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Equal recognition of self by mediators and suppressors while poor recognition leads to ignorance.

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Equal recognition of self by mediators and suppressors while poor recognition leads to ignorance.

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B.S., University of Kansas, 2006

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Abstract

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By Jennifer D. Hood

The strength of interaction of the T cell receptor (TCR) with peptide presented in the context of major histocompatibility complex (pMHC) is thought to influence the fate of T cells. For thymocyte development, TCR must bind self pMHC at a basal level to institute MHC restriction while any cell with extremely high affinity for self needs to be eliminated during negative selection to limit autoimmunity. Differential processing of ubiquitous antigens and induction of anergy is thought to prevent recognition of these selecting ligands in the periphery. High affinity interactions potentially drive thymocytes to become Foxp3-expressing regulatory $CD4^+$ T cells (Tregs). Once in the periphery, affinity influences the ability of cells to functionally respond to antigen, where in a polyclonal setting high affinity cells could out-compete lower affinity TCRs to dominate. We investigated T cell affinity for 3 different self antigens. Using a mouse model of autoimmunity against myelin, the affinity profiles of polyclonal self-specific $CD4^+$ conventional T cells (Tconvs) and Tregs were found to overlap and span a similar range. Both were dominated by lower affinity cells, but at the site of autoimmunity, there was skewing towards higher affinity cells in the Tconvs, which is contrary to what would be expected if Tregs are to be enriched for higher affinity self-specific cells. In a second system, determination of TCR affinity for a known endogenous positive selecting ligand revealed that it is lower than cognate agonist as expected. Lastly, a diabetogenic antigen (ChgA) was identified with its lower affinity leading to a lack of functional response to this self antigen. Moreover, analysis of TCR affinities for monoclonal transgenic T cells demonstrated that affinity can be dynamic changing with developmental or activation state. We found that DP thymocytes have the highest affinity followed by activated cells and then naïve peripheral cells regardless of antigen examined. Together these data demonstrate that there are important factors altering the effective affinity which is consistent with the differential sensitivity to stimulation of the various cell states. These results also highlight the potential importance of matched antigen specificities and similar affinities for Teffs and Tregs during autoimmunity.

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Chapter 1: Introduction

When at least two proteins interact there is a cascade of events that culminates in a cellular response. Understanding the biophysical parameters of this interaction provides insight into the intricacy of the immune response. The number of foreign antigens that exists greatly outnumbers the breadth of the diversity available for the T cell receptor (TCR). There is the added layer of complexity of needing those TCRs to remain ignorant or tolerant of self to limit autoimmunity. This selection of TCRs is accomplished in the thymus during a two-step process where self peptides are presented in the context of major histocompatibility complex (MHC). The TCR needs to be able to interact with MHC at a basal level to allow for MHC restriction, but any receptor that binds self too strongly needs to be eliminated or anergized to avoid overt autoimmunity. Better understanding of the affinities required to mediate selection could provide insights into why sometimes there is a breakdown in tolerance. Additionally, understanding the way in which TCRs discern self from non-self would allow for manipulation of the response to either: (a) boost immune responses to chronic infections leading to clearance of the pathogen or (b) dampen the response to restore tolerance during autoimmune diseases.

How affinity shapes the T cell repertoire

Developmental fate of thymocytes dependent on TCR affinity

Hematopoietic stem cells in the bone marrow give rise to the common lymphoid and common myeloid progenitors which are the sources of all the cells of the immune system. Lymphocytes (T, B and NK cells) differentiate from the common lymphoid progenitor, while all other leukocytes arise from the common myeloid progenitor. Initial differentiation occurs in the bone marrow, but T cells must travel to the thymus, a highly

specialized organ with unique architecture, to complete their development. Not only are T cells dependent on the thymus, but so too do thymic structures require interaction with thymocytes. When there is a block in maturation of thymocytes at their earliest stage, then the highly organized three dimensional (3-D) nature of the cortex is absent as are the strict distinctions between the cortex and medulla (1). If these mice are given bone marrow from different mice, it was shown that there is a stepwise progression in correcting the defects in the thymic architecture. Thymectomy of neonatal mice results in profound lymphopenia and immune dysfunction as demonstrated by acceptance of allogeneic skin grafts and inability to generate antibodies upon vaccination (2). The majority of these mice die from a wasting disease between two and four months of age. Ontogeny studies showed that T cells capable of lysis (cytotoxic T lymphocytes (CTLs)) or assisting B cells to produce antibodies (helper T cells) first appear in the thymus one day after birth while in the periphery there was a delay of four to six days (3-5). These studies hinted at the possibility that the thymus was required for the functional development of mature T cells that were capable of eliciting the interrogated functional readout.

Pre-thymocytes enter the thymus through high endothelial venules located in the cortex. They are termed double negative (DN) cells because they lack expression of co-receptors ($CD4^-CD8^-$). DN cells can be subdivided into 4 stages based on surface expression of CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Rearrangement of the gamma, delta and beta TCR chains begins at the DN2 stage and is completed by DN4 (6). Depending on which chains successfully rearrange, the thymocyte commits to either γδ or αβ T cell lineage, with the

former being thought of as an innate cell and the latter a member of the adaptive immune response. Once a β chain rearranges it pairs with pre-TCRα to determine if the rearrangement was successfully. If the pre-TCR is able to signal, then the TCR α chain locus begins to undergo recombination after a round of proliferation and the DN4 cell transitions to the double positive (DP) stage by upregulating CD4 and CD8.

DP thymocytes remain in the cortex to undergo positive selection, which is the process that ensures that the TCR is able to recognize antigen in context with the appropriate MHC. Mixed bone marrow chimeras showed that the radioresistant cortical thymic epithelial cells (cTECs) needed to express the correct MHC in order for positive selection to occur (7). Using unique machinery, cTECs generate "private" self peptides from ubiquitously expressed self proteins. For MHC class I, the proteasome incorporates the β5t subunit along with β1i and β2i subunits and is termed the thymoproteasome (8). Unlike the β5 and β5i subunits which produce cleaved peptides with hydrophobic C-termini, the β5t subunit has hydrophilic residues that line the S-1 pocket, thereby changing the specificity of the cleaved peptides (8). The cleaved self antigens lack a C-terminus that can effectively bind the hydrophobic pocket of MHC class I, which could help to limit the strength of signal received by the DP thymocyte through this instability thereby allowing for its selection. In addition to creating a weaker class I binding peptide, this differential processing of self by the thymoproteasome creates epitopes that would not be generated by the housekeeping and immunoproteasome which both cleave after hydrophobic residues (8). When mice lack the β5t subunit the frequency of $CD8^+$ thymocytes and splenocytes is greatly reduced without any changes in the frequency of $CD4^+$ T cells (8-10). In addition $CD8^+$ T cells that do develop have an altered repertoire

that is defective in allogeneic and antiviral responses (9). This β5t subunit is required for normal selection, because the defect in CD8 development occurs when the β5i subunit is knocked into the β5t gene, restricting its expression to cTECs (10).

cTECs express cathepsin L (CSTL) as one of the proteins involved in the degradation pathway of the invariant chain that binds MHC class II. Mice that are deficient for CSTL have decreased frequencies of $CD4^+$ thymocytes and splenocytes (11). This defect is most likely due to the over expression of surface CLIP:MHC class II molecules which are unable to mediate positive selection. The authors noted that like H-$2M^{-/-}$, $CSTL^{-/-}$ thymic epithelium was unable to mediate the positive selection of three different IA^b restricted TCR Tg mice (11). Deficiency in CSTL and H-2M would cause global defects in positive selection since they are essential for the proper processing of invariant chain and loading of peptides onto MHC class II respectively. To cleave endogenous self proteins for presentation, cTECs can use the thymus-specific serine protease (TSSP) although knocking out this gene didn't cause any quantitative differences in the frequency of $CD4^+$ T cells (12, 13). TSSP seems to be involved in the processing of select antigens in cTECs which impaired the positive selection of OT-II and Marilyn Tg cells (14), in addition to polyclonal responses to hen egg lysozyme (HEL) (15) and diabetogenic antigens (16, 17).

cTECs generate private peptides in order to ensure that TCRs are able to recognize antigens in the context of MHC. If the rearranged TCR is unable to interact with these private self peptides and MHC then they will die by neglect, while those that can will undergo negative selection. Although this process primarily occurs in the medulla, $CD11c^+$ dendritic cells (DCs) are capable of inducing apoptosis in SP

thymocytes present in the cortex (18). Thymic epithelial cells in the medulla (mTECs) are able to present thousands of self-proteins regardless of tissue restriction or gene silencing. To eliminate self-reactive thymocytes, they express the unique transcription factor termed autoimmune regulator (AIRE), which binds to and allows the promiscuous production of proteins that are normally tightly regulated. Deficiency in AIRE causes system-wide autoimmunity because of the inability to delete auto-reactive thymocytes to these tissue restricted antigens (TRAs). When a portion of ovalbumin (OVA) protein is placed under the rat insulin II promoter, which allows for expression of OVA in the thymus and periphery, $CD8^+$ transgenic T cells (OT-I) are deleted in the thymus (19).

Both positive and negative selection are dependent on how well the TCR is able to bind and interact with peptide:MHC (pMHC) complexes, the strength of which is thought to dictate the fate of developing thymocytes, known as the instructive or avidity/affinity selection model (see Figure 1.1). During positive selection, thymocytes that bind pMHC too weakly, i.e. low affinity, will be unable to accumulate enough signaling events through their TCRs to survive and will die by neglect. Basal recognition of self peptides in the periphery is required for the homeostasis of T cells, but this interaction is inadequate for full activation thereby maintaining peripheral tolerance to these ubiquitous self antigens. The unique processing of self antigens by cTECs helps to maintain this central tolerance, since the epitopes they generate are different from the antigens that would be presented in the periphery. For TCRs that interact too strongly, high affinity interaction, with self pMHC primarily in the medulla, the thymocyte will be negatively selected and undergo apoptosis to eliminate cells that could be activated in the periphery by self. It has been suggested that some of these higher affinity thymocytes can

survive negative selection by differentiating into thymic regulatory T cells (tTregs).

The premise that weak and strong interactions dictate the fate of developing thymocytes has been developed over many decades using TCR transgenic (Tg) mice with known antigen specificities. The employment of fetal thymic organ cultures (FTOCs) has greatly expanded our understanding of T cell development, because although they initially lack cells capable of lysis, culturing the thymic lobes *in vitro* allowed for the development of CTLs (20). Using FTOCs and altered peptide ligands (APLs) for OT-I $CD8^+$ T cells, it has been shown that cognate antigen mediates negative selection of thymocytes, while APLs can have differential effects depending on the strength of the interaction (21). Antagonist peptides, which can interfere with agonist stimulation but are non-stimulatory alone, can mediate positive selection of Tg thymocytes, while partial agonists can mediate both positive and negative selection. This study provided the groundwork for the current avidity selection model for thymocytes. Due to the unique experimental system crossing $CD8^+$ TCR transgenic mice to mice that lacked expression of β2-microglobulin (β2-M), allowed for an ideal FTOC to study the synchronous development of $CD8^+$ T cells upon the addition of various peptides and exogenous source of β2-M (22). Follow-up studies using other $CD8^+$ Tg mice provided further support of the avidity selection model although it was also shown that low enough concentrations of agonist peptide could allow for the emergence of $CD8^+$ T cells from FTOCs (23, 24).

However it was difficult to obtain similar results for $CD4^+$ T cells due to the lack of a good experimental system. In addition, there were conflicting data regarding the ability of antagonist peptides to mediate positive selection of $CD4^+$ TCR transgenic thymocytes (25-29). For N3.L2 thymocytes, known agonist and antagonists mediated

negative selection when the APLs were expressed at physiological levels in MHC class II expressing cells (27). In fact only one APL demonstrated increased positive selection of N3.L2 T cells, and this peptide had no effect on peripheral cells in addition to a lack of binding in surface plasmon resonance (SPR) experiments, which measures the specific interaction of proteins of interest (27). Similar results where obtained using a pigeon cytochrome C (PCC) specific $CD4^+$ TCR transgenic, where the previously identified antagonist APL was unable to induce positive selection of thymocytes and when it was supplied with the agonist peptide actually inhibited selection by PCC (28). These authors used a bioinformatics approach to identify likely endogenous positive selecting peptides on the basis that they would share similar sequence to the agonist peptide. This endogenous peptide was a weak agonist that was able to mediate selection of Tg cells. For two different moth cytochrome C- (MCC-) specific TCR Tgs, endogenous positive selecting ligands have been identified, and although the cells recognize the same MCC sequence, their endogenous ligands differ (30, 31). Both endogenous peptides were capable of boosting the response of their respective Tg cells in the presence of low doses of agonist peptides, which makes them coagonists.

$CD4^+$ T cells can differentiate into effector or regulatory T cells in the thymus. Initial studies using $CD4^+$ Tg mice crossed to mice which expressed the cognate antigen as a "neo-self" antigen lead to the hypothesis that only the highest avidity interaction could lead to the generation of Tregs. Jordan et al. showed that when they crossed their neo-self mice to a TCR Tg that was specific for an APL variant of hemagglutinin (HA) that had lower functional response to the parent HA peptide, there was no induction of Tregs, implying that only high functional avidity interactions were capable of generating

Tregs (32). These results were further corroborated in other neo-self models (33-35), thymic organ cultures with APLs (36), and using a Nur77-GFP reporter mouse which reads out the strength of TCR signal received by cells (37). However not all neo-self models allowed for the skewing of thymocyte development into the Treg lineage and instead lead to massive thymic deletion, which lead to questioning the validity of the avidity selection model (38, 39).

TCR repertoire studies comparing $CD4^+$ Teffs and Tregs have had mixed results; equally broad with significant overlap in sequences (40), Tregs have more diversity with distinct TCRs from Teffs (41), and Tregs and Teffs are enriched for non-self specificities without Tregs being preferentially self-reactive (42). When Treg TCRs are used to make Tg mice, unlike other Tgs examined previously, expression of the specific TCR only occurred when it was at low precursory frequencies in the thymus (43-45), which suggests that there is a limiting factor. To test this, DN thymocytes expressing TCRs with various avidities for OVA were injected into the thymi of RIP-mOVA mice, which express a membrane bound OVA under the control of the rat insulin promoter (46). There was a range of TCR avidities that were capable of developing Tregs, with the higher avidity TCRs giving the largest number of Tregs. The TCR avidities tested displayed an inverse relationship between precursor frequency and efficiency of Treg generation; however the two highest avidity TCRs at the lowest tested precursor frequency failed to support Tregs. In fact the lowest avidity TCR was most efficient at Treg generation at 100-fold lower precursor frequency than the higher avidity TCRs, even though the former only produced half as many Tregs as the latter. This could be intraclonal competition for a single selecting antigen that is expressed at low levels.

These studies lead to a modified thymocyte selection model requiring TCR-independent and dependent steps. The two-step model is similar to the instructive model in that strength of signal dictates survival, but Tregs and Teffs are capable of positively selecting over a range of affinities. Some studies have suggested that tTregs might still be enriched for higher affinity self reactive cells, because they are more resistant to apoptosis than Teffs. Although it has been shown that expression of Foxp3 induces a proapoptotic signature of increased levels of Puma, phosphorylated Bim and JNK-1/-2 and decreased levels of Bcl-2 and the dual specific phosphatases (DUSP1, DUSP5, and DUSP6) (47). This signature was specific to Foxp3 binding to the forkhead box binding domains in *Bbc3* (Puma)*, Bcbl2l1* (Bim)*, DUSP6,* and *Bcl-2*. Thymocytes which expressed Foxp3 needed to compete for limited IL-2 in order to mature and survive. The authors also showed that the proapoptotic signature observed in $Foxp3^{+}$ thymocytes was not due to mere high affinity engagement of the TCR, because AND TCR Tg $RAG2^{-/-}$ thymocytes that encountered their cognate PCC:IE^{k} complex in the thymus did not upregulate this signature or Foxp3 and instead expressed higher levels of Bim, Bcl-2, DUSP6 with decreased levels of Puma and unchanged levels of phoshorylated JNK (47).

More support for the two-step model came from studies looking at the TCR-independent signals required to allow maturation of thymocyte Treg precursors into mature Tregs. Supplementing cultures of thymic Treg precursors with IL-2 caused a modest increase in Treg maturation, although addition of ligands that could signal through tumor-necrosis factor (TNF) receptor superfamily (TNFRSF) members had an even more profound effect on Treg development (48). If neutralizing antibodies for TNFRSF members were added to thymic organ cultures, then there was a dearth of

phenotypically mature Tregs. Using the Nur77 reporter mouse, they showed that addition of GITRL-Fc protein to thymic cultures in graded doses, caused a progressive decrease in the MFI for Nur77 in the Tregs that developed, which was interpreted as the ability of TNFRSF ligation to compensate for lower affinity/avidity TCR interactions, thus broadening the range of TCR affinities expressed by the Tregs (48).

For a polyclonal repertoire, defining the signaling threshold required for thymocyte development into different fates is a difficult question to answer due to the inability to track individual TCR specificities through development linked to what antigens they had selected on, in addition to their agonist specificity. Using TCR Tg cells, we can define general rules regarding affinities required for selection, although the most pertinent information will be obtained when only naturally occurring positive and negative selecting ligands are assessed. Currently there are a limited number of identified positive selecting ligands as shown in Table 1.1. SPR analysis of OT-I TCR binding to endogenous positive selecting ligands showed that the affinity of this interaction was extremely low and beyond the normal level of detection at room temperature. Upon cooling to 10°C, K_D values were measured and showed that both positive selecting ligands had greater than 15x lower affinity than agonist peptide; this difference is greater than the lowest affinity APL previously known to induce positive selection which had been the basis of the avidity selection model (49). It is likely that most endogenous positive selecting ligands will be of lower affinity than the APLs that are capable of mediating positive selection.

TCR affinity in the periphery affords opportunity to alter the repertoire

Most thymocytes that survive selection will have a TCR specific for a foreign antigen, which they wouldn't have previously encountered in the thymus. One would expect that for any given antigen specificity, there would be a range of TCR affinities within the polyclonal response which should be normally distributed in a naïve repertoire if rearrangement and selection was unbiased. Unfortunately currently there is a lack of suitable techniques to directly measure the affinity ranges of a given antigen specificity from a naïve polyclonal response due to low frequency of cells that can recognize a given antigen. A study utilizing 20 different peptides presented in the context of IA^b showed that the number of tetramer positive cells present in a naïve mouse for a given antigen could range from five cells to over 500 depending on the antigen (50). Granted the precursor frequency would be greater since tetramers only stain the highest affinity cells and are unable to identify all of the antigen reactive cells (51-54). There is a strong correlation between the naïve precursor frequency and the size of the response post challenge with peptide (50). However the change in the affinity profile for a given specificity in a polyclonal response has not been determined to date.

Based on the current models of thymocyte selection, one would assume that for a given self antigen, especially if present in the thymus, the average affinity would be lower for self compared with a foreign antigen. In fact measurements of the affinities for MOG38-49:IA^b and gp66-77:IA^b during the peak of their respective immune responses (day 21 post disease induction for MOG and day 8 for gp66) did support this conclusion where the virus-specific $CD4^+$ T cell affinity on average was 1.3 logs greater than the self-reactive $CD4^+$ T cells (51). It is interesting to note that there are more MOG38-49:IA^b specific $CD4^+$ T cells than gp66-77:IA^b specific cells (250 compared to about 100

tetramer positive cells), but after challenge the affinities for gp66 tended to skew higher compared to MOG even though the precursor frequency was greater for the latter (50, 51). These data could lend support to the affinity maturation model, where the higher affinity clones preferentially out-compete lower affinity cells to dominate the response.

Classically, the affinity maturation model is predominately associated with B cells that undergo further DNA rearrangement to produce a B cell receptor with greater binding ability to the antigen, thereby leading to antibodies with higher affinity for their antigens (55, 56). However T cells don't typically rearrange their TCRs in the periphery, but there can be affinity maturation of the repertoire via preferentially proliferation of clones that outcompete lower affinity TCR-expressing cells for limited antigen. It is known that T cells are able to functionally respond to lower doses of cognate antigen as they become more functionally mature (57). This process shows that there is an increase in functional avidity for activated and memory cells compared to naïve responders. To answer the question of if there is selective survival of higher affinity clones a technique capable of measuring the affinity of the interaction of TCRs with their cognate antigen needed to be developed.

Multimeric forms of peptide in complex with MHC allowed for determination of the frequency of cells specific for a given antigen without needing to rely on functional readouts (58). Due to having multiple subunits, tetramers and other multimeric pMHC reagents are able to stain TCRs due to the accelerated kinetics of binding of subsequent bounds after the first binding event has occurred, which compensates for the fast off rates that limited monomeric reagents from staining (58, 59). Even though tetramers work through increased avidity, it has been shown that there is a positive correlation between

the ratio of tetramer bound to total TCR and the affinities that were measured for the same TCRs using SPR (60). This insight provided the first opportunity to try to answer the question of whether or not there is affinity maturation for T cells.

It was first shown that during the course of infection with *Listeria monocytogenes* and secondary challenge there appeared to be skewing of the $CD8^+$ T cell affinities over time for a dominant epitope (61). TCRβ staining from the blood of infected mice showed that there was different TCR chain usage depending on which time point was measured, which suggested that there was preferential survival of certain clones. They went on to show that the peak response of a secondary infection had a tighter peak and could stain with lower dilutions of tetramers compared to the primary peak response. Similar results were obtained by two other labs when $CD4^+$ T cells were examined (62, 63). Based on these data it appeared that there was affinity maturation, but it is known that tetramers can miss lower affinity cells which could still be present at these later time points (51). Therefore a closer examination of this process is needed by techniques that are capable of measuring the affinities for the entire antigen specific population and not just a subset.

How to measure the affinity of the entire response

To better refine the requirements for selection and affinity maturation, one must first consider the various parameters which have and should be used to characterize a peptide's ability to induce selection. Historically, functional readouts have been used for APLs and TCR Tg mice. With the advent of FTOCs, researchers were able to access the ability of a peptide to select, and then test the peripheral Tg cells for their functional responses to categorize the APLs. For $CD8^+$ Tgs there appeared to be a clear distinction that weaker APLs, antagonists and weak agonists, were capable of mediating positive

selection with none to limited negative selection, while agonists required extremely low doses to avoid negative selection (21, 23, 24). However as noted above the definition based on functional outcome did not hold true for CD4 Tg cells, where it was observed that only APLs that were null were capable of mediating positive selection (27). Another confounding factor is that even TCR Tg cells, which are a monoclonal population, are not all able to functionally respond upon demand (64-67).

Since the development of peptide-MHC tetramers (pMHC), they have been used as a measure of affinity, although avidity is the appropriate term since they have multimeric binding subunits that exhibit faster on-rates after a single pMHC has bound for the subsequent pMHC units (58-60). One inherent issue with pMHC tetramers is if the affinity of the interaction between the pMHC and TCR is low or has extremely fast off-rates, then detection of staining will be hindered. This has been demonstrated by TCR Tg cells, which are specific for their agonist, but fail to stain with tetramer (52); therefore tetramer decay studies and staining intensities (MFIs) will lack the sensitivity required to define the parameters for all responses.

Surface Plasmon resonance (SPR) has been used to measure the affinity of interaction between two proteins. One purified protein of interest is attached to a sensor, while the other is allowed to flow over the sensor in a fluid phase; binding is detected by the change in the deflection of light off the sensor due to binding between the two proteins of interest. Kinetic parameters of binding (on- and off-rates) in addition to affinities can be calculated. This technique requires the proteins to be purified, which is a tedious process and limits its applicability to studying polyclonal responses. Due to the fluid nature with which the proteins are able to interact, SPR is considered a three-

dimensional (3D) measure of affinity. Although studying protein-protein interactions isolated from any effects of other proteins which would normally be present in the plasma membrane of a cell can offer value, interpretations of the data can be difficult.

Some important factors missing from SPR measurements are the proteins being oriented properly for physiologically relevant interactions, and any other proteins that normally associate with them that could be needed for binding, like CD8 co-receptor. For determining the kinetics of antibodies binding to their antigens, SPR measurements are easier to interpret since the majority of antibodies mediate their functions when they are in solution and not bound to a surface membrane. For the TCR, which is only found in a cell's membrane, determining the kinetics of binding and strength of that bond while it and its cognate pMHC are in the context of membranes will be more insightful. Two independent labs published results using different ways to directly look at binding kinetics when the proteins were in the context of cell membranes; they found that two dimensional (2D) measures correlated better with functional outcomes than 3D parameters (68-70). One technique utilizes Förster resonance energy transfer (FRET) based microscopy to measure the binding kinetics while the other uses a micropipette based adhesion assay that measures the probability of a binding event. The advantage of the latter is that if pMHC monomers are available, then cells from a polyclonal response can be directly interrogated for their antigen specificity and binding kinetics without needing to further manipulate them, while the former relies on staining TCRs with a single chain antibody that binds the conserved region of the TCR that has either the acceptor or donor fluorophore and using a pMHC complex which has the reciprocal fluorophore directly conjugated to the peptide.

In the micropipette adhesion frequency assay, red blood cells (RBCs) are used as a biosensor by labeling their surfaces with pMHCs of interest through biotin-streptavidin interactions (71, 72). This allows for very stable expression of the desired pMHCs in the correct orientation on the RBCs due to specific biotin labeling of the MHC molecule at its base. Using different concentrations of biotin to coat the RBCs, a wide range of site densities for pMHCs can be obtained. The T cell and RBC are aspirated on opposing micropipettes under micromanipulation. To measure the binding kinetics, the T cell is brought into contact with the RBC and retracted using an electric piezo driven actuator controlled by a computer program to allow for consistent testing cycles, where the contact duration, resting period and speed of approach/retraction can be precisely varied. Measuring the adhesion frequencies at different time points and determining the time required for the adhesion frequency to reach half of its plateau value will yield the reverse rate, or off-rate (k_{off}), for that TCR pMHC. Using the site densities of the receptor and ligand, the effective 2D affinity can be calculated using the following equation:

$$A_c K_a = -\left(\frac{\ln(1 - P_a(\infty))}{m_r m_l} \right),$$

where A_cK_a is the effective 2D affinity, P_a is the adhesion frequency at an equilibrium time point, and m_r and m_l are the densities of TCRs and pMHCs respectively. For most T cells using an adhesion contact time of two seconds or greater is sufficient to reach equilibrium. An effective on-rate can then be calculated using the relationship of A_ck_{on} = A_cK_a X k_{off}.

For probing a polyclonal response the micropipette adhesion frequency assay is extremely useful. In addition to determining the kinetic properties of an individual TCR, it can be used to determine the frequency of antigen specific cells in a given population

by dividing the number of cells that bound the specific pMHC of interest by the total number of T cells tested. This allows one to determine what fraction of a response is generated to diverse antigens in a polyclonal response like a viral infection or during spontaneous autoimmune disease like type 1 diabetes. The size of the chamber assembly allows for multiple discrete populations of RBCs coated with different pMHCs or different densities of a given pMHC to further refine a T cell's antigen specificity. If the T cell tested doesn't recognize the first pMHC tested, then that RBC can be discarded and a new one from a different population can be aspirated to test the same T cell. This allows for multiple antigen specificities to be tested therefore determining what percent of cells recognize each given antigen during the immune response.

Role of CD4 T cells during autoimmunity

Experimental autoimmune encephalomyelitis as a model system to study Treg affinity

Experimental autoimmune encephalomyelitis (EAE) is an animal model that is used to study multiple sclerosis. It is typically induced in rodents by injecting an encephalitogenic peptide emulsified in complete Freund's adjuvant (CFA) (73, 74). Although the exact mechanism of action of the pertussis toxin is unknown, it does improve the disease incidence and consistency between experiments (75, 76). A few mechanisms have been proposed including: that pertussis toxin opens the blood brain barrier (77), preferentially induces Th1 cells (78), prevents anergy of the effector T cells (79), and reduces the frequency and suppresses the function of Tregs (80, 81). In C57BL/6 mice, the disease follows a primary progressive disease course where the mice develop symptoms rapidly and do not experience any major relapses. Typical EAE results in ascending paralysis that starts with a limp tail, followed by hindlimb weakness,

hindlimb paralysis, forelimb weakness, and can eventually result in death although it is not common.

Symptoms typically begin 12-15 days after the initial injection and the peak of disease is around day 21, when the mice have reached and maintain their maximal scores as shown in Figure 1.3 using the standard chronic induction protocol. The disease was thought to be mediated solely by Th1 $CD4^+$ T cells until it was shown that mice lacking T-bet, the key transcription factor for Th1 cells, that EAE was prevented (82). Follow-up studies showed that a new $CD4^+$ T cell subset which produced IL-17 might be important for EAE because antibody neutralization of this cytokine lead to reduced disease severity (83, 84). The strongest support for the role of Th17 cells came from IL-17 knockout mice, which had delayed onset and reduced severity compared to wild-type mice (85). In addition to $CD4^+$ T cells responding, disease induction with MOG35-55 is able to elicit a $CD8^+$ T cell response to MOG37-46 (86), however it was shown that induction with the minimal $CD8^+$ T cell epitope lead to mild disease, and both $CD4^+$ and $CD8^+$ T cells were needed for sustained CNS inflammation and normal disease severity (87).

In an acute model of EAE, the frequency of $CD4^+$ Teffs gradually increases from pre-symptomatic (day 10) to onset (day 13) through peak of disease (day 16) and returns to pre-onset numbers at disease remission (day 21), which is when the frequency of $CD4^+$ $Foxp3^+$ Tregs reaches its maximum amount (88). Using MOG:IA^b tetramers, the authors were able to show that at the recovery time point in the CNS there was one MOG specific Treg for every four MOG specific Teffs. They also showed that at peak disease the CNS Teffs were refractory to suppression by Tregs, which was attributed to TNF-α and IL-6 reversing the suppression mediated by Tregs. This Teff unresponsiveness can be

overcome by the adoptive transfer of additional Tregs prior to disease induction, which means that the ratio of Tregs to Teffs may be important to treating on-going disease (89). Another attractive reason to use EAE to investigate the affinity of Tregs is that there doesn't appear to be any appreciable epitope spread occurring and that the majority of the bulk $CD4^+$ T cells infiltrating into the CNS are specific for the inducing antigen (51, 90).

Using Tregs as possible therapeutics for autoimmunity is actively being studied. It has been shown that fewer antigen specific Tregs are needed as compared to bulk polyclonal Tregs (91, 92). This could be due to the preferential trafficking of the antigen specific Tregs to the site needed, competition for limited antigen needed for both Teffs and Tregs, or through the antigen specific Treg altering the APC to affect its ability to activate the Teffs. Intravital two-photon microscopy has shown that Tregs form stable interactions with APCs and their presence prevents Teffs from being able to stop and sample the antigens being presented (93, 94). In addition to competing for dwell time with APCs, it has been shown that Tregs are able to trans-endocytose CD80/CD86 from the surface of DCs via binding of CTLA-4 (95-99). Tregs are also able to prevent the maturation of DCs (100), and are capable of inducing apoptosis of APCs (101). Besides their potent effects on APCs, Tregs secrete IL-10 to modulate the cytokine milieu in addition to absorbing excess IL-2 to limit activation of Teffs (102, 103).

Tregs can develop in the thymus to become tTregs or in the periphery, conversion of Tconv cells exemplified by oral tolerance leads to pTregs. *In vitro* culture of naïve Tconv cells in the presence of TGF-β and IL-2 can lead to the induction of Foxp3 (104, 105), and addition of retinoic acid can improve the conversion efficiency (106-108). Discrimination of the different types of Tregs has been difficult. Helios, a member of the

Ikaros transcription factor family, has been suggested as a way to discern tTregs from pTregs and iTregs (109), although it has been shown that it can also be expressed by activated Tconv cells and iTregs that were generated using cognate antigen stimulation in the presence of TGF-β and IL-2 (110-112). Recently it was reported that Helios expression by antigen stimulated Tconv cells could be indicative of them being driven to tolerance since they were shown to be anergized (113). This role for Helios is consistent with its ability to bind to and epigenetically silence the IL-2 gene (114). It has also been argued that neuropilin 1 (Nrp1) expression can discriminate tTregs from pTregs (115, 116), although a study comparing the expression patterns of both markers suggested that Helios might be more reliable than Nrp1 to distinguish tTregs from pTregs (117).

In order for Tregs to be used as therapeutics they must be able to maintain the expression of Foxp3 and their anergic phenotype because it has been shown that there can be conversion of Tregs into ex-Foxp3's which act like activated memory cells (118). To assess the stability of Tregs, the methylation pattern of the Treg specific demethylated region (TSDR) can be determined. Because the TSDR is only needed to maintain but not for the induction of expression of Foxp3, mice that lack this region develop normally, are healthy, with normal numbers of Tregs (119). However if these cells are transferred into a lymphopenic environment or undergo multiple rounds of division in response to stimulus then Foxp3 is downregulated and systemic autoimmunity ensues (119, 120). Even though iTregs express similar levels of key markers like Foxp3 and CTLA-4, they exhibit little to no demethylation of the TSDR, which could suggest that they lack the inherent stability seen with tTregs and pTregs (121, 122). Addition of an inhibitor of methylation, azacitidine, to TGF-β induction of iTregs can lead to partial demethylation of the TSDR,

which does impart more stability to these cells (121). This is an important consideration for using Tregs as treatments.

Utilizing NOD mice to study diverse antigen specificities during spontaneous autoimmunity

Selective inbreeding of the cataract Shionogi strain of mice lead to the spontaneous development of diabetes in a subset of mice, which is how the non-obese diabetic (NOD) mouse evolved (123). This strain has provided novel insights into the disease development and a testing platform for therapeutics because of the many similarities to type 1 diabetes (T1D) in humans. A common susceptibility gene for human autoimmunity is the human leukocyte antigen (HLA) which presents peptides to $CD4^+$ T cells. NOD mice have an MHC class II allele that predisposes them to T1D; both HLA-DQ8 and IA^{g7} share a mutation in their beta chains of a key aspartic acid residue being substituted for a small uncharged amino acid, which disrupts a critical salt bridge between the alpha and beta chains near p9 of the peptide binding register (124, 125). This specific mutation is so critical that reintroduction of a beta chain expressing Asp57 severely reduced the incidence of spontaneous disease in female NOD mice from 60% to 12% by 30 weeks of age (126). Screening of peptide libraries has determined that IA^{g7} is a promiscuous peptide binder and it lacks strict rules for the MHC binding pockets like what has been observed for IA^b and IE^k (127).

Type 1 diabetes is an extremely complex disease with multiple auto-antigens identified in both mice and humans, although there are likely more targets that remain to be discovered. Diagnostically the antigen specificities and amounts of autoantibodies to known targets can be useful in early detection of disease. The earliest observed

autoantibodies are directed against epitopes from insulin and are present in the majority of type 1 diabetics, although not everyone with insulin autoantibodies will become diabetic (128). Autoantibodies to insulin (IAA), zinc transporter 8 (ZnT8A), glutamic acid decarboxylase 65 (GAD65), and islet antigen-2 (IA-2A) are commonly seen in patients and recently it was shown that ChgA is also able of eliciting an antibody response (129). The NOD model shares many of these same antigenic targets, which makes studying the disease progression in these mice extremely valuable. In fact ChgA was first identified as a diabetogenic antigen in NOD mice before it was determined that patients have antibodies specific for it as well (129, 130).

Identifying new autoantigens and their role in the NOD model might provide novel insight into the progression of the disease. Although it is argued that insulin is the initiating antigen in mice and men, not all mice which express TCRs specific for insulin will develop diabetes and those that due have delayed onset compared to other antigen specific TCRs (131). A study which included 16 different TCRs specific for numerous diabetogenic antigens, showed that only two TCRs, both specific for ChgA, had complete disease penetrance and with extremely rapid onset (131). In fact a subsequent study focusing on eight insulin specific TCRs determined that none were capable of inducing diabetes in 100% of the mice (132). Therefore an unbiased study to determine the order of appearance of antigen specificities is needed to help identify key checkpoints and possible therapeutic targets.

The NOD model also provides an opportunity to investigate how T cells that should have been tolerized to pancreatic antigens are able to escape central tolerance and instigate disease pathogenesis. Insulin is a model antigen for investigating this

circumvention by T cells to escape their education process and become self reactive. One unique feature of insulin is that there are two non-allelic genes on different chromosomes which encode it that lead to preproprotein forms that have 88% sequence homology, while the mature proteins are identical (133). These two genes have distinct tissue expression with *Ins1* being expressed only in the pancreas, while *Ins2* is expressed in the thymus, under the control of AIRE, in addition to the pancreas (134, 135). It was shown that the InsB9-23 epitope binds IA^{g7} in two different registers, one shifted by a single amino acid from the other, which allows for the formation of the peripheral immune response (136). Polyclonal cells that recognize the epitope presented when insulin is processed by APCs (InsB13-21), type A, are deleted in the thymus, while the type B cells, which are specific for the InsB9-23 peptide and not the naturally processed protein, survive thymic education and recognize InsB12-20. When a type B TCR was used to make a transgenic mouse, these T cells escaped negative selection and were able to directly migrate to the pancreas without being primed in the local lymph nodes (137). But they required high levels of the peptide to be expressed due to the instability of the register they recognize. These results again suggest that promiscuity of peptide binding to IA^{g7} can be a major culprit in the development of T1D.

It has been proposed that the multiple registers caused by weak peptide binding could explain why negative selection is incomplete in NOD mice (138). In addition to poor binding, it has been shown that post-translational modifications can be playing a crucial role in breaking tolerance. Two different diabetogenic antigens are known to elicit a stronger immune response once certain amino acids have been modified. Most of these modifications only occur during an inflammatory response when key enzymes are

expressed. Glucose-regulated protein 78 (GRP78) becomes citrullinated by peptidylarginine deiminase 2 (PAD2), which then becomes the target of autoantibodies and $CD4^+$ T cells responses (139). PAD2 mRNA is preferentially expressed in the islets of Langerhans from NOD mice compared to C57Bl/6 and NOR mice which are resistant to diabetes development, thus allowing for the generation of this neo-epitope. Three different T cell clones (BDC2.5, BDC10.1, and BDC5.10.3) produced more IFNγ in response to lower peptide concentrations of the newly identified autoantigen derived from chromogranin A (ChgA) after treatment of the peptide with transglutaminase (130, 140). Transglutaminase can mediate two different post-translational modifications: (a) the deamidation of glutamate to glutamine and (b) the formation of an isopeptide bond between a lysine and glutamine residue. To try to determine which reaction was mediating this increased in antigenic potential, a synthetic peptide of WE14 containing a glutamine in place of the glutamate was made, however it didn't boost the functional response. Processing of the higher molecular weight aggregates formed when transglutaminase mediated the isopeptide bond formation between lysine and glutamine are required to improve the antigenicity WE14. Therefore the exact peptide being presented is probably extremely complex and has not been identified to date.

One of the ChgA reactive TCRs, BDC2.5 has been extensively studied for decades even though the antigen specificity of this TCR remained elusive for almost thirty years. From a panel of diabetogenic T cell clones, the BDC2.5 TCR was picked to make a TCR transgenic mouse because it displayed rapid disease kinetics upon adoptive transfer and appeared to be highly restricted to an islet cell antigen when presented in the context of IA^{g7} (141). Another interesting feature of BDC2.5 is that it is specific for

mouse but not rat or human islet cells (142). Spontaneous disease is extremely low in BDC2.5 Tg mice, with only 12% becoming diabetic by 30 weeks of age, which is partly due to the expression of endogenous TCR alpha chains since there is an increase in spontaneous disease when mice are crossed to $C\alpha^{-/-}$ (143). BDC2.5 mice that lack RAG1 or Prkdc, an enzyme in the DNA double-stranded break repair machinery, exhibit rapid onset of disease before 5 weeks of age and 100% incidence ((143) and unpublished observations). Future studies with this TCR can offer novel insight into the initiation of disease especially in the context of how it recognizes cognate self antigen.

Even though both BDC2.5 and BDC10.1 recognize the same self-antigen, there are known differences between their TCR fine specificities (130, 140). BDC10.1 cells respond to the pS3 mimotope, while BDC2.5 can respond to pS3 and other mimotopes (17). In that study, only p2 and p3 of the TCR contact residues were different between the mimotopes that were tested. Another difference is the required endogenous positive selecting element for each since it was observed that BDC10.1 thymocyte development was dependent on the expression of the thymus-specific serine protease (TSSP), while BDC2.5 thymocytes matured in the absence of TSSP (17). This observation would be consistent with the previous findings that two MCC88-103-reactive T cell clones had different endogenous positive selecting ligands (30, 31). Closer examination of the affinities of BDC2.5 and BDC10.1 TCRs could explain the differences and similarities between these two clones.

Figure 1.1

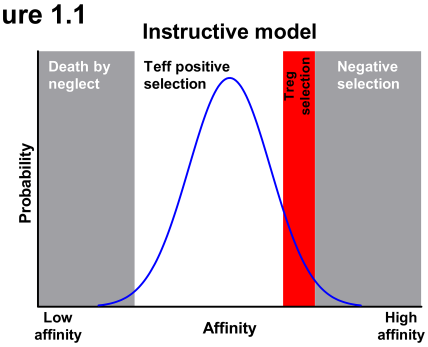

Figure 1.1 Instructive model of thymocyte selection. The functional outcome of selection is dependent on the strength of signal received by the thymocyte through the TCR. If there is little to no interaction with pMHC then the thymocyte will die by neglect, intermediate affinities produce CD4 and CD8 SP T cells that survived positive and negative selection. The highest affinity interactions result in apoptosis, which is known as negative selection, although it is thought that some $CD4^+$ SP thymocytes on the edge of the selection boundary are able to survive negative selection by deviating in the $Foxp3^+$ $CD4^+$ Treg lineage.

Figure 1.2

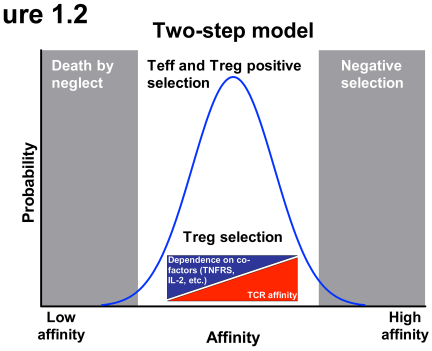

Figure 1.2 Two-step model of thymocyte development. Similar to the instructive model the lowest and highest affinity signals the cell to death either by neglect or apoptosis respectively. Teffs and Tregs are able to select on a range of affinities that overlap and it is thought that for Tregs a weak TCR signal can be supplemented via a co-factor like IL-2 or TNFRSF members.

Tg	CD4/CD8	Agonist	Endogenous positive selecting ligand	Stimulatory potential	Ref.
5C.C7	CD4	MCC	GP peptide	Coagonist	(30)
AND	CD4	MCC	gp250	Coagonist	(31)
OT-I	CD8	OVA	Cappa1	Null	(144)
			Catnb	Antagonist	

Table 1.1 Known endogenous positive selecting ligands for the indicated TCR transgenic mice. Stimulatory potential is based on the response of mature splenocytes to the peptides, where coagonist can increase the response to limited agonist stimulation, null is no known functional response, and antagonist is the ability of that peptide to inhibit the response to the agonist peptide.

Figure 1.3

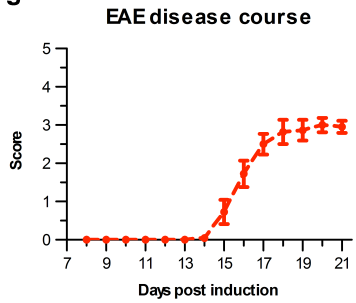

Figure 1.3 EAE disease course. Disease course in C57BL/6 mice scored on a 5-point scale. Data from one representative experiment with 11 mice.

Chapter 2: Regulatory and T effector cells have overlapping low to high ranges in TCR affinities for self during demyelinating disease

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http://www.jimmunol.org/content/195/9/4162.full

Abstract

Having regulatory T cells (Tregs) with the same Ag specificity as the responding conventional T cells is thought to be important in maintaining peripheral tolerance. It has been demonstrated that during experimental autoimmune encephalomyelitis there are myelin oligodendrocyte glycoprotein (MOG)-specific Tregs that infiltrate into the CNS. However the affinity of naturally occurring polyclonal Tregs for any self-antigen, let alone MOG, has not been analyzed in the periphery or at the site of autoimmune disease. Utilizing the highly sensitive micropipette adhesion frequency assay, which allows one to determine on a single-cell basis the affinity and frequency of polyclonal Ag-specific T cells directly *ex vivo*, we demonstrate that at peak disease MOG-specific Tregs were progressively enriched in the draining cervical lymph nodes and CNS as compared with spleen. These frequencies were greater than the frequencies measured by tetramer analysis, indicative of the large fraction of lower affinity T cells that comprise the MOG-specific conventional T cell (Tconv) and Treg response. Of interest, the self-reactive $CD4^+$ Tconvs and Tregs displayed overlapping affinities for MOG in the periphery, yet in the CNS, the site of neuroinflammation, Tconvs skew towards higher affinities. Most of

the MOG-specific Tregs in the CNS possessed the methylation signature associated with thymic-derived Tregs. These findings indicate that thymic-derived Treg affinity range matches that of their Tconvs in the periphery and suggest a change in TCR affinity as a potential mechanism for autoimmune progression and escape from immune regulation.

Introduction

A constant challenge for the immune system is balancing clearance of foreign pathogens while remaining tolerant of self. To limit overt auto-reactivity, central tolerance restricts the number of self-specific cells by the process of negative selection in the thymus, although it has been demonstrated that this process is imperfect (145). In the periphery, self-reactivity can be limited in several different ways, including, but not limited to, anergy, inability to access Ag, and suppression by regulatory T cells (Tregs). One of the earliest examples of regulatory T cells was demonstrated by pivotal work showing that depletion of $CD4^+$ $CD25^+$ T cells in mice lead to systemic autoimmunity (146). Later it was determined that Foxp3 is a key transcription factor expressed by Tregs and mutations in this transcription factor lead to multiorgan autoimmunity (147, 148).

Tregs arise in vivo by thymic selection (tTregs) or peripheral induction (pTregs). TCR affinity for self peptide-MHC complexes (pMHCs) during thymic education is thought to dictate the fate of developing thymocytes toward naive CD4+ conventional T cells (Tconvs) or Tregs, with the strongest interactions preferentially giving rise to Tregs. Early models of tTreg selection were based on the requirement of a strong interaction of the developing thymocyte, with self-antigen serving as an alternative to death via negative selection (32, 34). This implied that tTregs are of higher affinity than Tconvs for the same self-antigen, although tTreg selection is influenced by many additional factors, including the selecting niche size and availability of cofactors (43, 45, 149, 150). However, how these data translate to polyclonal responses has not been addressed to date because there is a dearth of information on naturally occurring tTreg TCRs with known self-antigen specificities.

Even in TCR transgenic mice, the Ag specificity that allowed for selection in the thymus may not be the same recognized in the periphery (31, 42). It has been shown that thymocytes are able to develop into both Tregs and Tconvs by selecting on the same self-peptide using the identical TCR (151). The limitations imposed by the selecting size niche did replicate when TCRs from three different tTregs were used to make retrogenic mice (43). When these TCRs were present at low clonal frequencies, they more efficiently developed into Tregs, although to date their Ag specificities remain elusive. If tTregs are enriched for self-reactivity, then identifying their specificities could prove difficult using current techniques such as pMHC tetramers. We and others have demonstrated that tetramers can greatly underestimate the frequency of self-specific T cells (51-54).

It has been shown that there are myelin oligodendrocyte glycoprotein (MOG)-specific Tregs that traffic to the CNS during experimental autoimmune encephalomyelitis (EAE) (88). They are still capable of suppression; however, Tconvs in the CNS have become refractory to the Tregs. The authors demonstrated that this similar ignorance of suppression could be achieved using naive Tconvs with exogenous IL-6 and TNF-α added to the coculture (88). In a study of relapsing and remitting multiple sclerosis patients, there was a significant correlation between the level of IL-6Rα expression and the ability of the Tconvs to escape suppression by Tregs (152). In addition to the Tconvs escaping regulation by Tregs, the presence of proinflammatory cytokines can actively recruit DNA methyltransferases to the Treg-specific demethylated region (TSDR), leading to de novo methylation at this site (120). The reintroduction of methyl groups at these CpGs makes Foxp3 expression less stable and impairs its inheritability upon

division into daughter cells (119). By BrdU labeling, it has been demonstrated that Tregs in the CNS during EAE are capable of dividing, so this could support a conversion of Tregs into memory-like effector T cells (88, 153).

Using Foxp3 reporter mice to discern the contributions of Tregs from Tconvs, we expanded on our previous studies demonstrating that autoimmunity is dominated by low-affinity T cells (51). Only approximately 10 and 30% of Tregs and Tconvs were Ag reactive in the CNS by tetramer and cytokine production, respectively, during EAE. However, when using the same pMHC monomer that is used for the tetramer, >80% of the Tregs and Tconvs from the CNS are MOG specific when measured by the micropipette adhesion frequency assay. Furthermore, this assay allowed the identification of how many MOG-specific Tregs were in the spleen (SPL), cervical lymph nodes (CLN), and CNS, defining enrichment of MOG-specific Foxp3+ T cells that have infiltrated into the CNS. All T cell responses are comprised of TCRs that span a range from lower to higher affinities. The overall affinity ranges in the periphery are similar between Tregs and Tconvs, with an enrichment of higher affinity Tconvs in the CNS. Based on a methylation signature and Helios expression, the Tregs in the CNS are likely derived from tTregs. Thus, the micropipette adhesion frequency assay allows one to define for the first time the distribution and affinity of polyclonal Tregs during autoimmune disease, revealing new insight into autoimmune disease progression and potential mechanisms of Treg development.

Materials and Methods

Mice

B6.Cg-Foxp3^{tm2Tch}/J (Foxp3 GFP) mice were obtained from The Jackson Laboratory and were bred in the Emory University Division of Animal Resources facility (154). C57BL/6 mice were obtained from National Cancer Institute. All experiments were approved by the Institutional Animal Care and Use Committee at Emory University.

EAE induction and isolation of cells

EAE was induced in 6- to 10-wk-old male or female mice by injecting s.c. 200 μg MOG_{35-55} emulsified in CFA containing 5 mg/ml heat-inactivated *Mycobacterium tuberculosis* (H37 RA, Difco) on days 0 and 7 and i.p. with 250 ng pertussis toxin (List Biologicals) on days 0 and 2 as described (155). Mice are scored and weighed daily starting at day 7 after induction using a 5 point scale: 0, no signs of disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness; 5, moribund.

Reagents

Cell culture media was composed of RPMI 1640 (CellGro) supplemented with 10% heat-inactivated FBS (Life Technologies), 4 mM $_L$-glutamine (CellGro), 0.01 M HEPES (CellGro), 100 μg/ml gentamicin (CellGro) and 20 μM 2-ME (Sigma-Aldrich). Phenol red-free reagents (RPMI 1640 and HBSS) were used in some experiments because phenol red-containing reagents can decrease enhanced GFP fluorescence by FACS and fluorescent microscopy (156). Experimental additive solution 45 (EAS45) was made as described previously (157). All EAS45 compounds were from Sigma-Aldrich except dibasic sodium phosphate, which was from Fisher Scientific. EAS45 was then further

supplemented with 1% BSA (Sigma-Aldrich). FACS wash consisted of 0.1% BSA and 0.05% Sodium Azide in PBS.

Isolation of CNS-infiltrating cells

Mice were sacrificed using CO_2 inhalation and were perfused with 1X Dulbecco's PBS (CellGro) via the left ventricle after the inferior vena cava was cut. For each mouse, the spinal cord and brain were combined and a single-cell suspension was obtained by pushing cells through a 100-μm cell strainer. Cells were collected from the interface of a 27%/63% discontinuous gradient of Percoll (GE Healthcare), washed two times with R10, and then counted on a hemocytometer to obtain the number of infiltrating cells.

Flow cytometry

Staining of Helios and Foxp3 was accomplished using the Foxp3 staining buffer set from eBioscience according to the manufacturer's protocol. For intracellular cytokine staining, cells were stimulated with PMA and ionomycin for 4 h in the presence of brefeldin A, as described previously, and then after staining surface markers, the cells were fixed and permeablized using the BD Cytofix/Cytoperm kit (BD Biosciences) followed by intracellular staining for cytokines (90). Tetramer frequencies were determined by staining cells with MOG_{38-48}:IA^b or hCLIP:IA^b for at least 6 h at 37°C in complete media as described previously (158). Data were collected on an LSR II (BD Biosciences) and then analyzed in FlowJo (Tree Star). Monomer-coated RBCs and T cells were stained to determine the absolute numbers of pMHC and TCR, respectively, on their surfaces using PE Quantibrite beads (BD Biosciences) as described previously (68). The following antibodies (clones) were purchased from a variety of vendors (BD Biosciences, eBioscience, BioLegend): Helios (22F6), Foxp3 (FJK-16s), CD8α (53-6.7), CD3ε (145-

2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD45.2 (104), TCRβ (H57-597), IA/IE (M5/114.15.2), IFNγ (XMG1.2), IL-17 (eBio17B7), and IL-10 (JES5-16E3).

Micropipette adhesion frequency assay

To determine the two-dimensional (2D) affinities and frequencies of MOG-specific cells, the micropipette adhesion frequency assay was conducted as previously described (51, 52, 68, 71, 90, 159-161). Human RBCs were coated with biotinylated pMHC monomers (National Institutes of Health Tetramer Core Facility). Binding events are scored manually by visualizing distension of the RBC membrane upon retraction of the T cell. After 25 or 50 touches, average adhesion frequencies are calculated and cells are considered to be Ag specific above the background of 0.1 binding frequency. The background binding frequency was set by using hCLIP:I-Ab–coated RBCs as a nonspecific binding control for the cells that had also been tested for binding to MOG:I-Ab. RBC pMHC density is manipulated to obtain adhesion frequencies between 0.1 and 1.0, which could be used to calculate an affinity. For example, cells that had an adhesion frequency of 1 were tested with a second RBC coated with a lower density of MOG:I-Ab to decrease the adhesion frequency and allow for measurement of the affinity. The number of Ag-specific cells divided by the total number of cells tested gives the frequency of MOG-specific cells. Effective 2D affinities (AcKa) are calculated using the following equation:

$$A_c K_a = \left(\frac{-\ln(1 - P_a(\infty))}{m_r m_l} \right)$$

where Pa is the adhesion frequency measured at an equilibrium contact time (≥ 2 s) and mr and ml are the densities of TCR and pMHC ligands, respectively. TCR densities are calculated for each T cell individually using the TCR level for the population, as determined by flow cytometry, and that cell's diameter, measured during the micropipette assay. For each independent experiment at least 14 cells were tested.

Bisulfite sequencing

Cells from male mice were used for methylation studies to avoid confounding results due to random X inactivation because Foxp3 is an X-linked gene. CD4+ Tconvs and CD4+ Foxp3+ Tregs were sorted from the indicated sources on a FACSAria II (BD Biosciences). DNA was extracted from cell pellets and bisulfite converted using a DNeasy blood and tissue kit (Qiagen) followed by an EpiTect bisulfite kit (Qiagen) or with the EZ DNA Methylation-Direct kit (Zymo Research) as per the manufacturers' protocols. Regions of interest were amplified using published primers and JumpStart Taq ReadyMix (Sigma-Aldrich) to determine the tTreg methylation (tTreg-Me) signature (122).

Gel-purified PCR products were subcloned using the TOPO TA cloning kit (Invitrogen), followed by blue/white screening with X-gal. Single-pass DNA sequencing was done by Beckman Coulter Genomics. Sequences were aligned with their in silico bisulfite-converted genomic sequences using a custom R script in Bioconductor Biostrings R package, and only those that contained all of the CpGs and had a bisulfite conversion rate >95% were included in the results (162).

Statistical analysis

Prism version 6 (GraphPad Software) was used for t tests and all ANOVAs with multiplicity-adjusted p values. Testing of normality via Q–Q plots, Shapiro–Wilk, and Jarque–Bera normality tests and comparison of affinity distributions using Kolmogorov–Smirnov were done using R script. A two-sided Fisher exact test was used to determine significance in the methylation patterns.

Results

Enrichment of MOG-specific Tregs and Tconvs in the CNS

At the peak of myelin destruction during EAE (day 21), approximately one in five CNS-infiltrating CD4+ T cells expresses Foxp3, the transcription factor that marks Tregs. This frequency is increased as compared with the Foxp3+ frequencies found in the draining CLN and SPL (Figure 2.1A, 14% each). Although a 1.5-fold enrichment in the frequency of Tregs from CNS over periphery occurred, this increase reflects changes in the total Treg population and not the frequency of MOG-specific Tregs. Additionally, there was a slight increase in the frequency of Tregs in the CLN and SPL during EAE compared with age-matched naive controls. Because Ag-specific Tregs are more effective than polyclonal Tregs at suppression, we sought to reveal the frequency of MOG-specific Tregs (91, 92).

To identify Ag-specific cells, pMHC tetramers were used to stain cells from Foxp3 GFP reporter mice that were sick with EAE. We observed approximately 4 and 8% of the Tconvs and Tregs, respectively, staining with tetramer in the CNS compared with $\leq$1% in the CLN and SPL at peak disease (Figure 2.2 and Fig. 2.1B). On average, MOG:I-A^b tetramers only detected <10% of the cells infiltrating the CNS as being specific for the inducing Ag (51, 88, 158). Another more sensitive method to enumerate polyclonal MOG-specific Tconvs and Tregs is the micropipette adhesion frequency assay. Individual cells within the polyclonal response were interrogated for their ability to specifically bind MOG:I-Ab–coated RBCs while not binding hCLIP:I-Ab–coated RBCs from the CNS of sick mice. Adhesion frequencies for MOG:I-Ab ranged from 0.1 to 1.0. However, an adhesion frequency of 100% (1.0) cannot be used to derive affinity from the

equation, so those T cells must be retested on RBCs coated with progressively lower Ag densities.

The adhesion frequency assay demonstrates specificity for the target Ag, as none of the cells that bound MOG:I-A^b displayed any adhesion >0.1 for hCLIP:I-A^b (Fig. 2.1C). Our previous work with monoclonal and polyclonal T cells has defined the 0.1 frequency as an operational cutoff for nonspecific binding, and it is considered the background level of the assay (51, 68, 160). Analysis of several tissues for Ag-specific T cells revealed similar adhesion frequencies and ranges in those frequencies for Tconvs and Tregs in the same tissue (Fig. 2.1D). Based on the adhesion frequencies, 80% of the CNS-infiltrating $CD4^+Foxp3^+$ T cells were specific for MOG, whereas the draining CLN and SPL displayed 70 and 15% reactivity for MOG, respectively (Fig. 2.1E). We also observed similar frequencies of MOG-specific Tconvs in the same tissues. These data demonstrate an additional specificity control of the assay, as not all T cells are able to interact with the MOG:I-A^b–coated RBCs (Fig. 2.1D).

These results parallel the increased numbers of Ag-reactive T cells that one would identify with specific tetramer but inclusive of the lower affinity T cells missed by all MHC class II tetramers (88). As measured by the micropipette assay, there are 8-fold more cells that are capable of recognizing MOG:IA^b. The frequency of MOG-specific Tregs as a percent of the total $CD4^+$ T cell compartment revealed a striking difference among the tissues with MOG-specific $Foxp3^+$ cells comprising ~18%, 11% and 2% within CNS, CLN and SPL, respectively (Fig. 2.1F). As expected from other reports, the intracellular cytokine assay also greatly underestimated the number of Ag-reactive T cells (Figure 2.3) (51, 52, 64-67).

Tregs and Tconvs have overlapping ranges of affinity for MOG but unique distributions in the CNS

We next determined how Treg affinities for MOG compared with Tconvs. In the CLN, the geometric mean 2D affinities of Tconvs and Tregs were the same (Fig. 2.4A, CLN Tconv, 1.446 [0.04940-11.42] and CLN Tregs, 1.349 [0.04670-14.35] X 10^{-5} μm^4 [range minimum to maximum]). The range in affinities was >100-fold for both cell types in the CLN. The greatest range in affinities was in the CNS with 560- and 2,450-fold for Tconvs and Tregs, respectively (Fig. 2.4A). This increased breadth in the CNS populations is due to enrichment of higher affinity cells. The CNS Tregs had a similar geometric mean affinity as the CLN cells, but the CNS showed enrichment for higher affinity cells as depicted by the increase in range (1.204 [0.09270-227.1] X 10^{-5}). Even though the CLN and CNS Tregs had the same mean affinity, the presence of high-affinity MOG-specific Tregs in the CNS was countered by an increase in low-affinity cells. However, the CNS Tconvs had a significant increase in the mean 2D affinity (4.631 [0.2159-121] X 10^{-5}) compared to CNS Tregs and cells in the periphery. Thus, in the CNS at peak disease, Tconv affinity is increased compared with CNS Tregs and peripheral MOG-specific Tconvs and Tregs.

Frequency distributions of the affinities were plotted and Gaussian curves fitted for CNS and CLN cells (Fig. 2.4B and 2.4C). The data fit to Gaussian curves, which could indicate that they are normally distributed. To determine whether the data are normally distributed, Q-Q plots were used to compare the experimental affinity distributions against a theoretical normal distribution with the same corresponding mean and SD. If the data are sampled from a normally distributed population, then the points

should fall along the plotted 45° reference line and normality tests will have a *p* value of >0.05. CNS Tconv affinities appear to be sampled from a normally distributed population as supported by Shapiro-Wilk and Jarque-Bera normality tests (*p* values of 0.2839 and 0.4397, respectively, Fig. 2.5A). The distribution of peripheral affinities also displayed the skewing and kurtosis expected of normal distributions as determined by the Jarque-Bera test (CLN Tconvs $p = 0.1484$, Tregs $p = 0.1971$). However they do deviate from normality as can be seen by the data on either end of the range not falling on the line in the Q-Q plots (Fig. 2.5C and D, Shapiro-Wilk CLN Tconvs $p = 0.02409$, Tregs $p = 0.002664$). Of interest, the CNS Treg affinity distribution deviated from normality as the corresponding Q-Q plot shows that the CNS Tregs are right-skewed (Fig. 2.5B) with poor Shapiro-Wilk and Jarque-Bera *p* values (*p* values of 0.002404 and 0.005418, respectively). Although we do not know the mechanism for this difference in Treg distribution at this time, it is interesting to speculate that Treg affinities are skewed at the site of active autoimmune disease.

To determine how similar two distributions are to each other, the Kolmogorov-Smirnov test can be used along with Q-Q plots. Comparison of Tconv and Treg distributions from the CNS using the Kolmogorov-Smirnov test, demonstrated a significant difference ($p = 6.978 \times 10^{-5}$) that can be visualized in the Q-Q plot where none of the points fall on the line (Fig. 2.5E). This shift is seen in the Gaussian curves where the CNS Treg curve is shifted to the left of the CNS Tconv curve (Fig. 2.4C). Although the CNS Treg affinity range is as wide as the CNS Tconv range, their distributions are significantly different (Fig. 2.4A and 2.5E). This difference in distribution for Tconvs and Tregs was specific to the CNS, as the same comparison of the

peripheral CLN populations revealed overlapping distributions (p value = 0.7724, Fig. 2.5F).

CNS Tregs are of thymic origin

We next wanted to determine the developmental origins of the CNS Tregs at peak disease. It has been published that adoptive transfer of highly purified naïve Tconvs from Foxp3 GFP reporter mice followed by EAE induction led to no pTregs generated (88). Helios was used as a marker of tTregs since it has been shown to be highly expressed by $Foxp3^+$ recent thymic emigrants while pTregs induced during oral tolerance lack Helios (109). Our data show >90% of the $Foxp3^+$ Tregs expressed Helios in the CNS (Fig. 2.6A and B). It has been reported that Helios expression can be induced under certain circumstances (110, 111); however we did not observe any *ex vivo* staining of Tconvs >6% in any organ during EAE (Fig. 2.7). To provide further evidence of the CNS Tregs being of thymic origin, a tTreg-Me signature was used. This methylation signature consists of key regions in *foxp3*, *tnfrsf18*, *ctla4*, and *ikzf4,* which are hypomethylated in tTregs compared to naïve Tconvs (122). Ohkura et al. (122) and others (163-167) have demonstrated that this methylation signature was not induced merely by the expression of Foxp3, because TGF-β induced Tregs failed to demethylate these regions, and their methylation was consistent with Tconvs.

We observed hypomethylation of these regions in Tregs but not Tconvs when cells from SPL of naïve mice were analyzed (Fig. 2.6C-D and Table I). Because our data validated the tTreg-Me signature, we assessed the methylation status of the CNS-infiltrating Tregs during EAE. We observed the same pattern of significant demethylation for the CNS $CD4^+$ $Foxp3^+$ Tregs whereas CNS Tconvs were mostly methylated (Fig.

2.6E-F and Table I). As an internal control for each experiment, *CD25* intron 1a was included since in resting Tconvs that lack CD25 expression, this region is methylated and it is demethylated in cells that express CD25 like Tregs and activated Tconvs (122). As expected, *CD25* was mostly methylated for Tconvs from naïve mice, whereas this region was predominately demethylated for Tregs (naïve and EAE mice) and Tconvs from the CNS of EAE mice (Fig. 2.6C-F). Based on these methylation data, we conclude that most Tregs in the CNS at peak EAE are of thymic origin. The region in *foxp3* analyzed was the TSDR that is required for stable *foxp3* expression and maintenance upon cell division (119). We observed high levels of methylation at the TSDR in the CNS Tregs (Fig 2.6E-F, *foxp3*). There was still a significant difference in the methylation pattern of the TSDR between the CNS Tconvs and CNS Tregs (Table 2.1).

Discussion

For EAE, tetramers and cytokines greatly underrepresented the frequency of MOG-specific Tconvs and Tregs present in the CNS when compared with the ability of those cells to specifically bind MOG:IA^b-coated RBCs in a micropipette adhesion frequency assay. We and others have demonstrated that especially for self-specific $CD4^+$ T cells in multiple autoimmune models, tetramers miss most Ag-specific cells (51-54, 161). If the cells have low affinity for their cognate pMHC, then they are unlikely to interact with tetramer. For example, we have shown that 2D2 transgenic cells do not stain with MOG:IA^b tetramer due to their extremely low affinity (52). Cytokine production in response to cognate peptide is able to identify more Ag-specific cells than tetramer (51, 53, 54, 161). In a parallel comparison, we showed that there are ~2.4-fold more CNS Tconvs capable of producing cytokines (IFN-γ and TNF-α) in response to MOG_{35-55} peptide compared to tetramer staining (51). During viral infection we also demonstrated that sorted $tetramer^-CD44^+$ T cells could produce cytokines after stimulation with the same Ag specificity as was in the tetramer (51). However, it is known that even cytokine production does not faithfully identify all of the Ag-specific cells as has been shown with cell lines and transgenic cells (64-67).

Therefore, to better assess the total number of Ag-specific cells in a polyclonal response, a more sensitive assay such as the micropipette adhesion frequency assay is needed. This assay allows for the biophysical interactions of proteins of interest to be measured as the probability of an adhesion event occurring when the receptor and ligand are allowed to interact (68, 71). Unlike surface plasmon resonance, which is considered a three-dimensional measure of affinity, 2D measures such as the micropipette adhesion

frequency assay were shown to better correlate with functional data (68, 69). Another advantage of the micropipette assay is the ability to rapidly measure the Ag specificity of a polyclonal response directly *ex vivo*, because intact T cells can be used to probe various pMHC monomer-coated RBCs. As shown in figure 2.1C, all of the cells that bound MOG:IA^b did not have any adhesions for hCLIP specificity control, demonstrating the exquisite discrimination of Ags in this assay. Additionally, analysis of a polyclonal response provides an internal control for Ag specificity in that not all of the cells tested will in fact bind the pMHC of interest, especially in sites such as the SPL which contain a diverse repertoire of Ag specificities.

The advantages of the 2D micropipette assay are multiple and greatly explain its increased sensitivity for identification of Ag- specific T cells. Many incorrectly assume that the RBC provides a simple increase in avidity extrapolating from work with pMHC tetramers. However, in the case of the RBCs, it is not a measure of avidity but an increase of valency (density of proteins) presented in their proper membrane context that increase the sensitivity of the assay. Avidity requires one binding event to alter the kinetic rates of subsequent binding events as occurs with pMHC tetramers and Abs. However, the increased number of pMHC molecules on the RBC only increases the probability of a binding event occurring, as demonstrated by multiple pMHC densities yielding different adhesion frequencies but the same affinity (68). Using monoclonal cells, it has been shown that when multiple densities of ligands are used for the same density of receptor, plots confirm that the assay is measuring interactions between a single receptor and ligand (68, 71, 159). This monovalent interaction measured by the micropipette assay has been observed for Fcγ receptors with IgG, TCR with pMHC, and CD8 with pMHC.

Noted also that surface plasmon resonance-based assays that measure affinity of purified proteins (three-dimensional measurements) requires the measurement of multiple molecules interacting to detect their signal and are not considered to be an avidity interaction. To solve for 2D affinity, the adhesion frequency must be measured at an equilibrium time point. The time required to reach equilibrium is determined by generating a curve where adhesion frequency is plotted against time. As time increases, the probability of binding events also increases before attaining a plateau level. The equilibrium frequency at the plateau level allows for derivation of the 2D affinity as the equation is defined by the adhesion frequency at equilibrium, density of the TCR, density of the pMHC and surface area of the interaction (see methods). All parameters except the surface contact area can be accurately measured. Because one cannot get an accurate assessment of the contact area, we report our data as relative 2D affinities that contain the contact area term. The density of the TCR takes into account differences in TCR numbers and cell size that may differ among responding cells at distinct point in their cell cycle, allowing measurement of the base 2D affinity for the TCR.

As expected, there were fewer MOG-specific cells in the SPL, with increased frequencies in the draining CLN for Tconvs and Tregs. We also determined that most of the CNS-infiltrating T cells (~80%) were in fact specific for MOG:IA^{b}, in contrast to less than half and ~10% by cytokine and tetramer staining, respectively (Figs 2.1E, 2.1B, and 2.3), which emphasizes the matched Ag specificities between Tregs and Tconvs. There were significantly more Tregs than Tconvs that stained with tetramer in the CNS, which could result from differences in the clustering or kinetics of TCR reorganization between Tregs and Tconvs. Because tetramer is an avidity-based reagent, these differences could

greatly affect staining. We have shown using bulk polyclonal cells that there appears to be an affinity cut-off that allows for tetramer staining; however, future studies are needed to determine whether these boundaries are different for Tregs and Tconvs or other Th cell subsets (51). In fact, we would argue that even a higher percentage of the CNS T cells are MOG specific, given that the micropipette assay will also miss extremely low-affinity cells represented by the 2D2 TCR Tg (52). The high frequency of MOG-specific cells in the CNS is not surprising since it is the site of Ag expression and inflammation. Similarly, the CLN collects APCs loaded with CNS Ags to prime T cell responses (168). Although these data cannot completely rule out the possibility of epitope spread, it would comprise a relatively small number of T cells at peak disease. We have also recently shown in a relapsing/remitting secondary progressive EAE model in NOD mice that >70% of the $CD4^+$ T cells are still specific for the inducing Ag (90), again suggesting that additional Ag specificities are not a required factor for chronicity.

Based on the methylation signature and Helios expression, we contend that the CNS Tregs are of thymic origin. They had similar hypomethylation of key regions previously identified to be only demethylated in tTregs and not in Tconvs or induced Tregs (122). This conclusion is also supported by the inability to identify pTregs generated during EAE (88). The TSDR region was mostly methylated in the CNS Tregs compared to naïve Tregs; however, all other gene regions were still hypomethylated. There is the possibility that these cells could be pTregs, but the origin of Tregs, whether thymic or peripherally induced, is a difficult question to answer, and to date there has not been a discriminating difference identified that faithfully and consistently discerns this distinction. It has been shown that in the presence of proinflammatory cytokines, STAT6

and STAT3 bind to the TSDR, recruiting DNA methyltransferases to this region, which could explain the high levels of methylation that we observed (120). During acute EAE, the TSDR is demethylated during disease recovery when the inflammatory milieu would be waning which could provide further support for the reason we saw methylation of this region during peak response in the chronic model (169).

One possible consequence of the TSDR being remethylated is lineage instability leading to the generation of pathogenic ex-Foxp3 cells (153). Mice that lack this region of DNA develop thymic Tregs that are capable of preventing the overt autoimmunity seen in mice deficient in Foxp3, but upon transfer to a lymphopenic host and subsequent homeostatic proliferation they lose Foxp3 expression (119). It has been demonstrated that Tregs in the CNS are capable of proliferation on the basis of BrdU incorporation (88). However, it was recently shown that the lineage instability due to lacking the TSDR requires multiple rounds of division before the loss of Foxp3 expression (120). Therefore, if the cells are not actively dividing, then they still could be stable, and upon the withdrawal of the inflammatory cytokines the TSDR could become demethylated again. In fact, a recent publication showed that the TSDR is actively demethylated in $Foxp3^+$ Treg thymocytes even in the presence of a late G1 inhibitor, which would allow for nondividing Tregs to demethylate this region to maintain lineage stability (170). To rule out the possibility of misidentification of Tregs due to differences in protein turnover for Foxp3 and GFP in the reporter mice, we repeated the experiments using Ab staining for Foxp3 to separate Tregs from Tconvs and obtained similar results. Future work will be done to determine the extent of any contribution to affinity by ex-Foxp3 cells.

At peak disease, there is a 1.5-fold enrichment of Tregs present in the CNS compared with peripheral sites. Additionally, there was an increase in the overall frequency of Tregs present in the periphery of EAE mice compared to age-matched naïve control mice. This result is consistent with a study that showed that peptide immunization could lead to *de novo* tTreg development of MBP-specific TCR Tg mice on a RAG-deficient background, which normally lack Tregs (171). It was shown that when the mice were thymectomized prior to peptide administration, there was only a slight delay in disease kinetics and the generation of Tregs was greatly impaired. Therefore, during EAE induction, it is possible that the MOG peptide required to induce disease is also leading to *de novo* tTreg development. The authors did note that when two doses of pertussis toxin were given, there was a marked decrease in the numbers of Tregs generated in their transgenic $RAG^{-/-}$ mice, which were not sufficient for disease prevention. When the frequency of MOG-specific Tregs as a percentage of total $CD4^+$ T cells is calculated, there is an enrichment of MOG-specific Tregs in the draining lymph node and at the site of autoimmunity, which is not appreciated when just the total percentage of $Foxp3^+$ cells is shown.

To our knowledge, this study is the first to report the affinities of tTreg cells for a self-antigen. The CNS Tregs had the largest range of affinity for MOG, but on average it was lower than CNS Tconvs. It is intriguing to speculate that this deviation in affinities between Tconvs and Tregs at the site of tissue inflammation is causative for chronic disease and escape from Treg mechanisms of peripheral tolerance. Although it has been hypothesized that Ag-specific tTregs would have high affinity for their cognate Ags, this question is extremely difficult to answer (32-37). The reason for this is the uncertainty of

whether the self-specific Treg that developed in the thymus selected on the same cognate Ag. For a cytochrome c-specific transgenic, a positive selecting ligand has been identified, and based on the fact that it is unable to induce proliferation of mature T cells on its own, we assume that it is lower affinity than the cognate agonist (31). Although we do not know the selecting peptide for the MOG-reactive T cells, the outcome is Tregs and Tconvs spanning an entire range of affinities. The concept that higher affinity TCRs are selected and survive to become tTregs for each self-selecting peptide is possible, but the identification of lower affinity TCRs for MOG and the wide range in affinity raises questions about this model of tTreg selection. Proof would have to come from analysis of polyclonal populations of T cells for which the self-selecting peptides are known, which is currently not possible. What we can take from these studies is that for the same self-antigen, Tconvs and Tregs have a range of affinities comprised of predominately low-affinity cells below the detection level of pMHC class II tetramers.

In conclusion, to our knowledge these data are the first to identify the frequency and affinity of Tregs for an endogenous self-antigen during autoimmune disease. We show that Ag-specific Tregs span a range of affinities for self that overlaps with Tconvs in the periphery, although in the CNS at the site of autoimmune disease the two populations have unique affinity distributions shifted to favor high-affinity Tconvs. The functional consequences for higher and lower affinity TCRs on Tregs need to be determined, but the altered Treg distribution pattern in the CNS potentially suggests mismatched TCR affinities as a mechanism for disease progression.

Acknowledgements

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Table 2.1. Two-sided Fisher's Exact *p* values for the data presented in Figure 2.6.

	Naïve Tconv vs. Naïve Treg	d21 CNS Tconv vs. d21 CNS Treg
Foxp3	3.73E-100	6.41E-08
Gitr	8.57E-27	2.20E-41
Ctla4	7.70E-37	3.44E-27
Eos	6.52E-12	0.000163
CD25	1.61E-32	0.006423

Figure 2.1

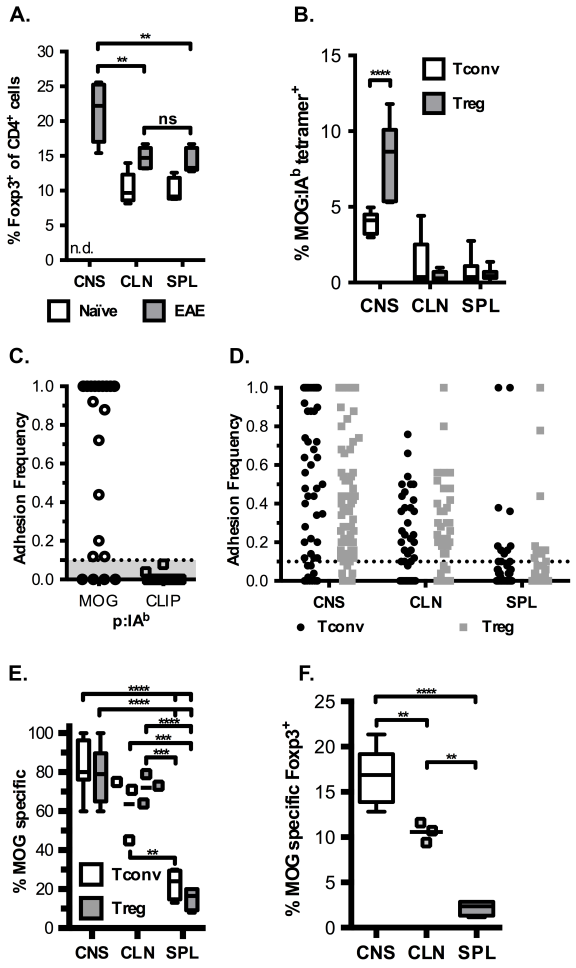

Figure 2.1 Enrichment of MOG-specific Foxp3$^+$ Tregs in the CNS during EAE. (A) Frequency of Foxp3-expressing CD4$^+$ cells in various organs from naïve mice (open box plots) and at peak disease (filled box plots). Data are from five experiments. ** p <0.01 (one-way repeated measures ANOVA; CNS versus CLN p = 0.0070; CNS versus SPL p = 0.0051). (B) Tetramer staining frequencies by organ during peak EAE for Tconvs and Tregs, with representative FACS plots shown in Figure 2.2. Data are from at least five independent experiments. **** p < 0.0001 (two-way ANOVA repeated measures). (C) Representative adhesion frequencies for individual CNS cells for MOG:IA^b– and hCLIP:IA^b–coated RBCs. All cells were tested for binding to both Ags, and data are from

two independent experiments, with 10 cells per each experiment tested. (D) MOG:IA^b adhesion frequencies for T cells isolated from indicated organs at peak EAE, where each dot represents an individual cell tested. Number of cells tested: CNS Tconvs – 65; CNS Tregs – 107; CLN Tconvs – 41; CLN Tregs – 40; SPL Tconvs – 55; SPL Tregs – 55. (E) Overall frequency of cells that recognize MOG from the indicated organs. For (D) and (E) data are from eight, three, and four experiments for CNS, CLN and SPL, respectively. For (E), two-way ANOVA comparing means for indicated cell type between tissues are shown: **** $p < 0.0001$ (CNS Tconvs versus SPL Tregs, CNS Tconvs versus SPL Tconvs, CNS Tregs versus SPL Tregs, CNS Tregs versus SPL Tconvs, CLN Tregs versus SPL Tregs), *** $p < 0.001$ (CLN Tconvs versus SPL Tregs, $p = 0.0004$; CLN Tregs versus SPL Tregs $p = 0.0003$), ** $p < 0.01$, CLN Tconvs versus SPL Tconvs, p = 0.0025, ns (Tconvs: CNS versus CLN; Tregs: CNS versus CLN). (F) The frequency of MOG-specific Tregs as a percent of total CD4$^+$ cells was determined by multiplying the percentage MOG-specific by the average frequency of Foxp3$^+$ cells in each location, one-way ANOVA, **** $p < 0.0001$ (CNS versus SPL), ** $p < 0.01$ (CNS versus CLN $p = 0.0078$, CLN versus SPL $p = 0.0020$). Box plots with minimum to maximum whiskers for all panels except (C) and (D), where each dot represents an individual cell, and for CLN in (E) and (F), where each dot represents an individual experiment because $n = 3$. n.d., not determined.

Figure 2.2

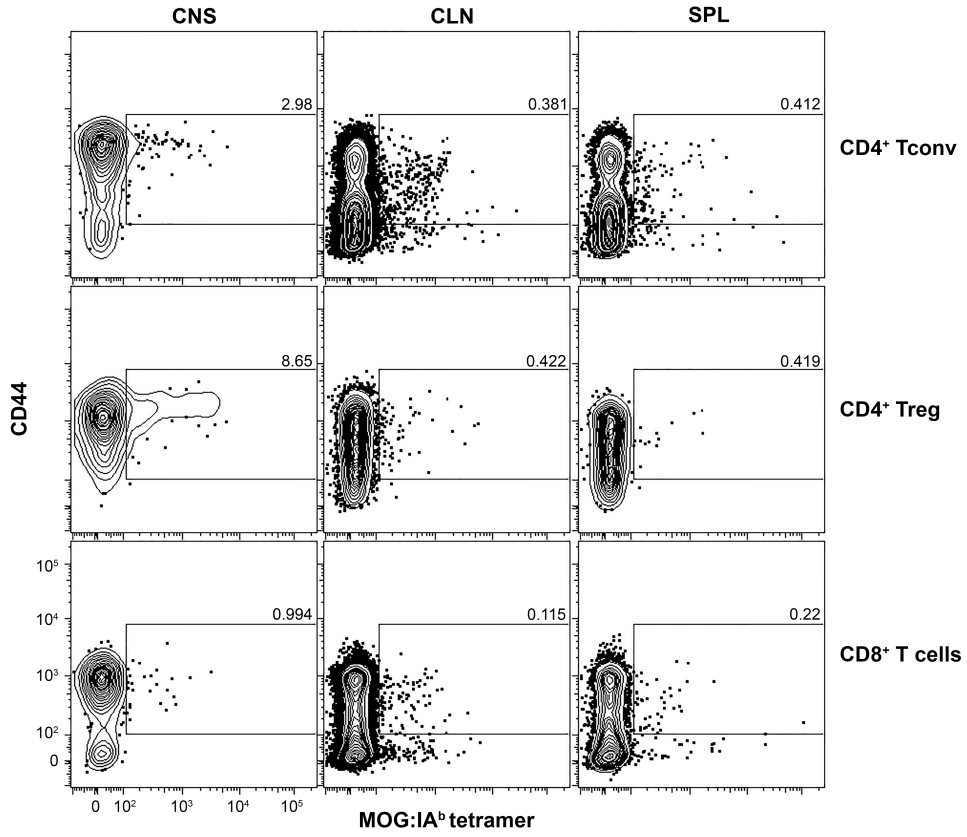

Figure 2.2 Representative FACS plots showing the staining of MOG:IA^b tetramers and CD44 for indicated cell types for the indicated organs isolated from mice at peak of EAE. The tetramer positive gates were drawn using the $CD8^+$ T cells as a negative control. Indicated cell types were gated as follows: $CD4^+$ Tconv ($Dump^-CD4^+$ Foxp3-GFP^-), $CD4^+$ Tregs ($Dump^-CD4^+$Foxp3-GFP^+), and $CD8^+$ T cells ($Dump^-CD8^+$). Dump consisted of CD11b, CD11c, and CD19.

Figure 2.3

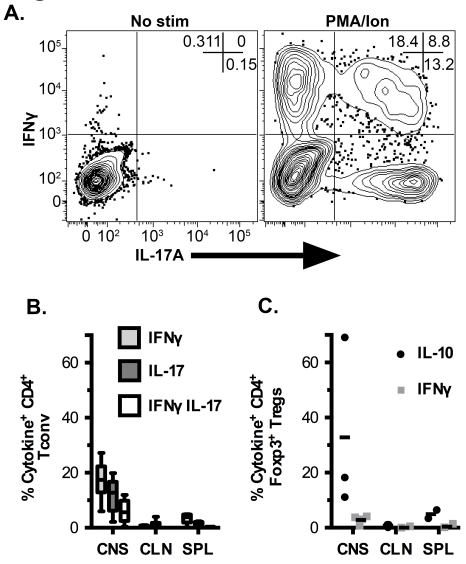

Figure 2.3 Production of cytokines by Tconvs and Tregs during EAE. (A) Representative flow plots showing IL-17A and IFN-γ production by CNS Tconvs. (B) Frequencies of single and double cytokine producing Tconvs in various organs at peak EAE upon stimulation with PMA and ionomycin. Data are from at least five independent experiments. (C) Percentage of Tregs producing IL-10 or IFN-γ in the various organs in response to PMA/ionomycin stimulation. Data are from three independent experiments, except for SPL, which is from two experiments.

Figure 2.4

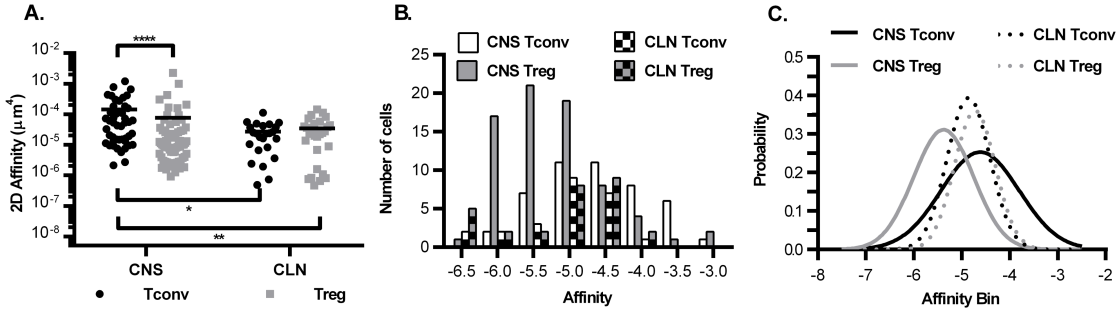

Figure 2.4 Tregs and Tconvs have overlapping ranges of affinity for MOG but unique distributions in the CNS. (A) The effective 2D affinities were derived from the adhesion frequencies for Tconv and Treg from Figure 2.1D, as described in Materials and Methods. Two-way ANOVA on geometric mean affinities, **** $p < 0.001$ CNS Tconvs versus CNS Tregs, ** $p = 0.0083$ CNS Tconvs versus CLN Tregs, * $p = 0.0222$ CNS Tconvs versus CLN Tconvs. Number of affinities: CNS Tconvs, 46; CNS Tregs, 73; CLN Tconvs, 24; CLN Tregs, 28. (B) Frequency distributions and (C) Gaussian curves were fitted to the affinity data from (A). Number of independent experiments by organ: CLN, three; CNS, eight.

Figure 2.5

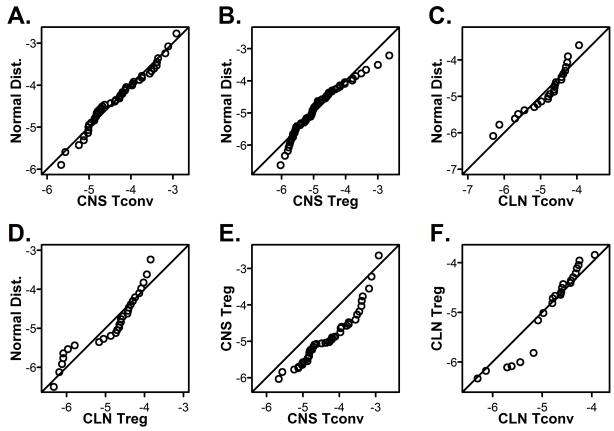

Figure 2.5 CNS Tconvs are normally distributed, unlike CNS Tregs and cells in periphery. (A-D) Q-Q plots were generated for (A) CNS Tconv, (B) CNS Tregs, (C) CLN Tconvs, and (D) CLN Tregs using the data from Figure 2.4A. Comparisons of the distributions of Tconv to Treg in the (E) CNS and (F) CLN were done by Q-Q plots.

Figure 2.6

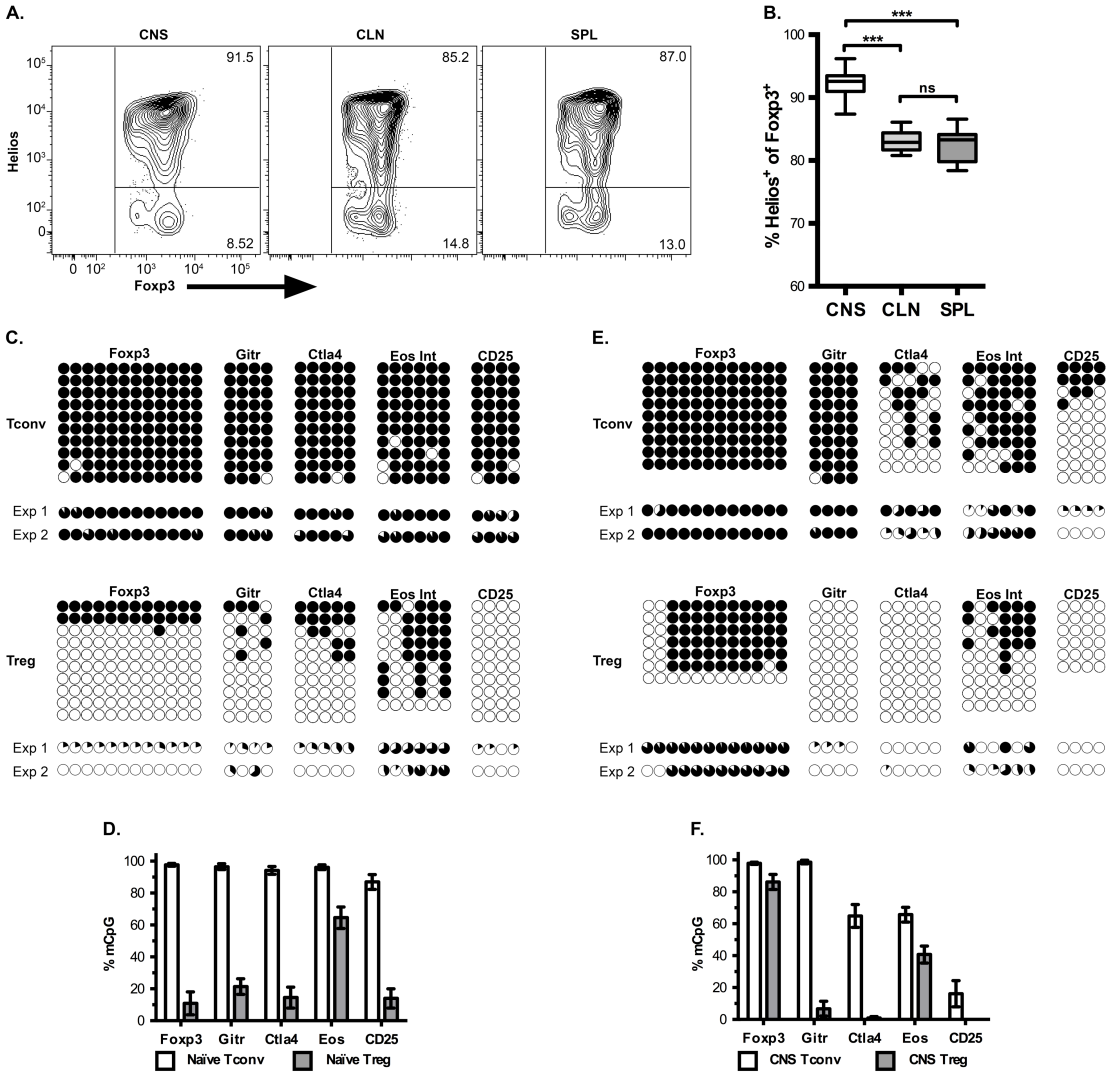

Figure 2.6 CNS Tregs are of thymic origin. (A) Representative flow plot for Helios expression by Tregs, related to Figure 2.7, showing Helios by Foxp3 expression for $CD4^+$ T cells. (B) Average frequency with SEM of Helios expression in $Foxp3^+$ $CD4^+$ cells in various organs at peak disease. Data are from seven independent experiments. *** $p <$ 0.001 (One-way repeated ANOVA) (CNS versus CLN, $p = 0.0003$, CNS versus SPL $p =$ 0.0002). (C-F) The methylation status within each gene region was determined for the tTreg-Me signature genes for $CD4^+$ Tconvs and Tregs from (C and D) splenocytes of naïve mice or (E and F) CNS-infiltrating cells from EAE mice. In (C) and (E), each

column represents an individual CpG within the region of interest, and each row is an individual clone sequenced, where a filled circle is a methylated CpG and an open circle is a demethylated CpG. Representative data from a single experiment are shown, and below are pie charts to indicate the methylation frequency at that CpG within each of the two experiments. (D) and (F) show total level of methylation for the CpGs for all clones and CpGs across that gene region. Data are from two independent experiments with at least nine clones sequenced per gene per experiment, except for d21 CNS Treg Foxp3 and CD25 which had at least four clones sequenced.

Figure 2.7

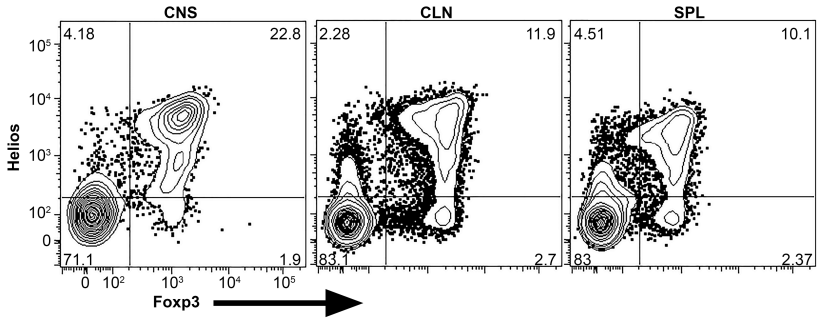

Figure 2.7 Helios *vs.* Foxp3 expression for $CD4^+$ T cells. Representative FACS plots showing the expression of Foxp3 and Helios of $CD4^+$ $Dump^-$ cells for the indicated organs isolated from mice at peak of EAE. Dump channel was made of CD11b, CD11c, and CD19.

Chapter 3 Affinity and lack of functional response suggests that Chromogranin A could be a selecting ligand for diabetogenic T cells.

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Abstract

Diabetogenic clones with various antigen specificities have been isolated and maintained for decades, although the identification of all the antigen specificities is still incomplete. The autoantigen recognized by the diabetogenic BDC2.5 and BDC10.1 clones was recently shown to be derived from chromogranin A (ChgA). Currently there are conflicting reports regarding the ability of two different epitopes to stimulate BDC2.5 cells, and we show that ChgA29-42 required millimolar amounts to elicit a minimal response, even when substitutions were made to the reactive cysteine residue. Using a highly sensitive micropipette adhesion assay we show that both BDC2.5 and BDC10.1 TCRs are specific for and possess different affinities for ChgA29-42. We tracked antigen reactivity of the BDC2.5 TCR through development from double positive (DP) thymocytes to naïve and activated $CD4^+$ splenocytes, and found that TCR affinities for antigen varied through the process of development and were highest for DP thymocytes followed by activated and naïve splenocytes. Determining the affinity of a different TCR transgenic (Tg) for its known endogenous positive selecting ligand, revealed that DP thymocytes and naïve splenocytes have a lower affinity for the selecting ligand compared to the agonist peptide as expected. Comparing the affinities of DP thymocytes and naïve cells, there is a trend that supports ChgA29-42 as a possible positive selecting ligand for BDC2.5 cells, which would explain the lack of functional response observed.

Introduction

Type 1 diabetes (T1D) is a complex T cell mediated autoimmune disease comprised of T cells with specificities for several self antigens. The BDC2.5 TCR is one of the most widely used tools to study T1D for its ability to infiltrate and destroy the pancreatic islet cells (141, 142, 172). When coupled with Rag1 mutation to inhibit endogenous TCR alpha chain rearrangement, the BDC2.5 Tg mice become diabetic extremely early with 100% penetrance (173). In a large study of 16 different diabetogenic TCRs with various antigen specificities, only BDC2.5 and BDC10.1 TCRs demonstrated 100% disease incidence, with BDC10.1 having the fastest progression to diabetes and is diabetogenic on the nonautoimmune background of C57Bl/6 mice (131). Both of these TCRs are very efficient at inducing disease, with BDC10.1 being the most diabetogenic TCR identified thus far and understanding the recognition kinetics for self antigen would be informative when compared to diabetogenic T cells with other self antigen specificities. Through the use of Chromogranin A (ChgA) knockout mice, it was determined that the BDC2.5, BDC5.10.3 and BDC10.1 clones lost their ability to produce IFN-γ when given islet cells from ChgA-/- mice, while an insulin specific clone maintained functionality (130). In patients with T1D, $CD4^+$ T cells reactive to ChgA have been identified (129). Thus ChgA appears to be a diabetogenic T cell antigen in mice and men.

There have been conflicting reports regarding the ability of BDC2.5 cells to respond to ChgA29-42 in addition to post-translational modification of WE14, the other epitope derived from ChgA, needed for full responsiveness (140, 174, 175). One possible explanation is that ChgA could be acting as a selecting element in the thymus, which

would anergize the cells to these antigens. Fetal thymic organ cultures (FTOCs) utilizing altered peptide ligands of hemoglobin demonstrated that only a null peptide was able to mediate positive selection, while the antagonist and agonist peptides caused negative selection of N3.L2 thymocytes (27). In fact by surface plasmon resonance, this peptide:MHC complex didn't show any detectable binding. Recently endogenous positive selecting ligands for two different moth cytochrome c specific transgenics were identified and even though the cells share the same agonist, their endogenous ligands were different (30, 31). The positive selecting ligands were unable to elicit a functional response from splenocytes on their own, but were able to act as coagonist to boost the response to suboptimal levels of MCC. Therefore ChgA could act as a selecting element for BDC2.5 cells, which is supported by studies showing that ChgA is expressed in the thymus by both cortical and medullary thymic epithelial cells (cTECs and mTECs) (176-178).

We wanted to use the highly sensitive micropipette adhesion frequency assay to determine antigen specificity for various diabetogenic T cells (51, 68). This assay measures TCR recognition of cognate peptide:MHC (pMHC) by deformation of a biosensor's membrane upon specific binding of the receptor to its antigen. The adhesion frequency can then be used to determine the percentage of cells specific for a given antigen and the effective two-dimensional (2D) affinities (51, 90). The comparisons performed to date show a direct correlation between 2D TCR affinity for antigen and the extent of functional T cell response (52, 68, 69, 160, 179-182). Using this assay it has been demonstrated that eight different insulin B specific clones have a range of affinities that correlate with their functional responses and disease induction (132).

In this report we show that BDC2.5 retrogenic and transgenic splenocytes do not functionally respond to ChgA29-42. Even though the peptide is nonstimulatory our analysis determined that two diabetogenic BDC TCRs recognized this self antigen with different affinities. Using transgenic mice, we demonstrate that DP thymocytes have a higher affinity than activated and naïve splenocytes for the same antigen. Comparing the affinities of DP thymocytes with naïve splenocytes for various ligands and cell types, we conclude that ChgA29-42 could be a selecting ligand for BDC2.5 since it has an extremely low affinity for it, similar to what we have measured for the known positive selecting ligand for AND TCR transgenic cells.

Materials and Methods

Mice

BDC2.5 and AND TCR transgenic (Tg) mice were bred in the Emory University animal facility and used between 5 and 20 weeks of age, with 9 weeks of age being the average age used. BDC2.5 and BDC10.1 retrogenic (Rg) mice were generated as previously described using retroviral producers encoding these TCRs (131, 183). For BDC2.5 Rg mice, reconstitution was verified by flow cytometric analysis 4-5 weeks post bone marrow transplant and healthy mice that exhibited engraftment and expression of the desired TCR were used for experiments. Due to the rapid onset of diabetes before full reconstitution with the BDC10.1 TCR, spleens were harvested 3-4 weeks post bone marrow transplant and analyzed separately. Only mice that exhibited normal TCR expression, as determined by flow cytometry, were used in experiments (131). All experiments were carried out in accordance with IACUC protocols at Emory University and St. Jude Children's Research Hospital.

Reagents and antibodies

Cell culture media R10 was RPMI-1640 supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine, 2 X 10^{-5} M 2-ME, 10 mM HEPES buffer, and 50 μg/ml Gentamicin sulfate. All media components were from Mediatech except FBS (Gibco) and 2-ME (Sigma Aldrich). Experimental additive solution 45 (EAS45) was prepared as described, sterile filtered and frozen in aliquots at -20°C until needed (157). Prior to use, EAS45 was supplemented with 1% BSA. All EAS45 components, including the BSA, were purchased from Sigma Aldrich except sodium phosphate dibasic which was obtained from Fisher Scientific. Antibodies and clones used: RT1B PE (Clone Ox-6,

detects IAg^7), TCRβ PE (H57-597), CD4 APC (RM4-5), Vα2 FITC (B20.1). All antibodies were from BD Pharmigen.

Peptides

All peptides were synthesized on a Prelude peptide synthesizer (Protein Technologies Inc.) using FMOC chemistry. Mimotope 1 – AHHPIWARMDA, ChgA29-42 – DTKVMKCVLEVISD. For ChgA29-42 35Cys variant peptides, the cysteine was changed to an alanine (35Ala), serine (35Ser), methionine (35Met), aspartic acid (35Asp), phenylalanine (35Phe), or 2-aminobutanoic acid (35Aba). Peptides were resuspended in molecular grade water at 3 mM concentrations and sterile filtered through a 0.22 μm syringe filter.

^{3}H-thymidine incorporation assay

Single cells suspensions of splenocytes (3 X 10^5 cells per well) were incubated in a 96 well flat bottom plate with the indicated peptide concentrations as described (184). After 48 hours of incubation, 0.4 μCi/well ^{3}H-thymidine was added for an additional 18 hours of incubation. The cells were then harvested with a Packard Filtermate 196 harvester onto filtermat A filters (Perkin Elmer). CPMs were obtained on a MicroBeta 1450 (Wallac) liquid scintillation counter and then stimulation indices were determined by dividing the CPMs at indicated concentration by the background CPMs obtained with no stimulation.

Micropipette adhesion frequency assay

Human red blood cells were biotinylated using X-NHS biotin and coated with peptide MHC class II (pMHC) monomers (NIH tetramer core facility) as published previously (68). RBCs were washed two times after each step. To quantitate the number

of pMHCs and TCRs on the surface of the cells, PE Quantibrite beads (BD biosciences) were used. An Olypmus IX71 microscope equipped with a 100X oil immersion objective and a 1.6X magnification chamber lens was used for these experiments. Cells were aspirated on opposing micropipettes and positioned by xyz linear translation systems equipped with 13 mm coarse micrometers (Newport Corporation) and one pipette had fine xyz positioning supplied by a hydraulic micromanipulator (MO-103, Narishige). The adhesion test cycle was controlled using Labview (National Instruments), which translated the zero, retraction, approach, and contact time into a repetitive movement of one of the pipettes by a piezo electric motor (P.840.1, Physik Instrumente). The average adhesion frequency (P_a) is determined from repeating the cycle for 25 or 50 touches and scoring the binding events. The frequency of antigen specific cells can be determined for the entire population by dividing the number of cells whose P_a was greater than the cutoff for nonspecific binding ($P_a > 0.1$) by the total number of cells tested. When the contact time used in the adhesion test cycle was in the equilibrium phase of the binding kinetic curve, the effective 2D affinity A_cK_a can be calculated using the following equation:

$$A_c K_a = \frac{-\ln(1 - P_a(\infty))}{m_{TCR} m_{pMHC}}$$

where $P_a(\infty)$ is the adhesion frequency at an equilibrium contact time (2-5 seconds), m_{TCR} and m_{pMHC} are the densities of TCR and pMHC on the T cell and RBC respectively.

Cell subset isolation

To isolate T cells for use in the micropipette assay, CD4 positive or negative selection kits were used from Miltenyi biotec, with the exception of DP thymocytes, which were isolated on the lack of expression of CD53 (185, 186). To generate activated cells to use in the micropipette assay, three million BDC2.5 Tg splenocytes were plated per well in

24 well plates and stimulated with 1 μM Mimotope 1 peptide for four days. Cells were then isolated by ficoll gradient centrifugation and enriched for $CD4^+$ cells using the same methods as above.

Statistical analysis

Prism 6 (GraphPad Software) was used to determine significance using either One-way ANOVA with Tukey's multiple comparisons and multiplicity adjusted P values or unpaired two-tailed t tests. For Q-Q plots, R console (R project for statistical computing) was used to draw plots and determine if there were statistical differences between the distributions. The Shapiro-Wilk test was used for the normality and the Kolmogorov-Smirnov test was used to test the similarity between two experimental distributions.

Results

BDC2.5 Rg and Tg cells lack functional responsiveness to ChgA29-42.

In the initial report identifying ChgA as a target in T1D, three different BDC clones, 2.5, 5.10.3 and 10.1, demonstrated specificity for ChgA protein (130). The precise peptide epitope for BDC2.5 is currently unclear due to conflicting reports of reactivity to two distinct epitopes (WE14 and ChgA29-42) within ChgA for transgenic T cells (130, 140, 174, 175), which is further complicated by conflicting reports regarding the ability of BDC2.5 Tg cells to respond to ChgA29-42 (140, 174, 175). One possible explanation is the oxidation of cysteine under neutral to basic conditions which can destroy the antigenicity of certain peptides (187-190). In addition to issues with peptide modification, the expression of endogenous TCR alpha chains on the NOD background could lead to a decreased functional response. Therefore we wanted to determine if BDC2.5 Rg NOD.*scid* splenocytes were capable of responding to ChgA29-42 or a variant peptide

lacking the cysteine. We used 2-aminobutanoic acid (Aba) that better mimics the size of cysteine while replacing the reactive thiol with a methyl group. Only extremely high doses of ChgA29-42 and ChgA 35Aba were able to elicit a minimal response from the cells (Fig. 3.1B). Due to the rapid onset of diabetes in BDC10.1 Rg mice occurring before full reconstitution, we were unable to test these cells for their functional response to ChgA.

We next tested a panel of peptides with various substitutions at aa35 to determine the best amino acid to replace the reactive cysteine while allowing for functional responses (Fig. 3.1A). Conservative substitutions of cysteine were: alanine (Ala), serine (Ser), methionine (Met) and 2-aminobutanoic acid (Aba) and for nonconservative substitutions phenylalanine (Phe) and aspartic acid (Asp) were used. As expected there was no proliferation when 35Phe and 35Asp variants were tested (Figure 3.1C). Of the conservative substitutions, Aba gave the best response (Fig. 3.1D), which even surpassed that of ChgA29-42 WT with Ala having an intermediate stimulatory capacity, although again this response was minimal and dependent on high doses of peptides. No proliferation was seen when Ser and Met were used (Fig. 3.1E), but it has been demonstrated that Ala and Ser substitutions for Cys may decrease the antigenicity of some peptides depending on whether the substitution is occurring at a TCR or MHC contact residue (188).

ChgA29-42 is recognized by two different diabetogenic clones.

Using altered peptide ligands, it has been shown that antagonist peptides, which are non-stimulatory on their own and actually limit the functional response to agonist during co-culture, can have measurable affinities for the TCR (68). Therefore we wanted

to determine if BDC2.5 and BDC10.1 TCRs were capable of binding ChgA in the context of IA^{g7}. We employed the use of peptide:MHC class II (pMHC) monomers that have the peptide covalently linked via their C-terminus to the MHC molecule via a flexible linker (191). Of note, the length of this linker can affect T cell activation and tetramer staining (192). WE14 (ChgA358-371) binds IAg^7 in a unique manner with nine of the carboxyl terminal amino acids binding outside of the groove leaving positions one through four unoccupied (130) and post-translational modification by transglutaminase increases the antigenicity of WE14 (140). Due to the unique peptide binding of WE14, which could be affected profoundly by the linker, we focused our studies on ChgA29-42 which completely fills the peptide groove with the aspartic acid (residue 42) binding in p9 (174) to determine BDC2.5 and BDC10.1 affinities.

TCR retrogenic (Rg) mice were used to probe ChgA29-42 reactivity because they allow for analysis of several TCRs in the absence of endogenous TCR alpha chain expression (183). Individual T cells were tested for their ability to specifically recognize ChgA29-42 in the micropipette adhesion frequency assay (71). Human RBCs were coated with pMHC monomers and used as a biosensor to detect TCR binding via the distension of the RBC membrane upon separation of the cells. The cycle of touching and retraction was repeated for 25 to 50 cycles to obtain an average adhesion frequency. Both BDC2.5 and BDC10.1 TCRs recognized ChgA29-42 as determined by having measurable adhesion frequencies (Figure 3.2A). The adhesion frequency can range from zero (no reactivity) to one (100% binding) for a given TCR depending on the densities of pMHC and TCR (68). The BDC2.5 Rg cells had a tighter, albeit lower density of TCR on their surfaces and this lower TCR expression is consistent with the initial characterization

of these two TCR Rgs (131). It should be noted that BDC10.1 Rg mice become diabetic rapidly and the active autoimmune response has likely contributed to the wide range in TCR expression (Fig. 3.2B).

Using the densities of pMHCs and TCRs along with the adhesion frequencies, the effective 2D affinities can be calculated as described (71). The micropipette adhesion frequency assay is considered a 2D measure of affinity since both the pMHC and TCR are anchored to cell membranes therefore confining their movement to only two planes (180). The equation solves for what is termed the effective affinity due to the inability to define the precise dimensions of the contact area. BDC2.5 Rg cells had ~13-fold higher affinity for ChgA29-42 compared to BDC10.1 Rg cells (mean 9.089 and 0.6987 X 10^{-4} μm^4, Fig. 3.2C). The majority of BDC2.5 and BDC10.1 cells possessed measurable binding (~90 and ~80% respectively) to the ChgA29-42:IAg^7 (Fig 3.2D and 3.2A). These frequencies are typical of what is observed for a monoclonal population with most of the variation due to the purity of the sample post MACS separation (68).

DP thymocytes have a higher affinity for the same cognate self antigen compared to peripheral cells.

We next wanted to examine the potential changes in antigen reactivity of BDC2.5 T cells as they progress from double positive (DP) thymocytes, to naïve splenocytes, to activated T cells. To obtain sufficient numbers for these assays, TCR transgenic (Tg) BDC2.5 mice were used. DP thymocytes were purified using CD53 depletion as described to obtain cells that should not have undergone any selection (185). T cells regulate the density of cell surface TCR expression depending on activation and developmental state. For BDC2.5 Tg cells, TCR density was lowest on DP thymocytes; it

increased just over fourfold on naïve and threefold on activated cells (DP - ~26, Naïve - ~120, Act. - ~81 TCR per μm^2, Fig. 3.3A). These levels of TCR expression on Tg cells are comparable to what we have observed for other transgenic mice (100-150) and on par for polyclonal T cells (51, 68). TCR density on DP thymocytes was similar between mice, while there was more variability observed the naïve splenocytes (Fig. 3.3A). The range in adhesion frequencies for DP thymocytes was tighter compared to naïve splenocytes (Fig. 3.3B), which translated into naïve Tg splenocytes possessing lower and expanded breadth of affinities (Fig. 3.3C and Table 3.1). DP thymocytes exhibited almost 40-fold higher affinity for ChgA29-42 compared to naïve splenocytes (mean 2.818 vs. 0.07406 X 10^{-4} μm^4, respectively). The affinity of activated cells was nine times higher compared to naïve splenocytes (mean 0.687 X 10^{-4} μm^4). These data imply that there are additional cell intrinsic factors affecting the effective 2D affinity, which could include clustering of receptors, membrane fluidity, and co-receptor densities.

BDC2.5 transgenic cells have higher affinity for mimotope than cognate self antigen.

2D affinities correlate with functional responses with higher affinities typically possessing greater function avidities (52, 68, 69, 160, 179-182). Before the identification of the ChgA self antigen for BDC2.5 cells, peptide library pools were screened for their ability to generate functional responses (193, 194). Mimotope peptides capable of eliciting strong responses were selected as surrogate stimulatory antigens; although these peptides tended to act more like superagonists when compared to the islet cells alone. Due to this robust functional response elicited by the mimotopes, BDC2.5 TCR likely has a high affinity for the peptides. Indeed for DP thymocytes and mature T cells (naïve or activated), mimotope 1 affinities were higher than their respective ChgA29-42 affinities

(DP – 3.109 vs. 0.2818, Naïve – 0.3239 vs. 0.007406, Act. – 0.7577 vs. 0.0687 X 10^{-3} μm^4 for mimotope 1 and ChgA29-42 respectively, Fig. 3.4A and Table 3.1). We observed the same trend of mimotope affinity varying based on the cell population tested. DP thymocytes had approximately tenfold and activated cells twofold higher affinity compared to naïve splenocytes (Fig. 3.4B). In comparison to our previous study, mimotope 1 affinities are more similar to a TCR Tg specific for a foreign antigen (LCMV specific SMARTA cells), while ChgA affinities more closely reflect those of a TCR Tg specific for a self antigen (neurofilament responsive 2D2 $CD4^+$ Tg) (52).

Decreased ChgA29-42 reactivity of peripheral BDC2.5 Tg cells coincides with increases in endogenous TCR alpha chain expression.

The frequency of ChgA29-42 specific cells from BDC2.5 Tg mice varied more than BDC2.5 Rgs, although it was only the self antigen and not the mimotope that displayed this drop in frequency (Fig. 3.5A and 3.2D). Tg DP thymocytes had greater ChgA29-42 reactivity than naïve splenocytes although it was not significantly different (~76% vs. ~58%). One possible explanation for the observed differences is that endogenous TCR alpha chains could be expressed at greater frequencies on naïve splenocytes as compared to DP thymocytes, thereby decreasing the number of ChgA specific TCRs available for recognition. Because of the absence of reagents to track the Tg alpha chain by flow cytometry, we tracked the frequency of Vα2 expressing cells. This same method was used to determine that BDC2.5 Tg T cells on the B6H2.g7 background have fewer endogenous TCR alpha chains as compared to being on the NOD background (195). The percentage of cells expressing Vα2 increased tenfold from the DP to CD4 single positive (SP) stage in the thymus (DP – 0.36% vs. SP – 3.59%, Fig. 3.5B).

In the periphery Vα2 frequency further increases to approximately 14%. Because $CD53^-$ DP thymocytes have not undergone any selection the likelihood of cells expressing dual TCRs is extremely low (185). One should note that this large percentage of Vα2 chain expression only reflects one of the possible TCR alpha chain families and it is likely that the overall frequency of endogenous TCR alpha expression is higher.

T cells have lower affinity for positive selecting ligand than agonist

FTOCs have shown that ligands with lower affinities are capable of mediating positive selection, while higher affinity interactions lead to negative selection. To date only OT-I TCR affinity for its endogenous positive selecting ligands has been measured; this interaction was so weak that binding could only be detected when the temperature was lowered during SPR measurements (49). We wanted to determine if the micropipette adhesion frequency assay was capable of detecting the binding of a $CD4^+$ T cell for its endogenous selecting ligand and compare it with the agonist's affinity. To this end AND Tg mice specific for moth cytochrome C were used. By the micropipette adhesion frequency assay binding to the endogenous positively selecting ligand gp250 was observed for both DP thymocytes and naïve splenocytes (Fig. 3.6A). The percent of naïve Tg cells that bound gp250 was lower than those that recognized MCC, while the DP recognition of both antigens was comparable, which was consistent with what was observed for BDC2.5 interacting with ChgA29-42. AND Tg affinity for its endogenous ligand gp250 was lower than that measured for MCC, with DP thymocytes having higher affinity than naïve splenocytes, similar to what was observed for BDC2.5 Tg cells (Fig. 3.6B and 3.6C). These data are consistent with the results from FTOCs using altered peptide ligands and with the recently measured affinities of OT-I for its two endogenous

positive selecting ligands. Based on the instructive model of thymocyte selection, there should be affinity cut-offs for peptides capable of mediated positive *vs.* negative selection. Although more points are needed to strengthen the argument, there does appear to be a linear relationship when the log affinities of DP thymocytes are plotted against the log affinities of naïve splenocytes for the same antigen (Fig. 3.6D). BDC2.5 Tg affinity for ChgA29-42 falls between AND affinities for gp250 and MCC, which could be indicative of ChgA functioning as a positive selecting ligand on the basis of affinity. To truly test this conclusion, FTOCs of thymi from ChgA$^{-/-}$ BDC2.5 Tg mice on the NOD background will need to be done, although ChgA may not be the only endogenous ligand capable of selecting BDC2.5 Tg cells, since it has been demonstrated that Tg cells can have more than one endougenous selecting ligand (144).

Discussion

This report demonstrates that ChgA29-42 is a peptide recognized by two different diabetogenic clones even though there is a lack of functional response by the one tested. Their TCRs have different affinities for ChgA29-42, with BDC2.5 having 13-fold higher affinity than BDC10.1. In comparison the eight insulin specific TCRs measured previously had a range in affinities spanning one log (10^{-3} to 10^{-4} μm^4 (132)). BDC2.5 Rg has an affinity for ChgA in the upper range of the measured insulin specific clones, while BDC10.1 is about half of a log lower than the lowest measured insulin specific TCR (9.089 and 0.6987 X 10^{-4} μm^4 for 2.5 and 10.1 respectively). It is interesting that the lower affinity BDC10.1 TCR has a faster progression to diabetes in retrogenic mice (BDC10.1 ID_{50} 27 days, BDC2.5 ID_{50} 52 days, where ID_{50} is the number of days post bone marrow transplant for 50% of the Rg mice to become diabetic (131)). TCR affinity for antigen is probably one of several factors that determine the aggressiveness of a given clone. Other kinetic parameters including the on rates, the force generated by the bond, and half-life can have an impact on the overall response (161, 179, 196, 197), in addition to availability of antigen and the impact of thymic education to self antigens (132, 198). We noticed with the insulin Rg mice, that the TCRs with the highest affinity also had lower levels of surface receptor (132). Due to the high affinity of the BDC2.5 TCR for self, those thymocytes which express low levels of receptor on their surfaces are able to survive selection which could then translate to delayed recognition of self in the periphery and activation of the cells.

BDC2.5 transgenic and retrogenic mice afforded an opportunity to determine if endogenous TCR alpha chain expression could affect the effective 2D affinities. Our Tg

mice are able to rearrange and express a secondary TCR alpha chain since they are bred to NOD mice, while Rg mice developed on the NOD.*scid* background can only express the TCR of interest. Rg cells had the highest affinity for ChgA29-42 compared to Tg cells (Table 3.1). DP Tg thymocytes had only a half log lower affinity compared to Rg splenocytes, while Tg naïve cells had almost two logs lower affinity. The expression of endogenous TCR alpha chains post selection in the thymus could be decreasing the apparent affinities and frequency of ChgA reactivity of naïve splenocytes by limiting the number of receptors capable of recognizing the antigen. It has been demonstrated in other transgenic mice that receptor editing and expression of endogenous TCR alpha chains can rescue cells from dying during thymic education (199-201). The extent to which T cells specific for self antigens mitigate self reactivity and autoimmune disease by TCR editing is still unknown, but seems to be active in our model. Since mimotope 1 is a higher affinity ligand that displays superagonistic properties it is possible that recognition predominately via TCR Vβ4 is sufficient for response. This possibility is highlighted by the fact that BDC2.5 and BDC5.10.3 both express identical CD3 regions on their TCR Vβ4 allowing them to robustly respond to the parent peptide mimotope 1, while BDC10.1 expresses Vβ15 and is unable to respond to this mimotope (194, 202).

This study also highlights the difference in affinities for the same TCR depending on the developmental and activation state of the cell. It has been known that activated cells have greater TCR avidities than naïve cells for dimeric pMHC complexes, which is due in part to the reorganization of the receptors to form clusters (203). We have shown that the effective 2D affinity differs between naïve and activated $CD8^+$ OTI T cells (68). Here we extend the findings by showing that DP thymocytes had a higher affinity for the

same antigen compared to naïve splenocytes, regardless of the antigen tested. In all cases, the cell possesses the ability to tune its receptor kinetics. At one level this provides the immune system and the T cells an additional mechanism for regulating the level of responses as changes to the number of TCRs expressed, TCR clustering, co-receptor expression and interaction, as well as integration with the actin cytoskeleton will affect overall affinity and responsiveness of a cell (204). Another possible explanation for the difference in affinities could be the different glycosylation patterns on DP thymocytes and naïve splenocytes. It has been shown that N-glycosylation can directly affect the signaling threshold needed for thymocyte selection (205).

Oxidation of critical amino acids required for antigen recognition has been suggested for the conflicting reports regarding the stimulatory capability of ChgA29-42 (174). It has been demonstrated the oxidization of cysteine residues at neutral to basic pH can ablate the antigenicity of some peptides (187). A common solution employed by many to avoid posttranslational modifications of cysteine, like oxidization or disulfide bridge formation, is to replace it with an alanine, serine or methionine. Our results support those of others where serine and methionine substitution for cysteine may not always conserve the stimulatory potential of the peptide (188-190). Although not often used, 2-aminobutanoic acid (Aba) can be used as a cysteine analog, and our results support that this might be the better substitution for a cysteine residue (188-190). Since the peptide is encoded for in the monomer construct, this would circumvent the issues with solubility and allow for the potentially unmodified peptide to be presented. In fact we see almost complete recognition of BDC2.5 Rg cells to the ChgA29-42:IAg7 monomer by micropipette which could support the hypothesis that potential oxidization

of the peptide results in the decreased functional response. Another possibility is that the cells are anergized to ChgA29-42 because of having selected on it in the thymus, which has been observed previously for endogenous selecting ligands (30, 31).

We show here that a known endogenous selecting ligand, gp250, for the MCC specific AND TCR Tg is recognized with lower affinity than the agonist by DP thymocytes and naïve splenocytes. Our data is consistent with another report that used SPR to determine that the interaction between OT-I's TCR and two known endogenous positive selecting ligands is a weaker interaction than that observed with agonist peptide (49). Since BDC2.5 Tg cells recognize ChgA29-42 with lower affinity than mimotope and ChgA is present in both cTECs and mTECs it is plausible that this epitope could be an endogenous selecting ligand for BDC2.5 (176-178). In fact when the affinities for DP thymocytes are plotted against naïve splenocytes for the various antigens there is a strong correlation that peptides which are higher affinity in one organ will be high affinity in the other (Fig. 3.6C). Based on the avidity selection model, it would be expected that there is an affinity cut-off between agonist and positive selecting ligands. ChgA29-42 affinities fall between that of AND Tg cells for gp250 and MCC, so it is possible that ChgA29-42 could be a positive selecting ligand for BDC2.5 cells. We would not expect ChgA29-42 to be a selecting ligand for BDC10.1 cells since it has recently been demonstrated that selection of these cells is dependent on thymus-specific serine protease, unlike BDC2.5 which developed normally when this protease was knocked out (17).

Type 1 diabetes is an extremely complex disease involving numerous antigen specificities including insulin, GAD65, insulinoma-associated protein 2 (IA-2), phogrin (IA-2b), and islet amyloid polypeptide (206). Posttranslational modifications of antigens,

alternative antigen processing and thymic selection pressures add additional layers of complexity to this disease. Insulin, the most prominent diabetogenic antigen, is a perfect example of the intricacy of this disease (206). It has been demonstrated for two different diabetogenic antigens that post-translational modifications boost responses and could explain how these cells that escaped thymic selection are now capable of breaking tolerance due to the generation of neo-antigens (139, 140). If ChgA29-42 is a positive selecting ligand, than it could need post-translational modification to allow the cells to escape the anergy that was programmed during thymic education. Our results provide further support that lower affinity interactions are needed to mediate positive selection.

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Cell type	Min	Max	Geo mean	Lower 95%	Upper 95%	n
BDC10.1 Rg SPL	0.112	14	0.6987	0.4882	1.0	40
BDC2.5 Rg SPL	2.39	30.3	9.089	6.612	12.5	20
BDC2.5 Tg DP	0.4240	8.62	2.818	1.996	3.979	20
BDC2.5 Tg SPL	0.00751	0.8160	0.07406	0.05591	0.09811	74
BDC2.5 Tg Act.	0.122	3.43	0.687	0.3704	1.274	17

All affinity values are X 10^{-4} μm^4

Table 3.1. Summary of ChgA29-42 affinity data, where n is the total number of T cells that had a measurable affinity for ChgA29-42. Rg –TCR retrogenic, Tg – TCR transgenic, SPL – naïve splenocytes, DP – DP thymocytes, Act. – activated splenocytes.

Figure 3.1.

A.

Name	29	30	31	32	33	34	35	36	37	38	39	40	41	42
ChgA29-42	D	T	K	V	M	K	C	V	L	E	V	I	S	D
ChgA 35 Ala							A							
ChgA 35 Ser							S							
ChgA 35 Met							M							
ChgA 35 Phe							F							
ChgA 35 Asp							D							
ChgA 35 Aba							X							

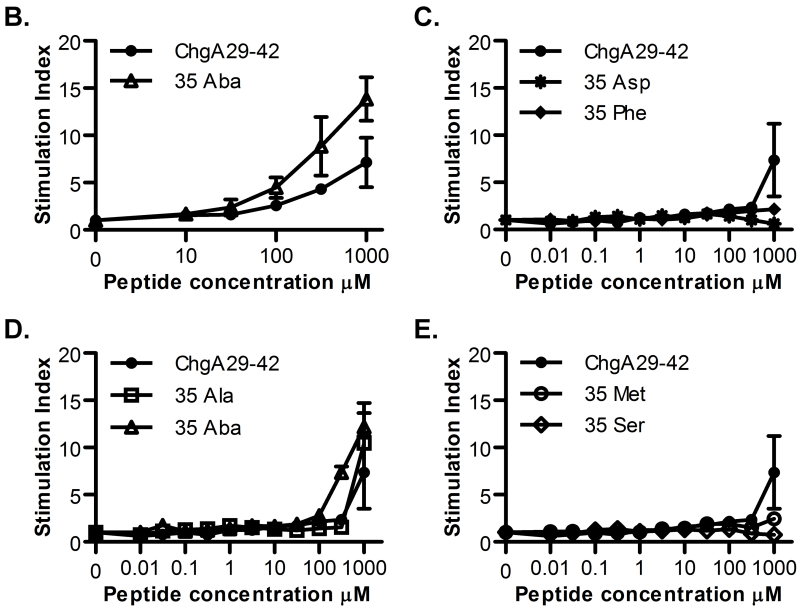

Figure 3.1. BDC2.5 Rg and Tg cells lack functional responsiveness to ChgA29-42. (A) Sequence using single letter amino acid codes for ChgA29-42 and the list of amino acid substitutions at position 35 to replace the cysteine with the indicated amino acids. X is 2-aminobutanoic acid (Aba). (B) Ex vivo proliferation of BDC2.5 retrogenic cells as measured by incorporation of tritiated thymidine. Data from three independent experiments, except half log concentrations, which are from two independent experiments. Stimulation index was determined by dividing the CPMs at indicated concentration by the background CPMs obtained with no stimulation. (C and D) BDC2.5 transgenic splenocytes tested against a panel of ChgA29-42 variant peptides, where the reactive cysteine was replaced with the indicated amino acids. Data from 4 independent

experiments for all peptides except ChgA29-42, 35 Aba, and 35 Ala, which are from 5 independent experiments. ChgA29-42 data are the same in panels C and D.

Figure 3.2.

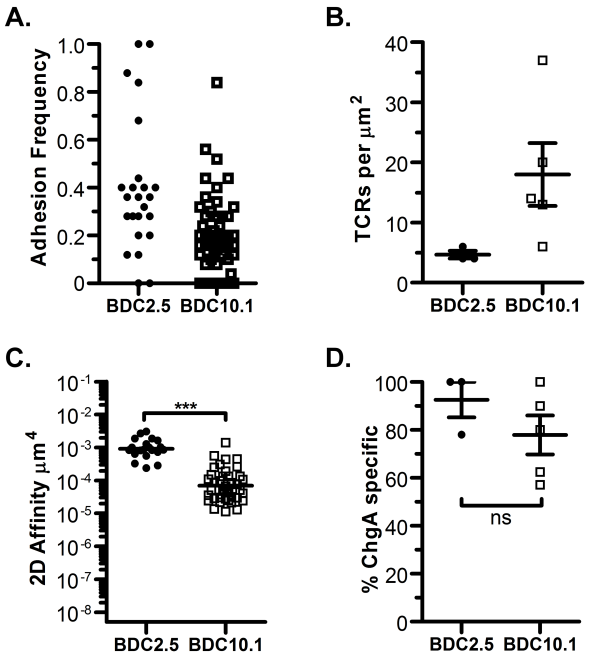

Figure 3.2. ChgA29-42 is recognized by two different diabetogenic TCRs. (A) BDC2.5 and BDC10.1 Rg cells specifically bind ChgA29-42:IAg^7 monomer coated red blood cells where each dot represents an individual T cell tested. (B) The TCR densities for each experiment were calculated as described in the methods. (C) Using the densities of TCRs (m_r) and ChgA29-42:IAg^7 molecules (m_l) the effective 2D affinities were calculated by the following equation $A_cK_a = - \ln(1-P_a)/m_rm_l$, with the line at the geometric mean affinity. The percent of cells specific for ChgA29-42 was calculated for each experiment by determining the number of cells that had an adhesion frequency above the nonspecific background binding divided by the total number of cells tested in that experiment. Data are from 3 (BDC2.5) and 5 (BDC10.1) independent experiments. Symbols in panels (A) and (C) represent individual cell pairs tested, while in panels (B) and (D) they are the individual experiments. Unpaired two-tailed t tests were used to

determine in panels B-D to determine significant differences. *** $p < 0.0001$, ns – not significant.

Figure 3.3.

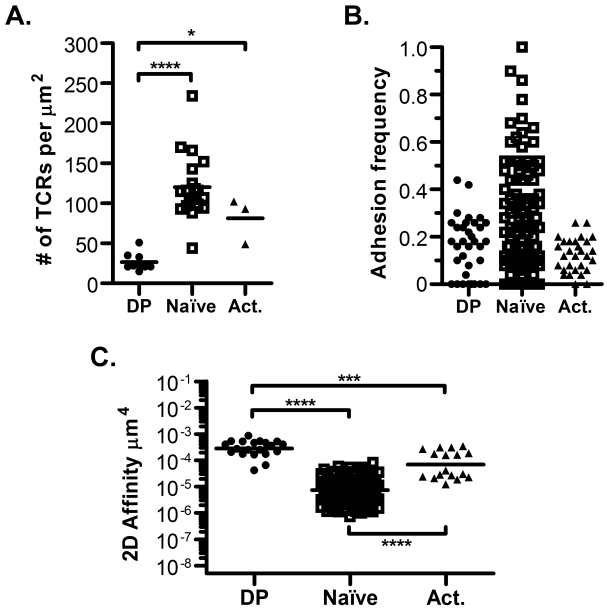

Figure 3.3. BDC2.5 transgenic T cells exhibit different affinities and TCR densities depending on the developmental state. (A) Variable levels of BDC2.5 TCR expression on the surface of the different cell types are listed in order from lowest to highest density: transgenic DP thymocytes (DP) < transgenic activated splenocytes (Act.) < naïve splenocytes (Naïve). (B) The adhesion frequencies for ChgA29-42 recognition by BDC2.5 Tg DP, Naïve and Act. cells. (C) Effective 2D ChgA29-42 affinities for DP, Naïve, and Act. cells were calculated as in Figure 3.2C. Each dot represents and individual T cell tested except in panel A, where each dot is an individual experiment. Data from at least 6 independent experiments except for activated cells, which is from 3 experiments. One-way ANOVA with Tukey's multiple comparisons tests with adjusted P values were calculated for panels A and C. * - $p = 0.0456$, *** - $p = 0.0004$, **** - $p < 0.0001$.

Figure 3.4.

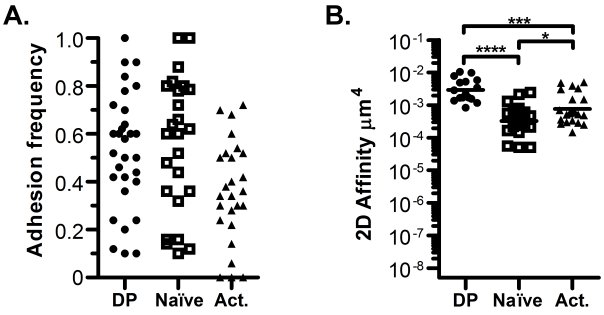

Figure 3.4. BDC2.5 Tg cells possess a higher affinity for a mimotope compared to cognate self antigen. (A) Adhesion frequencies of BDC2.5 Tg DP, Naïve and Act. cells recognizing mimotope 1. (B) Mimotope 1 affinities for each cell type were calculated as in figure 3.2C. * - $p = 0.0179$, **** - $p < 0.0001$ (One-way ANOVA with Tukey's multiple comparisons test and multiplicity adjusted P values). Each dot represents individual cells tested, data from 3 independent experiments per cell type.

Figure 3.5.

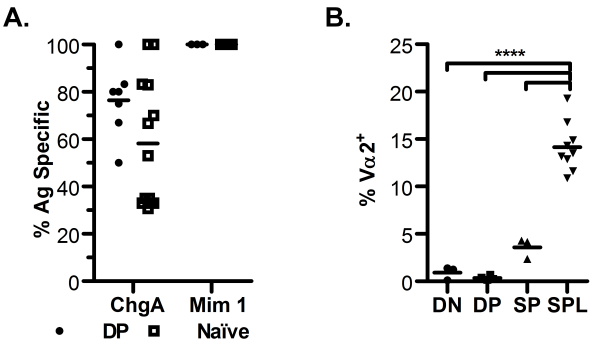

Figure 3.5. Drop in ChgA29-42 reactivity of naïve splenocytes as the expression of an endogenous TCR alpha chain increases as cells mature. (A) Frequency of antigen reactivity to either ChgA29-42 or mimotope 1 for the indicated BDC2.5 transgenic cell type. (B) Increased percentage of cells expressing an endogenously encoded TCR alpha chain as cells mature, **** - $p < 0.0001$ (One-way ANOVA). Thymocyte subsets: double negative (DN, $CD4^-CD8^-$), double positive (DP, $CD4^+CD8^+$), $CD4^+$ single positive (SP, $CD4^+CD8^-$). SPL ($CD4^+$ splenocytes). Each dot represents an individual experiment.

Figure 3.6.

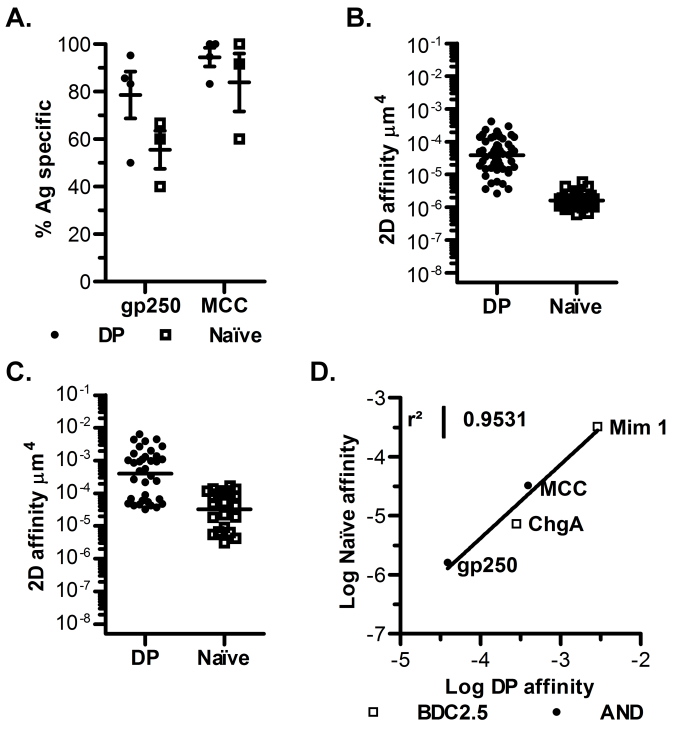

Figure 3.6. Endogenous positive selecting ligands are lower affinity compared to agonist peptides. (A.) Frequency of indicated antigen specificity for AND Tg DP thymocytes (filled circles) and naïve splenocytes (open squares), line is mean with SEM. (B. and C.) Effective 2D affinities of AND Tg DP thymocytes and naïve splenocytes for gp250 (B.) and MCC (C.). Number of experiments affinity measurements was 4 for DP thymocytes and 3 for naïve splenocytes. Total number of cells that had an affinity were: gp250 DP thymocytes – 50, gp250 naïve splenocytes – 28, MCC DP thymocytes – 35, MCC naïve splenocytes – 21. (D.) The log mean affinities for DP thymocytes were plotted against the matching log mean affinities for naïve splenocytes for the indicated antigens for BDC2.5 Tg cells (open squares) and AND Tg cells (filled circles). Linear regression line is plotted and the r^2 value is displayed.

Chapter 4: Discussion

A longstanding question in immunology is how the strength of the interaction between TCR and pMHC dictates the outcome. Affinity has been implicated to play important roles during thymocyte development and shaping the repertoire of the responding T cells during the course of an immune response. Monoclonal populations have allowed some generalities to be hypothesized, but how well these work for a polyclonal response is not yet known. In addition there are limited techniques to directly probe the affinity of the interaction and the most common one, surface plasmon resonance (SPR), is not applicable to polyclonal responses. Recently it was determined that the binding of the N3.L2 TCR with a positive selecting APL of hemoglobin could be measured by the micropipette adhesion frequency assay but not via SPR (27, 70). This result suggests that the micropipette adhesion frequency assay is more sensitive than SPR and might aid in identifying interactions between low affinity peptides that may not be capable of eliciting a functional response. SPR affinity measurements for two endogenous positive selecting ligands for OT-I required a lower temperature in order to slow the dissociation rate to allow for reliable detection (49). Therefore the micropipette adhesion frequency assay, which allows for the direct measurement of T cell affinity for their antigen *ex vivo,* will provide valuable insight into how the strength of recognition directly influences the immune response.

During thymocyte selection, TCR affinity has been proposed to directly dictate the developmental fate of a cell. FTOCs using APLs have shown that only weak interactions are capable of mediating positive selection, while stronger interactions lead to death of the cell via negative selection (21, 27, 28). It is also thought that affinity plays

a crucial role in determining if a $CD4^+$ thymocyte will upregulate Foxp3 and become a tTreg (32-37). The role that affinity has during thymic selection of polyclonal cells will be a difficult question to answer since a given antigen specificity will be at extremely low frequencies and there are many possible endogenous peptides which can be used for selection. Therefore studies determining the affinities of monoclonal cells for their endogenous positive and negative selecting ligands will afford the best opportunity to interrogate how affinity shapes the T cell repertoire during development.

The recent identification of an endogenous positive selecting ligand for AND TCR Tg cells allowed for the direct comparison of the affinity of this ligand compared to its agonist (31). Both DP thymocytes and naïve splenocytes had lower affinity for gp250, the endogenous ligand, compared to the agonist MCC, which mediates negative selection in FTOCs (10 and 20 fold for DP thymocytes and naïve splenocytes respectively, Chapter 3 and (29)). These data are consistent with measurements of OT-I's affinity for its endogenous positive selecting ligands being ~15-20 fold lower than the agonist OVA (49). Comparing the affinities of naïve N3.L2 splenocytes for agonist hemoglobin and the positive selecting APL A72 showed that A72 affinity was only 5.6 fold lower than agonist (70). In this system where only one TCR contact is different between the antigens, ligand discrimination was further enhanced when the bond was under force (70).

More measurements are needed, but based on the limited data to date it appears that weaker interactions are required to mediate positive selection, as first defined by FTOCs using APLs. Although the affinity for endogenous self is most likely lower than previously measured antagonists as demonstrated by 15-20 fold lower affinity for self ligands while APLs are typically less than 10 fold lower. (Chapter 3 and (21, 27, 28, 49,

70)). In addition to having a lower affinity for endogenous positive selecting elements, peripheral cells are unable to functionally respond to these ligands (30, 31). Therefore based on the low affinity and lack of functional response that BDC2.5 Tg cells have for ChgA29-42 it is plausible that ChgA is an endogenous ligand capable of mediating selection (Chapter 3). ChgA is expressed in thymic epithelia cells (TECs) at levels similar to those reported for *Sorl1*, which is the gene that encodes AND's endogenous positive selecting ligand gp250 (176-178). Support for this could come from FTOCs or thymic slice models for *in vitro* selection by addition of ChgA peptide to the culture. Additional proof could come from BDC2.5 Tg mice crossed onto $ChgA^{-/-}$ on NOD backgrounds to determine if selection is altered. However it is possible that the cells could still develop even in the absence of a given selecting ligand, since it has been shown that OT-I has two known endogenous selecting peptides, which is still probably a vast underestimation of the peptides capable of mediating positive selection (144).

If ChgA is identified as a positive selecting element for BDC2.5 Tg cells, then this could suggest that a posttranslational modification (PTM) could be essential for the breaking of tolerance imposed by the angery induced post education. For three different diabetogenic antigens, PTMs have improved the functional responses of T cells (139, 140, 207). The natural cleavage product WE14 from ChgA becomes more antigenic upon exposure to transglutaminase, although the exact nature of this PTM hasn't been elucidated since replacement of the glutamine residue with a glutamate wasn't able to recapitulate the increased functional response (140). Citrullination, a common PTM that has been implicated in playing a prominent role in arthritis (208), might be involved in breaking tolerance in type 1 diabetes (139). There are circulating auto-antibodies against

citrullinated glucose-regulated protein 78 (GRP78) in NOD mice and release of IFNγ from NOD T cells is only observed when the citrullinated protein is used as the antigen source. Although it has not been demonstrated to be a selecting ligand, it is intriguing to hypothesize that GRP78 could be an antigen that thymocytes are tolerized to because it is highly expressed in cTECs and mTECS and only through PTMs are T cell response able to be mounted against this ubiquitous self antigen (177, 178). T cells from patients with T1D recognized multiple epitopes within GAD65 that had underwent citrullination or deamidation of reactive lysines or glutamines respectively, again highlighting the potent ability of these neo-antigens to elicit a stronger immune response (207). GAD65 expression in the thymus has not been detected, so it is possible that these modifications might be instead increasing the stability of the peptide for the HLA molecule compared to the unmodified amino acids, which was the case for two of the GAD65 epitopes (177, 178, 207).

The sequence of ChgA29-42 contains multiple amino acid residues which can be modified posttranslationally. The aspartic acid residues (D29 and D42) can be converted to isoaspartic acid which has been shown to be antigenic in systemic lupus erythematosus (209). Another amino acid susceptible to multiple PTMs is lysine, K31 and K34, which can be carbamylated or acetylated. Conversion of lysine to homocitrulline during carbamylation has been implicated in generating neo-antigens in rheumatoid arthritis (210). Acetylation of lysine in a tumor antigen, p53, is capable of generating $CD4^+$ T cells specific for either the acetylated or nonacetylated form when peripheral blood mononuclear cells isolated from healthy individuals were stimulated *in vitro* with the respective forms of the antigen (211). ChgA29-42 also contains oxidation sensitive amino

acids, methionine (M33) and cysteine (C35). This allows for multiple sites within ChgA29-42 to be modified to possible generate a neo-antigen capable of generating a stronger immune response.

In fact it has been proposed that the reason for conflicting reports regarding the antigenicity of ChgA29-42 is due to the oxidation of cysteine at neutral to basic pHs (174). This peptide is difficult to dissolve due to its chemical composition of: six amino acids with hydrophobic side chains, three acidic residues, two basic side chains, two with polar uncharged side chains and the reactive cysteine. Overall the peptide is acidic with an isoelectric point of approximately four and contains greater than 40% hydrophobic residues. To increase solubility the pH can be raised however as it nears and then passes neutral the reactive thiol of Cys can be oxidized since it has a pKa of 8.14. It has been shown that oxidization can decrease the ability of T cells to recognize its antigen, therefore it is possible that if this residue is at a TCR contact position the reduced form is required for stimulation (187).

In an attempt to circumvent the issues with solubility and oxidization, a panel of variant peptides was generated where the reactive Cys was replaced with alanine, methionine, serine or 2-aminobutanoic acid (Aba), which is a non-proteinogenic amino acid that has a methyl group instead of the sulfhydryl group (Chapter 3). An advantage of 2-aminobutanoic acid over alanine is that it conserves the length of the side chain by the addition of the methyl group. Although these substitutions improved the solubility of the peptides, there wasn't an increase in the antigenicity as determined by proliferation assays (Chapter 3). These results could indicate that post-translational modification of the sequence as discussed above is needed to transform the peptide into an autoantigen or

that these cells are anergized to this epitope, which could be a consequence of central tolerance.

The developmental and activation status of a cell can cause the TCR affinity for its cognate antigen to vary (Chapter 3, (68)). One possibility is that there are differences in the contact area, which could greatly affect the 2D affinities that are reported since the exact area of contact is unable to be resolved and that term is left in the reported affinities. Although the cell membranes appear similar, there could be vast differences regarding how ruffled the membranes actually are, which would change the contact area. Another factor that could account for the differences in affinity is if the TCRs were in clusters on the membrane, since this would affect the local density thereby increasing the probability of an adhesion event occurring, thereby increasing the affinity. It has been shown that two different cholesterol depleting agents and an actin polymerization inhibitor drastically reduced the 2D affinities measured for two different $CD8^+$ Tg cells (68). Another possibility, as discussed in Chapter 3, is that for TCR Tg mice this difference could be due to the upregulation of endogenous TCR alpha chains. Figure 3.5 shows that the frequency of cells expressing Vα2, an endogenous alpha chain, is significantly higher for the naïve splenocytes compared to the different subsets present in the thymus. In fact the percent begins to increase at the $CD4^+CD8^-$ SP stage, which would be post-selection, while the affinities that were measured were for unsignaled DP thymocytes, naïve splenocytes and activated cells.

In an ongoing immune response, it has been hypothesized that there is affinity maturation, where the highest affinity cells will outcompete the lower affinity cells to become the dominate responders (212). Competition for antigen could be a strong driving

force during an immune response and it has been shown that when a monoclonal population is at high precursor frequencies then memory formation is impaired, while transfer of fewer antigen specific cells lead to robust memory formation (213). Previous studies have shown that there are low and high affinities cells present in both autoimmune disease and viral infection, with the latter being enriched for higher affinity cells (51). What is the functional consequence of both of these populations and does one contribute more to the response than the other is an important question to answer.

Although pMHC II tetramers miss the lower affinity cells, they could prove extremely useful in determining the functional capabilities of both high and low affinity cells (51). Since the CNS is predominately infilitrated by MOG specific Tconv and Tregs (Chapter 2 and (51)), tetramers could be used to sort out the high and low affinity cells. It has been shown that low affinity cells are just as capable of producing cytokines as high affinity cells, but it is not known if they produce the same amount (51). Determining if there are functional differences between low and high affinity cells and the dynamics that occur during a polyclonal response is paramount for the development of treatments for diseases.

Another important implication from the affinity maturation model is that overtime the immune response will be dominated by higher affinity clones because of their ability to outcompete their lower affinity counterparts. The affinity distributions of MOG-specific Tconvs and Tregs at the peak of EAE spanned a wide range without obvious skewing towards higher affinity clones (Figure 2.4). We did observe enrichment of higher affinity T cells in the CNS compared to the periphery, which is consistent with tetramer staining (Figs. 2.4 and 2.2). There are tetramer positive T cells present in the periphery,

but their frequencies are too low to allow for detection by micropipette without first enriching for this population. It is possible that affinity maturation has not had sufficient time yet to occur, and follow-up studies looking at chronic time points are needed. However in a relapsing remitting secondary progressive model of EAE, it was demonstrated that the affinity of CNS infiltrating T cells was actually highest on average at the acute time point as compared with remission, relapse and chronic time points, which ranged from 45 to 108 days post induction (90).

It has been suggested that tTregs would be enriched for self-reactivity and would be higher affinity for their cognate antigen, assuming that they had undergone selection on that antigen, than a Tconv cell recognizing the same antigen. To our knowledge, these studies provided the first direct measurements of affinities of Tconvs and Tregs for the same antigen (Chapter 2). Both populations displayed a wide range of affinities for MOG and the average affinity was actually higher for the Tconv cells than the Tregs that had infiltrated the CNS. One caveat of these studies is that we do not know if either cell type had selected on MOG in the thymus, so these data cannot directly imply how affinity shaped the cells during thymocyte development. However it is intriguing that both were dominated by lower affinity cells and that the majority of the responding cells were specific for the inducing antigen in the CNS. In fact there was significant enrichment of MOG specific Tregs in the CNS (18% of the $CD4^+$ T cells were MOG-specific Tregs) as determined by the micropipette adhesion frequency assay compared to the periphery (11% in CLN and 2% in spleen) which was not as dramatic when only tetramer staining was used to identify MOG specific Tregs (CNS – 8%, CLN and SPL – < 1%, Chapter 2).

If it is determined that high affinity Tregs are better suppressors than low affinity Tregs, then tetramer enrichment followed by *in vitro* expansion of a patient's cells could allow for an antigen-specific way to reestablish tolerance during autoimmunity. Even though tetramers only stain those cells that have the highest affinity this could still allow for enrichment of antigen specific Tregs which is important since it has been demonstrated that antigen specific are better at suppressing than polyclonal Tregs (91, 92). Another possibility is that lower affinity Tregs could be better suppressors than higher affinity cells because they would need to interact with APCs longer thereby limiting access of Tconvs to the antigen being presented, which has been shown that Tregs are capable of limiting the interactions between APCs and Tconv (93, 94).

It is also possible that both low and high affinity Tregs are capable of suppressing as equally well. Since it has been shown that both populations were capable of producing effector cytokines from Tconv cells, then it is plausible that high and low affinity Tregs would be capable of producing IL-10. Another mechanism of suppression is the ability of the Treg to change the APC to alter the response from Tconv cells. Tregs are able to prevent the maturation (100) and induce apoptosis of antigen loaded APCs (101). Through the binding of CTLA-4, Tregs are able to remove the co-stimulatory molecules CD80/CD86 from the surface of the APC in the process of trans-endocytosis (95-99). All of the effects on APCs would not necessarily be dependent on the affinity of the interaction of the TCR with its cognate antigen, since they are mediated by TCR independent mechanisms and the antigen specificity just allows for targeting of the desired specificity.

It has been demonstrated that during EAE the Tconv cells in the CNS have become refractory to suppression by the Tregs; the presence of proinflammatory cytokines, TNF-α and IL-6, were capable of repeating this phenotype of Tconv cells being ignorant of the Tregs (88). Based on the data that the TSDR is mostly methylated in CNS Tregs during peak EAE (Chapter 2) this could explain the inability to mediate suppression of CNS Tconv. In the presence of proinflammatory cytokines, it is known that there is recruitment of DNA methyltransferases to the TSDR, which would be able to remethylated this region (120). The TSDR is required for the maintenance and inheritability of Foxp3 (119), which is the key transcription factor required for Tregs (147, 148). Foxp3 directly regulates the expression of many of the factors required by Tregs for their suppressive capabilities. Therefore when the CNS Tregs were removed from the presence of the proinflammatory cytokines in co-cultures with either naïve Tg splenocytes or splenocytes from EAE mice, which weren't producing these cytokines, they could suppress these targets. However when interrogated for their ability to suppress CNS Tconv cells, which were still actively making TNF-α and IL-6, the key mechanisms required for suppression could have been dysregulated by the presence of these cytokines (88). To directly test this hypothesis the suppression assays would need to be repeated with neutralizing antibodies for TNF-α and IL-6.

Although we are unable to make direct statements regarding the affinity that was required for selection during development, we can still compare the affinity distributions to what would be expected based on the selection models. In the instructive model of thymocyte selection, it is proposed that Tconv cells would have a lower affinity for self, while Tregs would be enriched for higher affinity self-reactive TCRs. The two-step

model suggests that both cell types are capable of selecting over the same range of affinities for self. Unfortunately TCR repertoire studies have had conflicting conclusions regarding the antigen specificities for the two populations (40-42). Based on the affinity distributions obtained for Tconv and Tregs during EAE for the same self antigen, this data would support the two-step model of thymocyte selection (Chapter 2).

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