency results in a delay in tumor progression in mice bearing B16F10 tumors. The graph depicts the average tumor diameters in the two groups of mice in the experiment described in Figure 22a.

V.A.2. CD8 T cells were involved in the delay of tumor progression caused by MHC-II deficiency

Since CD8 T cells have been implicated in anti-tumor immune responses (123), we therefore asked whether CD8 T cells were involved in the delay of tumor progression caused by MHC-II deficiency. $5x10^5$ B16-OVA tumor cells were subcutaneously injected into the abdominal flank area of six 8 week-old $ia\beta^{neo/\Delta}$Tie2Cre$^+$ and two wild type litter mates. Three of the $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice received CD8 T cell depletion. CD8 T cell depletion accomplished using 200 μg/mouse of anti-CD8 antibody injected on the same day as the tumor challenge and 3 and 7 days later. Tumor sizes were monitored for 16 days only, as the mice that received CD8 T cell depletion had to be sacrificed due to large tumor burdens. $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice showed delayed tumor growth compared to the wild type mice (Figure 23a). However, tumors grew more rapidly in $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice that received CD8 T cell depletion compared to the wild type and the $ia\beta^{neo/\Delta}$Tie2Cre$^+$ mice that did not receive CD8 T cell depletion (Figure 23a-b). This result suggests that CD8 T cells are involved in the delay of tumor progression caused by MHC-II deficiency.

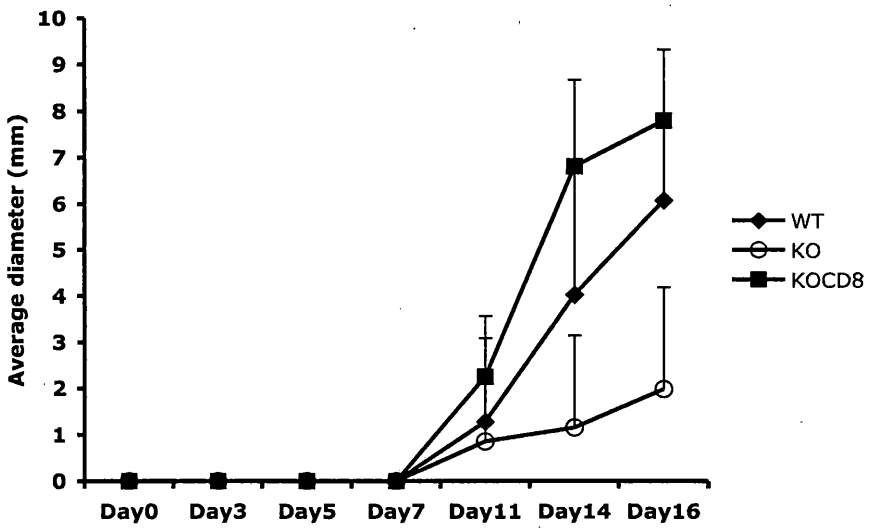


Figure 23a: CD8 T cells are involved in the delay of tumor progression caused by MHC-II deficiency. $5x10^5$ B16-OVA tumor cells were subcutaneously injected into the abdominal flank area of three CD8 T cell depleted $ia\beta^{neo/\Delta}Tie2Cre^+$(KOCD8), three $ia\beta^{neo/\Delta}Tie2Cre^+$(KO) and two wild type mice (WT). Tumors were monitored for 16 days. The tumor size was taken as the average of the perpendicular and vertical diameters for each group of mice.

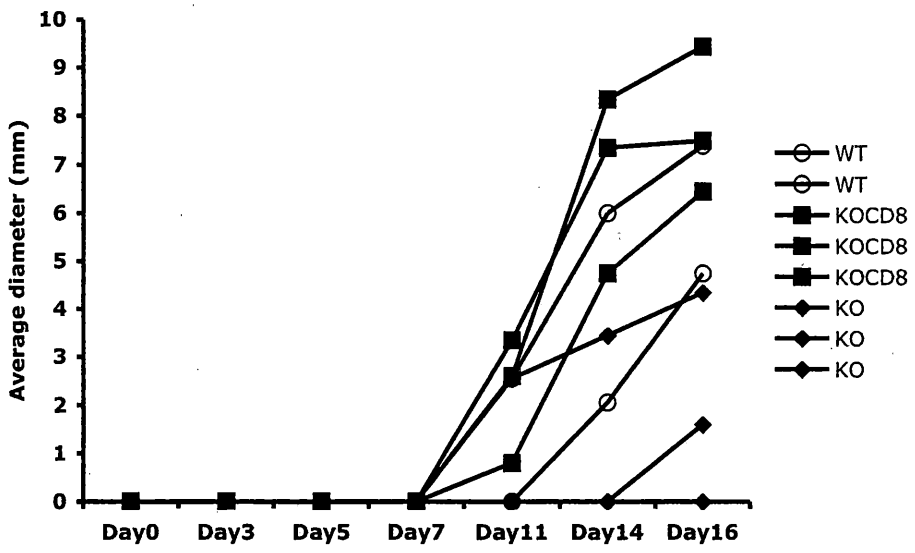


Figure 23b: CD8 T cells are involved in the delay of tumor progression caused by MHC-II deficiency. The graph depicts tumor growth for individual mice for the experiment described in Figure 23a.

VI. *In vivo* models to test the role of MHC-II dependent events in Treg mediated suppression

A. Hypothesis

$CD4^+CD25^+$ Tregs suppress the CD8 T cell mediated delay in tumor progression

Tregs are a double-edged sword in that, on the one hand, they are important for maintaining peripheral tolerance, without which autoimmune disease manifests, but on the other hand, they hamper anti-tumor immune responses (73-77). One mechanism employed by Tregs to interfere with anti-tumor immune responses is suggested to be via their ability to suppress CD8 T cell cytotoxicity in a TGF-β dependent manner (82). The model and readout for the following experiments follows reports in which pre-activated OT-I CD8 T cells, as opposed to naïve CD8 T cells, induced tumor rejection in mice bearing B16-OVA tumors (104, 124), and experiments in which antigen-specific $CD4^+CD25^+$ T cells co-injected with CD8 T cells resulted in the suppression of tumor rejection (82, 84, 125, 126). Therefore, we developed a model in which the role of MHC-II dependent events mediated by Tregs in the suppression of CD8 T cell tumor rejection could be tested. We first tested this system using B6 mice before employing conditional MHC-II knockout mice.

VI.A.1. Activated CD8 T cells induce a delay in tumor progression

Naïve OT-I CD8 T cells adoptively transferred into tumor bearing mice have been shown to be tolerized and failed to protect mice from tumor growth (124). However, the injection of activated OT-I CD8 T cells caused a delay in tumor progression (104, 124). Using activated OT-I CD8 T cells, we repeated these experiments using B16-OVA tumor cells. Seven B6 mice were subcutaneously injected with $5x10^5$ B16-OVA cells into the left abdominal flank area. Three days later, $4x10^6$ pre-activated OT-I $CD8^+$ T cells were adoptively transferred into 3 mice with 4 mice serving as controls. Tumor growth was monitored every other day for 23 days starting on day 10 after tumor challenge. B6 mice that received only the B16-OVA cells showed rapid tumor growth, and by day 18 the mice had to be sacrificed due to large tumor burdens (Figure 24). There was about a 10-day delay in tumor growth in mice that received pre-activated OT-I CD8 T cells but tumors eventually grew despite the transfer of the OT-I CD8 T cells (Figure 24). These results confirm observations by others (104, 124) indicating that pre-activated CD8 T cells cause a delay in tumor progression.

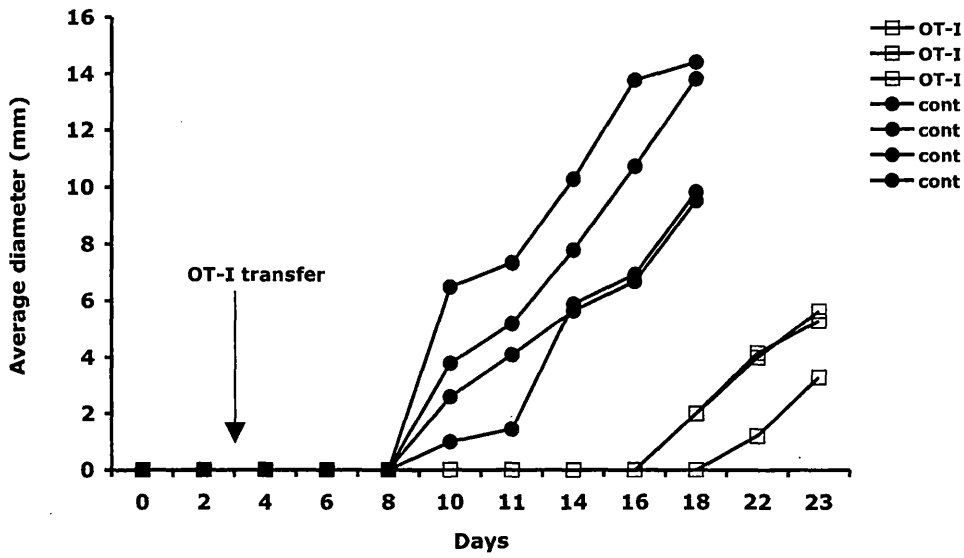


Figure 24: Activated OT-I CD8 T cell cause a delay in tumor progression. $5x10^5$ B16-OVA tumor cells were subcutaneously injected into the abdominal flank area of 8 week-old B6 mice. Three days later, 4 x 10^6 pre-activated OT-I $CD8^+$ T cells were intravenously injected into three B6 mice while four mice served as controls. Mice were monitored for tumor growth for 23 days and the tumor size was taken as the average of the perpendicular and vertical diameters for each mouse.

VI.A.2. OT-II Tregs inhibited the CD8 T cell mediated delay in tumor progression

After establishing that pre-activated OT-I CD8 T cells induced a delay of tumor progression, we tested the role of Tregs in inhibiting the CD8 T cell mediated delay in tumor progression. B6 mice were subcutaneously injected with $2x10^5$ B16-OVA cells into the left abdominal flank. Three days later, three mice received $10x10^6$ pre-activated OT-I CD8 T cells, two mice received the same number of pre-activated OT-I CD8 T cells plus $2x10^5$ sorted OT-II Tregs ($V\alpha2^+CD25^+CD4^+CD11c^-CD8^-$) from OT-II x Act-mOVA mice and three mice were left untreated. Tumor growth was monitored for 24 days and the tumors measured every other day. Pre-activated OT-I CD8 T cells transferred alone caused a 7 day delay in tumor growth compared to mice and were left untreated that showed rapid tumor growth (Figure 25). There was a marginal but not insignificant suppression of the tumor delay when OT-I CD8 T cell were co-transferred with Tregs, perhaps due to the high numbers of OT-I CD8 T cells and low numbers of Tregs transferred (Figure 25). Therefore, the number of T cells must be optimized in this system before using it to test the role of MHC-II dependent events in Treg mediated suppression of CD8 T cell delay in tumor progression.

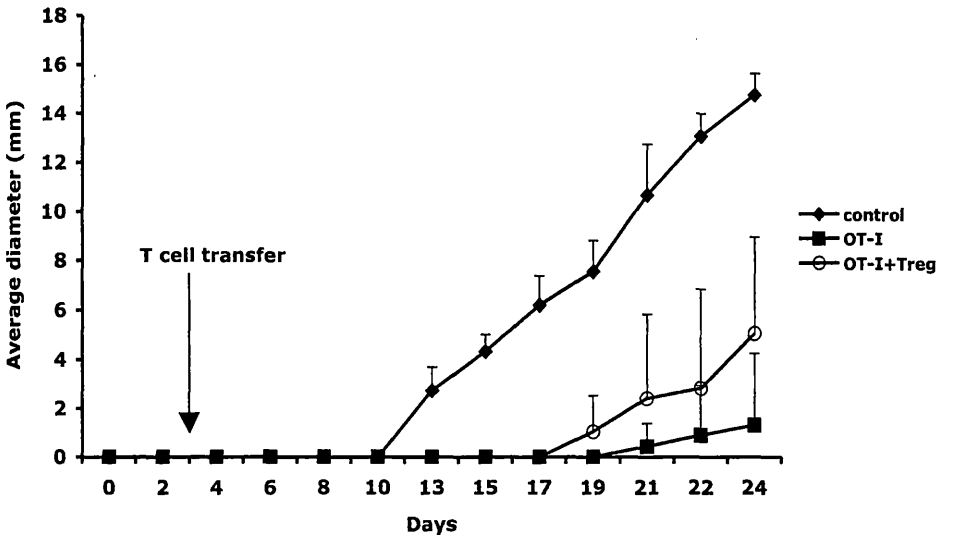


Figure 25: OT-II Tregs cause a marginal but not a significant suppression of OT-I CD8 T cell mediated tumor rejection. 2 x10^5 B16-OVA tumor cells were subcutaneously injected into the abdominal flank area of eleven 10 week-old B6 mice. Three days later, 1x10^7 pre-activated OT-I CD8 T cells were either injected alone (5 mice) or with 2 x10^5 OT-II Tregs from OT-II x Act-mOVA (2 mice). Mice were monitored for tumor growth for 24 days and the tumor size was taken as the average of the perpendicular and vertical diameters for each group of mice.

B. Hypothesis

$CD4^+Foxp3^+$ Tregs suppress CD8 T cell in vivo cytotoxic killing

In vitro, Tregs suppress T cell proliferation and effector molecule production in both CD4 and CD8 T cells (22). *In vivo,* however, studies suggest that Tregs do not interfere with CD8 T cell priming, proliferation, secretion of effector molecules and even homing to draining lymph nodes (82). It appears that Tregs interfere with the late phase of CD8 T cell effector function (82). Mempel et al. elegantly demonstrated these observations using multiphoton intravital microscopy in TDLNs of anesthetized mice (107). Tregs inhibited CD8 T cell cytotoxic killing of target cells in part by inhibiting granule exocytosis as measured by the lysosomal marker CD107a rather than by inhibiting effector molecules such as IFN-γ (107). We, therefore, adapted this *in vivo* cytotoxic killing assay as described by Mempel and colleagues (107), first using B6 mice but with the goal of using the system with conditional MHC-II knock out mice.

VI.B.1. OT-IIGFP$^+$ Tregs inhibit CD8 T cell in vivo cytotoxic killing

This experiment utilizes activated Tregs with the assumption that they still need to be activated in an antigen-specific manner to exert suppression *in vivo*. Sorted $1.5x10^6$ OT-IIGFP$^+$ Tregs ($V\alpha2^+GFP^+CD44^{hi}CD8b^-CD11c^-$) from OT-IIFoxp3^{gfp} mice were activated on an immobilized anti-CD3/anti-CD28 (1 μg/ml) plate in complete medium with 100 U/ml of IL-2 for 4 days. Cells were then rested in complete medium supplemented with 10 U/ml of IL-2 for an additional day. At the end of this culture period, $3.13x10^6$ activated OT-IIGFP$^+$ Tregs cells were obtained. Naïve OT-I CD8 T

cells ($CD8\alpha^{+}CD122^{lo}CD43^{lo}CD4^{-}CD11c^{-}$) were then sorted and $1x10^{5}$ cells were intravenously injected via the tail vein of two B6 mice while three B6 mice also received $5x10^{5}$ activated OT-IIGFP^{+} Tregs. One day later, $6x10^{6}$ irradiated T cell depleted Act-mOVA splenocytes (on B10.BR background) were also intravenously injected into the mice. The splenocytes express membrane bound ovalbumin protein and serve to activate the adoptive transferred T cells. Seven days later, a mixture of a 1:1 ratio of B6 splenocytes (see Materials and Methods), one half labeled with 0.2 μM CFSE ($CFSE^{lo}$) and pulsed with SIINFEKL peptide (target peptide) and the other half pulsed with 2 μM CFSE ($CFSE^{hi}$) without peptide, was intravenously injected into the mice. The spleens and lymph nodes were harvested 8 hours later and analyzed by FACS. The FACS plots used to calculate the specific lysis are shown in Figure 26a. The specific lysis of target cells in the mouse that received naïve OT-I CD8 T cells was 45% in the lymph nodes and 38% in the spleen (Figure 26b). When naïve OT-I CD8 T cells were co-transferred with the activated OT-IIGFP^{+} Tregs the specific lysis decreased to 15% in the lymph nodes and 2% in the spleens suggesting that OT-IIGFP^{+} Tregs interfered with CD8 T cell cytotoxic killing (Figure 26b).

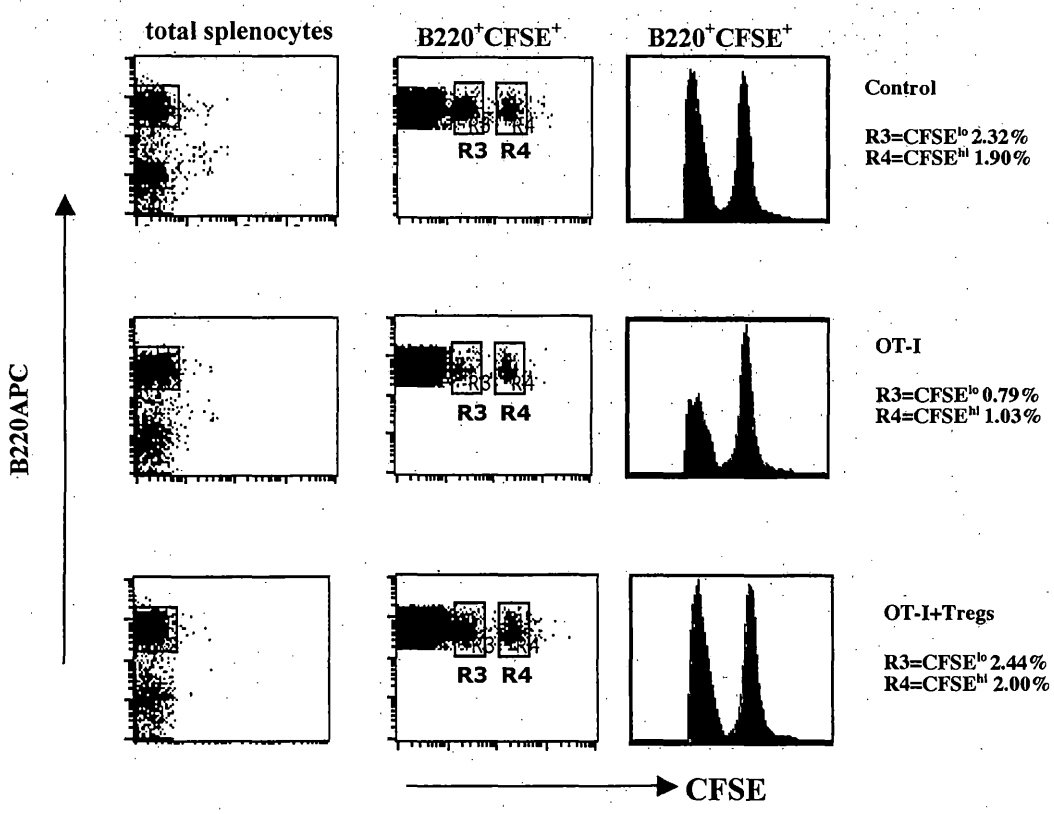


Figure 26a: OT-IIGFP$^+$ Tregs inhibit CD8 T cell cytotoxic killing. 1x10^5 naïve OT-I CD8 T cells were intravenously injected into B6 mice with or without 5x10^5 activated OT-IIGFP$^+$ Tregs. One day later 6x10^6 irradiated T cell depleted Act-mOVA splenocytes were intravenously injected into the mice. Seven days later, a mixture of a 1:1 ratio of B6 splenocytes (CFSE^{lo}:CFSE^{hi}) were intravenously injected into the mice. Spleens and lymph nodes were harvested 8 hours later and stained for B220APC (1:500) and analyzed on a FACS calibur. Depicted are spleen cells gated on B220$^+$ and CFSE. CFSE^{lo} cells are represented by gate R3 and CFSE^{hi} cells are represented by gate R4.

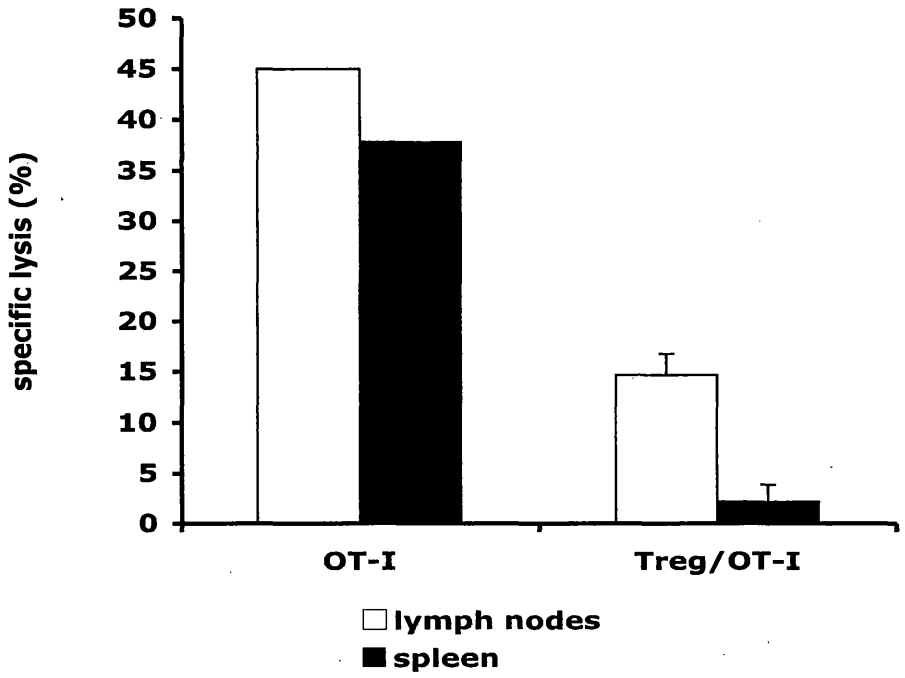
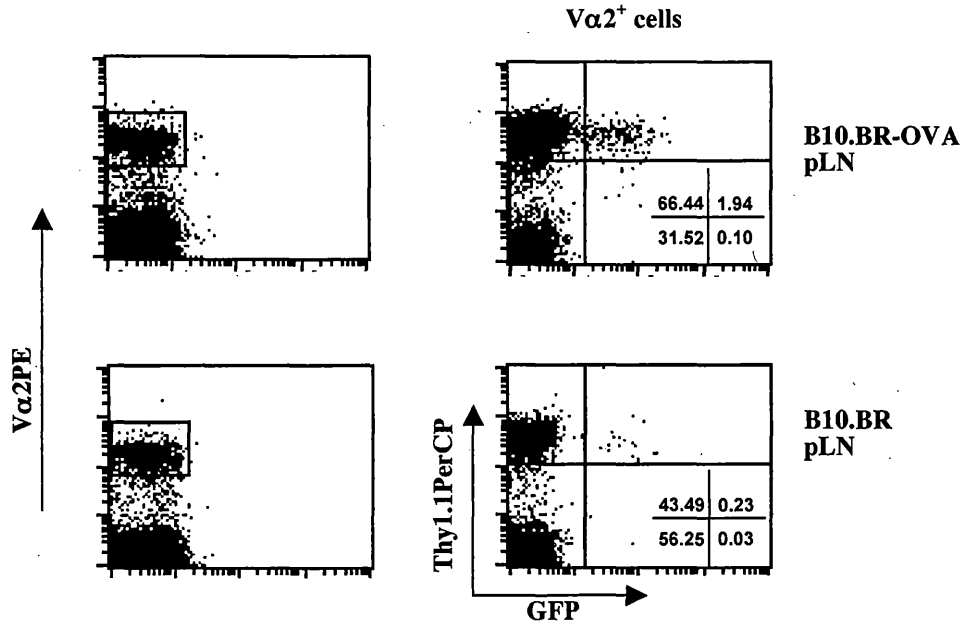


Figure 26b: OT-IIGFP^{+} Tregs inhibits CD8 T cell cytotoxic killing. 1x10^{5} Naïve OT-I CD8 T cells were intravenously injected into B6 mice with or without 5x10^{5} activated OT-II Tregs. One day later 6x10^{6} irradiated T cell depleted Act-mOVA splenocytes were also intravenously injected into the mice. Seven days later, a mixture of a 1:1 ratio of B6 splenocytes, CFSE^{lo} (pulsed with SIINFEKL peptide):CFSE^{hi} were intravenously injected into the mice. The spleen and lymph nodes were harvested 8 hours later and stained for B220APC (1:500) and analyzed on a FACS calibur. The percent of the specific lysis was determined with the formula described in the Materials and Methods section using percentages from gate R3 and R4 as shown in Figure 26a using spleen cells as an example.

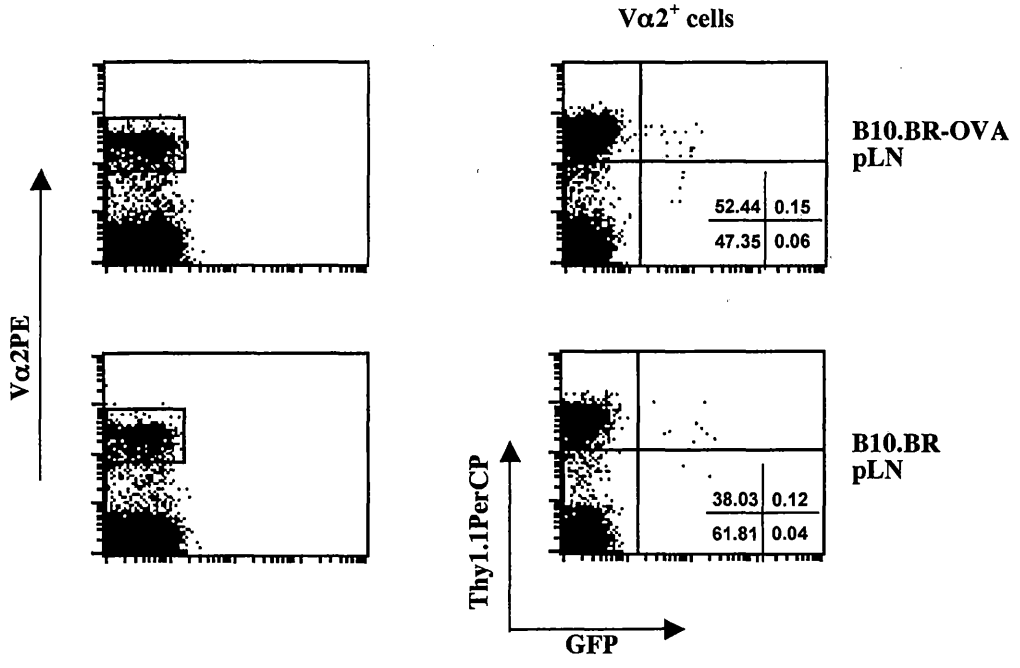
VI.B.2. OT-IIGFP$^+$ Treg accumulation in local lymph nodes is MHC-II dependent

We sought to modify the above model of *in vivo* cytotoxic killing to investigate the role of Treg mediated suppression at local sites such as draining lymph nodes rather than systemically. We first determined whether or not Tregs accumulate at local lymph nodes in a MHC-II dependent manner. Two *ia*$\beta^{neo/\Delta}$Tie2Cre$^+$ and two wild type mice were intravenously injected with 40x10^6 total cells from the spleen and pooled lymph nodes of OT-IIFoxp3^{gfp} mice. Instead of using Act-mOVA splenocytes as described in Figure 26, we loaded ovalbumin protein by osmotic shock into B10.BR splenocytes. After the osmotic shock, most of the B10.BR splenocytes die, allowing the host APCs to present and activate the adoptively transferred cells. A day after the injection of cells from the OT-IIFoxp3^{gfp} mice, 10^7 B10.BR-OVA splenocytes were injected into the right hind footpad of the mice, with the left footpad injected with B10.BR cells that were not loaded with ovalbumin protein serving as controls. Footpad injection allows draining of antigen to the popliteal lymph nodes. Popliteal lymph nodes were harvested 7 days later and cells analyzed by FACS. OT-IIGFP$^+$ Tregs failed to accumulate in the popliteal lymph nodes of *ia*$\beta^{neo/\Delta}$Tie2Cre$^+$ mice and wild type mice that received B10.BR control cells (Figure 27). OT-IIGFP$^+$ Tregs, however, accumulated in the popliteal lymph node of wild type mice that received B10.BR-OVA cells but not in the popliteal lymph node of *ia*$\beta^{neo/\Delta}$Tie2Cre$^+$ mice (Figure 27). This result suggests that OT-IIGFP$^+$ Tregs accumulate in draining lymph nodes in a MHC-II dependent manner.

MHC-II Wild type mice:



Conditional MHC-II knockout mice:



VII. Discussion

MHC-II deficiency results in a delay in tumor progression

We demonstrated that MHC-II dependent events are involved in the delay in tumor progression caused by MHC-II deficiency. Conditional MHC-II knock out mice bearing B16-OVA tumors showed a delay in tumor growth compared to wild type controls. The delay in tumor progression was not distinct to the B16-OVA tumors because of the presence of ovalbumin protein, since the same results were obtained with B16F10 tumors, which did not express the neo-antigen. This result suggests that the presence of the neo-antigen was not necessary for tumor delay in the absence of MHC-II. CD8 T cells were shown to be involved in the anti-tumor response as depletion of this T cell subset resulted in rapid tumor growth in the conditional MHC-II knock mice. There are not many examples in the literature of tumor growth in MHC-II deficient mice. Studies in which complete MHC-II knockout mice were challenged with highly immunogenic tumor cells produced two different results, probably due to the location of the tumor challenge (127, 128). Den Boer et al. observed tumor rejection in MHC-II$^{-/-}$ mice but not B6 wild type mice when tumor cells transformed by the human adenovirus type 5 early region 1 (Ad5E1A) oncogene were injected subcutaneously into the flank area (127). When these same tumors were intracamerally injected in the anterior chamber of the eye of MHC-II$^{-/-}$ mice, tumor rejection was not observed whereas B6 control mice showed tumor regression (128).

Unlike our model of conditional MHC-II deficient mice, MHC-II knock out mice do not have Tregs, and so this model precludes analysis of the potential for MHC-II independent Treg suppression. Based on these results, it is still unclear as to the role of MHC-II dependent events in tumor progression. However, the overall role of lymphocytes in tumor immune surveillance has been clearly established in earlier experiments that utilized mice such as RAG-$2^{-/-}$ mice bearing MCA-induced sarcomas (129).

It is very intriguing and somewhat unexpected that conditional MHC-II deficient mice show this delay in tumor progression. This delay provides a window of opportunity during which some of the mechanisms of tumor-induced tolerance can be investigated. These mice differ from conventional MHC-II knockout mice in that they still contain their own naïve CD4 T cells and Tregs. It is possible that during tumor progression, there is a lack of Treg activation and recruitment to TDLNs in these mice, thus allowing CD8 T cell cytotoxicity to be more effective, as evidence suggests that Tregs suppress CD8 T cell cytotoxic killing (82, 107). However, CD8 T cell effector functions might not of long duration as these mice still develop tumors. CD4 T cell help has been shown to be important in CD8 T cell clonal expansion and function, in the generation of CD8 T cell memory (130-134) and in anti-tumor CTL responses (135-137). Therefore, due to the absence of MHC-II, effective CTL and memory responses may not be maintained.

One mechanism of tumor-induced tolerance involves creating an immunosuppressive environment by recruiting tolerogenic DCs or inducing DCs to be suppressive (62). Therefore, during the early phase of tumor development, factors that are responsible for the induction of immunosuppression in such cells could be MHC-II dependent and thus less effective. IDO^{+} pDCs for example have been shown to

contribute to tumor-induced tolerance by inhibiting T cell responses (71). It has not been investigated but if IDO induction were MHC-II dependent, then IDO^{+} pDCs would be less suppressive in conditional MHC-II knockout mice. To begin to answer this question, immunohistochemical analysis of TDLNs from conditional MHC-II knockout mice challenged with B16-OVA tumors and litter mate controls would allow us to know whether IDO induction is MHC-II dependent or not. Other tolerogenic DCs, such as pDCs, would be less suppressive if the suppressive mechanisms operating in these cells were MHC-II dependent. Bone marrow-derived APCs have been shown to induce CD4 T cell tolerance in tumors (65, 66). It is possible that in MHC-II deficient mice during the early phase of tumor growth, CD4 T cell are not tolerized but face other consequences as a result of the lack of MHC-II. If the above factors were true, the overall effect of the disruption of MHC-II dependent events would be a less suppressive tumor microenvironment. A growth kinetic studies of tumor bearing conditional MHC-II knockout mice that receive MHC-II sufficient APCs compared to wild type untreated control mice would allow us to assess if adding back APCs that are MHC-II sufficient restores tumor growth. Thus, further experiments are needed to answer these questions.

Since conditional MHC-II deficient mice lack expression of MHC-II on most peripheral APCs, it would be of interest to delineate the effects of MHC-II deficiency on mice lacking MHC-II on a specific subset of APCs. Since we already have $ia\beta^{neo}$ mice (loxP-flanked MHC-II β chain gene mice), crossing these mice with appropriate Cre mice would allow the deletion of MHC-II on subsets of APCs. We have preliminary data on some of these mice generated in the laboratory. We had generated Ly6C-Cre mice, with the aim of deleting MHC-II on pDCs. However, these mice showed deletion not only in

pDCs but also in B cells and $CD11c^+$ DCs suggesting that the Ly6C promoter is not specific to pDCs. $ia\beta^{neo/neo}$CD11cCreGFP mice were also generated in the laboratory by crossing CD11cCreGFP mice (138) with $ia\beta^{neo}$ mice. These mice showed a 97% loss of MHC-II in $CD11c^+$ DCs but incomplete deletion in lymphoid pDCs and a substantial loss in B cells. Another Cre recombinase knockin mouse that has been developed in the laboratory is a CD8α-CreGFP mouse that when crossed with $ia\beta^{neo}$ mice showed about 70% loss of MHC-II on $CD8\alpha^+$ DCs, but deletion in some $CD4^+$ DCs was also observed. Although to date deletion of MHC-II on distinct DC subsets has not been successful, MHC-II deletion on B cells has been reported by the generation of $ia\beta^{neo/neo}CD19^{cre/+}$ mice (139). B16-OVA challenged in these mice resulted in tumor growth at the same rate as their wild type littermates. Thus, the delay in tumor growth in conditional MHC-II knockout mice does not appear to be due to the loss of MHC-II from B cells.

Models to test the role of Treg mediated suppression *in vivo*

We describe two models that can be used to test the role of MHC-II dependent events in Treg mediated suppression. The first model tests the hypothesis that Tregs suppress the CD8 T cell mediated delay in tumor progression. Activated OT-I CD8 T cells were co-injected with or without OT-II Tregs from OT-II x Act-mOVA mice. Activated OT-I CD8 T cells induced a delay in tumor progression that was not significantly reversed by OT-II Tregs. Thus, this experiment must be optimized, especially in terms of the number of OT-I CD8 T cells used and also the number of Tregs. Chen et al. used a 1:1 ratio of Tregs to CD8 T cells and showed that Tregs reversed CD8 T cell mediated tumor rejection (82). Others have shown that a 1:10 ratio (Treg:CD8 T cells) was effective at reversing CD8 T cell mediated tumor rejection (84).

At the time when this experiment was performed, Foxp3^{gfp} mice were not yet available and therefore the number of Tregs for adoptive transfer experiments was limited. With the generation of OT-IIFoxp3^{gfp} mice, increased numbers of Tregs can now be obtained for adoptive transferred. Also, harvest of activated Tregs from OT-IIFoxp3^{gfp} mice using the protocol described in the Materials and Methods section could greatly enhance the number of Tregs for adoptive transfer, although only a 2 fold increase in Treg numbers is obtained with this protocol. Expanded Tregs in other systems have been shown to drain to lymph nodes in an antigen-specific manner (103). Thus, activated OT-II Tregs are expected to accumulate in draining lymph nodes in an antigen-specific manner. In view of this idea, one modification of the system would be to look at suppression in TDLNs as opposed to measuring the tumor sizes, for which the tumor cells would have to be injected into the anterior-medial area of the thigh, rather than the abdominal flank area, in order to drain to inguinal lymph nodes. Therefore, the first experiments would be to investigate the homing of activated Tregs and activated CD8 T cells to TDLNs and analyze their phenotype. Since we used activated CD8 T cells, and Tregs have been shown to suppress CD8 T cell cytotoxic killing, we would then perform experiments to analyze Treg mediated inhibition of CD8 cytotoxicity in the TDLNs as described by others (82, 107). These experiments will first be performed in B6 mice and then in conditional MHC-II knockout mice to investigate the role of MHC-II dependent events in Treg suppressive function *in vivo*.

The second model proposed to test the role of MHC-II dependent events in Treg mediated suppression *in vivo* is one that examines Tregs suppression of CD8 T cell cytotoxic killing using Act-mOVA splenocytes to activate the transferred cells. This

system utilized irradiated T cell depleted Act-mOVA splenocytes, allowing the host APCs to present antigen to the T cells. We show in preliminary data that OT-II Tregs suppressed CD8 T cell *in vivo* cytotoxic killing of target cells in B6 mice. However, this experiment must be repeated with greater mouse numbers in each group, especially the group that received CD8 T cells. In order to optimize this system, investigating the role of Treg mediated suppression at a local site would be beneficial. Thus, we would examine lymph nodes targeted with antigen rather than of systemically. Treg homing and phenotype at these local sites will be analyzed. Figure 27 suggests that OT-II Tregs accumulated in popliteal lymph nodes in a MHC-II dependent manner, when whole splenocytes from OT-IIFoxp3^{gfp} mice were used and B10-BR-OVA cells were injected into the footpad of the mice. Activated OT-II Tregs will be used in this system and thus we would analyze accumulation of these cells at popliteal lymph nodes. Therefore, when CD8 T cells are transferred with OT-II Tregs, it is expected that CD8 T cells would show robust killing of target cells. These experiments therefore will provide a better understanding of MHC-II dependent events in Treg function *in vivo*. Therefore, knowledge of the mechanisms of Treg mediated suppression in the context of tumor-induced tolerance could lead to strategies for potent anti-tumor immunotherapy.

VIII. Summary

Using an *in vitro* system we showed that Treg suppressed of CD8 T cell proliferation and effector molecule production in a MHC-II dependent manner. *In vivo*, MHC-II deficiency resulted in a delay in tumor progression that involved CD8 T cells. This delay in tumor progression provides a window of opportunity during which the mechanisms of tumor-induced tolerance could be investigated. We described two *in vivo* models in which the role of MHC-II dependent events in Treg mediated suppression could be tested. Additionally, our results showed that Treg accumulation in draining lymph nodes was MHC-II dependent. Since the biggest challenge in tumor immunotherapy is the immunosuppressive environment created by tumors, inhibiting MHC-II dependent events could prove to be beneficial in certain cancer patients. Specifically, targeting Treg activation and accumulation in TDLNs and the tumor microenvironment using MHC-II blocking antibodies could break tumor tolerance. This would subsequently lead to blocking Treg differentiation and suppressive functions. Thus, in these studies it is essential to further define the antigen specificity of Tregs in tumors. Specific MHC-II deletion of tolerogenic DCs in TDLNs and the tumor microenvironment could also lead to anti-tumor immunity. Furthermore, a combination of these novel targeting schemes with traditional immunotherapy regimes could be greatly beneficial. Therefore, the MHC-II pathway could be a potential target in designing therapies that seek to inactivate Tregs or tolerogenic DCs to enhance anti-tumor immunity.

IX. References

1. Abbas, A. K., and A. H. Lichtman. 2003. *Cellular and Molecular Immunoloty.* Saunders, Philadelphia
2. Hogquist, K. A., T. A. Baldwin, and S. C. Jameson. 2005. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 5:772-782.
3. Jones, L. A., L. T. Chin, D. L. Longo, and A. M. Kruisbeek. 1990. Peripheral clonal elimination of functional T cells. *Science* 250:1726-1729.
4. Rocha, B., and H. von Boehmer. 1991. Peripheral selection of the T cell repertoire. *Science* 251:1225-1228.
5. Walker, L. S., and A. K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol* 2:11-19.
6. Schwartz, R. H. 2003. T cell anergy. *Annu Rev Immunol* 21:305-334.
7. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531-562.
8. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.
9. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3:253-257.
10. Lohr, J., B. Knoechel, and A. K. Abbas. 2006. Regulatory T cells in the periphery. *Immunol Rev* 212:149-162.
11. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
12. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
13. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4:337-342.
14. Fontenot, J. D., and A. Y. Rudensky. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6:331-337.
15. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.
16. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in

CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172:5149-5153.

17. Wan, Y. Y., and R. A. Flavell. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* 102:5126-5131.
18. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329-341.
19. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10:1969-1980.
20. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287-296.
21. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 164:183-190.
22. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 6:338-344.
23. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.
24. Nakamura, K., A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* 172:834-842.
25. Oida, T., L. Xu, H. L. Weiner, A. Kitani, and W. Strober. 2006. TGF-beta-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. *J Immunol* 177:2331-2339.
26. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194:629-644.
27. Piccirillo, C. A., J. J. Letterio, A. M. Thornton, R. S. McHugh, M. Mamura, H. Mizuhara, and E. M. Shevach. 2002. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 196:237-246.
28. Niedbala, W., X. Q. Wei, B. Cai, A. J. Hueber, B. P. Leung, I. B. McInnes, and F. Y. Liew. 2007. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* 37:3021-3029.
29. Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450:566-569.
30. Tang, Q., and J. A. Bluestone. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 9:239-244.

31. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100:8886-8891.
32. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235-247.
33. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 198:249-258.
34. Li, M. O., and R. A. Flavell. 2008. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* 28:468-476.
35. Bluestone, J. A., and Q. Tang. 2005. How do CD4+CD25+ regulatory T cells control autoimmunity? *Curr Opin Immunol* 17:638-642.
36. Tarbell, K. V., L. Petit, X. Zuo, P. Toy, X. Luo, A. Mqadmi, H. Yang, M. Suthanthiran, S. Mojsov, and R. M. Steinman. 2007. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J Exp Med* 204:191-201.
37. Tarbell, K. V., S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman. 2004. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* 199:1467-1477.
38. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192:303-310.
39. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M. L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4:1206-1212.
40. Mellor, A. L., B. Baban, P. Chandler, B. Marshall, K. Jhaver, A. Hansen, P. A. Koni, M. Iwashima, and D. H. Munn. 2003. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J Immunol* 171:1652-1655.
41. Tadokoro, C. E., G. Shakhar, S. Shen, Y. Ding, A. C. Lino, A. Maraver, J. J. Lafaille, and M. L. Dustin. 2006. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 203:505-511.
42. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7:83-92.
43. Jensen, P. E. 2007. Recent advances in antigen processing and presentation. *Nat Immunol* 8:1041-1048.
44. Bevan, M. J. 2006. Cross-priming. *Nat Immunol* 7:363-365.
45. Belz, G. T., G. M. Behrens, C. M. Smith, J. F. Miller, C. Jones, K. Lejon, C. G. Fathman, S. N. Mueller, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J Exp Med* 196:1099-1104.

46. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192:1685-1696.
47. Rock, K. L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev* 207:166-183.
48. Shen, L., and K. L. Rock. 2006. Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Curr Opin Immunol* 18:85-91.
49. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* 199:9-26.
50. Cresswell, P., A. L. Ackerman, A. Giodini, D. R. Peaper, and P. A. Wearsch. 2005. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev* 207:145-157.
51. Trombetta, E. S., and I. Mellman. 2005. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* 23:975-1028.
52. Bryant, P., and H. Ploegh. 2004. Class II MHC peptide loading by the professionals. *Curr Opin Immunol* 16:96-102.
53. Robinson, J. H., and A. A. Delvig. 2002. Diversity in MHC class II antigen presentation. *Immunology* 105:252-262.
54. Villadangos, J. A., P. Schnorrer, and N. S. Wilson. 2005. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol Rev* 207:191-205.
55. Steinman, R. M., D. Hawiger, K. Liu, L. Bonifaz, D. Bonnyay, K. Mahnke, T. Iyoda, J. Ravetch, M. Dhodapkar, K. Inaba, and M. Nussenzweig. 2003. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann N Y Acad Sci* 987:15-25.
56. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* 196:1627-1638.
57. Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, and M. C. Nussenzweig. 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* 194:769-779.
58. Mahnke, K., Y. Qian, J. Knop, and A. H. Enk. 2003. Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 101:4862-4869.
59. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat Rev Immunol* 6:476-483.
60. Tan, J. K., and H. C. O'Neill. 2005. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J Leukoc Biol* 78:319-324.
61. Rutella, S., S. Danese, and G. Leone. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 108:1435-1440.

62. Rabinovich, G. A., D. Gabrilovich, and E. M. Sotomayor. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25:267-296.
63. Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky. 1998. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 95:1178-1183.
64. Overwijk, W. W., M. R. Theoret, S. E. Finkelstein, D. R. Surman, L. A. de Jong, F. A. Vyth-Dreese, T. A. Dellemijn, P. A. Antony, P. J. Spiess, D. C. Palmer, D. M. Heimann, C. A. Klebanoff, Z. Yu, L. N. Hwang, L. Feigenbaum, A. M. Kruisbeek, S. A. Rosenberg, and N. P. Restifo. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 198:569-580.
65. Cuenca, A., F. Cheng, H. Wang, J. Brayer, P. Horna, L. Gu, H. Bien, I. M. Borrello, H. I. Levitsky, and E. M. Sotomayor. 2003. Extra-lymphatic solid tumor growth is not immunologically ignored and results in early induction of antigen-specific T-cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Res* 63:9007-9015.
66. Sotomayor, E. M., I. Borrello, F. M. Rattis, A. G. Cuenca, J. Abrams, K. Staveley-O'Carroll, and H. I. Levitsky. 2001. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood* 98:1070-1077.
67. Zou, W., V. Machelon, A. Coulomb-L'Hermin, J. Borvak, F. Nome, T. Isaeva, S. Wei, R. Krzysiek, I. Durand-Gasselin, A. Gordon, T. Pustilnik, D. T. Curiel, P. Galanaud, F. Capron, D. Emilie, and T. J. Curiel. 2001. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med* 7:1339-1346.
68. Hartmann, E., B. Wollenberg, S. Rothenfusser, M. Wagner, D. Wellisch, B. Mack, T. Giese, O. Gires, S. Endres, and G. Hartmann. 2003. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res* 63:6478-6487.
69. Salio, M., M. Cella, W. Vermi, F. Facchetti, M. J. Palmowski, C. L. Smith, D. Shepherd, M. Colonna, and V. Cerundolo. 2003. Plasmacytoid dendritic cells prime IFN-gamma-secreting melanoma-specific CD8 lymphocytes and are found in primary melanoma lesions. *Eur J Immunol* 33:1052-1062.
70. Vermi, W., R. Bonecchi, F. Facchetti, D. Bianchi, S. Sozzani, S. Festa, A. Berenzi, M. Cella, and M. Colonna. 2003. Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. *J Pathol* 200:255-268.
71. Munn, D. H., and A. L. Mellor. 2004. IDO and tolerance to tumors. *Trends Mol Med* 10:15-18.
72. Munn, D. H., M. D. Sharma, D. Hou, B. Baban, J. R. Lee, S. J. Antonia, J. L. Messina, P. Chandler, P. A. Koni, and A. L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest* 114:280-290.

73. Viguier, M., F. Lemaitre, O. Verola, M. S. Cho, G. Gorochov, L. Dubertret, H. Bachelez, P. Kourilsky, and L. Ferradini. 2004. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 173:1444-1453.
74. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M. L. Disis, K. L. Knutson, L. Chen, and W. Zou. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942-949.
75. Woo, E. Y., H. Yeh, C. S. Chu, K. Schlienger, R. G. Carroll, J. L. Riley, L. R. Kaiser, and C. H. June. 2002. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 168:4272-4276.
76. Woo, E. Y., C. S. Chu, T. J. Goletz, K. Schlienger, H. Yeh, G. Coukos, S. C. Rubin, L. R. Kaiser, and C. H. June. 2001. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61:4766-4772.
77. Chattopadhyay, S., N. G. Chakraborty, and B. Mukherji. 2005. Regulatory T cells and tumor immunity. *Cancer Immunol Immunother* 54:1153-1161.
78. Munn, D. H., and A. L. Mellor. 2006. The tumor-draining lymph node as an immune-privileged site. *Immunol Rev* 213:146-158.
79. Sakaguchi, S., T. Takahashi, S. Yamazaki, Y. Kuniyasu, M. Itoh, N. Sakaguchi, and J. Shimizu. 2001. Immunologic self tolerance maintained by T-cell-mediated control of self-reactive T cells: implications for autoimmunity and tumor immunity. *Microbes Infect* 3:911-918.
80. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163:5211-5218.
81. Yu, P., Y. Lee, W. Liu, T. Krausz, A. Chong, H. Schreiber, and Y. X. Fu. 2005. Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 201:779-791.
82. Chen, M. L., M. J. Pittet, L. Gorelik, R. A. Flavell, R. Weissleder, H. von Boehmer, and K. Khazaie. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* 102:419-424.
83. Ercolini, A. M., B. H. Ladle, E. A. Manning, L. W. Pfannenstiel, T. D. Armstrong, J. P. Machiels, J. G. Bieler, L. A. Emens, R. T. Reilly, and E. M. Jaffee. 2005. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med* 201:1591-1602.
84. Antony, P. A., C. A. Piccirillo, A. Akpinarli, S. E. Finkelstein, P. J. Speiss, D. R. Surman, D. C. Palmer, C. C. Chan, C. A. Klebanoff, W. W. Overwijk, S. A. Rosenberg, and N. P. Restifo. 2005. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 174:2591-2601.

85. Shimoda, M., F. Mmanywa, S. K. Joshi, T. Li, K. Miyake, J. Pihkala, J. A. Abbas, and P. A. Koni. 2006. Conditional Ablation of MHC-II Suggests an Indirect Role for MHC-II in Regulatory CD4 T Cell Maintenance. *J Immunol* 176:6503-6511.
86. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2:301-306.
87. Bensinger, S. J., A. Bandeira, M. S. Jordan, A. J. Caton, and T. M. Laufer. 2001. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J Exp Med* 194:427-438.
88. Bienvenu, B., B. Martin, C. Auffray, C. Cordier, C. Becourt, and B. Lucas. 2005. Peripheral CD8+CD25+ T lymphocytes from MHC class II-deficient mice exhibit regulatory activity. *J Immunol* 175:246-253.
89. Bochtler, P., C. Wahl, R. Schirmbeck, and J. Reimann. 2006. Functional adaptive CD4 Foxp3 T cells develop in MHC class II-deficient mice. *J Immunol* 177:8307-8314.
90. Stephens, G. L., J. Andersson, and E. M. Shevach. 2007. Distinct subsets of FoxP3+ regulatory T cells participate in the control of immune responses. *J Immunol* 178:6901-6911.
91. Andersson, J., I. Stefanova, G. L. Stephens, and E. M. Shevach. 2007. CD4+CD25+ regulatory T cells are activated in vivo by recognition of self. *Int Immunol* 19:557-566.
92. Huang, C. T., C. J. Workman, D. Flies, X. Pan, A. L. Marson, G. Zhou, E. L. Hipkiss, S. Ravi, J. Kowalski, H. I. Levitsky, J. D. Powell, D. M. Pardoll, C. G. Drake, and D. A. Vignali. 2004. Role of LAG-3 in regulatory T cells. *Immunity* 21:503-513.
93. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *J Immunol* 180:5916-5926.
94. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol* 3:33-41.
95. Sauer, B. 1998. Inducible gene targeting in mice using the Cre/lox system. *Methods* 14:381-392.
96. Hashimoto, K., S. K. Joshi, and P. A. Koni. 2002. A conditional null allele of the major histocompatibility IA-beta chain gene. *Genesis* 32:152-153.
97. Koni, P. A., S. K. Joshi, U. A. Temann, D. Olson, L. Burkly, and R. A. Flavell. 2001. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J Exp Med* 193:741-754.
98. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.
99. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76:34-40.

100. Ehst, B. D., E. Ingulli, and M. K. Jenkins. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3:1355-1362.
101. Falo, L. D., Jr., M. Kovacsovics-Bankowski, K. Thompson, and K. L. Rock. 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. *Nat Med* 1:649-653.
102. Asselin-Paturel, C., G. Brizard, J. J. Pin, F. Briere, and G. Trinchieri. 2003. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* 171:6466-6477.
103. Tang, Q., K. J. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199:1455-1465.
104. Schuler, T., and T. Blankenstein. 2003. Cutting edge: CD8+ effector T cells reject tumors by direct antigen recognition but indirect action on host cells. *J Immunol* 170:4427-4431.
105. Liu, K., T. Iyoda, M. Saternus, Y. Kimura, K. Inaba, and R. M. Steinman. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med* 196:1091-1097.
106. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 171:131-137.
107. Mempel, T. R., M. J. Pittet, K. Khazaie, W. Weninger, R. Weissleder, H. von Boehmer, and U. H. von Andrian. 2006. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity* 25:129-141.
108. Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389-400.
109. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* 167:1137-1140.
110. Chai, J. G., J. Y. Tsang, R. Lechler, E. Simpson, J. Dyson, and D. Scott. 2002. CD4+CD25+ T cells as immunoregulatory T cells in vitro. *Eur J Immunol* 32:2365-2375.
111. Klein, L., J. Emmerich, L. d'Cruz, K. Aschenbrenner, and K. Khazaie. 2005. Selection and behavior of CD4+ CD25+ T cells in vivo: lessons from T cell receptor transgenic models. *Curr Top Microbiol Immunol* 293:73-87.
112. Kawahata, K., Y. Misaki, M. Yamauchi, S. Tsunekawa, K. Setoguchi, J. Miyazaki, and K. Yamamoto. 2002. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J Immunol* 168:4399-4405.
113. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 3:756-763.
114. Kretschmer, K., I. Apostolou, E. Jaeckel, K. Khazaie, and H. von Boehmer. 2006. Making regulatory T cells with defined antigen specificity: role in autoimmunity and cancer. *Immunol Rev* 212:163-169.

115. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162:5317-5326.
116. Clarke, S. R., M. Barnden, C. Kurts, F. R. Carbone, J. F. Miller, and W. R. Heath. 2000. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunol Cell Biol* 78:110-117.
117. Singh, N., Y. Seki, M. Takami, B. Baban, P. R. Chandler, D. Khosravi, X. Zheng, M. Takezaki, J. R. Lee, A. L. Mellor, W. B. Bollag, and M. Iwashima. 2006. Enrichment of regulatory CD4(+)CD25(+) T cells by inhibition of phospholipase D signaling. *Nat Methods* 3:629-636.
118. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743-4748.
119. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203:1701-1711.
120. Tuovinen, H., J. T. Salminen, and T. P. Arstila. 2006. Most human thymic and peripheral-blood CD4+ CD25+ regulatory T cells express 2 T-cell receptors. *Blood* 108:4063-4070.
121. Zhou, G., and H. I. Levitsky. 2007. Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance. *J Immunol* 178:2155-2162.
122. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27:635-646.
123. Masopust, D., V. Vezys, E. J. Wherry, and R. Ahmed. 2007. A brief history of CD8 T cells. *Eur J Immunol* 37 Suppl 1:S103-110.
124. Dalyot-Herman, N., O. F. Bathe, and T. R. Malek. 2000. Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed APC. *J Immunol* 165:6731-6737.
125. Antony, P. A., C. M. Paulos, M. Ahmadzadeh, A. Akpinarli, D. C. Palmer, N. Sato, A. Kaiser, C. Heinrichs, C. A. Klebanoff, Y. Tagaya, and N. P. Restifo. 2006. Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol* 176:5255-5266.
126. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R. F. Wang. 2005. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* 309:1380-1384.
127. den Boer, A. T., G. J. van Mierlo, M. F. Fransen, C. J. Melief, R. Offringa, and R. E. Toes. 2005. CD4+ T cells are able to promote tumor growth through inhibition of tumor-specific CD8+ T-cell responses in tumor-bearing hosts. *Cancer Res* 65:6984-6989.
128. Schurmans, L. R., L. Diehl, A. T. den Boer, R. P. Sutmuller, Z. F. Boonman, J. P. Medema, E. I. van der Voort, J. Laman, C. J. Melief, M. J. Jager, and R. E. Toes.

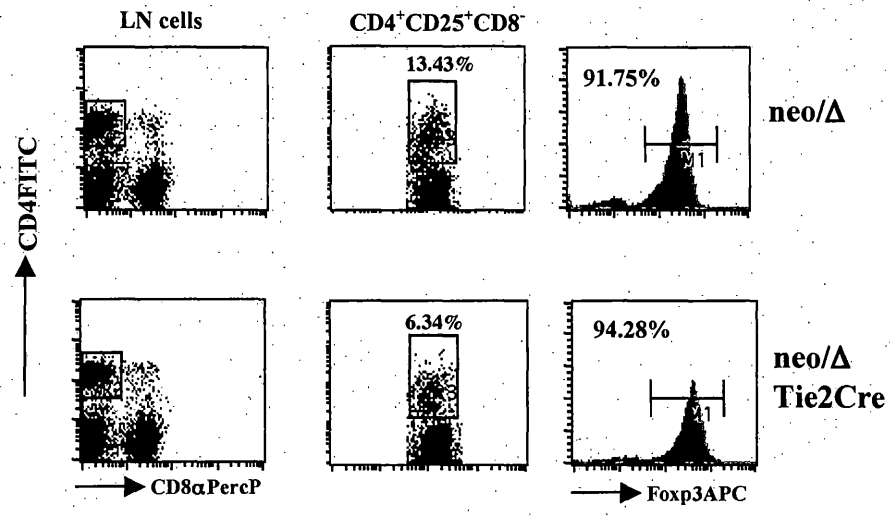
2001. Rejection of intraocular tumors by CD4(+) T cells without induction of phthisis. *J Immunol* 167:5832-5837.

129. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The three Es of cancer immunoediting. *Annu Rev Immunol* 22:329-360.
130. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu Rev Immunol* 25:171-192.
131. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421:852-856.
132. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337-339.
133. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300:339-342.
134. Wang, J. C., and A. M. Livingstone. 2003. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. *J Immunol* 171:6339-6343.
135. Gerner, M. Y., K. A. Casey, and M. F. Mescher. 2008. Defective MHC class II presentation by dendritic cells limits CD4 T cell help for antitumor CD8 T cell responses. *J Immunol* 181:155-164.
136. Kennedy, R., and E. Celis. 2008. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol Rev* 222:129-144.
137. Marzo, A. L., B. F. Kinnear, R. A. Lake, J. J. Frelinger, E. J. Collins, B. W. Robinson, and B. Scott. 2000. Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J Immunol* 165:6047-6055.
138. Stranges, P. B., J. Watson, C. J. Cooper, C. M. Choisy-Rossi, A. C. Stonebraker, R. A. Beighton, H. Hartig, J. P. Sundberg, S. Servick, G. Kaufmann, P. J. Fink, and A. V. Chervonsky. 2007. Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. *Immunity* 26:629-641.
139. Shimoda, M., T. Li, J. P. Pihkala, and P. A. Koni. 2006. Role of MHC class II on memory B cells in post-germinal center B cell homeostasis and memory response. *J Immunol* 176:2122-2133.

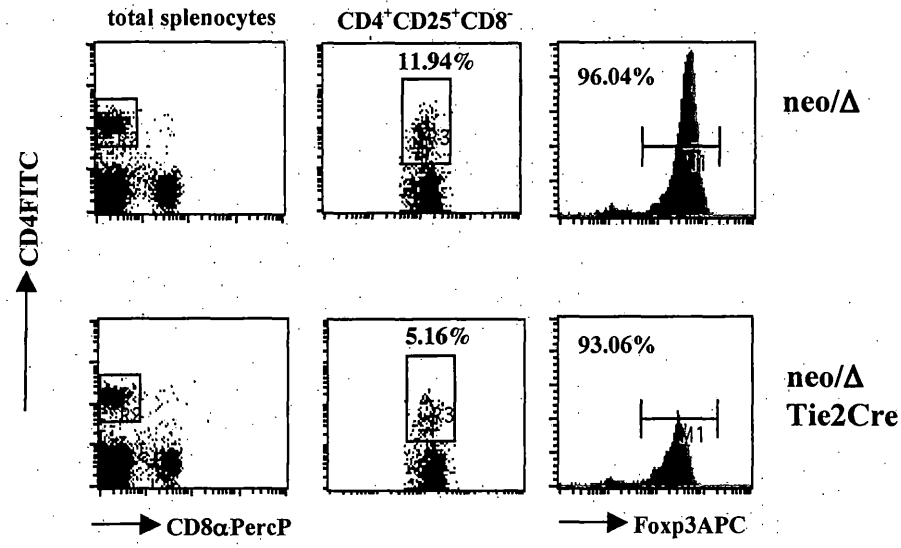
X. Appendix

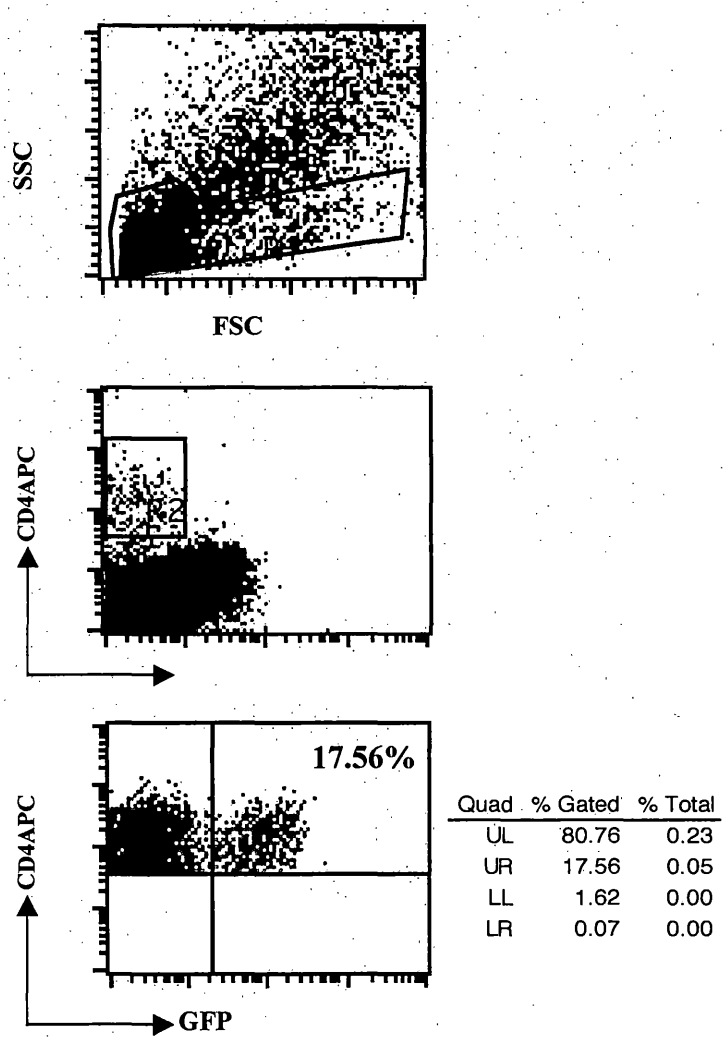
Appendix Figure 1: $CD4^+Foxp3^+$ Tregs are present in $ia\beta^{neo/\Delta}$ $TIE2Cre^+$ mice. Spleen and lymph nodes from 8 week-old $ia\beta^{neo/\Delta}$ $TIE2Cre^+$ mice and their wild type littermates were isolated and prepared as described in Materials and Methods section. Cells where stained for CD4FITC (1:500), CD25PE (1:250) and CD8αPercP (1:250), fixed and the next day intracellularly stained with Foxp3APC (1:100).

A. Lymph nodes

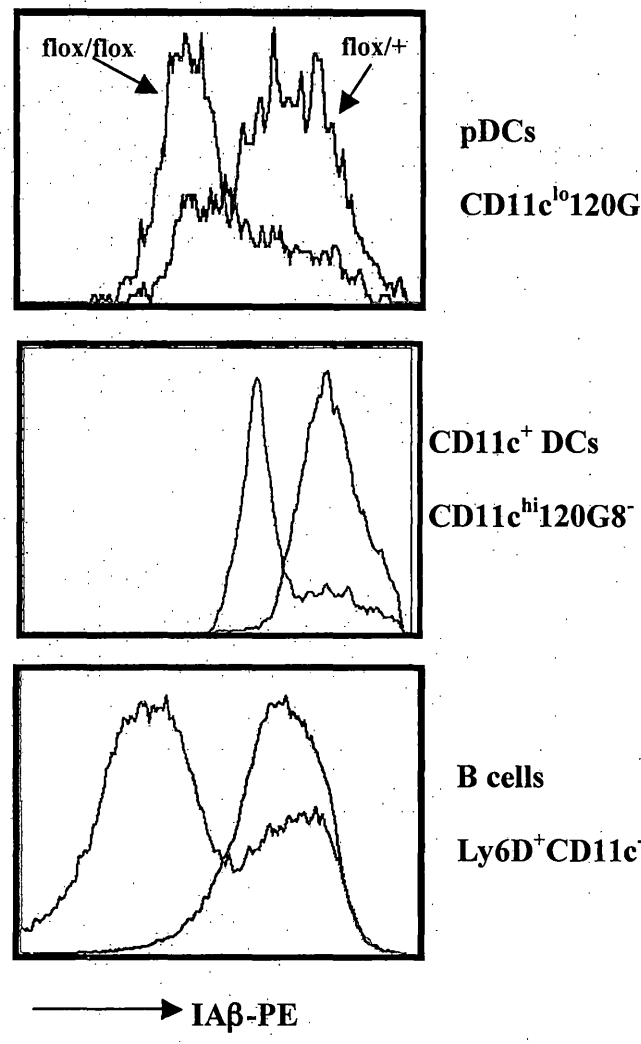


B. Spleen



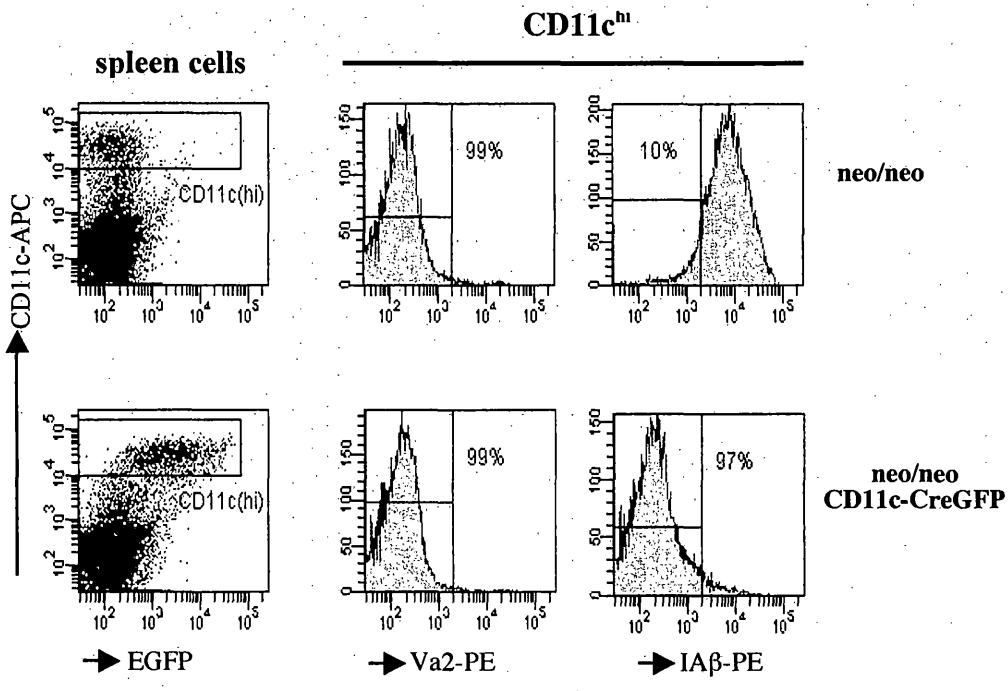


Appendix Figure 2: $CD4^+Foxp3^+$ Tregs are present in the tumor mass of B16F10 tumors. $Foxp3^{gfp}$ mice were subcutaneously injected with $5x10^5$ B16F10 tumors into the right abdominal flank area. Ten days later the tumor was isolated and digested as described in the Materials and Methods section. After the digestion tumor cells were stained with CD4APC (1:500). The third dot plot displays cells gated from R1 + R2. Cells were analyzed on a FACS calibur.



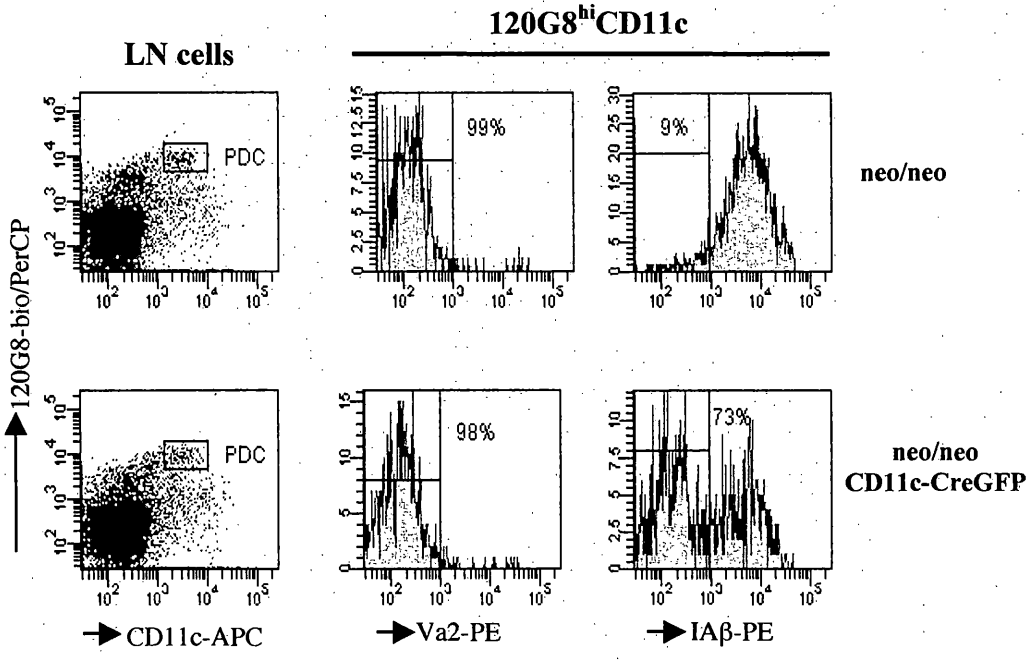
Appendix Figure 3: MHC-II deletion in iaβ $^{flox/flox}$Ly6c-Cre mice. Spleens from iaβ $^{flox/flox}$Ly6c-Cre (KO) and iaβ $^{flox/+}$Ly6c-Cre (WT) mice were isolated and DNase and collagenase treated for 30 minutes at 37°C. Cells were then treated as described in Materials and Methods. Cells were stained with 120G8Bio (1:500) / Ly6DBio (1:5000) / StreptAPC(1:500), CD11cFITC (1:250) and IAβPE (1:1000) and analyzed on a FACS Calibur. The histogram depicts MHC-II expression of knock out mice (green line) and wild type mice (red line).

A



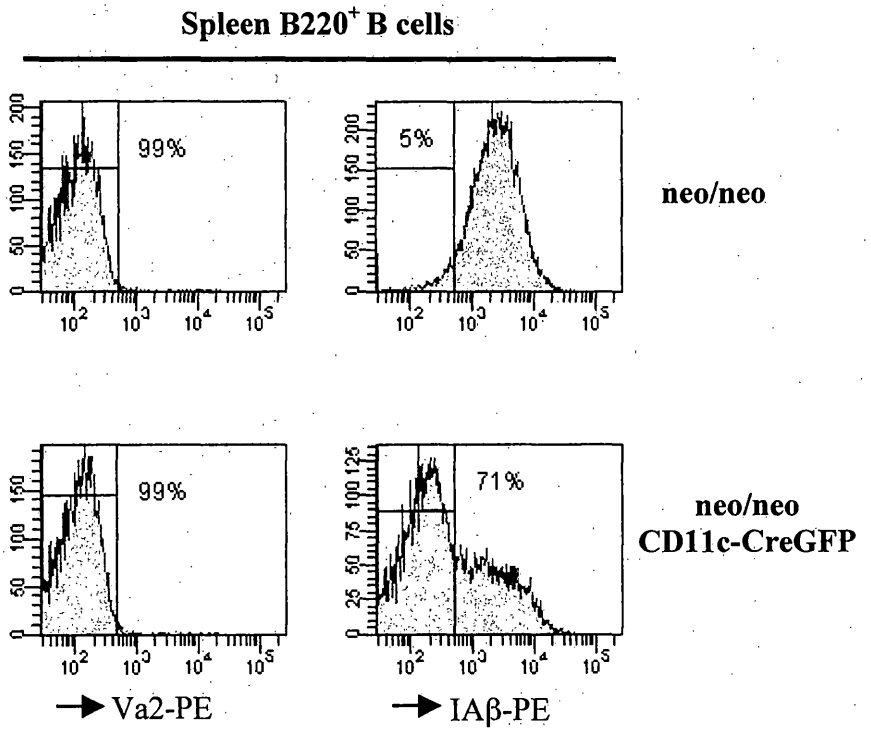
Appendix Figure 4a: Loss of MHC-II on virtually all CD11c$^+$ DCs from ia$\beta^{neo/neo}$CD11cCreGFP mice. Spleens from aged matched ia$\beta^{neo/neo}$CD11cCreGFP mice and wild type littermates were isolated and prepared as described in Materials and Methods. Cells were stained with 120G8Bio (1:500) / StreptPE (1:500), CD11cAPC (1:250), CD8αPerCP (1:250) and IAβPE (1:1000), and Vα2PE (1:1000) was used as an isotype control. Cells were analyzed on a FACS Canto. Data were obtained in collaboration with Dr. Pandelakis Koni.

B



Appendix Figure 4b: Incomplete deletion of MHC-II on lymphoid pDCs from ia$\beta^{neo/neo}$CD11cCreGFP mice. Lymph node cells were prepared and stained as in Figure 36a and analyzed on a FACS Canto. Data were obtained in collaboration with Dr. Pandelakis Koni.

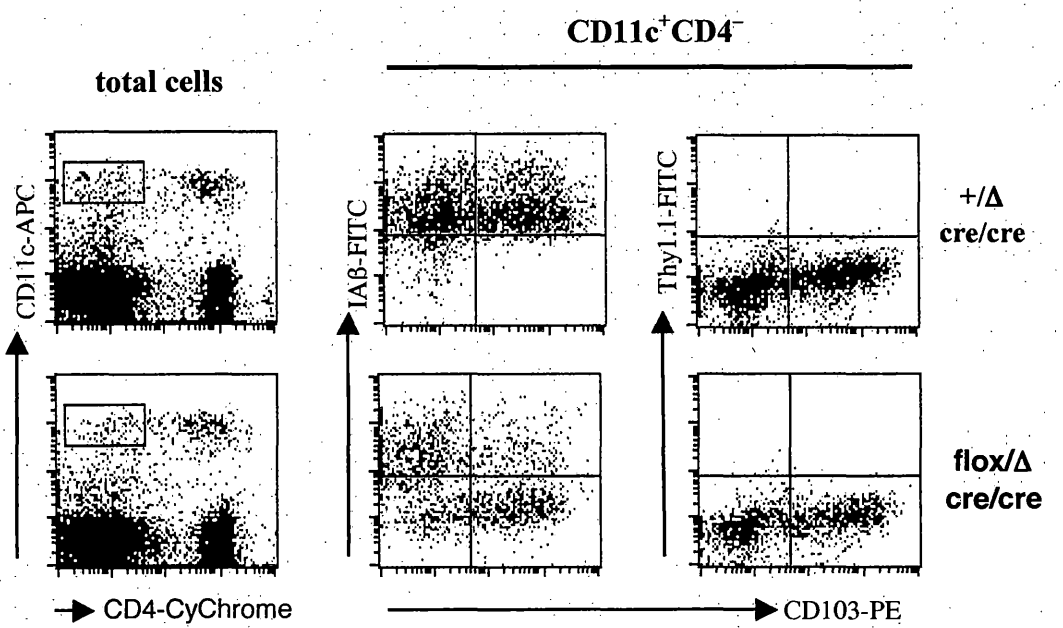
C

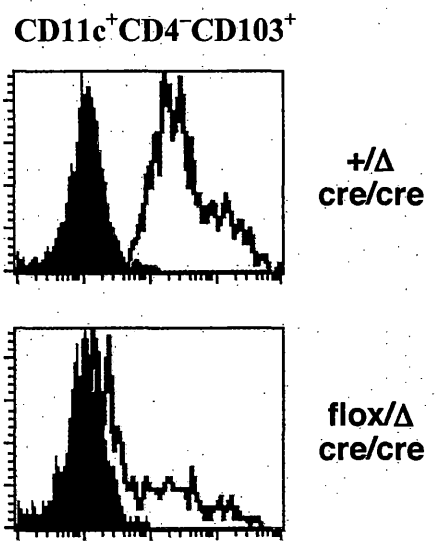


Appendix Figure 4c: Substantial loss of MHC-II B cells from ia$\beta^{neo/neo}$CD11cCreGFP mice. Spleen cells from Figure 36a were also analyzed for B cells. Data were obtained in collaboration with Dr. Pandelakis Koni.

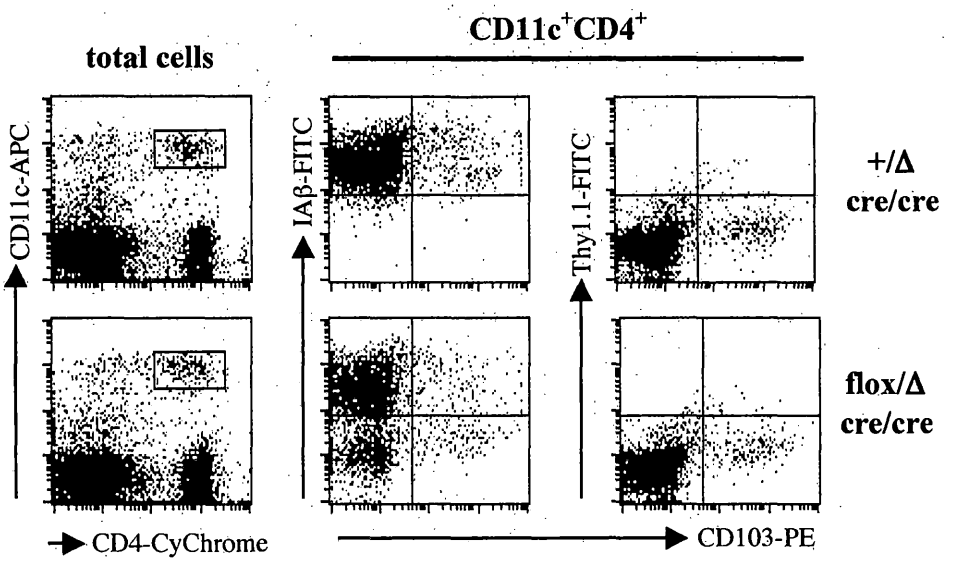
Appendix Figure 5a: Substantial but incomplete loss of MHC-II from CD8α$^+$ DCs. Spleens from age-matched CD8α-CreGFP knockin mice and wild type littermates were isolated and prepared as described in Materials and Methods. Cells were stained with CD11cAPC, CD4cychrome, CD103PE and IAβFITC and Thy1.1FITC was used as an isotype control. The CD8α^{cre} allele caused low expression of CD8α, therefore CD8α$^+$ DCs were defined using CD103 instead as most of these cells are CD8α$^+$. There was a 70% loss of MHC-II on CD11c$^+$CD103$^+$CD4$^-$ DCs. Data were obtained in collaboration with Dr. Pandelakis Koni.

A

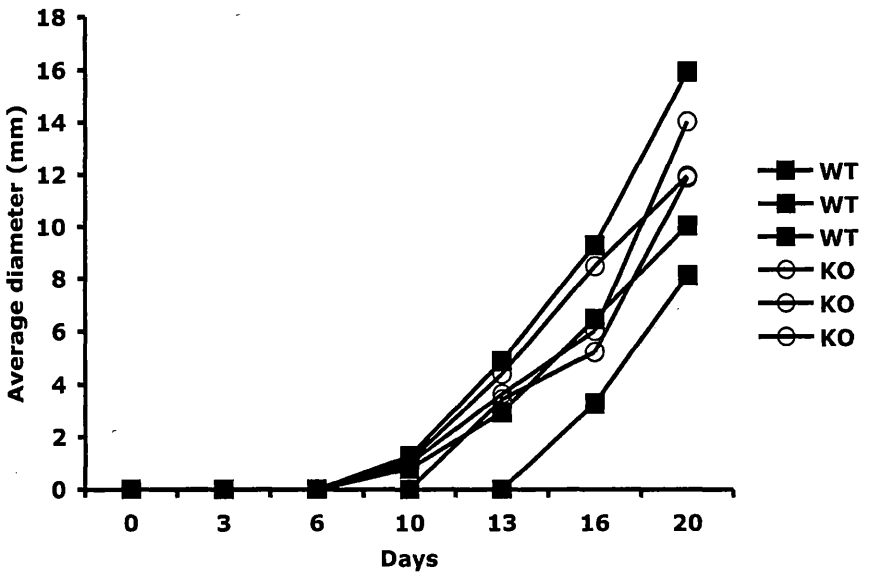

CD11c+CD4−
total cells
CD11c-APC
IAβ-FITC
Thy1.1-FITC
+/Δ
cre/cre
flox/Δ
cre/cre
CD4-CyChrome
CD103-PE


CD11c+CD4−CD103+
+/Δ
cre/cre
flox/Δ
cre/cre

B



Appendix Figure 5b: The CD8α^{cre} allele causes loss of MHC-II from about one-third of CD4$^+$ DCs. Cells from Figure 33a were also analyzed for CD4$^+$ DCs. About 20% of CD11c$^+$ CD4$^-$CD103$^-$ DCs showed loss of MHC-II. Data were obtained in collaboration with Dr. Pandelakis Koni.



Appendix Figure 6: MHC-II deficiency on B cells did not cause a delay in tumor progression. ia$\beta^{neo/neo}$CD19Cre/+ mice (KO) and their wild type littermates (WT) were injected with 5x10^5 B16-OVA cells subcutaneously into the abdominal flank area. Tumor growth was monitored for 20 days. The tumor size was taken as the average of the perpendicular and vertical diameters. ia$\beta^{neo/neo}$CD19Cre/+ mice and their wild type littermates(139) were provided by Dr. Shimoda.