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DENDRITIC CELL REGULATION OF PERIPHERAL TOLERANCE IN POLYCLONAL T CELL REPERTOIRES

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Revati F. Masilamani

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DENDRITIC CELL REGULATION OF PERIPHERAL TOLERANCE IN POLYCLONAL T CELL REPERTOIRES

Revati F. Masilamani, Ph.D.
The Rockefeller University 2008

Dendritic cells (DCs) play a pivotal role in determining whether the outcome of the immune system's encounter with antigen will be immunity or tolerance. Using an antibody against the DEC-205 receptor, antigens have been delivered specifically to DCs *in vivo*. Under steady state conditions, such presentation of antigen leads to peripheral tolerance in transgenic T cells, either by deletion, anergy or the induction of regulatory T cells. We wanted to examine whether delivery of autoantigens to DCs using this approach, could be used to tolerize autoreactive polyclonal T cells, thereby preventing autoimmunity in mouse models. We succeeded in inducing tolerance to the myelin oligodendrocyte protein and preventing disease in the experimental autoimmune encephalomyelitis model. However no halt or delay in onset of autoimmune diabetes was observed when insulin was targeted to DCs in the non-obese diabetic (NOD) mouse model. Also, foreign antigen-specific T cell responses could not be abolished by targeting antigen to DCs in the NOD mouse. These results strongly suggested that establishing peripheral tolerance in disease-prone polyclonal repertoires such

as in the NOD model, would be far more challenging than the previously studied tolerance in non-autoreactive transgenic models had been. We recognized that success in DC-targeting-based autoimmune therapy would first require a better understanding of tolerance in non-disease prone polyclonal T cell repertoires. Towards that end, we examined T cell tolerance in C57BL/6 mice. In the steady state, targeting ovalbumin (OVA) to DCs resulted in polyclonal CD4 and CD8 T cell tolerance. This tolerance was non-deletional and characterized by persistence of T cells that produced IFNγ, but no IL-2. CD4 dependent antibody production by B cells *in vivo* was abrogated. Also, both CD4 and CD8 proliferative responses *in vitro* were abolished. Subsequent to tolerization, depending on the strength of the costimulatory stimulus that the CD4 and CD8 T cells are exposed to, tolerance can be reversed both *in vivo* and *in vitro*. Thus our results demonstrate that while tolerance in non-autoreactive polyclonal repertoires in steady state DC environments can be achieved, reversal of the tolerized state can also occur. This suggests that in autoreactive T cell repertoires in chronically inflamed DC environments, the prevention or treatment of autoimmune disease is a challenge that will require comprehensive understanding of the balance between immunity and tolerance.

I dedicate this thesis to my grandmother who gave me my first lessons in atomic chemistry, who taught me the nature of light and who answered my first questions on what a cell is. She inspired me, challenged me, and always believed in me. And to my parents for their love and their wisdom, and for being there for me for every hill and mountain I climbed.

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Chapter 1

Introduction

Central Tolerance

The immune system has evolved to recognize foreign antigen from self antigen so that it can respond to invaders and protect the host, while remaining tolerant to self. The continual ability to make this distinction is fundamentally important in avoiding self-destructive immune responses and was recognized as such by Ehrlich and Morgenroth at the turn of the last century (1). They referred to this as horror autotoxicus. Owen and Hasek (2, 3) demonstrated that encounter with foreign tissue during embryonic development leads to life-long tolerance against the antigens expressed in the tissue. These results were extended by Billingham and Medawar, when they demonstrated acquired tolerance to foreign blood cells and skin grafts in animals that had been exposed during neonatal development to tissues from the donor(4). These results were interpreted to mean that the developing immune system was malleable and if a foreign subtsance was introduced early enough, it would induce tolerance rather than immunity. Burnet, in 1957, synthesized these experimental findings into the theory of clonal selection, which postulated that lymphocytes with receptor

specificities against antigens present during embryonic development, would be selectively eliminated by a process of deletion. To this day Burnet's theory forms the fundamental framework for understanding how immunological tolerance is induced.

The clonal selection theory has been directly validated by studies that observed the deletion of T cells in the thymus, bearing T cell receptors that recognize endogenous antigens in the context of the Major Histocompatibility (MHC) Class I and II molecules (5-7). Immature autoreactive thymocytes enounter their cognate antigen during development and get deleted by apoptotic cell death (8) that ensures the absence of these clones from the mature repertoire that enters the periphery from the thymus. This process, referred to as negative selection, was thought to be mediated by bone marrow- derived antigen presenting cells (APCs) (9, 10), likely thymic dendritic cells (DCs). Subsequently thymic medullary DCs were shown to mediate thymic deletion both *in vitro* (11) and *in vivo* (12).

Central tolerance to self-antigens has to account for two categories of antigens: those that are ubiquitously expressed and those that have tissue specific distribution (13). It has been reported that many tissue specific

antigens are expressed promiscuously (14, 15) in medullary thymic epithelial cells (mTECs) under the regulation of AIRE (autoimmune regulator), a gene identified from patients with a rare autosomal recessive disorder known as autoimmune polyglandular syndrome type 1 (APS-1). Affected patients develop spontaneous autoimmune disease targeted pimarily at endocrine organs (16). In mice deficient in AIRE expression in mTECs, the expression of a subset of tissue specific antigens is abrogated, resulting in the failure of negative selection in the thymus (17-20).Studies suggest that in addition to AIRE there are other, as yet unknown factors that direct expression of tissue specific antigens in mTECs and are thus involved in central tolerance (18). It has been shown that mTECs can directly mediate negative selection of CD8 T cells against tissue specific antigens by presentation on their MHC Class I molecules. Thymic DCs on the other hand can acquire tissue specific antigens from mTECs either by uptake of apoptotic cells, or nibbling of surface membranes of live cells, then process for MHC class I and class II presentation, and delete both CD8 and CD4 T cells (21).

Negative selection in the thymus, while presenting a sophisticated and coherent model for self tolerance, cannot explain every instance of tolerance

encountered through the life of the animal. For instance, some self antigens are only expressed in the body after the stage of early T cell development in the thymus (22), and most innocuous environmental antigens that are tolerated are also unavailable for antigen presentation during negative selection. Additionally, it has been shown that some low-affinity autoreactive T cells escape thymic negative selection because the strength of signal transduction upon TCR engagment by the cognate antigen-MHC complex is below the threshold required for induction of apoptosis (23).The fact that despite these limitations of thymic negative selection, and the documented presence of autoreactive T cells in the periphery (24, 25) most individuals do not succumb to autoimmunity, suggested the existence of extrathymic mechanisms to enforce tolerance.

Peripheral Tolerance

It was observed that mature T cells could be tolerized to non-self antigens that were either injected (26, 27) or expressed as endogenous antigens (28) independent of thymic selection. This form of extrathymic tolerance was called peripheral tolerance and both CD4 and CD8 T cells were shown to be potential targets of this form of tolerance (27, 29, 30). Peripheral tolerance

of T cells was shown to abrogate both T-helper dependent antibody production (31) as well as autoimmune disease (29, 32).

The initial challenge to the study of peripheral tolerance was how to exclude the involvement of thymic mechanisms in the analysis of tolerance induction. An early solution to the problem, was the use of antibodies that blocked the presentation of antigens on endogenous MHC molecules during thymic development (33). Another approach involved the transfer of H-Y specific TCR transgenic T cells into male recipients that express the H-Y antigen. Transferred T cells intially proliferated in response to the H-Y antigen in the periphery, but were later subjected to extrathymic deletion and anergy (28). Similar results were obtained in models that studied T cell response to novel, extrathymic antigens, transgenically expressed under tissue specific promoters (34-36). While T cell production and survival in the thymus was unchanged in these experiments, autoreactive clones in the periphery were deleted, thus making a strong case for a mode of tolerance that was solely regulated in the periphery.

That there is an intricate regulation of peripheral tolerance, was underscored by studies where the transgenic expression of novel proteins in the periphery

did not result in tolerance (37, 38) and sometimes even led to autoimmunity (39). It was observed that newly made peripheral proteins were ignored by antigen- specific T cells, but could cause autoimmunity if their expression was concurrent with viral infection or ectopic expression of IL-2 (40-42). A significant observation was that the expression levels of novel proteins could influence the fate of antigen specific T cells. If a protein was expressed at high levels, it favored the deletion of T cells (43-46). All these findings point to an extremely delicate balance between the regulation of tolerance and immunity that depends on multiple checkpoints. Antigen presenting cells as the initiators of the cascade that leads to an immune response are therefore at the apex of this regulation.

Dendritic Cells in Immunity and Tolerance

As antigen presenting cells, dendritic cells (DCs) have been revealed in the last two decades to be critical to the balance between tolerance and immunity. Soon after their discovery (47-49), dendritic cells, so called because of their long astral processes, were shown to be potent stimulators of T cells. They were able to initiate primary immune responses (50-53), induce polarization in T cells, to generate Th1 and Th2 responses (54), and most recently Th17 responses (55) and were used successfully for

therapeutic immune reponses in human patients (56). DCs efficiently process peptides from soluble antigens (57) and also present them on both class I and class II complexes *in vitro* (57, 58) and *ex vivo* (59). DCs also uptake dying cells and process and present their antigens to T cells (60, 61). Recently in work described below DCs have been targeted *in vivo* with Class I and Class II antigens, for processing and presentation to CD4 and CD8 T cells (62-64).

DCs are found throughout the body so they come into contact with antigens in tissues all over. They are present in skin (65), the airways (66), blood (67), lymphatics (68), lymphoid organs (48) and interstitial parenchymal spaces (69). Dendritic cells are an ephemeral population with a life-span of a few days (49, 70), however they are highly mobile and migrate upon antigen enounter to lymphoid organs (71). Also it was recently shown that DCs divide in situ in peripheral tissues, and peptide-MHC complexes are transferred to the daughter DCs (72).

DCs use several types of receptors for antigen uptake such as Fc receptors of Type I, II (73) and III (74), complement receptors (75), scavenger receptors (76) and C-type lectin receptors (77, 78). In recent years, the C-type lectin

receptors have been targeted by genetically engineered antibodies carrying antigens for specific delivery to DCs. The first lectin receptor to be targeted was the DEC-205 receptor, which has an unusual trafficking pathway that passes through late endosomes leading to effective antigen presentation (79). Subsequently the DC-SIGN receptor (80), DCIR2 (63), and Langerin (81), of the lectin family of receptors, have been targeted for antigen delivery to the DCs.

The initiation of immunity by DCs is regulated by the presence of costimulatory, activating molecules and receptors on their surface as well as the induction of cytokines. Some of the important costimulatory molecules are Ig family members such as CD80 and CD86 (82), B7-DC (83), PD-L1 (84), PD-L2 (85) and TNF family member CD30 (86). These molecules form ligand-receptor pairs with their counterparts on T cells such as CD28 (87), CTLA-4 (88) and PD-1 (84). DCs express several classes of receptors such as Toll-like receptors (TLRs), Tumor Necrosis Factor family receptors (TNF-Rs) and cytokine receptors. The TLRs are involved in the recognition of signals associated with infection and tissue damage, and are therefore seen as a link between innate and adaptive immunity. The binding of their pathogenic ligands such as lipopolysaccharide (89, 90) peptidoglycans, CpG

(91) and others, lead to a cascade of downstream signaling resulting in the activation of transcription factors such as NF-κB involved in survival, differentiation and inflammation responses (92). In addition to their role in antigen uptake, Fc receptors can also trigger both activatory and inhibitory signaling cascades (93-95). CD40 is an important TNF family receptor that is involved in feedback activation of DCs by CD4 helper cells that upregulate CD40L after intial activation (96, 97). Upon activation DCs upregulate the production of pro-immune cytokines such as IL-12 (75) and IFNα (98). The concerted involvement of all these DC activation pathways, leads to the activation of T cells and consequent immunity.

The role for DCs in tolerance was demonstrated by the targeting of antigens to the DEC-205 receptor in the absence of adjuvant (64). An antibody against the DEC-receptor expressed mainly on CD8 DCs, was cloned and fused to a peptide from hen egg lysozyme (HEL) (αDEC/HEL). When DCs were targeted *in vivo* with this construct in the absence of adjuvant, they presented antigen to HEL-specific T cells and the consequence of this steady state presentation was the deletion of the T cells and ultimately tolerance against future challenge with HEL. In contrast if the αDEC/HEL was delivered along with an inflammatory stimulus, the consequence of antigen

presentation to T cells was persistent activation and immunity. The model that emerged from this paradigmatic study was that the context in which DCs *in vivo* present antigen to T cells, governs the fate of the T cells. Under normal steady state conditions, defined as the absence of inflammation or infection, the presentation of antigen by DCs leads to T cell tolerance. In contrast, under conditions of inflammation and tissue injury, the DCs acquire an activated phentotype and the presentation of antigen to T cells in this context leads to immunity against the antigen (99).

Recently the DCIR2 molecule on the CD8- subset of DCs was targeted for antigen delivery by a monoclonal antibody and the consequence of such targeting was tolerance. Using transgenic T cells against OVA, the presentation efficiencies of the DEC-205 and DCIR2 receptors was compared. It was demonstrated that the DCIR2 targeting strategy leads to superior Class II presentation, while the DEC-205 targeting strategy leads to better Class I presentation of antigen to T cells (63).

Mechanisms of Peripheral Tolerance

DCs can induce tolerance in T cells by several different mechanisms. T cell ignorance defined as the lack of antigen recognition was observed in several

cases of endogenously expressed novel antigens (40, 41) and was postulated to be responsible for some forms of tolerance. However as a mechanism it is a very limited explanation for the life-long tolerance against a wide-range of self and harmless non-self antigens that an animal constantly encounters.

Deletion is a characteristic mechanism of peripheral tolerance observed among responding T cells (28, 33, 35, 45, 46). A characteristic feature of such deletion is an initial clonal expansion, preceeding the disappearance of autoreactive T cells. Peripheral deletion has some resemblance to activation induced cell death, an important pathway to terminate immune responses. There have been examples of Fas induced death of T cells in tolerance (100) and TNF involvement (101), but other mechanisms of deletion may exist that are yet to be discovered.

Anergy as a form of tolerance involves a state of unresponsiveness of T cells, that otherwise remain viable and persist for long periods of time in an organism (102). Initially anergy that resulted from incomplete signaling to the T cells was reported. In such cases anergy was maintained by a block in the Ras/MAP kinase pathway, could be reversed by IL-2 or anti-OX40

signaling, and usually did not result in the inhibition of effector functions (82, 103, 104).

Other forms of anergy have now been shown that require complete initial activation of the T cells by stimulatory CD28 and inhibitory CTLA-4 signaling (105). We have shown that upon antigen targeting to steady state DCs, transgenic T cells against the myelin oligodendrocyte protein were rendered anergic. This anergy required initial activation of T cells and was characterized by the upregulation of CD5. Upon blocking with αCD5 antibody, we could restore antigen-specific responsiveness of the T cells *in vivo*. Thus CD5 in this model, acts as an inhibitor of T cell signaling (106). CD5 is a glycoprotein that acts as both a positive and negative regulator of T cell activation (107-111). The intracellular domain of CD5 contains sequences resembling both an immunoreceptor tyrosine-based activatory motif (ITAM) and an inhibitory motif (ITIM), that may explain the dual nature of CD5 function(112).

One of the pathways of anergic tolerance involves an early block in tyrosine kinase activation, which predominantly inhibits calcium mobilization, and an independent mechanism that blocks signaling through the IL-2 receptor

(102). The reduction in IL-2 levels has been shown to be an important element in antigen-specific anergy, and results in an anti-proliferative state of the T cells (113-115). Thus anergy has several pathways, and is an important mechanism for tolerance in the periphery.

Regulatory T cells (T regs) have become an intensely studied subset of T cells in recent years. It was observed that early thymectomy of mice causes general autoimmune disease (116). This observation was explained by an apparent decrease of CD4+CD25+ cells in thymectomized animals, which was later confirmed by successful prevention of autoimmunity by transfer of syngenic CD4+CD25+ cells (116). *In vitro*, CD4+CD25+ cells were shown to suppress proliferation of other cells by IL-2 production in a cell contact-dependent manner that also required TCR activation of the CD4+CD25+ cells. Recently it has been shown that antigen-loaded mature DCs can induce expansion of CD4+CD25+ T cells *in vitro*, and that these cells can actively suppress CD4+CD25- proliferation in response to splenic APCs (117). This approach was then used to expand CD4+CD25+ transgenic T cells from a non-obese diabetic strain to prevent and treat autoimmune diabetes (118, 119). CD4+CD25+ suppressors specifically express the transcription factor Foxp3, which regulates their development and

function(120). Mutations in Foxp3 lead to widespread autoimmunity (120-122). It was shown that antigen targeting to the DCs in minute doses in steady state *in vivo*, led to the conversion of CD4+CD25- effector cells into CD4+ CD25+ Foxp3+ T cells (123). These cells require IL-2 for their maintenance and activation and the lack of IL-2 results in reduced numbers of these cells *in vivo* (124-126). In contrast to the requirement of cell contact for *in vitro* suppression, there are many reports that indicate that cytokines such as IL-10 and TGF-β are needed *in vivo* for mediating suppression or conditioning a suppressive milieu (127, 128). Another subset of T regs is made under tolerogenic conditions and produces IL-10 which has been shown to have suppressive function *in vivo* in conditions of inflammation (129). T regs are therefore an important component of the regulation of T cell tolerance *in vivo*.

Thus the establishment and maintenance of tolerance in the periphery is intricately regulated by the interdependence of DCs and T cells and their relationships with other cells of the immune system.

Dendritic Cells and Polyclonal T cell Tolerance:

Questions and an Outline of Experimental Systems

To study peripheral tolerance DCs were targeted by antigen using an αDEC-205 antibody, and transgenic T cell models were used to study the outcome of such presentation of antigen. It was observed that under steady state, presentation of antigens to the T cells led to T cell tolerance and depending on the model studied, tolerance was achieved by deletion (62, 64), anergy (106), or the induction of regulatory T cells (123). Based on these studies, we decided to examine whether such targeting of antigen could lead to T cell tolerance in an autoimmune disease model and whether such tolerance would result in prevention and treatment of the disease.

Autoimmunity is the breakdown of tolerance against self, and subsequent tissue destruction by autoreactive T cells and B cells. While genetic pre-disposition to autoimmunity leads to the selection of autoreactive T cells during thymic selection, peripheral tolerance check points also break down in individuals that are autoimmune. Thus the induction of peripheral tolerance by a strategy such as the one described above would help to keep in check autoreactive T cells in the periphery.

The animal models we chose for these studies, were the experimental autoimmune Encephalomyelitis model (EAE) (130, 131), an induced, acute mouse model for mutiple sclerosis, and the Non-Obese-Diabetic Mouse (NOD) (132), which is a spontaneous, chronic model for autoimmune Type 1 diabetes. The myelin oligodendrocyte peptide (MOG) (35-55) was the autoantigen against which tolerance was examined in the EAE model. In the NOD model, the 9-23 peptide from the β chain of insulin was used for targeting therapy.

Our results showed that targeting of MOG antigen to the DCs in the EAE model leads to tolerance and prevention of progression to disease. In the NOD model, however chronic targeting of insulin could not prevent development of disease. The difference in therapy outcomes in the two models, highlighted the need for a better understanding of tolerance in polyclonal repertoires since all the previous studies on the outcome of antigen targeting to DCs had been done in transgenic T cell models. As the first step in understanding polyclonal tolerance, we examined the C57BL/6 model, a non-disease prone repertoire, for tolerance to CD4 and CD8 antigens from Ovalbumin.

The results described in this thesis, show that antigen targeting to DCs in non-disease prone mice leads to polyclonal CD4 and CD8 tolerance. This tolerance is non-deletional, as the cells persist with alterations in their effector cytokine profiles. The tolerized CD4 T cells, lose the ability to make IL-2, but not IFNγ. Tolerized CD4 T cells are unable to provide help to B cells for antibody production and thus humoral tolerance is established. CD4 and CD8 cells that have been tolerized *in vivo* lose their ability to proliferate in response to antigen. Stimulation with αCD28 *in vitro* and αCD40 *in vivo* leads to a reversal of tolerance.

The reversible nature of the tolerance induced in a non-disease polyclonal repertoire suggests that establishment and maintenance of tolerance in disease models will be a challenge, since the immune environment in such cases is chronically inflamed. Antigen presentation in that context by DCs, to low affinity disease-prone polyclonal T cells could potentially lead to immunity or reversal of tolerance. These results provide important insight for the future development of successful DC-based therapeutic strategies for autoimmune disease.

Chapter 2

Materials and Methods

Mice: All mice used in experiments were maintained under specific pathogen free conditions. 5-8 week old B6.SJL (CD45.1), NOD/LtJ, and C57BL/6 mice were purchased from Jackson Laboratory. B6.H2^{g7} were maintained by breeding. The mice were used according to institutional guidelines. Protocols were approved by the Institutional Animal Care and Use Committee at The Rockefeller University.

Mice were injected subcutaneously with protein in complete freund's adjuvant (CFA) and intraperitoneally (i.p) with chimeric antibodies.

Immunizations: Mice were primed either once i.p. or multiple times with 5μg αDEC/peptide antibodies, or with 5μg αDEC/peptide antibody in conjunction with 50μg αCD40 (clone IC10) (as indicated in Results) or the same doses of αCD40 alone or with 100μg of LPS-free OVA (Seikagaku Corp.) precipitated in alum (Pierce Chemical Co.)). Antibodies were elicited by boosting the mice once i.p. with 1μg OVA-NP_{11}, which is a conjugate of

11 molecules of the hapten (4-hydroxy-3-nitrophenyl) acetyl (NP) with the carrier protein (OVA).

EAE induction: C57BL/6 females 6-8 weeks old, were injected with 100µg MOG peptide in CFA (Difco) (200µl total) divided into two parts and injected into each flank. This was immediately followed by i.p injection with 200ng Pertussis Toxin (List BioLabs) in PBS. Another dose of Pertussis Toxin (PTX) was injected 48 hours later. CFA used for injection was enriched with mycobacterium TB (10ml CFA +40 mg M. TB from Difco).

Diabetes Experiments: For all diabetes experiments, development of diabetes was monitored with chemstrips (Roche Applied Science), which detect urine glucose above 150 mg dL^{-1}. A mouse was considered diabetic on the first of three consecutive readings of high urine glucose. Diabetes was monitored from the time mice were 11 weeks old, until they were 25-30 weeks old.

Adeno/GAG p41 immunization: Mice received one dose ($1x10^7$ PFU per mouse) of recombinant adenovirus/GAG p41 intramuscularly (i.m).

Construction and production of the hybrid antibodies: The entire cDNA encoding chicken OVA was cloned in frame with the carboxyl terminus of the heavy chain of mouse αDEC-205 or isotype control III/I0 antibodies. Likewise, a 52 base pair sequence comprising amino acids 9-23 from the insulin β chain peptide, was cloned into the antibodies to produce αDEC/INS or III/I0/INS. Hybrid antibodies were produced by transient transfection in 293T cells using calcium phosphate as described previously (64), and the fusion antibodies were purified on Protein G columns (GE Healthcare). They were assayed for binding to DEC-205 expressed stably on the surface of CHO cells, provided by C.G. Park (The Rockefeller University, New York, NY). Titrations ranging from 5μg/ml to 0.01μg/ml were tested, and the binding was detected using a secondary αmouse IgG.

Peptide libraries: Peptides for OVA and GAG p41 were synthesized in collaboration with the Proteomics Resource Center, The Rockefeller University. For *in vitro* restimulation assays, the OVA peptide 75 (epitope 265-279 EKLTEWTSSNVMEER) was used, which consists of both Class I and Class II epitopes. For GAG p41 experiments, the entire peptide library of GAG p41 was pooled.

Intracellular cytokine staining: Intracellular IFNγ and IL-2 production by CD4 and CD8 T cells after immunization, was evaluated using bulk splenocytes incubated with 2 μM of the OVA or GAG p41 peptide or medium alone in the presence of 2 μg/ml of costimulatory •CD28 antibody (clone 37.51). Cells were cultured for 6 h in the presence of brefeldin A (BD Biosciences), incubated with CD16/CD32 antibody to block Fc receptors, and stained with anti-mouse CD4 (clone GK 1.5), CD8 (clone 53-6.7), and CD3 (clone 145-2C11) antibodies for 15 min at 4°C. After fixation with Cytofix/Cytoperm Plus (BD Biosciences), cells were stained for intracellular IFNγ- (XMG1.2) for 15 min at room temperature. All mAbs were purchased from BD Biosciences. Data was collected using FACSCalibur and analyzed using FlowJo software (Tree Star).

In vitro Proliferation Assay: We used CFSE (10^7 cells/ml, 1 μM, 10 min, 37°C; Invitrogen) dilution to assess proliferation of primed T cells in response to antigen. Bulk splenocytes were labeled with CFSE, and added at 500, 000 cells per well, in the presence of OVA peptide (2 μg/ml), medium alone, or •CD3 (0.1 μg/ml) and •CD28 (2 μg/ml; positive control) for 4 days in 1 ml round-bottom tubes.

ELISA: For the detection of NP- or OVA-specific antibodies, high-binding ELISA plates (Costar) were coated overnight with 5 μg/ml NP_2-BSA (Biosearch Technologies) or OVA protein (Sigma) in PBS. Plates were then washed three times with PBS-Tween 20 0.02% and blocked with PBS-BSA 1% for 1 h at room temperature. Serial dilutions of the sera in PBS-BSA 0.25% were incubated for 2 h at room temperature and visualized with goat •mouse IgG Fc-specific antibody conjugated to horseradish peroxidase (1:2,000; Jackson ImmunoResearch Laboratories) followed by colorimetric assay using 1-Step ABTS. OD_{405} was measured using a $VERSA_{max}$ microplate reader (Molecular Devices). Titers represent the highest dilution of serum showing an OD_{405} >0.1. The results are presented as the $\log_{10}$ antibody titer of each individual mouse.

LUMINEX Assay for cytokines: 14 days after immunization, bulk splenocytes were isolated and cultured with or without αCD28 in the presence of peptide or medium alone for 72 hrs. The supernatants were then harvested and assayed on the LUMINEX instrument using the Biosource Multiplex Bead Immunoassay kit for detection of TNFα, IFNγ, IL-17, IL-10, and IL-4.

Statistical Analysis: All comparisons between groups used Welch's t-test to reject the null hypothesis that the means of the groups being compared are identical. The Welch's t-test is a modification of the standard Student's t-test which can be used to compare groups with different variances (133). The null hypothesis was rejected if the p-value was less than 0.05. Analysis was performed using MATLAB (Mathworks, MA). Error bars represent one standard error of the mean.

Chapter 3

Results Part I

Targeting of antigen to DCs in an autoimmune disease model

The ability to establish peripheral T cell tolerance in transgenic models against antigens targeted to DCs, suggested that a similar targeting approach could be used to deliver self-antigens to DCs for presentation to autoreactive polyclonal T cells in mouse models of autoimmune disease. If such an approach resulted in tolerance of autoreactive polyclonal T cells, it would be a promising advance towards therapy of autoimmune disease.

We chose the mouse model of experimental autoimmune encephalomyelitis (EAE), to examine the consequences of targeted self-antigen delivery to DCs. EAE resembles the human neurodegenerative disorder- multiple sclerosis (MS), and is mediated by the destruction of the neuronal myelin sheath by T cells that are specific for myelin epitopes. In mice, EAE can be induced by immunization with myelin oligodendrocyte (MOG) peptide 35-55. This immunization leads to the activation of MOG-specific CD4 T cells, which then home into the central nervous system by infiltrating the blood-brain barrier, ultimately leading to neuronal destruction (131). We

decided to examine the consequences of targeted delivery of MOG peptide to DCs, on the pathology of the disease.

Daniel Hawiger, designed hybrid DC targeting antibodies that consisted of the MOG 35-55 peptide fused to the carboxyl terminus of the cloned αDEC (referred to as αDEC/MOG) and isotype control (III/10 /MOG) heavy chains (Figure 1A). Using these antibodies we demonstrated that MOG- specific transgenic CD4 T cells, could be tolerized by targeting MOG peptide to steady state DCs for presentation to T cells (106). We therefore wanted to determine whether αDEC/MOG targeting to DCs would lead to the prevention of MOG peptide-mediated disease in the EAE model. Disease was induced by activating MOG peptide-specific T cells by injection of MOG peptide in CFA, followed by injection of Pertussis Toxin (PTX) to breech the blood-brain barrier. C57BL/6 mice were injected with αDEC/MOG or isotype control III/10/MOG or PBS and 7 days later immunized with 100μg MOG peptide in CFA subcutaneously, followed by two doses of PTX. The condition of the mice was monitored daily. Within two weeks after immunization with MOG peptide in CFA followed by PTX, controls injected with PBS or III/10/MOG had developed symptoms of EAE. In contrast, all but one of the αDEC/MOG- treated mice remained

completely disease-free, and the one mouse that developed symptoms showed only very mild disease (Figure 1B). The mice were monitored for the next 10 days and no significant change in their condition was detected.

To examine whether the prevention of disease in αDEC/MOG treated mice, was due to some form of T cell tolerance, we assessed homing capacity of CD4 T cells to the spinal cords in tolerized versus control mice. While increased numbers of CD4 T cells were detected in spinal cords from control treated mice that showed symptoms of EAE, no such cells were found in spinal cords from mice that were treated with αDEC/MOG prior to immunization with MOG peptide in CFA and PTX (Figure 1C). Thus treatment with αDEC/MOG blocked the homing capacity of CD4 T cells to the spinal cord and effectively prevented induction of EAE.

The success of the dendritic cell targeting strategy to prevent disease in EAE which is an induced acute autoimmune model, encouraged us to attempt the challenge of therapy in a spontaneous chronic mouse model of autoimmunity. The model that we chose was the Non Obese Diabetic (NOD) mouse which closely resembles human automimmune type I diabetes in its pathology, immunology and genetics.

Our goal was to induce antigen specific T cell tolerance by delivering diabetogenic antigens to DCs in NOD mice, and thereby delay or prevent the development of diabetes. We chose the 9-23 insulin β chain peptide as our antigen for delivery to DCs since it has been reported in many studies to be the immunologically dominant epitope in the pathology of the disease in both mice and humans and there is a high degree of conservation of this epitope across species (134). To deliver this antigenic insulin epitope to DCs *in vivo*, we generated hybrid antibodies with the insulin peptide fused to the carboxyl terminus of the heavy chain of either the cloned αDEC (referred to as αDEC/INS) or the III/10 Isotype control (referred to as III/10/INS) heavy chain. The DNA constructs encoding these hybrid antibodies were transiently transfected into 293T cells, and the protein was purified from the supernatant (Figure 2A).

To test whether the hybrid αDEC antibody bearing the insulin peptide was able to bind to the DEC-205 receptor, we did an *in vitro* binding assay on CHO cells that stably expressed the DEC-205 receptor on their surface. The αDEC/INS antibody was able to bind the DEC-205 receptor even at

concentrations as low as 0.1μg/ml, while the III/I0/INS antibody showed no binding even at a concentration of 5μg/ml (Figure 2B).

To determine whether αDEC/INS targeting altered the onset of diabetes, we treated groups of 10 NOD females each, beginning at age 4 weeks, with αDEC/INS, or III/10 INS or PBS every 10 days. We injected doses ranging from 15μg to 0.005μg of αDEC/INS and III/I0/INS in multiple independent experiments. Mice were tested for urine glucose levels every week for upto 25- 30 weeks. While the first experiment seemed to suggest that lower doses of αDEC/INS could retard the onset of diabetes (Figure 3A), this was not borne out by subsequent experiments (Figure 3B and C). We did not observe an abrogation or significant retardation in onset of diabetes. Thus delivery of insulin peptide to DCs in NOD mice did not result in protection against development of diabetes.

In summary, delivery of the MOG antigen to steady state DCs led to the absence of CD4 T cell accumulation in the spinal cord and the prevention of disease in the EAE model. In the NOD model however, delivery of insulin antigen to the DCs failed to block the development of diabetes. There are many possible reasons for this striking difference in outcomes between the

two disease models. One important difference is that in the NOD model, antigen is being delivered to DCs that are in a chronically inflamed environment, in contrast to the EAE model where inflammation only sets in upon induction of disease. Also in EAE which is an induced model of disease, the T cells do not see their antigen, until immunization, while in NOD, the insulin antigen is constantly in circulation from well before treatment begins. These and other considerations on the difference between the two disease models and its implications for treatment are discussed in detail in Chapter 6.

Figure 1. Vaccination with αDEC/MOG prevents EAE in C57BL/6 wild type mice.

A) Schematic representation of the αDEC/MOG hybrid antibody.

B) Groups of 10 C57BL/6 mice each, were injected with either PBS or 15μg of αDEC/MOG or III/I0/MOG, as indicated. 8 days after antibody or PBS injection the mice were challenged with 100μg MOG peptide in CFA s.c. along with Pertussis Toxin i.p. The mice were monitored for disease symptoms daily and scored on days 14 and 21 post-immunization. The following clinical scale was used: 0- no clinical signs, 1- flaccid tail, 2-hind limb weakness and abnormal gait, 3- complete hind limb paralysis, 4- complete limb paralysis. The scores were the same on days 14 and 21. Results represent scores from day 21.

C) Some of the animals from the experiment described above were sacrificed on day 21 after disease induction and their spinal cords were removed, processed and analysed by flow cytometry. Histograms show intensity of staining with αCD4 APC among cells in the lymphoid gate.

Figure 1

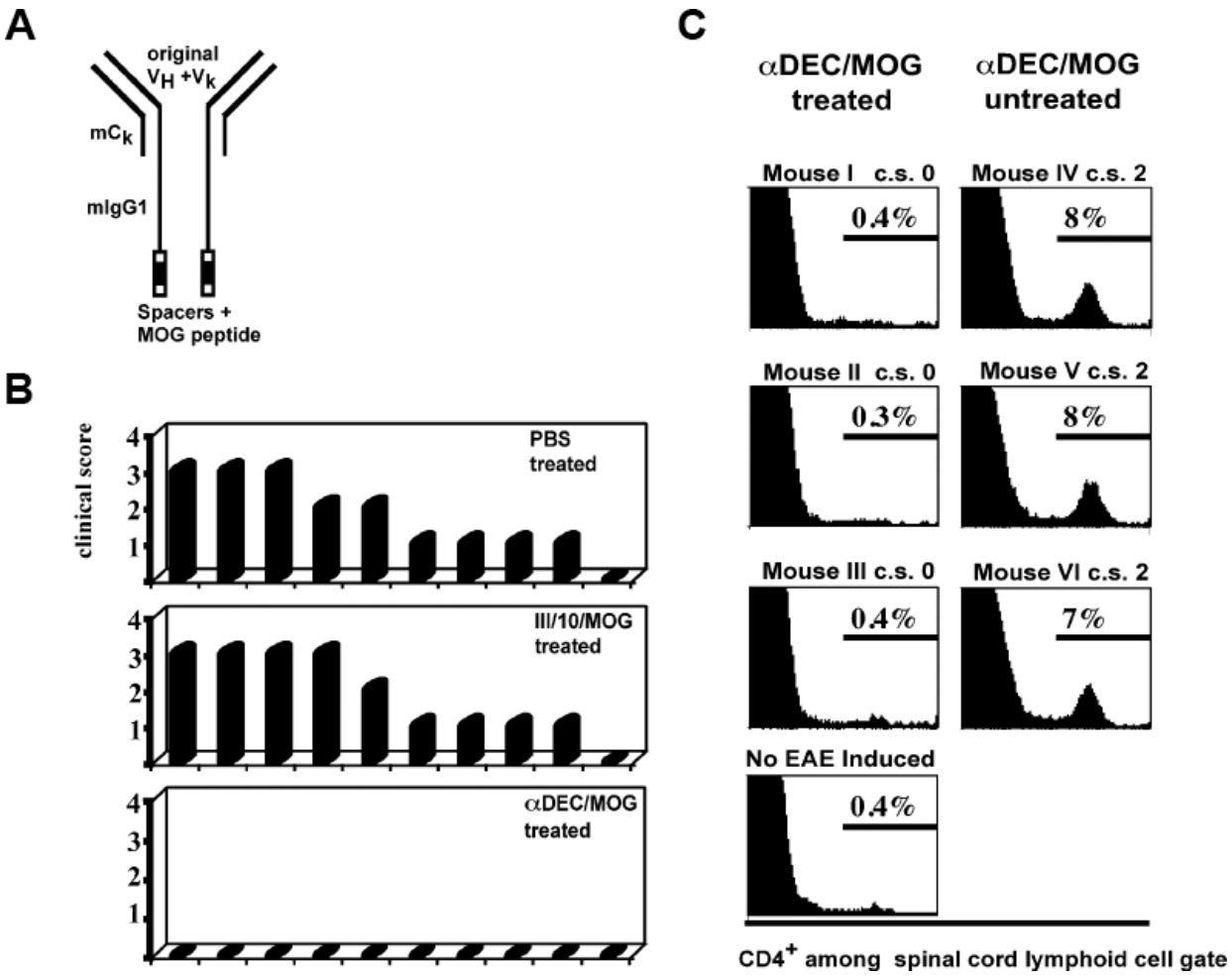

A
original
V_H +V_k
mC_k
mIgG1
Spacers +
MOG peptide
B
clinical score
PBS
treated
III/10/MOG
treated
αDEC/MOG
treated
C
αDEC/MOG
treated
αDEC/MOG
untreated
Mouse I c.s. 0
0.4%
Mouse IV c.s. 2
8%
Mouse II c.s. 0
0.3%
Mouse V c.s. 2
8%
Mouse III c.s. 0
0.4%
Mouse VI c.s. 2
7%
No EAE Induced
0.4%
$CD4^+$ among spinal cord lymphoid cell gate

Figure 2. Characterization of the hybrid αDEC/INS and III/I0/INS antibodies.

A) SDS PAGE analysis of purified αDEC/INS and III/I0/INS hybrid antibodies followed by Coomassie staining.

B) αDEC/INS and III/I0/INS antibodies were tested for *in vitro* binding to DEC-205 , expressed on CHO cells (Titration range 5μg/ml - 0.01 μg/ml). Histogram plots show mean fluorescence intensity of staining with secondary •mouse IgG.

Figure 2

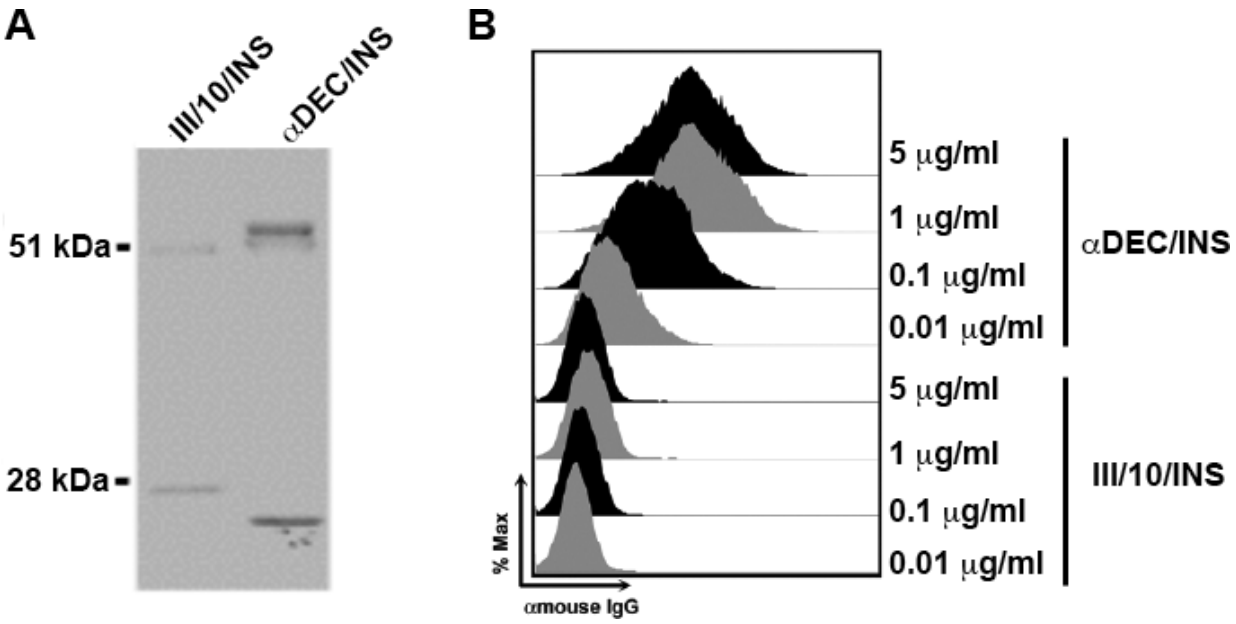
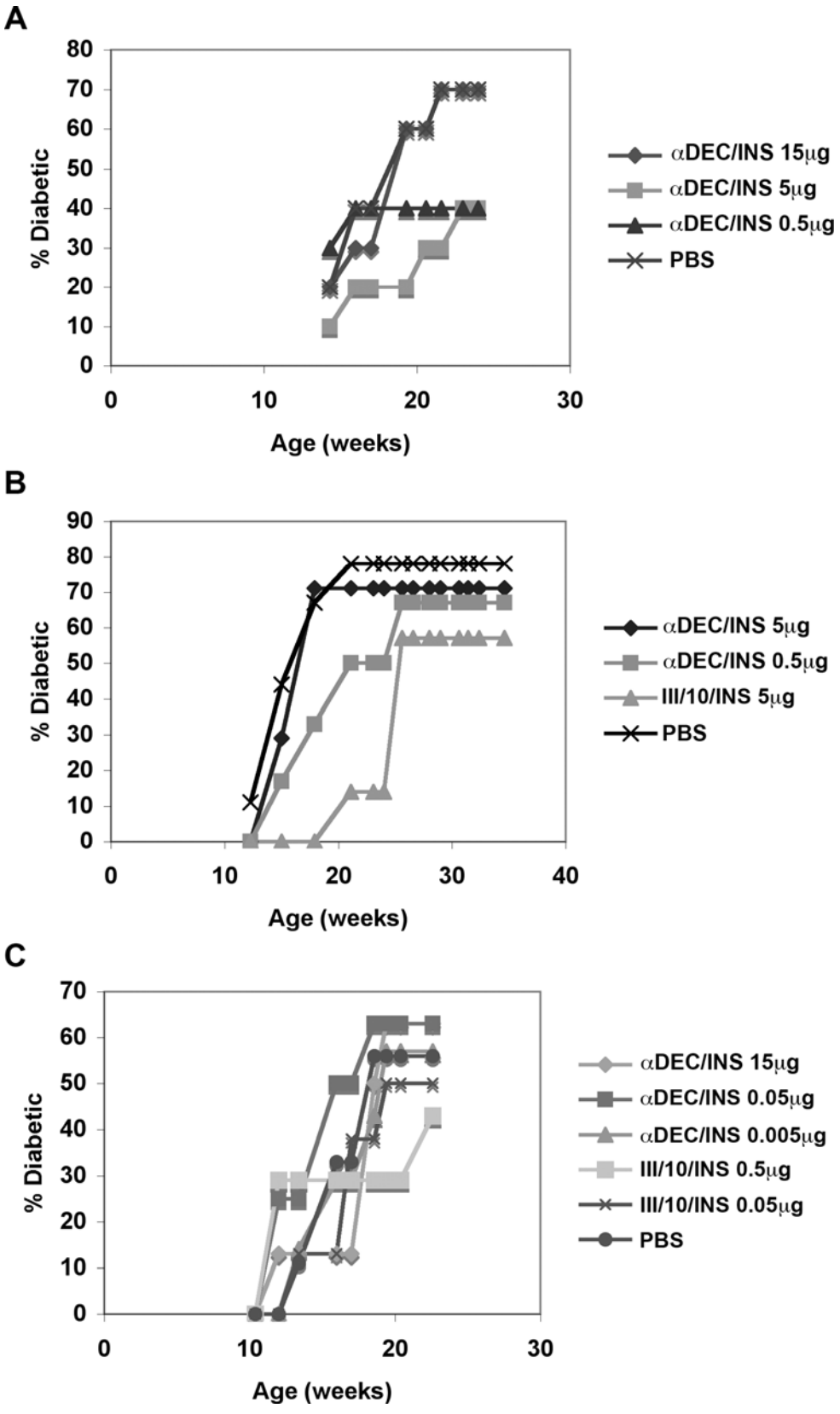

A
III/10/INS
αDEC/INS
51 kDa
28 kDa
B
5 μg/ml
1 μg/ml
0.1 μg/ml
0.01 μg/ml
αDEC/INS
5 μg/ml
1 μg/ml
0.1 μg/ml
0.01 μg/ml
III/10/INS
% Max
αmouse IgG

Figure 3. Chronic treatment with αDEC/INS did not prevent or delay onset of diabetes.

(A, B and C) Groups of 10 NOD females each, were injected every other week, beginning at 4 weeks of age, with the indicated doses of αDEC/INS, III/I0/INS or PBS till they were 25 weeks old. Beginning at age 11 weeks, the mice were tested weekly for urine glucose levels, and a positive was scored if the mouse tested glucose high, two consecutive times in a row. Graphs represent 3 independent experiments with different doses of antibodies.

Figure 3


A
% Diabetic
Age (weeks)
αDEC/INS 15μg
αDEC/INS 5μg
αDEC/INS 0.5μg
PBS
B
% Diabetic
Age (weeks)
αDEC/INS 5μg
αDEC/INS 0.5μg
III/10/INS 5μg
PBS
C
% Diabetic
Age (weeks)
αDEC/INS 15μg
αDEC/INS 0.05μg
αDEC/INS 0.005μg
III/10/INS 0.5μg
III/10/INS 0.05μg
PBS

Chapter 4

Results Part II

Targeting of Antigen to DCs in NOD mice

Prior to the studies described in this thesis, the consequences of antigen targeting to DCs had only been examined using transgenic T cell models. Our findings detailed in the previous chapter revealed the complexities of achieving tolerance in spontaneous wild type disease models with chronic inflammation such as NOD. We therefore decided to dissect the consequences of DC- targeted antigen delivery to polyclonal T cell repertoires at a cellular level in the NOD model. Since in NOD mice, the study of tolerance to insulin is complicated by a variety of factors, both genetic and immunological, we decided to first simply ask whether polyclonal T cells in NOD mice could be tolerized against a harmless foreign antigen by DC targeting.

Towards this end, we set out to identify a foreign antigen that could generate detectable antigen specific T cell responses in a polyclonal NOD repertoire. The NOD MHC Class II molecule I-A^{g7}, has an unstable peptide binding groove due to a non-aspartic acid substitution at position 57 of the β chain

(135), which precludes high affinity binding with many antigenic epitopes. To find an antigen whose epitope could be presented by the I-A^{g7}, we screened a panel of hybrid αDEC antibodies bearing different antigenic proteins and peptides (HIV GAG p41, circumsporozoite protein from *Plasmodium yoelii*, ovalbumin protein from chicken egg, and myelin oligodendrocyte peptide) in B6.$H2^{g7}$ mice. The B6.$H2^{g7}$ congenic strain has the NOD $H2^{g7}$ locus ($H2^{g7}$ = K^d, I-A^{g7}, I-E^{null} and D^b) introgressed onto the C57BL/6 background (136). As controls we used C57BL/6 mice, known to generate either strong CD4 or CD8 T cell responses against all the antigens screened.

We immunized the mice with 5μg of hybrid αDEC antibody in conjunction with 50μg of αCD40 antibody, as had been reported in C57BL/6 mice for the induction of robust T cell responses (137, 138). The αCD40 antibody acts as an agonist for the CD40 receptor expressed on DCs. This interaction bypasses the need for the CD40 ligand on activated CD4 T cells to bind the receptor. αCD40 thus acts as a potent stimulator of DC maturation and thereby immunity. Two weeks after immunization bulk splenocytes isolated from the immunized mice were restimulated *in vitro* with αCD28 and antigenic peptides. Intracellular IFNγ responses were then assayed by flow

cytometry. The p41 protein of HIV GAG (GAG p41) was the only antigen amongst those tested that generated detectable IFNγ responses in CD4 T cells in B6.H2^{g7} (Figure 4A), while all other antigens (Figure 4B and data not shown) did not. We therefore chose to target GAG p41 to DCs to probe polyclonal T cell immunity and tolerance in NOD.

NOD, B6.H2^{g7} and C57BL/6 mice were immunized with αDEC/GAG p41 along with αCD40. Two weeks after immunization IFNγ and IL-2 levels in CD4 T cells were measured (Figure 5A, B and C). C57BL/6 mice made strong IFNγ responses, while the responses in B6.H2^{g7} were significantly lower (p value = 0.007). Interestingly, the IFNγ responses in NOD mice were even lower than those of the B6.H2^{g7} (p value=0.025) (Figure 5D). The diminished IFNγ responses of B6.H2^{g7} mice compared to the C57BL/6 mice confirm studies by others, showing that the I-A^{g7} is a defective MHC molecule that leads to poor antigen presentation and dampened T cell responses (139). The additional reduction in IFNγ responses in NOD CD4 T cells points to non-I-A^{g7} defects in the mounting of immunity against foreign antigen in this strain. The defects are likely to lie in either the DCs or CD4 T cells or both, since the targeted antigen delivery strategy uniquely involves

these subsets. These results correlate with previous studies that have shown defects *in vitro* in APCs and CD4 T cells from NOD (140, 141).

The NOD mouse has a recombinant MHC Class I locus consisting of H-2K^{d} and H-2D^{b}. To determine whether the CD8 T cell compartment in NOD is also defective, we decided to immunize the mice with a recombinant adenovirus carrying the GAG p41 protein (Adeno/GAG p41), which had previously been reported to generate strong H-2K^{d} restricted IFNγ responses in BALB/c mice (138). NOD mice and BALB/c controls were immunized with a single intramuscular dose of the Adeno/GAG p41, and 2 weeks later bulk splenocytes were restimulated *in vitro* with GAG p41 peptides and assayed for intracellular IFNγ (Figure 6). The level of IFNγ made by the CD8 T cells in NOD was comparable to BALB/c, suggesting that unlike the CD4 T cell responses, the CD8 T cell responses against foreign antigen are not deficient in NOD.

Having established detectable immune responses against the foreign GAG p41 antigen in the NOD CD4 polyclonal repertoire, we wanted to know whether targeting the antigen to DCs in the absence of adjuvant would lead to antigen-specific tolerance in NOD T cells, as had been observed in the

transgenic T cell models. A measure of such tolerance would be the abrogation of cytokine production in response to immunogenic challenge with antigen. We treated NOD mice with 5μg of αDEC/GAG p41, and waited 7 days for tolerance to be established, in accordance with empirical observations from the studies in transgenic models. The T cells were then subjected to immunogenic challenge in the form of αDEC/GAG p41 in conjunction with αCD40. 14 days after the challenge, bulk splenocytes were isolated and restimulated *in vitro* with GAG p41 antigenic peptides and IFNγ production was measured. NOD mice pretreated with the tolerizing regimen of αDEC/GAG p41 showed no reduction in IFNγ levels in response to challenge, when compared to control mice that had only received the challenge (Figure 7).

The previous studies on T cell tolerance achieved by DC targeted antigen delivery had been done in models that involved adoptive transfer of transgenic T cells in wild-type mice (62, 106). The success in establishing tolerance in these model T cells, had led us to believe that antigen-specific T cells from autoreactive polyclonal repertoires could be tolerized in a similar fashion. The failure to achieve antigen-specific tolerance in the NOD mice, in the experiments detailed here, emphasized the challenges to achieving

tolerance in disease-prone repertoires that have multiple defects in their DCs and T cell compartments. These results also strongly brought into focus the many important differences between polyclonal T cell repertoires and the more simplistic transgenic models that had been studied before. Polyclonal T cells have a wide range of affinities and specificities compared to the high – affinity, single epitope specificity of transgenic T cells. Another major difference in these experimental systems is that while adoptive transfer of transgenic T cells involves following the fate of an unreplenishing population of cells, wild type models have cells constantly emigrating from the thymus and being turned over. A detailed discussion of these differences and their potential impact on the establishment of tolerance is presented in chapter 6. The results described above, brought us to the conclusion that any attempt towards the challenge of tolerizing autoreactive disease-prone repertoires, would have to be based on a thorough understanding of tolerance in non-disease prone polyclonal repertoires.

Figure 4. αDEC/GAG p41 generates IFNγ responses in B6.H2^{g7} mice.

B6.H2^{g7} mice were immunized i.p. with 5μg of either αDEC/GAG p41 (A) or αDEC/OVA (B) in conjunction with 50μg αCD40. 2 weeks later, bulk splenocytes were restimulated *in vitro* with either αCD28 alone, or GAG p41 peptides with αCD28, and cells were stained for intracellular IFNγ. Plots are gated on CD3 cells.

Figure 4

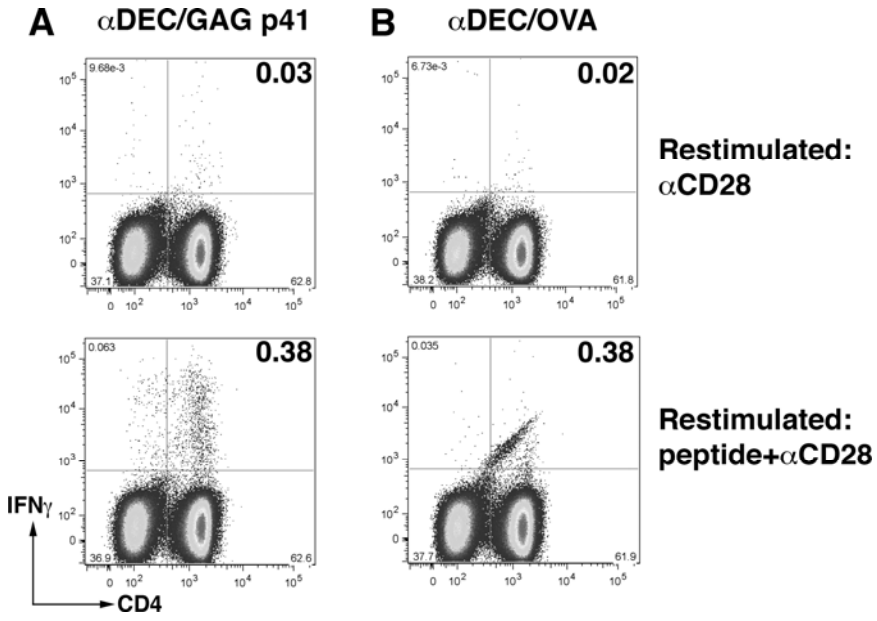

A
αDEC/GAG p41
B
αDEC/OVA
9.68e-3
0.03
37.1
62.8
6.73e-3
0.02
38.2
61.8
Restimulated:
αCD28
0.063
0.38
36.9
62.6
0.035
0.38
37.7
61.9
Restimulated:
peptide+αCD28
IFNγ
CD4

Figure 5. αDEC/GAG p41 generates IFNγ responses in CD4 T cells in NOD mice.

A) C57BL/6, B6.H2^{g7} and NOD mice (3 per group) were immunized i.p. with αDEC/GAG p41 (5μg) in αCD40 (50μg). 2 weeks later bulk splenocytes were isolated and restimulated *in vitro* with GAG p41 peptides. After 6 hours, cells were fixed and stained for intracellular IFNγ. Plots are gated on CD3 cells.

B) Percentage of IFNγ producing T cells in C57BL/6, B6.H2^{g7} and NOD mice. Values represent means from 3 independent experiments as shown in (A) with 3 mice per group. Error bars represent one standard error of the mean. p-values were computed using Welch's t-test (refer to Chapter 2).

Figure 5

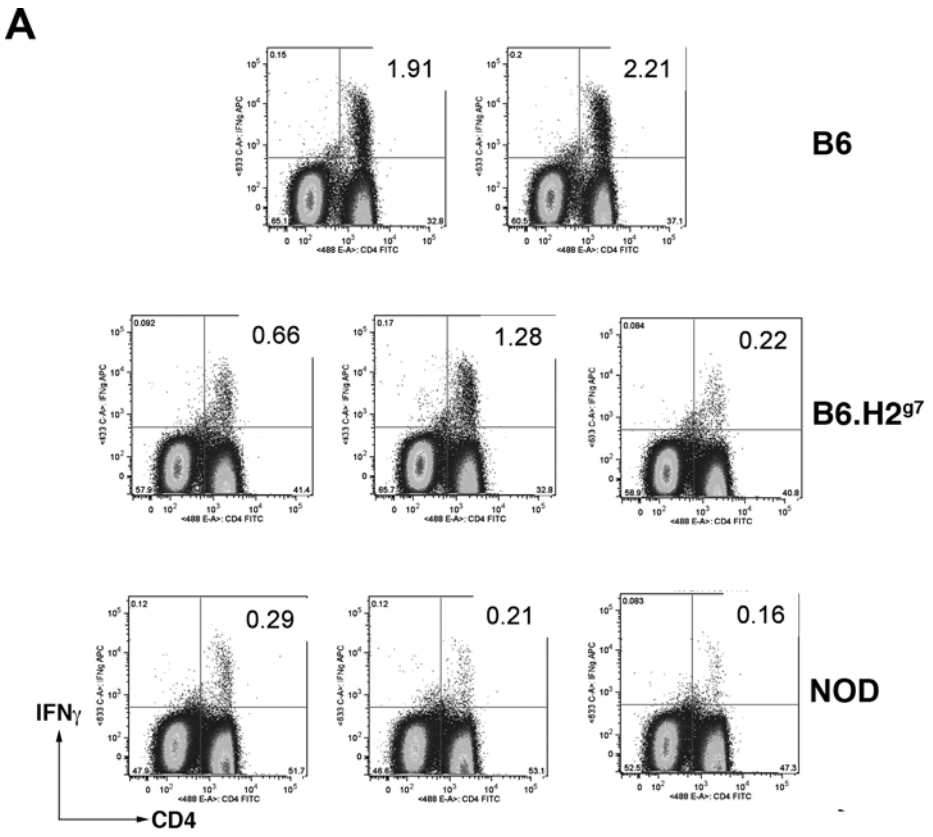

A
1.91
2.21
B6
0.66
1.28
0.22
B6.H2g7
0.29
0.21
0.16
NOD
IFNγ
CD4
B
p=0.007
p=0.025
%IFNγ+ T cells
B6
B6.H2g7
NOD

Figure 6. Adeno/GAG p41 generates IFNγ responses in CD8 T cells in NOD.

NOD and BALB/c mice were vaccinated using one i.m. dose ($1X10^7$ PFU) of Adeno/GAG p41. 14 days later, bulk splenocytes were isolated and restimulated with GAG p41 peptide for 6 hrs, and intracellular IFNγ was measured by flow cytometry. Plots are gated on CD3 cells.

Figure 6

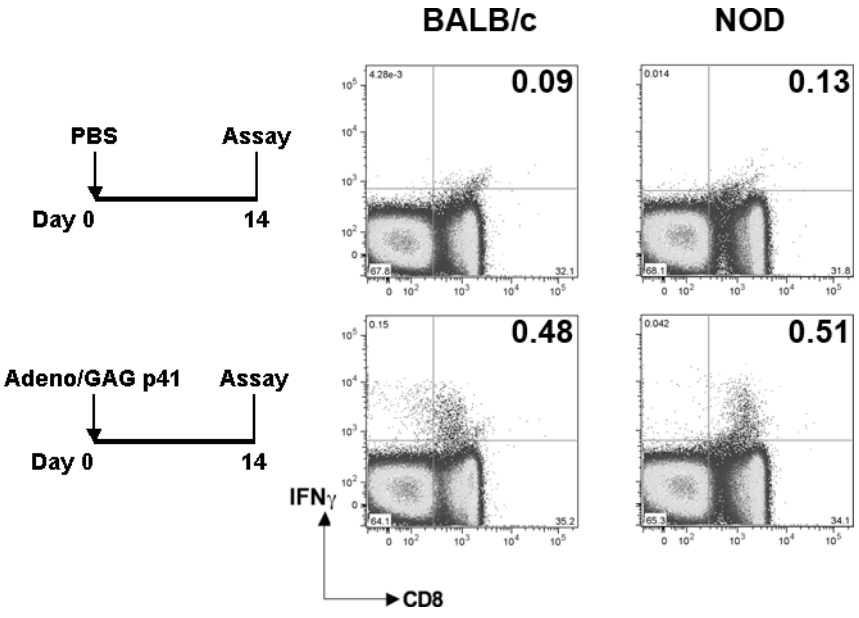
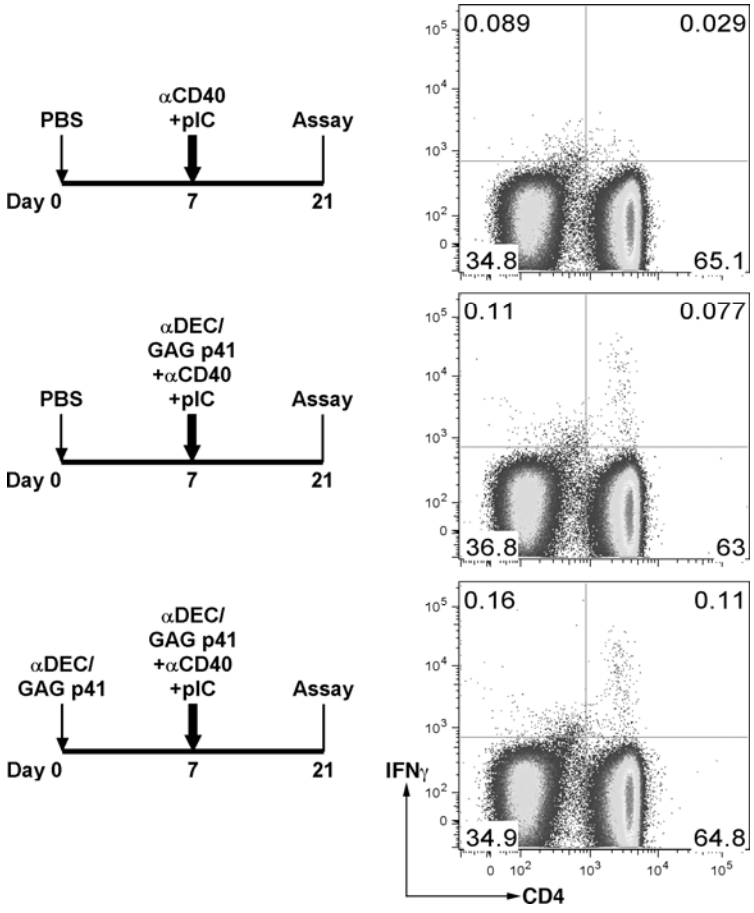

BALB/c
NOD
PBS
Assay
Day 0
14
Adeno/GAG p41
Assay
Day 0
14
0.09
0.13
0.48
0.51
IFNγ
CD8

Figure 7. αDEC/GAG p41 does not tolerize αGAG p41-specific CD4 IFNγ responses in NOD mice.

NOD mice were injected i.p. with PBS or αDEC/GAG p41 (5μg). 7 days later, mice were immunized with αDEC/GAG p41 (5μg) and αCD40 (50μg). 14 days later bulk splenocytes were restimulated with GAG p41 peptide for 6 hrs, and intracellular IFNγ was measured by FACS. Plots are gated on CD3 cells.

Figure 7


αCD40
+pIC
PBS
Assay
Day 0
7
21
αDEC/
GAG p41
+αCD40
+pIC
PBS
Assay
Day 0
7
21
αDEC/
GAG p41
αDEC/
GAG p41
+αCD40
+pIC
Assay
Day 0
7
21
0.089
0.029
34.8
65.1
0.11
0.077
36.8
63
0.16
0.11
34.9
64.8
IFNγ
CD4

Chapter 5

Results Part III

Tolerance in non-disease polyclonal repertoires

Our results from the studies in the previous chapters emphasized the need to dissect the pathways and mechanisms of DC- mediated tolerance in polyclonal T cell repertoires in non-disease models, as the basis for progress towards achieving therapeutic tolerance in disease models. Towards this end, we chose to study tolerance in wild type C57BL/6 mice, with chicken egg ovalbumin (OVA) as the model antigen. Apart from being one of the most characterized antigens, OVA has the rare advantage of possessing both well-defined MHC Class I and II epitopes. In choosing it for these studies, we therefore hoped to gain insight into the similarities and differences between the regulation of peripheral tolerance in the CD4 and CD8 T cell compartments.

To deliver OVA protein to DCs *in vivo*, a hybrid antibody was produced as a fusion protein with the full length OVA protein added to the carboxyl terminus of the heavy chain of the cloned αDEC (referred to as αDEC/OVA) antibody (Figure 8A). The DNA construct encoding this

hybrid antibody was transiently transfected into 293T cells, and the protein was purified from the supernatant (Figure 8B).

We tested binding of hybrid αDEC antibody bearing the OVA protein, to the DEC-205 receptor in an *in vitro* assay. We used CHO cells expressing the DEC-205 receptor on their surface. αDEC/OVA was able to bind the DEC-205 receptor even at concentrations as low as 0.1μg/ml (Figure 8C).

We immunized C57BL/6 mice with 5μg of αDEC/OVA in conjunction with αCD40, to see whether we could detect measurable cytokine responses in the polyclonal repertoire against the CD4 and CD8 T cell epitopes of OVA. 2 weeks later we isolated bulk splenocytes and restimulated *in vitro* with a mix of αCD28 and peptide 75 (epitope 265-279) consisting of overlapping Class I and II epitopes of OVA. αCD28, the TCR-independent T cell costimulator (142) , was used to enhance T cell responses to facilitate detection. In response to immunization with αDEC/OVA, the CD4 T cells make low but detectable levels of IFNγ, and medium to high levels of IL-2 (Figure 9A and B). In the case of CD8 T cells, IFNγ responses are very strong and IL-2 responses are undetectable. (Fig. 9C and data not shown).

We also tried immunizations with DC maturation stimuli milder than αCD40, specifically the TLR ligands polyIC and LPS, and Complete Freund's Adjuvant (CFA). These adjuvants had been shown to generate robust transgenic T cell responses in conjunction with DC targeted antigen, (62, 106) and our hope was that they would also prove effective in generating polyclonal responses in our assays. However, no detectable cytokine production was seen in response to any of these adjuvants (data not shown) leading us to conclude that in order to visualize primary polyclonal responses, a potent adjuvant such as αCD40 was necessary.

Having established detectable polyclonal CD4 and CD8 T cell cytokine responses against immunogenic OVA, we wanted to examine whether targeting the antigen to DCs in the absence of adjuvant would lead to antigen-specific tolerance. A measure of such tolerance would be the abrogation of cytokine production in response to subsequent immunogenic challenge with antigen. We treated C57BL/6 mice with 5μg or 20μg of αDEC/OVA and waited 10 days for tolerance to be established. The T cells were then subjected to immunogenic challenge in the form of 5μg αDEC/OVA in conjunction with αCD40. 14 days after the challenge, bulk

splenocytes were isolated and restimulated *in vitro* with αCD28 and the OVA peptide 75 bearing the Class I and II epitopes, and IFNγ and IL-2 production was measured. Neither CD4 nor CD8 T cells from mice pretreated with the tolerizing regimen of αDEC/OVA, showed reduction in the levels of IFNγ in response to challenge, when compared to IFNγ levels from control mice that had only received the challenge (Figure 10A and data not shown). Interestingly however, the CD4 T cells from mice pretreated with the tolerizing regimen of αDEC/OVA, showed a reduction in IL-2 levels when compared to control mice that had only received the challenge. (Figure 10B). The results were similar for mice treated with 5μg or 20μg of αDEC/OVA preceeding immunization. Thus polyclonal wild type CD4 T cells, when exposed to antigen in the absence of adjuvant are not deleted but persist, and can respond to subsequent immunogenic challenge by producing high levels of IFNγ, but can no longer make IL-2. This outcome is very different from what we see in adoptively transferred transgenic T cells after exposure to tolerizing antigen, where the T cells are either deleted, or persist having lost all ability to make cytokines.

We decided to examine a panel of cytokines to see whether in addition to IL-2, T cells exposed to tolerizing antigen lost or gained the ability to make

other cytokines. We treated the mice with 5μg of αDEC/OVA in the absence of adjuvant and 10 days later, immunized them with αDEC/OVA in conjunction with αCD40. 14 days later, we isolated bulk splenocytes and cultured them with peptide for 72 hours, after which the supernatants were tested on a high-throughput LUMINEX assay for IFNγ, IL-10, IL-17, IL-4, IL-12 and TNFα. Mice that had received the tolerizing regimen prior to challenge, made low but detectable levels of IL-10 while mice that had received only the challenge did not. All the other cytokines examined such as TNFα were produced at comparable levels in both groups of mice (Figure 11 and data not shown). Thus in polyclonal T cells, the exposure to antigen under tolerizing conditions alters the cells' effector cytokine profile. The cells continue to make IFNγ, but lose the ability to make IL-2, while gaining the ability to make IL-10.

IL-10 is a anti-inflammatory cytokine induced under tolerogenic conditions in certain subsets of suppressor T cells (129). Thus the presence of this cytokine in mice exposed to the tolerizing regimen, suggested a skewing of the Th1 polarized IFNγ response towards a pro-tolerance cytokine milieu. We looked to see whether there was an increase in the numbers of CD4+CD25+Foxp3+ cells, however we failed to see any difference in

immunized mice compared to mice that had been exposed to αDEC/OVA before immunization (data not shown).

To determine whether the alteration in effector cytokines upon tolerizing antigen exposure, resulted in functional tolerance for the CD4 T cells, we decided to examine antibody responses in these mice. B cells need CD4 T cell help in order to make antibodies, and therefore if the CD4 T cells in our mice are functionally tolerized, we would hypothesize that antibody production in these mice would be diminished. C57BL/6 mice that are immunized with either αDEC/OVA in conjunction with αCD40, or OVA protein in alum followed by a boost 14 days later with NP-OVA, generate OVA-specific and NP-specific antibodies, the peak titres of which are between 14 and 21 days after the boost. Since αCD40 is a very potent adjuvant, we worried that tolerized T cells exposed to it upon immunization, might reverse tolerance. Alum on the other hand is an appropriately mild adjuvant, and has been shown to produce detectable antibody titres, albeit one order of magnitude lower than those produced by αCD40 (137). We therefore chose to use both immunization protocols in mice pretreated with tolerizing OVA, and expected to see a more dramatic reduction of antibody titres in response to alum immunization as compared to αCD40

immunization. We treated groups of 8 mice each, with either PBS, a single dose of αDEC/OVA or three sequential doses of αDEC/OVA. The duration between each dose of αDEC/OVA was 10 days. 10 days after the final dose, we immunized the mice with either αDEC/OVA in conjunction with αCD40 or OVA in alum. 14 days later, the mice were boosted with NP-OVA, and bled at day 14 post-boost. Anti-OVA and anti-NP antibody titres were then measured using a capture ELISA with either OVA, or NP conjugated to BSA respectively. We found that mice exposed to tolerizing antigen (single or multiple doses) prior to immunization, showed between one and two log diminished antibody titres, with both immunization regimens. The reduction in antibody titres was greater in the case of the alum immunization, as compared to the αCD40 immunization (Figure 12) Our results indicate that treatment with αDEC/OVA in the absence of adjuvant leads to a special form of anergy in polyclonal repertoires, where the CD4 T cells experience not just an alteration in cytokine profiles, but also a loss in functional ability to aid the B cells in mounting humoral responses.

We then decided to examine *in vitro* proliferative responses to antigenic restimulation in CD4 and CD8 T cells from mice that had been exposed to tolerizing antigen. We treated the mice with αDEC/OVA and 10 days later,

immunized with αDEC/OVA in conjunction with αCD40. 14 days later, bulk splenocytes were labeled with CFSE and pulsed with peptide with or without αCD28 *in vitro*. 3 days later, cells were assayed for proliferation by flow cytometry. We observed that both CD4 and CD8 cells that had been exposed to tolerizing antigen prior to immunization, when pulsed with αCD28, made stronger proliferative responses when compared to cells from mice that had received only immunization. In contrast in the absence of αCD28, tolerized mice showed a complete abrogation of proliferation in both the CD4 and CD8 compartment (Figure 13 and 14). We therefore conclude that tolerizing exposure to antigen in the polyclonal repertoire leads to functional tolerance in both CD4 and CD8 T cells, one measure of which is *in vitro* proliferation. αCD28, a potent stimulator of TCR-independent signaling in T cells, reverses proliferative arrest in the tolerized mice. There are two explanations for this observation. Either, the αCD28 is reversing anergy in the tolerized T cells, by pushing them into cycle, or it is activating a hypo-responsive population of antigen-specific T cells, that had hitherto been unresponsive to the antigen. The observation that cells from tolerized mice that have seen antigen twice, i.e. during tolerization as well as immunization, make stronger proliferative responses in the presence of αCD28 than cells from immunized mice that have seen antigen only once,

seems to suggest that the former explanation is more likely. Thus tolerized CD4 and CD8 T cells, appear to be in a state of anergy that can be reversed upon exposure to αCD28 mediated costimulation.

The results in this chapter show that polyclonal CD4 and CD8 T cells can be tolerized by antigen delivered to DCs in the absence of inflammation. The mechanisms and phenotype of this tolerance is very different from that seen in the previously examined transgenic T cell models. Unlike transgenic T cells which are either deleted or completely anergized, with no effector responses *in vivo* and *in vitro*, polyclonal T cells persist after tolerance but alter their cytokine profile. They continue to make IFNγ in response to immunogenic challenge, but lose the ability to make IL-2, while gaining the ability to make IL-10. The functional outcome of this tolerance is the loss of CD4 T cell help to the antibody producing B cells. Thus antibody titres in tolerized mice are significantly reduced. αCD40 as an adjuvant seems to reverse tolerance to some extent, since in mice immunized with alum, there is a more dramatic reduction in antibody titres compared to mice immunized with αCD40. Tolerized CD4 and CD8 cells lose their ability to proliferate *in vitro* in response to peptide restimulation. However restimulation in the presence of αCD28 appears to reverse the proliferative block on the anergic

T cells *in vitro*. These results suggest that this form of polyclonal tolerance is very delicately held in balance and strong stimulation of the T cells under conditions such αCD40 *in vivo* or αCD28 *in vitro* can reverse this tolerance in both CD4 and CD8 subsets.

This susceptibility to reversal of T cell tolerance in wild type mice as compared to transgenic models, could be explained by the nature of polyclonal T cells. These cells range in affinities and specificities, with the average affinity being lower than that of most high affinity, single specificity transgenic T cells. In the case of transgenic T cells, strong signaling during high affinity interactions with tolerizing antigen leads to deletion or complete anergy. In contrast, in lower affinity polyclonal T cells, weaker strength of signaling during interactions with tolerizing antigen may lead to partial anergy that can be reversed under conditions of strong costimulation. Thus polyclonal T cell tolerance has different mechanisms and differing dynamics of establishment and reversal compared to transgenic models, and the understanding of these differences is an important step towards achieving tolerance in disease prone polyclonal repertoires and the therapy of autoimmune disease.

Figure 8. Design and characterization of the αDEC/OVA hybrid antibody.

A) Schematic representation of the αDEC/OVA hybrid antibody.

B) SDS PAGE analysis of purified αDEC/OVA hybrid antibody followed by Coomassie staining. αDEC was used as a loading control.

C) αDEC/OVA antibody was tested for *in vitro* binding to DEC-205 expressed on CHO cells (Titration range 5μg/ml - 0.01 μg/ml). Histogram plots show mean fluorescence intensity of staining with secondary αmouse IgG.

Figure 8

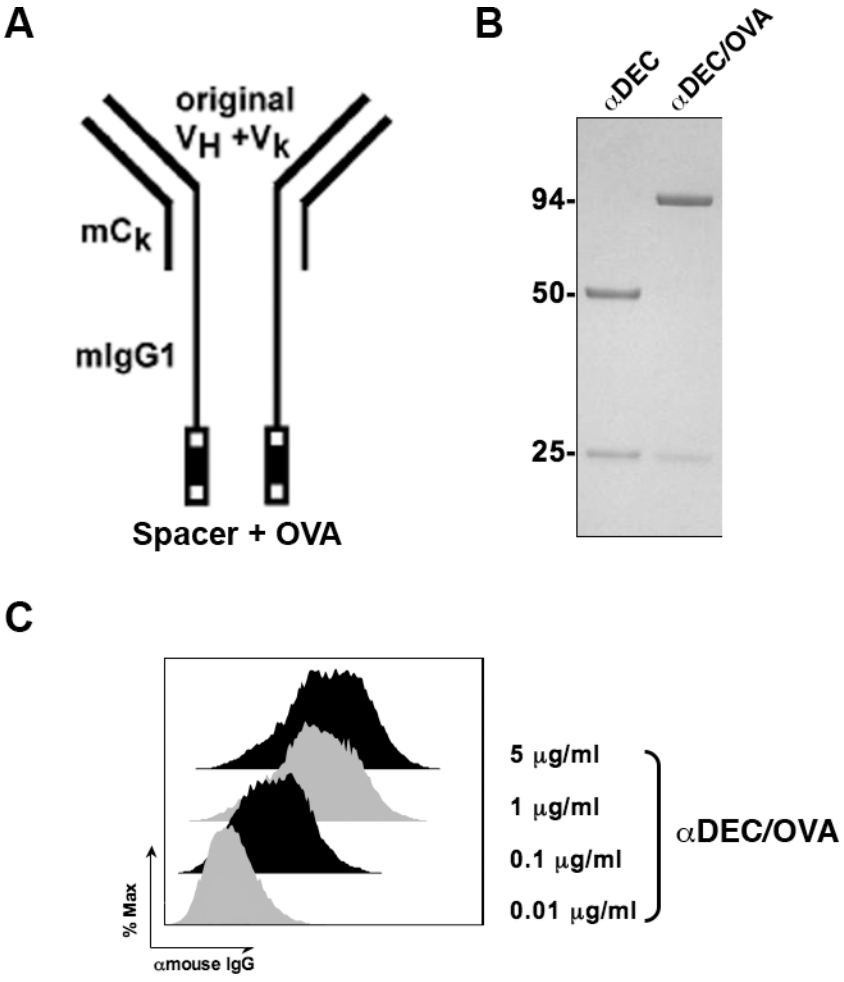

A
original
V_H +V_k
mC_k
mIgG1
Spacer + OVA
B
αDEC
αDEC/OVA
94-
50-
25-
C
5 μg/ml
1 μg/ml
0.1 μg/ml
0.01 μg/ml
αDEC/OVA
% Max
αmouse IgG

Figure 9. αDEC/OVA generates detectable IFNγ and IL-2 responses in CD4 and CD8 cells in C57BL/6 mice.

C57BL/6 mice were immunized i.p with αDEC/OVA (5μg) along with αCD40 (50μg). Two weeks later bulk splenocytes were isolated and restimulated *in vitro* with OVA peptide 75 containing Class I and II epitopes. After 6 hours cells, were fixed and stained for intracellular IFNγ and IL-2. Plots are gated on CD3 cells.

(A) IFNγ in CD4 cells. (B) IL-2 in CD4 cells. (C) IFNγ in CD8 cells.

Figure 9

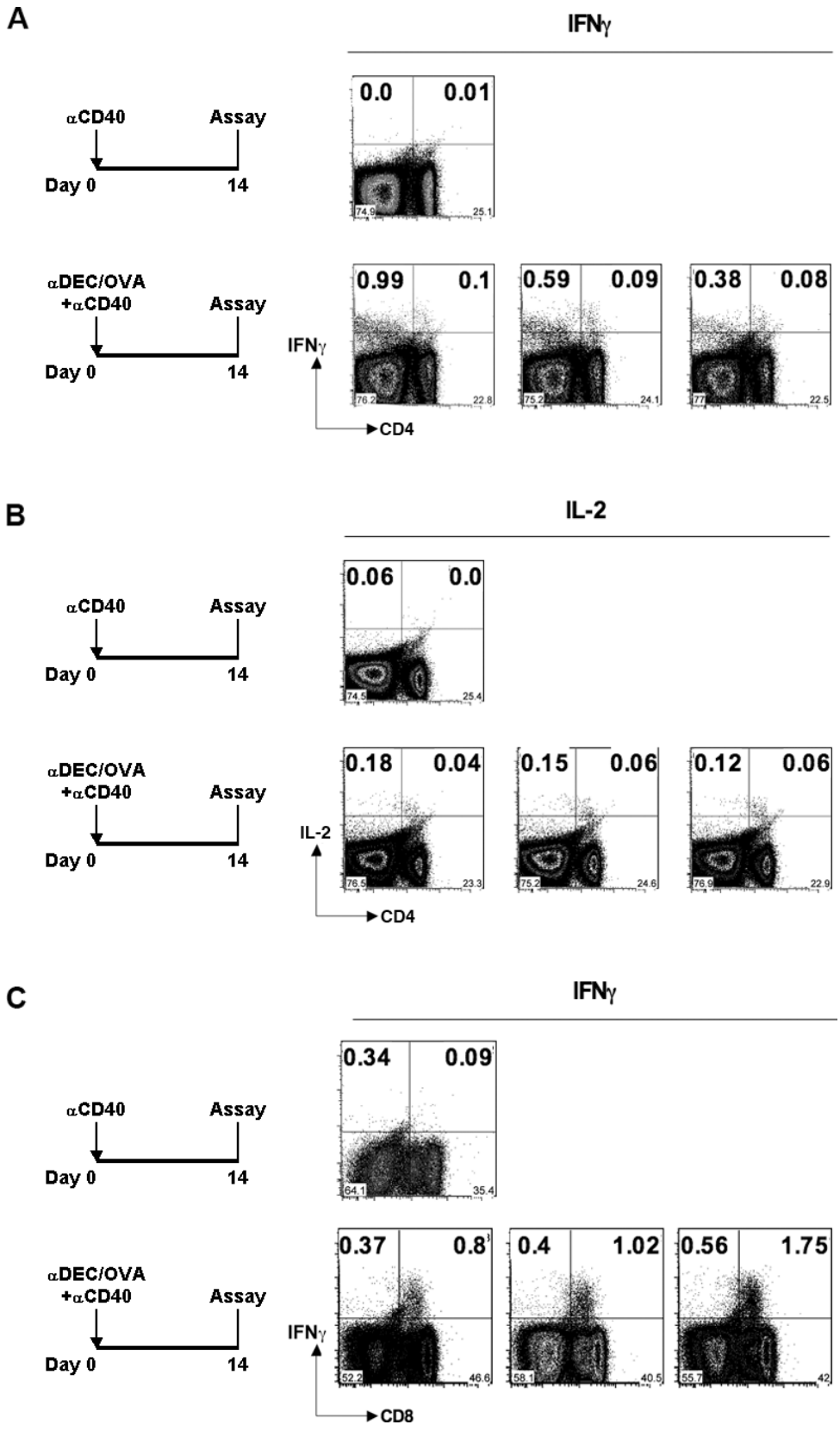

A
IFNγ
αCD40
Assay
Day 0
14
0.0
0.01
74.9
25.1
αDEC/OVA
+αCD40
Assay
Day 0
14
0.99
0.1
76.2
22.8
0.59
0.09
75.2
24.1
0.38
0.08
77
22.5
IFNγ
CD4
B
IL-2
αCD40
Assay
Day 0
14
0.06
0.0
74.5
25.4
αDEC/OVA
+αCD40
Assay
Day 0
14
0.18
0.04
76.5
23.3
0.15
0.06
75.2
24.6
0.12
0.06
76.9
22.9
IL-2
CD4
C
IFNγ
αCD40
Assay
Day 0
14
0.34
0.09
64.1
35.4
αDEC/OVA
+αCD40
Assay
Day 0
14
0.37
0.8
52.2
46.6
0.4
1.02
58.1
40.5
0.56
1.75
55.7
42
IFNγ
CD8

Figure 10. CD4 T cells from C57BL/6 mice treated with αDEC/OVA prior to immunization, show reduced levels of IL-2 but not IFNγ, following tolerization regimen.

C57BL/6 mice were injected with αDEC/OVA (5μg). 10 days later, mice were immunized with αDEC/OVA (5μg) in conjunction with αCD40 (50μg). 14 days later, bulk splenocytes were isolated and restimulated *in vitro* for 6 hrs, with αCD28 and the OVA peptide 75 bearing the Class I and II epitopes. Intracellular IFNγ (A) and IL-2 (B) was measured by flow cytometry. Plots are gated on CD3 cells.

Figure 10

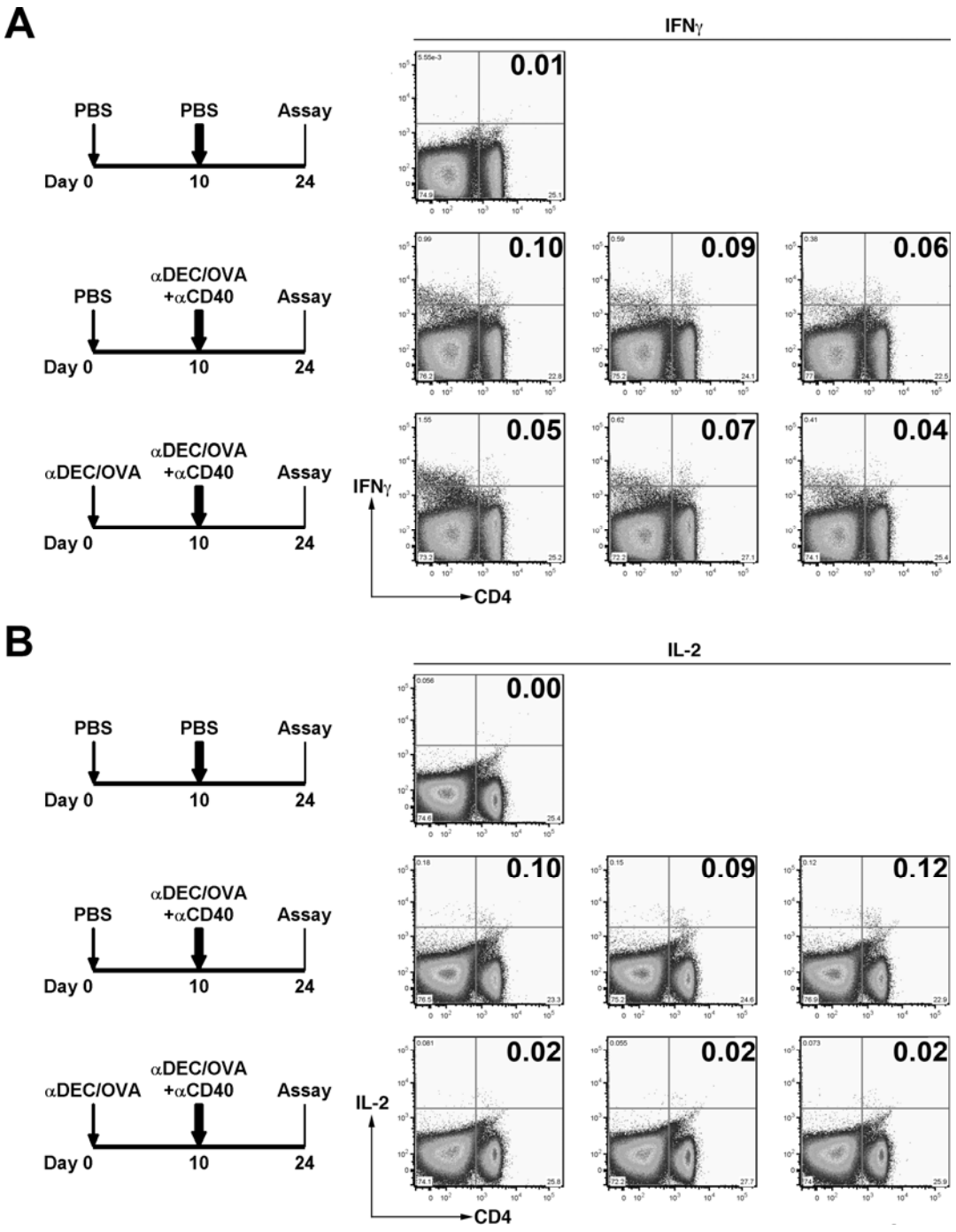

A
IFNγ
PBS
PBS
Assay
Day 0
10
24
0.01
αDEC/OVA
+αCD40
0.10
0.09
0.06
αDEC/OVA
0.05
0.07
0.04
IFNγ
CD4
B
IL-2
0.00
0.10
0.09
0.12
0.02
0.02
0.02
IL-2
CD4

Figure 11. CD4 T cells from C57BL/6 mice treated with αDEC/OVA prior to immunization, show increased levels of IL-10, following tolerization regimen.

C57BL/6 mice were injected with PBS or αDEC/OVA (5μg). 10 days later, mice were immunized with αDEC/OVA (5μg) in conjunction with αCD40 (50μg). 14 days later, bulk splenocytes were isolated and restimulated *in vitro* for 72 hrs, with medium or the OVA peptide 75 bearing the Class I and II epitopes. Supernatants were then assayed by LUMINEX for IL-10 and TNFα. Values represent mean cytokine concentrations with 4 mice per group. Error bars represent one standard error of the mean.

Figure 11

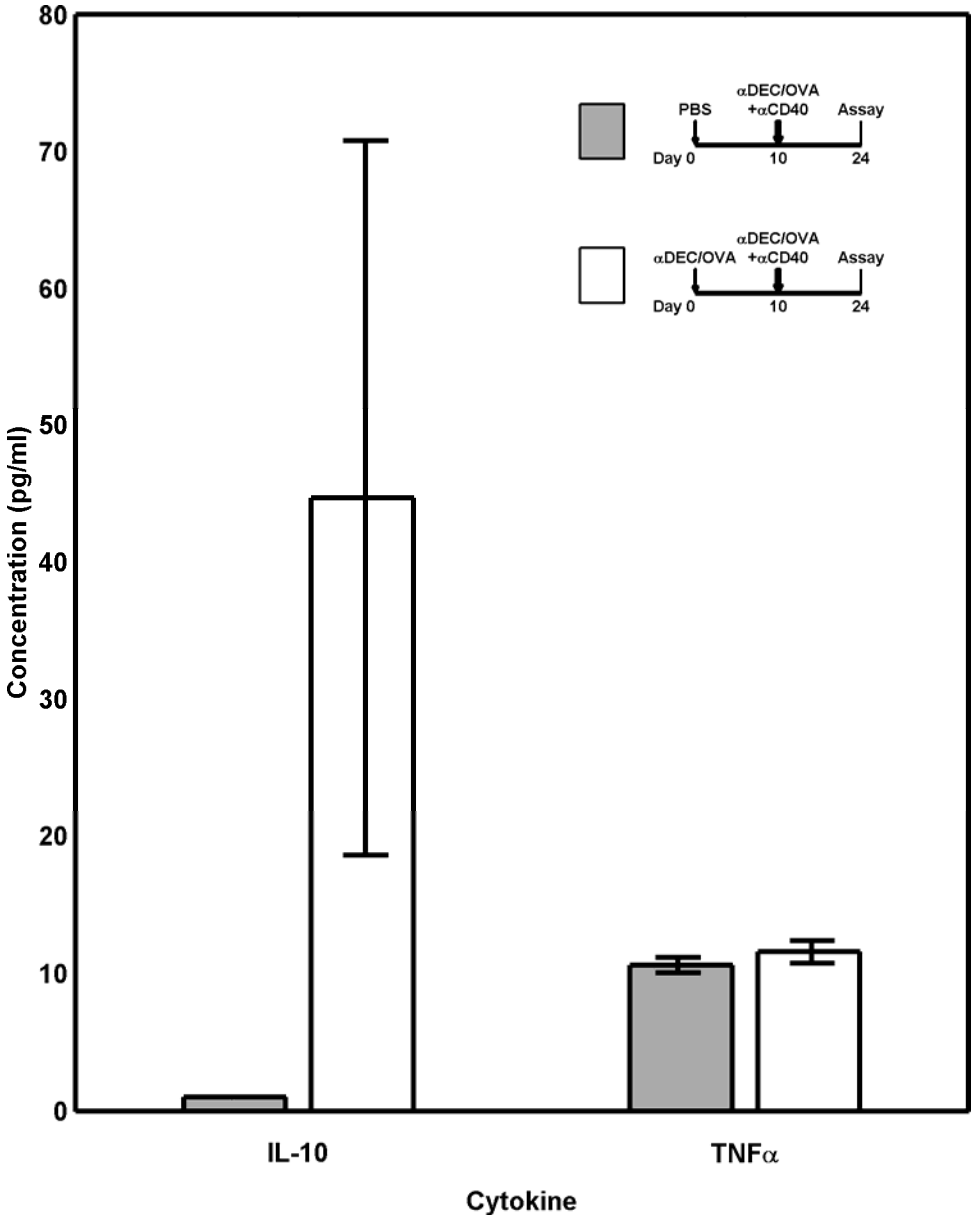

80
70
60
50
40
30
20
10
0
Concentration (pg/ml)
αDEC/OVA
PBS
+αCD40
Assay
Day 0
10
24
αDEC/OVA
αDEC/OVA
+αCD40
Assay
Day 0
10
24
IL-10
TNFα
Cytokine

Figure 12. Single or multiple doses of αDEC/OVA prior to immunization, abrogates antibody production in C57BL/6 mice.

Schematic representation of the protocols for tolerance induction, followed by immunization with αDEC/OVA along with αCD40 (A), or OVA in alum (D).

Groups of 8 mice each were treated with either PBS, a single dose of αDEC/OVA or three sequential doses of αDEC/OVA at 10 day intervals. 10 days after the final dose, mice were immunized with either αDEC/OVA along with αCD40, or OVA in alum. 14 days later, the mice were boosted with NP_{11}-OVA. Mice were bled 14 days post-boost. Symbols represent the log of αOVA titres (B and E) and αNP titers (C and F) of individual mice as measured by ELISA. Horizontal bars depict the mean of the log of the antibody titers for each group.

Figure 12

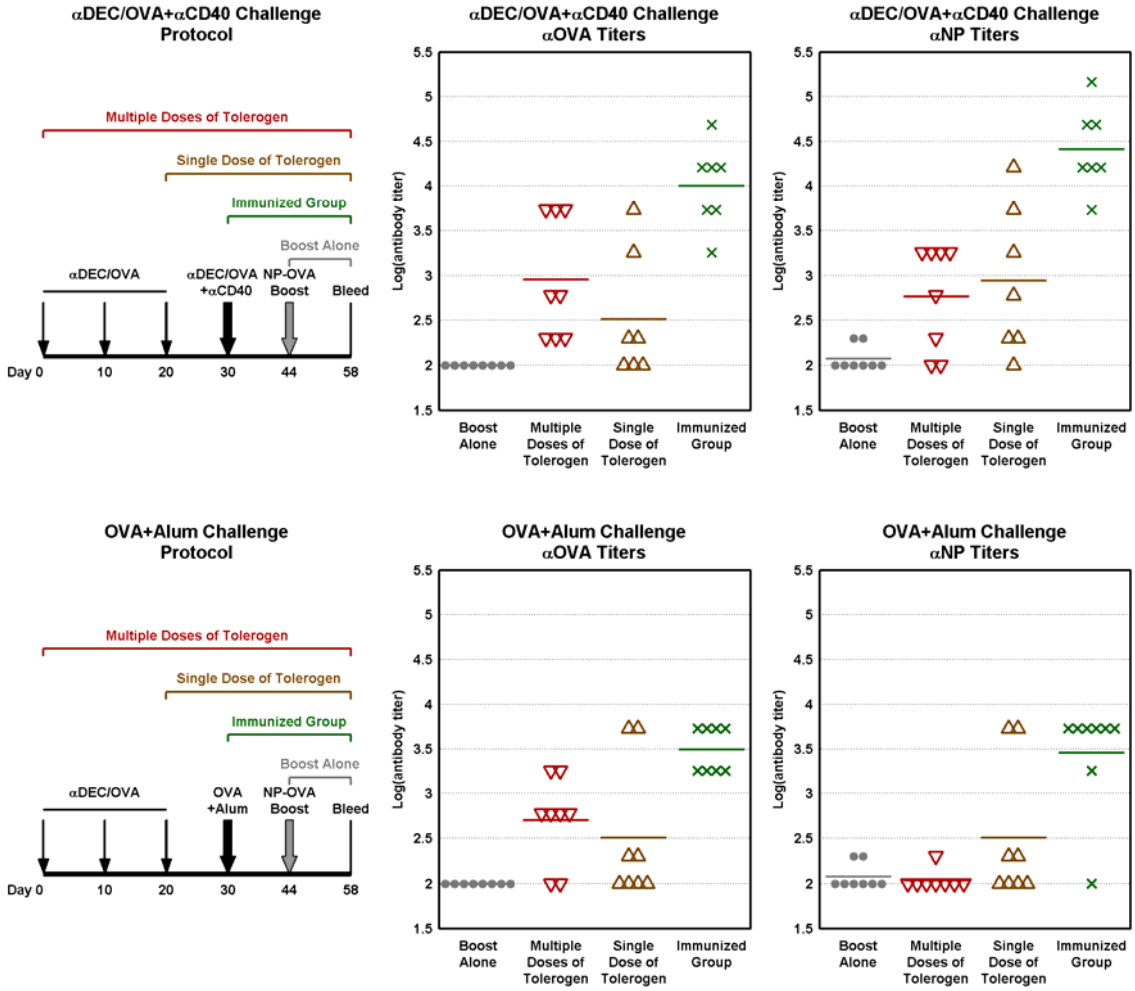

αDEC/OVA+αCD40 Challenge Protocol
Multiple Doses of Tolerogen
Single Dose of Tolerogen
Immunized Group
Boost Alone
αDEC/OVA
αDEC/OVA +αCD40
NP-OVA Boost
Bleed
Day 0
10
20
30
44
58
αDEC/OVA+αCD40 Challenge αOVA Titers
αDEC/OVA+αCD40 Challenge αNP Titers
Log(antibody titer)
5.5
5
4.5
4
3.5
3
2.5
2
1.5
Boost Alone
Multiple Doses of Tolerogen
Single Dose of Tolerogen
Immunized Group
OVA+Alum Challenge Protocol
OVA +Alum
OVA+Alum Challenge αOVA Titers
OVA+Alum Challenge αNP Titers

Figure 13. CD4 T cells from mice treated with αDEC/OVA prior to immunization, do not proliferate *in vitro* in response to antigen.

Mice were treated with PBS or αDEC/OVA (5μg) and 10 days later, immunized with αDEC/OVA (5μg) along with αCD40 (50μg). 14 days after immunization, bulk splenocytes were labeled with CFSE and pulsed with peptide with or without αCD28 *in vitro*. 3 days later, cells were assayed for proliferation by flow cytometry. Plots are gated on CD3+CD4+ cells. Identical gates have been applied to all samples.

Figure 13

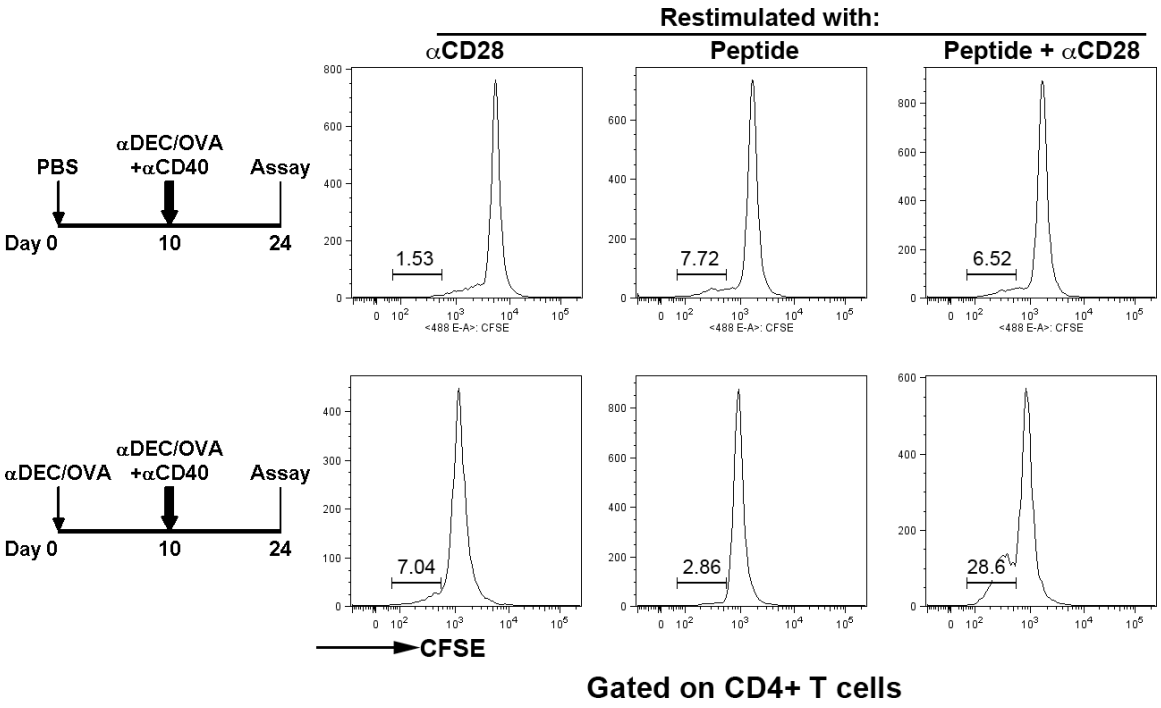
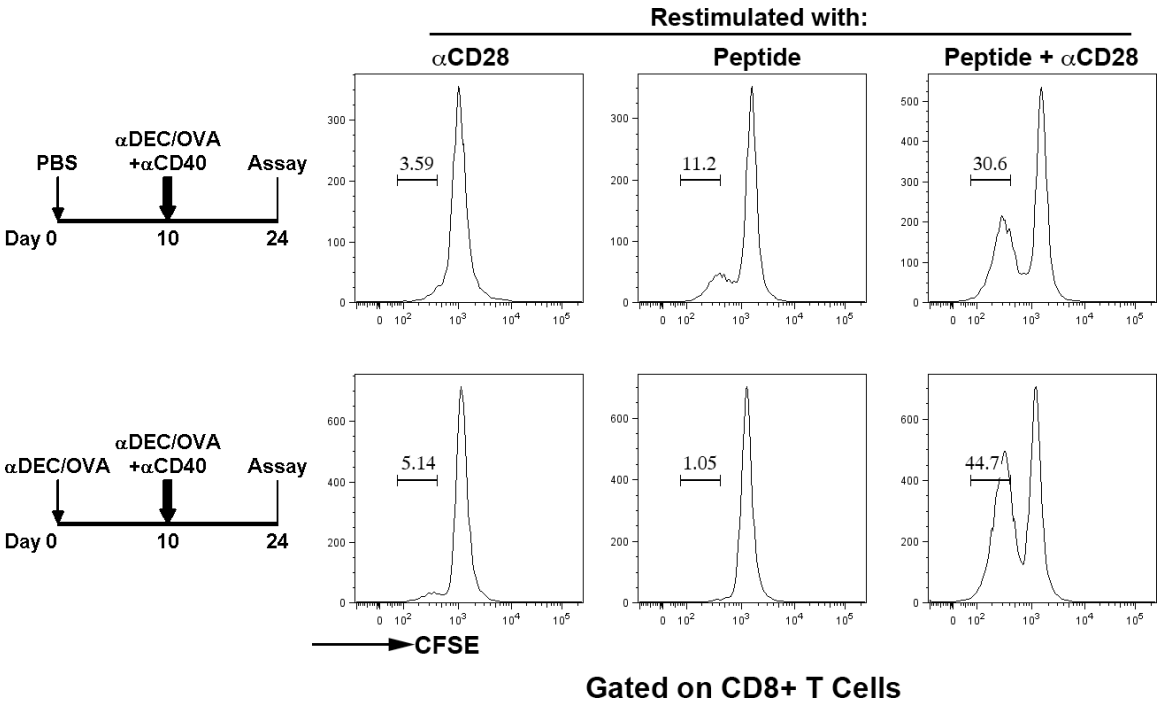

Restimulated with:
αCD28
Peptide
Peptide + αCD28
PBS
αDEC/OVA
+αCD40
Assay
Day 0
10
24
1.53
7.72
6.52
αDEC/OVA
αDEC/OVA
+αCD40
Assay
Day 0
10
24
7.04
2.86
28.6
CFSE
Gated on CD4+ T cells

Figure 14. CD8 T cells from mice treated with αDEC/OVA prior to immunization, do not proliferate *in vitro* in response to antigen.

Mice were treated with PBS or αDEC/OVA (5μg) and 10 days later, immunized with αDEC/OVA (5μg) along with αCD40 (50μg). 14 days after immunization, bulk splenocytes were labeled with CFSE and pulsed with peptide with or without αCD28 *in vitro*. 3 days later, cells were assayed for proliferation by flow cytometry. Plots are gated on CD3+CD8+ cells. Identical gates have been applied to all samples.

Figure 14


Restimulated with:
αCD28
Peptide
Peptide + αCD28
PBS
αDEC/OVA +αCD40
Assay
Day 0
10
24
3.59
11.2
30.6
αDEC/OVA
αDEC/OVA +αCD40
Assay
Day 0
10
24
5.14
1.05
44.7
CFSE
Gated on CD8+ T Cells

Chapter 6

Discussion

The intricate regulation of the immune system's ability to distinguish self from non-self is at the heart of what protects the animal from infection while preserving it from self-destruction. The establishment and maintenance of tolerance to self is a process that begins during fetal development and continues through the life of the animal. The negative selection of autoreactive T cells in the thymus, is a principal mechanism for the establishment of tolerance, nevertheless not every autoreactive T cell is deleted in the thymus. Some make it to the periphery, and in genetically pre-disposed individuals or special circumstances of inflammation and infection, can lead to autoimmunity. The establishment and maintenance of peripheral tolerance is therefore critical for the prevention of autoimmune disease.

An important fork in the divergent paths to immunity and tolerance in the periphery is the presentation of antigen to the T cells by the DCs. The context of the DCs during this presentation has been shown to be critical in the decision between tolerance and immunity. We have devised a strategy to deliver antigens specifically to DCs in situ, based on a genetically

engineered hybrid antibody comprising the variable region specific for the DEC-205 receptor on DCs, a constant region that was mutated to prevent binding to Fc receptors, and antigenic protein or peptide fused to the carboxyl terminus of the heavy chain. Using models of adoptive transfer of transgenic T cells into wild type mice, it was shown that antigen presentation by DCs in the steady state leads to tolerance, while presentation during inflammation leads to immunity. The fate of tolerized transgenic T cells was either deletion, or complete anergy. The anergy observed in these models was complete paralysis of the T cells, where they ceased to proliferate or make cytokines in response to subsequent challenge with immunizing antigen both *in vivo* and *in vitro*.

These results in transgenic models suggested a promising novel approach to prevention and therapy of autoimmune diseases. Our rationale was that delivering autoantigens to DCs using the targeting strategy would lead to tolerance of autoreactive T cells in the periphery, and the consequent prevention of progression to autoimmune disease. To evaluate this strategy of DC- targeted antigen therapy, we chose the experimental autoimmune encephalomyelitis (EAE) mouse model for human multiple sclerosis (MS). MS is an autoimmune condition affecting the central nervous system, with a

relapsing-remitting or progressive course that often leads to paralysis or even death. It is characterized by de-myelination of the neuronal sheath and the presence of multiple scarred areas in the brain infiltrated by T cells and macrophages. The EAE model of multiple sclerosis, is an acute model induced by immunization with brain specific proteins and peptides (143, 144), including a peptide from myelin oligodendrocyte glycoprotein (pMOG 35-55) (145).

To deliver MOG peptide to DCs *in vivo*, hybrid antibodies were produced with the MOG peptide fused to the carboxyl terminus of the cloned αDEC antibody (αDEC/MOG) (Figure 1A). Our results demonstrate that treatment of C57BL/6 mice with αDEC/MOG prior to immunization with MOG peptide, protects them from developing disease (Figure 1B). Also treatment with αDEC/MOG prevents accumulation of effector CD4 T cells in the spinal cord (Figure 1C).

Our success in the prevention of EAE, led us to then investigate the therapeutic potential of the DC based antigen delivery strategy in the Non-Obese Diabetic (NOD) mouse model. The NOD mouse is an important model of autoimmune Type 1 diabetes because it shares many genetic,

immunological and pathological similarities with the human disease (146-150). The destruction of the insulin producing β cells in the pancreatic islets by autoreactive CD4 and CD8 T cells leads to the disease. Diabetes onset typically occurs at 12 to 14 weeks of age in female mice and slightly later in male mice. Beginning as early as 3-4 weeks of age, both female and male NOD mice demonstrate mononuclear infiltrates that surround the islet (peri-insulitis). These infiltrates progress and invade the islets (insulitis) over the subsequent few weeks. 60%-80% of females and 20- 30% males ultimately progress to overt diabetes (146).

One of the most important loci in the genetic susceptibility to diabetes in NOD, is H-2^{g7}, the MHC locus. The MHC Class II I-A^{g7} molecule encoded by this locus has a low-affinity peptide binding groove, which has been suggested to be involved in defective negative selection of autoreactive T cells in the thymus (151, 152). Some of the major autoantigens implicated in the pathogenesis of T1D are insulin, glutamic acid decarboxylase, insulinoma-associated protein 2, IGRP and heat shock protein 60 (134).

To evaluate the therapeutic potential of the dendritic cell- targeted antigen delivery strategy in the chronic spontaneous diabetes model, the 9-23

peptide of the β chain of insulin was selected as the antigen of choice. This peptide has both CD4 and CD8 immunodominant epitopes that have been implicated in the disease (134). A hybrid antibody was produced with the insulin peptide fused to the carboxyl terminus of the cloned αDEC antibody (αDEC/INS) (Figure 2A). Chronic treatment of NOD mice with αDEC/INS starting at age 5 weeks, failed to delay or prevent onset of diabetes (Figure 3A, B and C).

The failure of the autoantigen targeting strategy in NOD in contrast to EAE, pointed to the complexities of the NOD disease model and the challenge of tolerizing disease-prone polyclonal repertoires. The EAE model is an acute induced model of autoimmunity, which means that the system is in a non-inflamed steady state conducive to the induction of tolerance, until the time when immunity- induced inflammation sets in. Also until induction of disease, the MOG specific T cells are ignorant of their antigen, since it is sequestered behind the blood-brain barrier. Thus when the MOG antigen is presented for the first time, by steady state DCs to MOG-specific T cells in the periphery, the outcome is tolerance.

In striking contrast, the NOD model, which mimics progressive human autoimmune disease, has a chronically inflamed immunological environment from the time the mice are as young as 3-4 weeks old. Thus the DCs in this model are most likely in a chronic state of activation, while the insulin-specific T cells encounter their circulating antigen via the DCs even before treatment with αDEC/INS begins. Also in the EAE model the pathogenic T cells that are induced by MOG peptide immunization, are of a single specificity, and are addressed by the MOG peptide delivered through DC targeting. In the NOD model of spontaneous autoimmunity however, pathogenesis is caused by autoreactive T cells of varying islet specificities. We had hoped that since the 9-23 epitope in insulin is immunodominant, tolerizing autoreactive T cells against this epitope would suppress T cells with other specificities as well (153). However, therapy in NOD using DC based delivery of antigens, might require a combination of islet antigens in order to be successful.

Our results made us realize that achievement of peripheral tolerance in NOD is complicated by several factors, and insights into successful DC-based therapeutic strategies would have to come from a thorough understanding of DC regulation of peripheral T cell responses in NOD.

To examine polyclonal T cell tolerance in NOD without the complicating variables associated with autoantigens, we asked whether foreign antigen-specific peripheral tolerance could be achieved. The antigen that was chosen for targeting to the DCs was the HIV GAG p41 protein which generated detectable IFNγ responses in the I-A^{g7}-restricted CD4 compartment in B6.$H2^{g7}$ mice (Figure 4A). Consistent with *in vitro* studies by others (139), the presence of the I-A^{g7} molecule in the C57BL/6 background (B6.$H2^{g7}$) severely dampens T cell responses in comparison to those in wild type C57BL/6 mice. However, the NOD mice appear to have additional suppression of T cell responses compared to the B6.$H2^{g7}$ mice (Figure 5). These results indicate that not only is the MHC II in NOD mice defective, but there are likely other defects in the DC and CD4 T cell compartments. Others have reported defects in *in vitro* antigen presentation and costimulation by NOD APCs. There have also been reports of enhanced cytokine effector responses upon non-specific TCR stimulation in NOD as compared to B6.$H2^{g7}$ (141, 152). Our results *in vivo* however show dampened IFNγ responses to immunizing antigen targeted to NOD DCs. In the future it would be interesting to dissect whether the *in vivo* defect in responsiveness that we see, lies in the DC or the CD4 compartment.

To examine tolerance against the foreign GAG p41 antigen, NOD mice were pretreated with αDEC/GAG p41 without adjuvant, prior to immunization with αDEC/GAG p41 in conjunction with αCD40. This treatment with αDEC/GAG p41 does not alter the ability of CD4 T cells to make IFNγ in response to immunogenic challenge (Figure 7). Thus the effector cytokine response in NOD was not altered by DC targeted antigen delivery, under the conditions examined.

The failure to achieve tolerance in the disease- prone NOD repertoire, brought into strong focus the need to study polyclonal tolerance in a non-disease prone model. Thus far all the studies on antigen targeting to DCs have involved transgenic T cell models. In those simplistic systems tolerance resulted in either deletion or complete anergy of the T cells, characterized by a loss of proliferation and cytokine production. However, polyclonal models have several complicating factors. Firstly, polyclonal T cells respond to several different epitopes in an antigen, while transgenic T cells respond to a single one. Thus tolerizing transgenic T cells is less challenging. Secondly, for the same epitope specificity, there is a wide range of affinities amongst poylconal cells, while transgenic T cells are of single specificity and high

affinity. There is evidence that high affinity T cells upon interaction with tolerizing antigen get deleted and thus tolerized, while lower affinity T cells are not deleted but persist, and under certain conditions of inflammation respond to immunogenic stimuli (154). Thirdly, experiments involving transgenic T cells have always been carried out by adoptively transferring these cells into a wild type mouse and then tracking their fate. This is therefore a closed system which receives no input from the thymus, unlike polyclonal wild type repertoires that are constantly being replenished from the thymus (155). In a system of induced peripheral tolerance such as the one studied here, the influx of naïve antigen specific T cells from the thymus, although infrequent, could potentially lead to a reversal of tolerance upon challenge. For all these reasons, establishment and maintenance of tolerance in polyclonal models is considerably more difficult to achieve when compared to tolerance in transgenic T cells.

We therefore decided examine in detail, the establishment and maintenance of wild type polyclonal tolerance, to glean insights into the pathways of immune regulation. C57BL/6 mice were studied for responses against the CD4 and CD8 epitopes of OVA. Treatment with αDEC/OVA prior to immunization with αDEC/OVA in conjunction with αCD40, leads to an

alteration in the cytokine profile of the CD4 and CD8 T cells. Both CD4 and CD8 T cells continue to make IFNγ while IL-2 levels are diminished in the CD4 compartment (Figure 10). IL-2 is required to maintain proliferative responses during clonal expansion in response to antigen challenge and for T cell survival *in vivo*. In the absence of IL-2, effector CD4 and CD8 T cells cannot survive long enough to become memory (156-158). Thus despite the fact that the tolerized T cells continue to make IFNγ in the short-term (14 days), the lowered ability to make IL-2 probably impacts the longevity of these cells, ultimately leading to death.

Since its discovery IL-2 was thought to be solely a pro-immune cytokine, until that role was challenged by studies showing that mice deficient in IL-2 or its receptor components develop lymphoproliferative diseases and autoimmunity (159, 160). Recent reports have explained this immuno-suppressive role of IL-2, by demonstrating that it is crucial to the development, survival and function of CD4+CD25+ T regulatory cells (Tregs) (161). These results are now widely interpreted to be indicative that IL-2 is crucial only for tolerance and not for immunity *in vivo* (161). Our results however suggest that IL-2 might be required to maintain T cell responses *in vivo* as functional tolerance correlates with the absence of this

cytokine. There maybe a intricate balance between the effects of IL-2 on Tregs versus effector T cells *in vivo*. Depending on whether IL-2 levels are limiting, tolerance or immunity dominate. To test if the abrogation of T cell responsiveness that we see in our system, is due to the loss of IL-2, we plan to examine the consequences of supplying exogenous IL-2 *in vivo*. We hypothesize that if the loss of IL-2 is responsible for the tolerance established *in vivo*, then exogenous IL-2 would result in rescue of T cell responsiveness.

To examine whether other cytokines produced by the T cells were altered after tolerizing regimen of antigen, we tested a panel of different cytokines. Tolerized T cells gained the ability to make low levels of IL-10, when compared to immunized T cells. None of the other cytokines tested were altered after exposure to the tolerizing regimen (Figure 11). IL-10 is a pro-tolerance cytokine made by a subset of regulatory T cells under tolerogenic conditions, that has been shown to suppress inflammatory responses *in vivo* (129). We did not find an increase CD4+CD25+Foxp3+ regulatory cells in mice that had been tolerized, indicating that the tolerance we see is possibly mediated by an alteration in the cytokine mileu and the balance between effector and suppressor cytokines such as IL-2 and IL-10.

CD4 T cells that have been exposed to tolerizing antigen prior to immunogenic challenge, lose their ability to provide help to B cells for antigen-specific humoral responses. Mice receiving αDEC/OVA prior to immunization had significantly diminished antibody titres compared to mice that had only been immunized (Figure 12). Therefore targeting of antigen to DCs in the steady state leads to polyclonal CD4 T cell tolerance, as well as tolerance of the humoral responses against antigen.

The antibody titres were more diminished in the case of tolerized mice that had been immunized with OVA in alum, compared to mice that had received αDEC/OVA along with αCD40 (Figure 12). Thus the exposure of tolerized polyclonal CD4 T cells to αCD40, which is a potent activator of DCs, appears to induce some degree of reversal of tolerance *in vivo*. This result is supported by a study on polyclonal CD8 T cells showing that high affinity T cells are deleted in response to tolerizing antigen, while low affinity cells are not deleted but persist, and can be reactivated upon potent costimulation (154).

CD4 and CD8 cells pretreated with the tolerizing regimen, failed to make proliferative responses *in vitro* in response to peptide restimulation, demonstrating that as a consequence of tolerance, T cells lose their ability to cycle. This can be explained by the loss of IL-2, since it has been shown to be indispensable for *in vitro* proliferation and differentiation of T cells (162). Surprisingly, when CD4 and CD8 T cells were pulsed *in vitro* with peptide in the presence of αCD28, the cells made stronger proliferative responses in the tolerized mice compared to the immunized mice (Figure 13 and 14). Thus both CD4 and CD8 T cells that are tolerized, fail to proliferate *in vitro* upon restimulation with antigen. However, αCD28 can reverse the abrogation in proliferation. There are two explanations for this observation. Either, the αCD28 is reversing anergy in the tolerized T cells, by pushing them into cycle, or it is activating a hypo-responsive population of antigen-specific T cells, that had hitherto been unresponsive to the antigen. The observation that cells from tolerized mice that have seen antigen twice, i.e. during tolerization as well as immunization, make stronger proliferative responses in the presence of αCD28 than cells from immunized mice that have seen antigen only once, seems to suggest that the former explanation is more likely. Thus tolerized CD4 and CD8 T cells, appear to be in a state of anergy that can be reversed upon exposure to αCD28 mediated

costimulation. αCD28 is a potent costimulator of T cell activation that is involved in stabilization of cytokine mRNA and long-lasting IL-2 secretion and proliferation. Thus in the case of the tolerized CD4 and CD8 T cells, αCD28 might be removing the proliferative block on the cells, by restoring IL-2 secretion (142).

Concluding Remarks

Our results demonstrate that polyclonal T cell tolerance in a wild type repertoire can be achieved by targeting of antigen to DCs in the steady state. This tolerance seems to be a form of non-deletional, partial anergy. Unlike anergic transgenic T cells, these cells do not completely lose their ability to make cytokines. Instead they continue to make IFNγ while losing the ability to make IL-2, and gaining the ability to make IL-10. This partial anergy can be reversed with strong costimulatory stimuli, such as αCD40 *in vivo* and αCD28 *in vitro*.

This susceptibility to reversal of T cell tolerance in wild type mice as compared to transgenic models, could be explained by the nature of polyclonal T cells. Polyclonal cells range in affinities and specificities, with the average affinity being lower than that of most high affinity, single

specificity transgenic T cells. In the case of transgenic T cells, strong signaling during high affinity interactions with tolerizing antigen leads to deletion or complete anergy. In contrast, in lower affinity polyclonal T cells, weaker strength of signaling during interactions with tolerizing antigen may lead to partial anergy that can be reversed under conditions of strong costimulation.

Our results with polyclonal T cell tolerance in a non-disease prone repertoire, provide an important understanding of the mechanisms and regulation of peripheral tolerance in wild type mice. They offer critical insight into why the establishment and maintenance of tolerance in models with chronic inflammation and low affinity T cell repertoires such as NOD, is a challenge. This understanding is an important step towards the successful design of antigen-specific DC based therapeutic strategies for prevention and treatment of autoimmune disease.

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