FACTORS REGULATING THYMIC DENDRITIC CELL HOMEOSTASIS AND FUNCTION

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ABSTRACT

Nicholas A. Spidale: Factors regulating thymic dendritic cell homeostasis and function
(Under the direction of Roland Tisch)

The immune system must balance the generation of a diverse T cell repertoire responsive to various pathogens versus the prevention of tissue destruction by self-antigen-specific T cells. Thymic dendritic cells (DC) are known to eliminate self-antigen-specific thymocytes. Despite this, little is known about the factors regulating thymic DC homeostasis and antigen presentation, which were investigated in this study.

In the first part of our study, it was hypothesized that factors within the thymus regulate thymic DC homeostasis. Specifically, the role of CD4⁺ and CD8⁺ single-positive thymocytes (SP) was investigated. In mice lacking SP, significantly fewer DC resided in the steady-state thymus, and those DC displayed a less mature phenotype (e.g. reduced expression of major histocompatibility [MHC] and T cell costimulatory molecules) and a markedly reduced T cell stimulatory capacity compared to wild-type thymic DC. When CD4⁺SP or CD8⁺SP were individually restored, thymic DC activation status was only recovered under conditions where antigen-specific interactions with SP occurred. During antigen-specific interactions, the restoration of thymic DC activation status depended on CD40 ligand from CD4⁺SP but not CD8⁺SP. Thus, thymic DC homeostasis is regulated by antigen-specific interactions with CD4⁺SP or CD8⁺SP by distinct molecular mechanisms.
To promote thymic tolerance, thymic DC must acquire and present cognate antigens to thymocytes; for example, through “nibbling” of antigens from thymic epithelial cells (TEC). However, the mechanism by which this occurs is largely unknown. Using an in vitro co-culture system, thymic DC nibbling of MHC was investigated. Thymic DC efficiently nibbled MHC from TEC and DC, but not from B cells. It was hypothesized that the cell surface organization of MHC may regulate nibbling by thymic DC. Specifically, the role of lipid rafts was analyzed. Thymic DC acquired lipid rafts from TEC, DC, and B cells. Interestingly, when another B cell surface molecule, immunoglobulin M (IgM), was actively clustered to lipid rafts, nibbling of IgM occurred. These data suggest that cell surface organization of clustered molecules may facilitate acquisition by thymic DC via nibbling. Together, the studies presented herein provide novel insight into the factors regulating thymic DC function and T cell central tolerance.
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* Note that abbreviations are used identically in the singular and plural

<table>
<thead>
<tr>
<th>Ab</th>
<th>Antibody</th>
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<tbody>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor for antigen</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL6/J mouse</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>CDP</td>
<td>common DC precursor cell</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>Ctrl</td>
<td>control</td>
</tr>
<tr>
<td>CTxB</td>
<td>Cholera Toxin B subunit</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DN</td>
<td>CD4/CD8 double-negative thymocyte</td>
</tr>
<tr>
<td>DP</td>
<td>CD4/CD8 double-positive thymocyte</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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</table>
EpCAM  epithelial cell adhesion molecule
FACS  fluorescence activated cell sorting
FCS  fetal calf serum
Flt3  fms-like tyrosine kinase receptor 3
FoxP3  forkhead box p3 protein
GM-CSF  granulocyte macrophage colony-stimulating factor
HA  influenza hemagglutinin peptide
IFN  interferon
Ig  immunoglobulin
IL  interleukin
i.p.  intraperitoneal
i.v.  intravenous
LN  lymph node
LPS  lipopolysaccharide
mAb  monoclonal antibody
MACS  magnetic activated cell sorting
mg  milligram
µg  microgram
MHC  major histocompatibility complex
mL  milliliter
mM  millimolar
µM  micromolar
µm  micrometer or micron
mTEC  medullary thymic epithelial cell
NOD  nonobese diabetic mouse
PBS  phosphate-buffered saline
pDC  plasmacytoid dendritic cell
PRR  Pattern recognition receptor
PTA  peripheral tissue antigen
qPCR  quantitative polymerase chain reaction
RANK  receptor activator of nuclear factor kappa B
sBDC  super BDC agonist peptide
SEM  standard error of the mean
SIRPα  Signal regulatory protein alpha
SP  single-positive thymocyte
SR-A  scavenger receptor type A
TCR  T cell receptor for antigen
TEC  thymic epithelial cell
Teff  effector T cell
TGFβ  transforming growth factor beta
Th  T helper cell
TLR  Toll-like receptor
TNF  tumor necrosis factor
TNFSF  tumor necrosis factor superfamily
Treg  regulatory T cell
TSLP  thymic stromal lymphopoietin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>tTreg</td>
<td>thymus-derived regulatory T cell</td>
</tr>
<tr>
<td>TW</td>
<td>TransWell</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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CHAPTER 1: Introduction

1.1 Self/Non-Self Recognition

The fundamental responsibility of the immune system is to discern between the benign self and potentially pathogenic non-self or altered self, and many mechanisms exist to facilitate this process. The immune system is broadly segregated into two arms: innate and adaptive immunity. Each of these arms serves both unique and collaborative roles during an immune response. The innate immune system responds rapidly to stimuli, and is often responsible for priming of a subsequent adaptive immune response (1). Self/non-self discrimination is achieved through the function of various pattern recognition receptors (PRR), including members of the toll-like receptor (TLR) family (2), nucleotide-binding oligomerization domain receptor family (3), and RIG-I-like receptor family (4). These PRR typically recognize common, conserved, and often repetitive structures found ubiquitously among pathogens, including bacteria, viruses, and fungi, but not found as part of endogenous self-proteins or host cells. In contrast, the adaptive immune system responds more slowly to stimuli, typically requiring several days to mount an initial response. However, adaptive immune responses are highly specific, with an individual T or B cell typically recognizing only one antigen (Ag). Self/non-self recognition in the adaptive immune system is due to the expression of unique Ag receptors, namely the T cell receptor (TCR) or B cell receptor (BCR). The TCR recognizes small antigenic peptides presented in the context of a major histocompatibility complex (MHC) molecule on the surface of Ag-presenting cells (APC) (5). The BCR recognizes three-dimensional soluble Ag, or Ag bound to receptors such as complement receptors or Fc receptors, that binds directly to the BCR (6). While an individual T or B cell generally recognizes only one Ag, many unique T and B cells are generated,
affording immense diversity of Ag recognition (7, 8). Importantly, adaptive immunity is further characterized by the generation of long-lived immunological memory (9, 10). Memory immune responses can be rapidly recalled upon infection with a previously encountered pathogen, providing enhanced responsiveness and highly effective protection against infection. Thus, proper self/non-self discrimination is an essential feature of an effective immune system.

1.2 Immunological Tolerance

A critical feature afforded when self/non-self recognition properly functions is the establishment of immunological self-tolerance. Tolerance takes many cell type-dependent forms; in the context of this study, T cell tolerance, specifically of T cells bearing a conventional heterodimeric \(\alpha\beta\) TCR, is most important.

1.2.1 Central T cell Tolerance

An early checkpoint for enforcement of T cell tolerance occurs during T cell development in the thymus. Immature T cells, referred to as thymocytes, will spend up to 2-3 weeks in the thymus before entering the peripheral immune system as mature T cells (11). Throughout this time period, thymocytes progress through various stages of development, and many thymocytes (>95%) will die within the thymus (12). All thymocytes originate from blood-borne hematopoietic precursor cells (13), which seed the thymus and are the source of the earliest stage of identifiable thymocytes present in the thymic cortex, CD4⁺CD8⁻ double-negative (DN) thymocytes. To progress through this stage, thymocytes must successfully complete beta selection, which entails productive rearrangement of TCR\(\beta\) chain loci and subsequent cell surface expression of TCR\(\beta\) chain along with an immature pre-TCR\(\alpha\) chain
Completion of beta selection promotes upregulation of the two T cell coreceptor molecules, CD4 and CD8, and differentiation into CD4^+CD8^+ double-positive (DP) thymocytes. DP thymocytes will begin to rearrange TCRα chain loci, and cells that express a productive TCRα chain, and thus a complete αβ TCR, will enter into positive selection. Positive selection tests the ability of DP thymocytes to interact with self-MHC displayed by cortical thymic epithelial cells (cTEC), the primary APC in the thymic cortex (16). The ability to recognize self-MHC is critical to T cell function, as Ag encountered in the peripheral immune system will be presented in the context of this same self-MHC. Thus, this process “positively” selects for thymocytes expressing a functional TCR.

After successful positive selection, thymocytes undergo a number of developmental events. These include differentiation of DP into either CD4^+CD8^- or CD4^-CD8^+ single-positive (SP) thymocytes and expression of certain chemokine receptors and adhesion molecules that facilitate trafficking into the other subanatomical niche within the thymus, the medulla (17). In the thymic medulla, thymocytes are scanned for reactivity to self-Ag. Thymocytes that strongly recognize self-Ag presented by medullary thymic epithelial cells (mTEC) (18), dendritic cells (DC) (19), and possibly B cells (20) undergo apoptosis in a process called negative selection (21). Negative selection ensures that self-reactivity is purged from the T cell repertoire. T cells that do not strongly recognize self-Ag fail to receive a pro-apoptotic signal, and subsequently emigrate from the thymus to populate the peripheral immune system as mature T cells. Alternatively, cells that recognize self-Ag with “intermediate” strength may be induced into a distinct T cell lineage, namely regulatory T cells (Treg) that express the forkhead box P3 (FoxP3) transcription factor (22). These cells are critical for the maintenance of peripheral immune tolerance (discussed below) and the
resolution of T cell responses after eradication of a pathogen. While the extensive process of thymocyte development can be quite effective, full deletional tolerance of all self-reactivity is not typically achieved (23). Thus, additional mechanisms of tolerance must exist.

1.2.2 Peripheral T cell Tolerance

While the overwhelming majority of self-reactive T cells are purged during negative selection, some self-reactive cells escape this process. If left unchecked, these T cells could potentially cause tissue destruction leading to autoimmunity. However, several mechanisms of peripheral tolerance exist to prevent this from occurring. Efficient activation of naïve T cells requires signals induced by the TCR following binding of peptide-MHC complexes and by the co-stimulatory molecule CD28 via binding of APC-derived CD80 or CD86 (24).

Additionally, the pro-survival cytokine interleukin- (IL-) 2 promotes T cell proliferation, as the IL-2 receptor is upregulated upon TCR ligation (25). Subsequently, additional cytokines are typically required to foster T cell effector (Teff) differentiation. For example, interleukin-(IL-) 12 promotes generation of interferon (IFN) γ-producing T helper (Th) 1 cells (26), while IL-4 promotes generation of IL-4-, IL-5-, and IL-13-producing Th2 cells (27).

Stimulation of naïve T cells through the TCR alone, without CD28 costimulation or cytokine signals, results in incomplete activation and T cell anergy (28), a state of functional nonresponsiveness. On the other hand suppression of established pathogenic Teff is mediated primarily by Treg. Treg typically produce immunomodulatory cytokines, such as IL-10 and transforming growth factor beta (TGFβ), which block Teff reactivity (29). Furthermore, Treg can directly suppress Teff via expression of various inhibitory cell surface receptors (30), and can also limit Teff activation by preferentially occupying APC and thus limiting Teff APC access (31). Finally, suppression of Teff also occurs through APC, especially tolerogenic DC.
Steady-state DC exhibit an immature phenotype marked by expression of low levels of T cell costimulatory molecules, such as CD80 and CD86, and the lack of production of proinflammatory cytokines (32). Thus, T cells that interact with immature DC do not receive strong activating signals. Additionally, DC can produce immunomodulatory cytokines to dampen T cell activation (33). Together, these mechanisms of peripheral tolerance help prevent autoimmunity that could be caused by self-reactive T cells that have escaped central tolerance.

1.3 Dendritic cells as central orchestrators of tolerance and immunity

 Implicit throughout the description of immune tolerance is the complete dependence of T cells on APC for both development and peripheral activation. While many cell types can be APC under certain conditions, DC are the primary, professional APC of the immune system (34). DC constitutively express both MHC I and MHC II, which endows DC with the ability to effectively stimulate both CD8+ and CD4+ T cells, respectively. However, as mentioned above, steady-state immature DC do not prime T cell immunity. In fact, targeting Ag to resting DC induces potent, long-lived Ag-specific T cell tolerance (35). In order to prime an effective T cell response, DC must first be activated. DC express a broad array of PRR (36), permitting the recognition of many diverse pathogens. Additionally, immature DC are highly phagocytic and thus capable of acquiring extracellular Ag for processing and presentation to T cells (37). However, the context of Ag acquisition is critical to the resulting DC function. In the absence of activating signals, such as those through PRR or other danger signals, DC phagocytosis of extracellular debris, especially in the form of apoptotic cells, further reinforces DC quiescence (38). Indeed, DC that bind apoptotic cells become
refractory to subsequent inflammatory stimuli (39, 40), and elimination of cellular receptors for apoptotic cells abrogates this phenomenon (41).

Once the proper inflammatory context has been perceived, a complex process of DC activation and maturation occurs. Ingested Ag will be processed and loaded onto MHC molecules, which are shuttled to the cell surface (42, 43), leading to robust upregulation of cell surface MHC expression. Moreover, inflammatory stimuli significantly upregulate the costimulatory molecules CD80 and CD86 on DC (36), thus allowing DC to provide the necessary costimulatory signal to T cells. Depending on the initial inflammatory stimuli, various cytokines including tumor necrosis factor (TNF) and IL-12 will be produced as well (44). As a whole, this process promotes maturation of DC from maintainers of tolerance to primers of T cell immunity. Furthermore, DC:T cell interactions result in feedback to DC, for example via T cell-expressed CD40 ligand (CD40L) interaction with DC-expressed CD40, which extends DC activation and promotes DC survival (45). However, most DC have a relatively quick turnover rate (3-5 days for most lymphoid-resident DC) (46, 47), which may help ensure that DC-driven T cell immune responses do not continue indefinitely. This resolution phase of an immune response is critical; a rampant, unchecked immune response may lead to unnecessary and potentially harmful damage to the host. Indeed, DC themselves are also responsive to immunomodulatory cytokines, for example IL-10 and TGFβ (48–50). Overall, DC activation and maturation is a complex and heavily regulated process, and the tight regulation of DC maturation places DC at the core of both tolerance and immunity.
1.4 DC development and heterogeneity

While all DC possess many common characteristics, DC are in fact a heterogeneous population of cells that are typically divided into multiple subsets, the divergence of which occurs at various points during DC development. Like all hematopoietic cells, DC are derived from hematopoietic stem cells residing in the bone marrow (BM) (51). DC initially diverge from lymphoid (T, B, and natural killer) cells upon the development of the macrophage and DC precursor cell, which gives rise to both monocyte/macrophage lineage cells and DC. Subsequent differentiation leads to a common DC precursor (CDP) cell that develops into the 2 major DC subsets: classical or conventional DC (cDC) and plasmacytoid DC (pDC) (52). Before development into fully differentiated cDC, CDP will become pre-cDC. Unlike all prior developmental stages, pre-cDC can be found in both the BM and secondary lymphoid organs such as the spleen and lymph nodes (LN). At this stage, pre-cDC are heavily dependent upon recognition of the fms-like tyrosine kinase receptor-3 (Flt3) ligand to achieve full differentiation into cDC (53). Subsequently, cDC lose expression of Flt3 and are most reliably identified by expression of the pan-DC surface marker CD11c (integrin αx), which is expressed by both cDC and pDC, albeit at lower levels on pDC. This developmental process lays the foundation for further functional specialization of terminally-differentiated DC subsets.

Many different subsets can be identified within the cDC population, and the features that distinguish these populations vary from organ-to-organ. In secondary lymphoid organs such as the spleen and LN, cDC are broadly delineated into resident and migratory cDC. Resident cDC are always present, and are typically divided into 2 major subsets based on the
expression of CD8α (54). While surface marker expression is the means to separate these populations, the subsets are considered distinct due to unique functional capacities. For example, CD8α⁺ cDC are well-known for the ability of cross-presentation (55), a mechanism by which extracellular Ag—which is typically processed for loading onto MHC II—is shuttled into the MHC I processing pathway for subsequent presentation to CD8⁺ T cells. Additionally, CD8α⁺ cDC are also highly efficient at the uptake of apoptotic cells (56). On the other hand, CD8α⁻ cDC may be specialized for presentation of Ag to CD4⁺ T cells (57). Migratory DC enter LN through lymph vessels draining the associated tissue site. It is noteworthy that most migratory DC express particularly high levels of MHC II, perhaps indicating that the ability to migrate to the LN is preferentially bestowed upon activated DC (58).

pDC are also resident in secondary lymphoid organs and appear to be specialized in antiviral defense. Unlike most cDC populations, which express moderate levels of MHC II in the steady-state, resting pDC express minimal MHC II. Upon encountering the appropriate signal, especially viral signatures such as double-stranded or single-stranded RNA or unmethylated CpG DNA, pDC can mature and adopt many of the surface features of mature cDC, especially high levels of MHC and costimulatory molecule expression (59). However, the most notable contribution of pDC to antiviral immunity is through the production of type I IFN (60), which promotes an antiviral state in host cells that helps defend against viral infection. Hence, DC heterogeneity is an important feature that allows these cells to tailor the immune response in a manner appropriate for the given situation.
1.5 DC in central tolerance

While DC are highly effective at initiating an adaptive T cell response, DC also make crucial contributions to T cell central tolerance during development in the thymus. The first indication of this came from a seminal work by Marrack and colleagues. In this study, it was shown definitively that BM-derived cells were sufficient to induce tolerance to self-MHC (61). A second critical study showed that MHC expression in the thymic medulla was essential for the maintenance of T cell self-tolerance (62). Though both important early studies, each study also had its shortcomings. While BM-derived cells were shown to be sufficient to induce self-MHC tolerance, the identity of the BM-derived population remained a mystery. In the second study, MHC expression was targeted only to the thymic cortex, thus providing positive selection of T cells but no tolerizing input from medullary APC populations. However, several different APC populations inhabit the thymic medulla, including mTEC and thymic DC. Definitive proof that thymic DC contribute to negative selection of thymocytes was then provided by a study using targeted expression of MHC II only to DC (19). Importantly, this study also showed that thymic DC could not induce positive selection of thymocytes, further emphasizing that positive selection was the realm of cortical APC such as cTEC and negative selection the realm of medullary APC such as mTEC and DC. These critical studies provide a foundation upon which subsequent refinements to our understanding of the role of APC in central tolerance have been made.

1.6 Thymic DC subsets

As in other lymphoid organs, thymic DC are heterogeneous. This fact complicated early studies attempting to delineate distinct populations of thymic DC. Attempts to
segregate thymic DC into the commonly used CD8α+ and CD8α− populations were confounded by the observation that thymic DC pickup CD8α from thymocytes (63). By use of a second cell surface molecule, signal regulatory protein alpha (SIRPα), it was discovered that two distinct populations of thymic cDC exist: a SIRPα+CD8αlo population and SIRPα− CD8α+ population (64). Additionally, pDC are found in the thymus (65), yielding 3 major subsets of thymic DC. Many subsequent studies have focused on the unique properties of these 3 populations.

1.7 Thymic DC ontogeny

As with all other DC, thymic DC are hematopoietic in origin and dependent on BM precursors for their development. However, whereas most lymphoid resident DC complete full development within the bone marrow itself (51), thymic DC were initially described as developing from an intrathymic precursor that also gives rise to T cells (66). While it is generally accepted that thymic CD8α+ cDC develop intrathymically, whether the immediate precursor also retains T cell potential has been challenged (67). Nonetheless, the intrathymic origin of this DC population suggests that it is specialized for functions necessary for T cell tolerance. On the other hand, thymic SIRPα+ cDC and pDC do not develop within the thymus but rather are migratory populations derived from the bloodstream (68–70). Implications of this observation for central tolerance will be discussed below. In whole, it is tempting to speculate that this diversity in thymic DC origins exists as a mechanism to expand the range of Ag to which thymic DC can induce self-tolerance.
1.8 Thymic DC and antigen acquisition

Thymic DC are widely considered to be primary contributors to the clonal deletion of self-reactive thymocytes; thymic DC expression of MHC II is sufficient to purge self-reactive CD4+ T cells (19). How thymic DC acquire Ag for presentation in the thymus remains poorly understood. Thymic DC retain high expression of proteosomal proteins necessary for Ag processing and presentation (71, 72). Thus, thymic DC should be capable of constitutive processing and presentation of intracellular self-Ag via MHC I. It is also tempting to speculate that the large number of dying thymocytes would provide a source of extracellular self-Ag for presentation via MHC II. However, this still leaves a significant portion of self-Ag—particularly those unique to specific tissues—unexpressed by thymic DC. Many peripheral tissue Ag (PTA) are expressed in the thymus under control of the autoimmune regulator (AIRE) protein (73). While thymic DC may express AIRE (74), the level of expression is significantly reduced compared to mTEC, the primary AIRE-expressing cell population in the thymus. However, AIRE-dependent PTA may be acquired by DC via intracellular transfer of PTA from mTEC (75–78). Indeed, acquisition of mTEC-derived Ag by thymic DC plays an important role in central tolerance, though mTEC are also capable of direct presentation of Ag for central tolerance induction (79). The range of Ag to which DC can induce central tolerance is further expanded by the ability of DC to acquire Ag via the bloodstream (80–82). Finally, migratory DC populations ferry peripheral Ag to the thymus for presentation to developing thymocytes (69, 70, 83). Thus, thymic DC acquire Ag in diverse ways to facilitate the elimination of self-reactive thymocytes.

It is worthwhile to note that, while DC are sufficient to induce tolerance to self-MHC and are capable of clonal deletion of self-reactive thymocytes, proof that DC are absolutely
required for the maintenance of central tolerance remains controversial. This is primarily derived from two analogous studies in which constitutive ablation of DC was achieved (84, 85). In one study, T cell development was normal in the absence of DC, but the mice succumbed to a fatal myeloproliferative syndrome (84). It was concluded that thymic DC are therefore dispensable for T cell development and central tolerance. In the second study, an increased frequency of CD4SP thymocytes was observed and CD4+ T cells infiltrated multiple organs, resulting in fatal autoimmunity in DC-less mice (85). This indicated that thymic DC are critical for the maintenance of central and peripheral T cell tolerance. While the results of these two studies remain contentious, it should be noted that the mice used in these studies were generated independently. Moreover, the two studies show differing deletion efficiencies of DC subpopulations. These factors could very well explain the discrepant findings. Thus, the relative contribution of thymic DC and other medullary APC populations requires continued investigation.

1.9 Thymic DC and thymic Treg development

Elimination of self-reactive thymocytes via clonal deletion is only one way in which thymic processes help ensure self-tolerance. Another important function of the thymus is to promote the development of FoxP3-expressing thymus-derived Treg (tTreg, formerly referred to as natural Treg) (22, 86). DC have been shown to induce tTreg development, though the factors involved may vary in mice versus humans. In humans, DC conditioned by thymic stromal lymphopoietin (TSLP) can induce tTreg in a mechanism involving Ag-specific MHC-TCR interaction and CD80/CD86 costimulation (87). This function is not unique to thymic cDC; human thymic pDC can also induce tTreg (88, 89). In the case of
thymic pDC, it seems that TSLP or CD40L “license” pDC to induce tTreg. It is currently unclear whether additional factors are involved in the licensing of human thymic DC (cDC or pDC) to induce tTreg. It is also unclear whether other human thymic APC populations induce tTreg development. In murine studies, it has become clear that tTreg development is fostered by either thymic DC or mTEC (79, 90–93). Unlike with human DC, the ability to induce tTreg development seems to be mostly restricted to the SIRPα+ cDC population (94). The reasons for this specialization are unclear, however thymic SIRPα+ cDC are more mature than CD8α+ cDC or pDC and thus may more efficiently provide TCR ligation and costimulatory signals, both of which are critical for tTreg development (92, 95, 96). Thus, whether by clonal deletion of self-reactive thymocytes or induction of tolerance-promoting tTreg cells, thymic DC make crucial contributions to the maintenance of T cell homeostasis.

1.10 Cellular crosstalk in the thymus

Since both clonal deletion and tTreg cell induction are thought to require intermediate to high strength signals via the TCR and possibly costimulatory molecules (97), it is reasonable to speculate that expression of MHC and costimulatory molecules in the thymus may be a tightly regulated process. This is certainly the case for mTEC, which have been extensively studied in this context. It has been shown that mTEC progress through a step-wise development process, which ultimately results in the generation of fully mature CD80hiMHC IIhiAIRE+ mTEC (so-called mTEC*hi) (98). However, this process is not under intrinsic mTEC control but rather requires input from various different thymic cell populations. The majority of this input comes in the form of ligation of various tumor necrosis factor superfamily (TNFSF) receptors expressed by mTEC progenitors. One of the
major TNFSF family members involved in mTEC development is receptor activator of nuclear factor kappa B (RANK) and its ligand, RANKL. Mice deficient in the expression of either RANK or RANKL have sharply reduced overall numbers of mTEC and mTEC\textsuperscript{hi} (99–102). It is tempting to speculate that RANKL/RANK interactions may be the earliest form of crosstalk critical to mTEC development. This interaction can be provided by lymphoid tissue inducer cells and invariant γδ T cell precursors (99, 102), both of which inhabit the embryonic thymus very early in development, prior to most conventional thymocyte populations. RANKL can also be provided by positively-selected αβ thymocytes (101), and thus likely contributes to mTEC development at various stages of mouse ontogeny. Subsequently, CD40/CD40L become critical in controlling mTEC cellularity, possibly through promoting mTEC proliferation (100, 103, 104). It is unsurprising that CD40L controls later stages of mTEC development; significant CD40L expression is not detected until the TCR\textsuperscript{+}CD4\textsuperscript{+}CD8\textsuperscript{−} stage of αβ thymocyte development (101, 104). Importantly, T cells generated in mice lacking RANK and CD40 likely have increased autoreactivity; adoptive transfer of peripheral T cells into athymic nude mice resulted in autoimmunity (100). Overall, cellular crosstalk is an important process necessary for normal mTEC development and the maintenance of immune homeostasis.

1.11 Goals of this study and study findings

It has become clear that thymic DC are crucial contributors to T cell central tolerance and Treg development. While many of the mechanisms that regulate T cell development and thymic selection remain incompletely understood, it is generally accepted that the strength of signal received by developing thymocytes is critical in determining the fate of these cells.
Certainly, intrinsic properties of a given TCR will play a strong role in regulating the relative strength of signal perceived by a thymocyte. However, the activation status—levels of expression of T cell stimulatory molecules such as MHC and CD80/86—of thymic APC is also expected to impact thymocyte selection. Despite this, very little is known about the factors that regulate thymic DC homeostasis. In the first part of our studies, we show that thymic DC homeostasis is regulated by interactions with SP thymocytes. In TCRα-deficient (NOD.TCRα−/−) mice, which lack SP thymocytes, thymic DC phenotype, function, and cellularity were significantly reduced relative to wild-type (WT) NOD thymic DC.

Subsequently, we show that induction of Ag-specific interactions between thymic DC and either CD4+ or CD8+ thymocytes fully restored thymic DC phenotype via CD40L-dependent and CD40L-independent mechanisms, respectively. Together, these findings uncover a previously unknown mechanism of thymocyte:DC crosstalk that is necessary for normal thymic DC homeostasis.

Another topic in thymic DC biology that remains poorly understood is the nature of the Ag thymic DC present to thymocytes. Unlike mature mTEC, thymic DC are not known to ectopically express a significant amount of PTA. Notably, however, thymic DC are critical for the presentation and functional deletion of thymocytes specific for at least some mTEC-expressed PTA in model systems via acquisition of mTEC Ag (“nibbling” or “cross-dressing”). Though the significance of this process in vivo has been demonstrated, it is unclear whether specific mechanisms govern the acquisition of Ag by thymic DC via nibbling. In the second part of our studies, we investigated the mechanisms by which thymic DC nibbling occurs primarily through the use of an in vitro culture system relying on differential MHC haplotype expression. Using this system, thymic DC acquisition of
functional MHC I and MHC II from mTEC and other DC but, surprisingly, not from splenic B cells was readily observed. Interestingly, membrane fragments known to be enriched in MHC II, namely lipid rafts, could be acquired from both mTEC and B cells. When a B cell surface molecule, immunoglobulin (Ig) M, was induced into lipid rafts by antibody (Ab) crosslinking, DC could acquire IgM. Therefore, dense clustering of surface molecules into lipid rafts may facilitate nibbling of such molecules by thymic DC.
CHAPTER 2: Antigen-specific thymocyte feedback regulates homeostatic thymic conventional dendritic cell maturation

2.1 Summary

Thymic dendritic cells (DC) mediate self-tolerance by presenting self-peptides to and depleting autoreactive thymocytes. Despite a significant role in negative selection, the events regulating thymic DC maturation and function under steady-state conditions are poorly understood. We report that crosstalk with thymocytes regulates thymic conventional DC (cDC) numbers, phenotype, and function. In mice lacking TCR-expressing thymocytes, thymic cDC were reduced and exhibited a less mature phenotype. Furthermore, thymic cDC in TCR transgenic mice lacking cognate antigen expression in the thymus were also immature; notably, however, thymic cDC maturation was reestablished by an Ag-specific cognate interaction with CD4+ or CD8+ single-positive thymocytes (SP). Blockade of CD40L during Ag-specific interactions with CD4SP but not CD8SP limited the effect on cDC maturation. Together, these novel findings demonstrate that homeostatic maturation and function of thymic cDC is regulated by feedback delivered by CD4SP and CD8SP via distinct mechanisms during a cognate Ag-specific interaction.

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2.2 Introduction

Thymocyte maturation and self-tolerance is induced by interactions with thymus-resident APC. Cortical thymic epithelial cells promote the positive selection of CD4⁺CD8⁺ double-positive thymocytes (DP), whereas medullary thymic epithelial cells (mTEC), which express multiple peripheral-tissue Ag, drive negative selection of autoreactive single-positive thymocytes (SP) (16). Thymic DC also induce negative selection of self-reactive thymocytes (19, 70, 105, 106) as well as promote natural regulatory T cell development (87, 88, 94, 106). Moreover, ablation of DC in mice has been shown to lead to either autoimmunity due to aberrant thymic tolerance (85) or dysregulation of peripheral immune homeostasis (84, 107), highlighting the broad regulatory function of DC.

DC in the thymus include three major subsets: CD8α⁺ cDC, signal regulatory protein α⁺ (SIRPα⁺) cDC, and plasmacytoid DC (pDC) (64). Migratory SIRPα⁺ cDC and pDC ferry peripheral self-Ag to the thymus to mediate negative selection (69, 70, 83). Additionally, SIRPα⁺ and intrathymically-developed CD8α⁺ cDC uptake soluble blood-borne Ag and subsequently process and present self-epitopes to thymocytes (64, 80–82). Furthermore, thymic DC can acquire Ag expressed by mTEC (75–78), which expands the self-Ag pool presented by thymic DC. Interestingly, thymic DC express elevated levels of MHC and costimulatory molecules, which correlate with enhanced T cell stimulatory capacity relative to resting peripheral (e.g. splenic) DC (94, 106, 108, 109). The latter is expected to enhance the efficacy of thymic negative selection. Little is known, however, about the factors that regulate thymic DC homeostasis. In this article, we show that increased activation and maturation of steady-state thymic DC required Ag-specific interactions with CD4SP or CD8SP. CD4SP-derived feedback, but not CD8SP-derived feedback, was abrogated by
blockade of CD40L. Thus, DC:thymocyte crosstalk is critical for the maintenance of thymic DC phenotypic activation and function.
2.3 Materials and Methods

2.3.1 Mice

NOD/LtJ (NOD), NOD/BDC2.5, NOD.TCRα−, NOD/BDC2.5 x NOD.TCRα− (BDC2.5/TCRα−), and NOD.Clone 4 TCR transgenic x NOD.scid (CL4.scid) mice have been described (110, 111) and were housed in specific pathogen-free facilities at the University of North Carolina at Chapel Hill. All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

2.3.2 Cell isolation and culture

Thymi from 3- to 6-wk old mice were digested with 1 mg/mL collagenase D and 20 µg/mL DNAse I (Roche) for 30 min at room temperature. EDTA was included for the final 5 min. DC were enriched using an OptiPrep gradient (Axis-Shield) and purified (>95%) via fluorescence-activated cell sorting (FACS). Splenic T cells were purified using a CD4+ Isolation Kit (Miltenyi Biotec).

To measure T cell proliferation, T cells were labeled with 5 µM CellTrace Violet (Invitrogen), co-cultured with peptide-pulsed DC at a 1:10 DC:T cell ratio for 3 d, and assessed by FACS. Division Index was calculated with FlowJo software (TreeStar).

2.3.3 FACS

Monoclonal Ab (mAb) and streptavidin were purchased from BD Biosciences, eBioscience, BioLegend, or Invitrogen. Cells were incubated with αCD16/32 (2.4G2) to block Fc receptors prior to Ab staining. Dead cells were excluded using propidium iodide, DAPI, or LIVE/DEAD stain (Invitrogen). For IL-12 (p40) staining, DC were cultured for 2-4
h with 10 µg/mL Brefeldin A (Sigma-Aldrich), fixed and permeabilized with BD Cytofix/Cytoperm solutions. Data were acquired on a BD LSR II and analyzed with FlowJo software.

2.3.4 DC localization

Thymic sections (7 µm) were stained with rabbit αKeratin 5 (Covance), biotin αDEC-205 (BioLegend), and Alexa Fluor 647 αCD11c (eBioscience) followed by Alexa Fluor 488 goat αrabbit IgG and Alexa Fluor 594 streptavidin (both from Invitrogen). Images (10x) were acquired using the 10x/0.45 numerical aperture objective of an LSM 710 spectral laser-scanning confocal microscope and ZEN software (Zeiss). Image analysis was performed in ImageJ: channels were split and converted to binary with automatic thresholding (negative = 0; positive = 255). Mean intensity of CD11c was measured for a given area of Keratin 5+ medulla or DEC-205+ cortex. This was performed for 3 separate medulla and cortex zones per image, averaged, and recorded as 1 data point.

2.3.5 mAb production and peptides

The αCD40L blocking mAb MR1 (ATCC CRL-2580) and αCD40 agonist mAb 1C10 were produced in-house. Control animals received either PBS or rat IgG2a isotype control (2A3, BioXCell). The sBDC mimetic peptide (RTRPLWVRME) and influenza hemagglutinin (HA, IYSTVASSL) peptides were produced at ≥95% purity by the UNC High-Throughput Peptide Synthesis and Array Facility.
2.3.6 Analysis of BrdU incorporation

To analyze DC turnover, mice received an initial intraperitoneal (i.p.) injection of 1 mg 5-bromo-2’-deoxyuridine (BrdU) and then 0.8 mg/ml BrdU in sterile drinking water containing 1% glucose to overcome taste aversion. Water was replaced daily throughout the assay duration. BrdU was detected via FACS using the PE BrdU Set (clone 3D4, BD Biosciences) following previously described methods (46, 112). Gating for BrdU+ cells was always based on cells stained with the αBrdU mAb but not administered BrdU. Isotype control staining gave similar results (unpublished observations).

2.3.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Unless otherwise indicated, significance was calculated by ANOVA with Bonferroni posttest.
2.4 Results

2.4.1 SP are necessary for normal thymic cDC numbers, phenotype, and function

Similar to C57BL/6 mice (94, 106, 108, 109) (Fig. 2.1), we found that NOD mouse thymic DC expressed significantly higher levels of MHC I, MHC II, CD40 and CD86, but not CD80, compared to resting splenic DC, and also more efficiently stimulated CD4+ and CD8+ T cell proliferation (Fig. 2.2). We hypothesized that the increased activation and maturation status of thymic DC is regulated by cognate interactions with SP. Accordingly, thymic DC from NOD.TCRα−/− mice, in which CD4SP and CD8SP development is blocked were studied. While overall thymus cellularity is unaffected by TCRα deficiency (113, 114), the frequency and number of thymic CD11chi cDC were reduced 3- to 5-fold in NOD.TCRα−/− versus NOD mice (Fig. 2.3A,B); pDC were unaffected, however. We also investigated whether DC localization was disrupted in the NOD.TCRα−/− thymus, which lacks an orderly medulla (101, 103, 114). As expected, the majority of CD11c+ thymic DC resided in the well-organized medulla of NOD thymi. In comparison, NOD.TCRα−/− thymi contained only small, disorganized medullary “islands” in which CD11c+ cells were enriched (Fig. 2.3C,D). Further analyses confirmed that both cDC and pDC predominantly localized to the medulla in NOD and NOD.TCRα−/− thymi (Fig. 2.4). Thus, the organization of DC in NOD.TCRα−/− thymi mirrors that of the decreased and disrupted medulla, suggesting that thymic DC cellularity is linked to medulla size.

Next, the activation and functional status of thymic DC in NOD.TCRα−/− mice were assessed. NOD.TCRα−/− versus NOD thymic cDC expressed decreased MHC II, CD40 and CD86 whereas CD80 levels remained unchanged (Fig. 2.3E). Interestingly, constitutive expression of IL-12, a cytokine implicated in the deletion of CD4SP (115, 116) was readily
detected in NOD thymic cDC, especially CD8α+ cDC; however the frequency of IL-12-producing thymic CD8α+ cDC was decreased 2-fold in NOD.TCRα−/− mice (Fig. 2.3F). ELISA confirmed that IL-12p70 was secreted by thymic DC (N.A. Spidale and R. Tisch, unpublished observations). To test DC stimulatory capacity, CD8α+ cDC, SIRPα+ cDC, and pDC were FACS-sorted from NOD and NOD.TCRα−/− thymi, pulsed with sBDC agonist peptide, and then co-cultured with BDC2.5 CD4+ T cells. NOD CD8α+ cDC induced significantly more BDC2.5 CD4+ T cell proliferation than NOD.TCRα−/− CD8α+ cDC (Fig. 2.3G,H). BDC2.5 CD4+ T cell proliferation was also increased by NOD versus NOD.TCRα−/− SIRPα+ cDC although this trend did not reach statistical significance. Thymic pDC from either NOD and NOD.TCRα−/− mice induced only low levels of proliferation. Overall, these data indicate that thymic cDC but not pDC number, phenotype, and function are significantly altered in the absence of SP. With the latter in mind our subsequent efforts focused on thymic cDC.

2.4.2 Ag-specific feedback regulates homeostatic thymic cDC maturation

Mature CD4+ and CD8+ T cells provide distinct modes of feedback to regulate peripheral DC maturation and effector function during a cognate immune response. Accordingly, whether CD4SP and CD8SP have distinct effects on thymic cDC homeostasis was studied using BDC2.5/TCRα−/− and CL4.scid mice in which only CD4+ T cells or CD8+ T cells develop, respectively. Despite a significant SP thymocyte pool, thymic cDC from either BDC2.5/TCRα−/− or CL4.scid mice resembled NOD.TCRα−/− thymic cDC (Fig. 2.3), marked by a less mature phenotype (Fig. 2.5A,B) and reduced frequency of IL-12+ cells (Fig. 2.5C,D) compared to wild-type NOD thymic cDC.
In BDC2.5/TCRα−/− and CL4.scid mice thymocytes undergo positive but minimal (if any) negative selection due to the lack of thymic expression of the cognate Ag. This suggested that thymic cDC phenotype was regulated by Ag-dependent interactions with thymocytes. To test this BDC2.5/TCRα−/− and CL4.scid mice were injected with cognate peptide, sBDC and HA, respectively, to facilitate direct interaction between DC and thymocytes. As expected, thymocyte apoptosis and activation were detected after peptide injection (Fig. 2.6). Thymic cDC from BDC2.5/TCRα−/− mice injected with 10 μg sBDC exhibited enhanced activation relative to controls (Fig. 2.5E). Interestingly, at a reduced dose of 1 μg sBDC, only SIRPα+ cDC in BDC2.5/TCRα−/− mice upregulated MHC and costimulatory molecules (Fig. 2.5E), suggesting increased sensitivity to CD4SP feedback and/or enhanced uptake and presentation of soluble Ag. Similarly, thymic cDC expression of MHC and costimulatory molecules was increased in an HA dose-dependent manner in CL4.scid mice (Fig. 2.5F). Importantly, we confirmed that activation of peripheral T cells is not responsible for thymic cDC activation using an adoptive transfer model wherein Ag-specific T cells were present in peripheral tissues but not the thymus (Fig. 2.7A,B). No effect on thymic cDC activation was observed when peripheral T cells were activated by administration of cognate Ag (Fig. 2.7C,D). Notably, similar if not greater levels of serum IFNγ were detected in T cell-recipient NOD.TCRα−/− mice compared to BDC2.5/TCRα−/− and CL4.scid mice after Ag injection (Fig. 2.7E), indicating comparable levels of peripheral T cell stimulation. Overall these findings, coupled with a report indicating that thymic DC, not mTEC, primarily acquire i.v. injected peptide (80), demonstrate that robust peripheral T cell activation has no effect on thymic cDC, and that thymic cDC activation and maturation are dependent on an Ag-specific cognate interaction with CD4SP or CD8SP.
2.4.3 A role for CD40/CD40L in CD4SP feedback to thymic cDC

Peripheral DC activation and maturation are regulated in part by CD40 signaling induced by binding to CD40L expressed by activated T cells. We hypothesized that CD40/CD40L play a similar role in thymic DC:thymocyte crosstalk. First, NOD.TCRα−/− mice were injected with an agonist αCD40 mAb. Expression of MHC II, CD40, CD80, and CD86 by thymic cDC was increased in αCD40 mAb-treated NOD.TCRα−/− mice (Fig. 2.8A). Thymic cDC from NOD mice treated with αCD40 exhibited little increase in maturation despite higher initial CD40 expression (Fig. 2.3E, 2.8A). Thus, ligation of CD40 alone was sufficient to induce thymic cDC activation to some extent. To test whether CD4SP regulated thymic cDC maturation via CD40L during Ag-specific interactions, BDC2.5/TCRα−/− mice were injected with a blocking αCD40L mAb prior to peptide administration. CD40L blockade strongly limited upregulation of MHC and costimulatory molecules by CD8α+ cDC and, to a lesser degree, by SIRPα+ cDC (Fig. 2.8B). In contrast, CD40L blockade had little effect on MHC and costimulatory molecule upregulation by thymic cDC in HA-injected CLA.scid mice (Fig. 2.8C). Overall, these data show a role for CD40 ligation by CD4SP but not CD8SP, and indicate additional feedback mechanisms contribute to murine thymic DC function.
2.5 Discussion

Our study demonstrates that cognate interactions with SP are critical for regulating thymic cDC homeostasis, including thymic cDC abundance (Fig. 2.3A,B). Notably, analyses of BrdU incorporation revealed no difference in the rate of thymic DC turnover in NOD.TCRα−/− versus NOD mice (Fig. 2.9). This suggests that reduced thymic cDC cellularity in NOD.TCRα−/− mice is due to a developmental defect in the absence of SP, which may be especially relevant for intrathymically-derived CD8α+ cDC. It is possible that mTEC, which are also reduced in TCRα−/− mice (101, 103, 117), contribute to thymic cDC cellularity and maturation. mTEC may provide a niche through production of chemokines such as XCL1 and CCL8 or other factors that regulate thymic cDC localization, recruitment, and/or maturation (78, 81, 118). However, thymic DC numbers are normal in β2M- or MHC II-deficient mice, the latter exhibiting a reduced mature mTEC pool (103, 117). These findings illustrate two key points: either CD4SP or CD8SP are sufficient to sustain DC homeostasis, consistent with our findings in peptide-treated BDC2.5/TCRα−/− and CL4.scid mice (Fig. 2.4), and thymic cDC homeostasis is maintained in the absence of mature mTEC. This second scenario is supported by findings demonstrating that thymic cDC maturation is unaffected in NOD (Fig. 2.10) and B6 (78) mice lacking Aire expression and thus mature mTEC. Therefore, mTEC appear to have only a limited effect on thymic cDC homeostasis. On the other hand, these observations support our model that thymocyte feedback is the key factor regulating thymic cDC numbers and maturation.

We propose that SP regulate thymic cDC homeostasis based in part on our observations obtained with TCRα−/− mice (Fig. 2.3). TCRα−/− mice though, also lack TCRαβ+DP that may contribute to thymic cDC feedback. While the inability of DC to induce
positive selection *in vivo* (19) likely precludes feedback from the majority (>95%) of DP, post-positive selection CD69\textsuperscript{hi}/TCR\(\alpha\beta\)\textsuperscript{+} DP may provide feedback to thymic cDC. Positive selection induces the migration of CD69\textsuperscript{hi}/TCR\(\alpha\beta\)\textsuperscript{+} DP into the medulla (119), which then may interact with thymic cDC. CD69\textsuperscript{hi} DP express *Cd40lg* mRNA (101, 103), which we show is associated with feedback mediated by MHC II-restricted thymocytes (Fig. 2.8B). However, levels of *Cd40lg* mRNA expression are reduced ~10-fold compared to CD4SP (101), and <4% of CD69\textsuperscript{hi} DP express surface CD40L, compared to nearly 30% of CD4SP (104). Consequently, we favor a dominant role for SP in feedback to thymic cDC due to a numerical advantage, uniform medullary localization, and significantly higher frequency of CD40L\textsuperscript{+} cells relative to TCR\(\alpha\beta\)\textsuperscript{+} DP. Nevertheless, future work is needed to address if TCR\(\alpha\beta\)\textsuperscript{+} DP indeed contribute to the maintenance of thymic cDC homeostasis.

Whereas cDC were regulated by SP, thymic pDC were not. Little is known about the factors regulating thymic pDC homeostasis. For example, CCL25, which regulates CCR9-dependent pDC migration to the murine thymus (83), is produced by thymic stroma in an AIRE-independent manner (78), and may explain the normal pDC numbers in the NOD.TCR\(\alpha\)\textsuperscript{-/-} thymus (Fig. 2.3A,B). Regulation of migration may be a primary means of thymic pDC regulation. Migration of activated versus immature pDC to the thymus is significantly reduced (83), which may prevent tolerance induction against foreign Ag during infection. Despite an immature phenotype and poor *ex vivo* stimulatory capacity of thymic pDC (Fig. 2.3E,G,H), peptide-loaded pDC transferred i.v. have been shown to delete Ag-specific SP (83). One interesting scenario is that pDC ferry peripheral self-Ag to the thymus, which is then “transferred” to cDC that stimulate negative selection. A similar process of Ag transfer occurs from mTEC to DC (75–78).
Analyses of NOD.TCRαβ thymic cDC revealed decreased activation status, IL-12 production, and T cell stimulatory capacity compared to NOD thymic cDC (Fig. 2.3E-H). DC maturation was regulated by a cognate Ag-specific interaction with either CD4SP or CD8SP (Fig. 2.6E,F). Similarly, SP thymocyte feedback is critical for mTEC differentiation, but is mediated exclusively by CD4SP (103). Intriguingly, CD4SP and CD8SP regulated thymic cDC homeostasis by distinct mechanisms; CD4SP- but not CD8SP-mediated effects were CD40L-dependent (Fig. 2.8). The latter is not surprising since CD8SP express low levels of CD40L mRNA relative to CD4SP (101, 103). Of keen interest is defining the nature of CD8SP-mediated feedback, as well as determining if thymic cDC subsets are regulated by distinct mechanisms. Full characterization of the molecular interactions occurring during thymocyte:DC crosstalk will help define the events that influence the efficacy of thymic negative selection, and may reveal novel mechanisms by which DC subsets and thymic stromal cells contribute to thymocyte development.
Figure 2.1. Enhanced phenotype and function of thymic versus splenic DC in C57BL/6 (B6) mice. (A) Analysis of MHC and costimulatory molecule expression by B6 thymic and splenic DC subsets (n=4). (B) Thymic and splenic DC subsets were FACS-sorted, pulsed with OVA\textsubscript{323-339} peptide, and then co-cultured with CellTrace-labeled OT-II splenic CD4\textsuperscript{+} T cells. (C) Constitutive IL-12 production by DC from B6 mice was assessed as described in Materials and Methods. Error bars represent SEM. *, P<0.05; **, P<0.01; ***, P<0.001 by two-way ANOVA with Bonferroni posttest. Data are representative of at least two experiments.
Figure 2.2. NOD thymic DC are phenotypically and functionally more mature than splenic DC. Gating scheme for all analyses of DC from NOD mouse (A) thymus and (B) spleen following isolation as described in Materials and Methods (Section 2.3). (C-D) Comparison of MHC I (H2D^b, H2K^d), MHC II (IA^a^b), CD40, CD80, and CD86 expression by NOD thymic and splenic DC gated as shown in Panels A and B (n=4). Data are representative of at least four experiments. (E-F) Thymic and splenic cDC subsets were FACS-sorted as Live/CD11c^{hi}/SIRPα^+/CD8α^-/lo (SIRPα^+ cDC) or Live/CD11c^{hi}/SIRPα^-/CD8α^+/CD8α^- (CD8α^+ cDC), pulsed with (E) 10 ng/mL sBDC or (F) 10 ng/mL IGRP peptide, then co-cultured with 2.5x10^5 CellTrace Violet-labeled (E) BDC2.5 CD4^+ or (F) 8.3 CD8^+ T cells for 66-72 hours. Proliferation of Live/Thy1.2^+/CD4^+ or CD8^+ T cells was determined via
CellTrace Violet dilution. Plasmacytoid DC induced no proliferation under the conditions tested (data not shown). Graphed data are pooled from two independent experiments. All error bars represent SEM. *, $P<0.05$; ***, $P<0.001$ by two-way ANOVA with Bonferroni posttest.
Figure 2.3. Dysregulation of thymic DC in NOD mice lacking SP. (A) Frequency and (B) absolute number (±SEM) of thymic cDC and pDC in NOD and NOD.TCRα−/− thymi (n=8 each). (C) Staining of thymic sections for Keratin 5+ mTEC, DEC-205+ cTEC, and CD11c+ DC. (D) Quantification of mean CD11c intensity per unit area (±SEM) in the thymic medulla and cortex (n=12 sections each). (E) Analysis of MHC and costimulatory molecule expression by NOD and NOD.TCRα−/− thymic DC. Data are representative of 4 experiments. (F) Constitutive intracellular IL-12 expression (±SD from 3 experiments) by thymic cDC from NOD and NOD.TCRα−/− mice. (G) DC subsets were FACS-sorted from NOD and NOD.TCRα−/− thymi and BDC2.5 CD4+ T cell proliferation measured. Data are gated on
live/Thy1.2+/CD4+ cells from representative co-cultures with 10^{-2} \mu g/ml sBDC-pulsed DC subsets. (H) Division Index (±SEM) calculated from cells proliferating in Panel G. Data represent 3 pooled experiments. *, \ P<0.05; ***, \ P<0.001.
Figure 2.4. cDC and pDC more heavily localize to the medulla vs. the cortex in both NOD and NOD.TCRα⁻/⁻ thymi. A) Thymic sections from NOD and NOD.TCRα⁻/⁻ mice were stained with biotin αCD11c, purified rabbit αKeratin 5, and Alexa Fluor 647 PDCA-1, then Brilliant Violet 421 streptavidin and Alexa Fluor 546 goat anti-rabbit. Images were acquired on an LSM 710 confocal microscope using ZEN software (Zeiss). The dimensions of each image are approximately 2.4 mm x 2.4 mm. Images were analyzed in ImageJ as follows: first, channels were split then converted to binary with automatic thresholding (negative = 0, positive = 255). Pixels where PDCA-1 and CD11c overlapped were subtracted from the total CD11c image using the Image Calculator to create a new CD11c⁺PDCA-1⁻ (cDC) image. This image was then overlaid with the binary PDCA-1 (pDC) image and binary Keratin 5 image to create a new image showing cDC, pDC, and Keratin 5 (images shown are the result of this process). B) Mean intensity per unit area of cDC and pDC was calculated in the medulla and cortex of NOD and NOD.TCRα⁻/⁻ thymic sections (n=8/group, 2 sections from each of 4 individual thymi). ***, P<.001 by two-way ANOVA with Bonferroni posttest.
Figure 2.5. Ag-specific interactions with SP regulate thymic DC activation status. (A-B) MHC and costimulatory molecule expression by thymic cDC isolated from NOD and (A) BDC2.5/TCRα^−/− or NOD and (B) CL4.scid mice. (C-D) Constitutive IL-12 production by thymic cDC from NOD and (C) BDC2.5/TCRα^−/− or (D) CL4.scid mice. (E) BDC2.5/TCRα^−/− or (F) CL4.scid mice were injected i.v. with sBDC or HA, respectively, or PBS (Ctrl), and 16-18 h later MHC and costimulatory molecule expression by thymic DC measured. Values are expressed as fold change in mean fluorescence intensity versus Ctrl (normalized to 1). All asterisks represent comparison to Ctrl. Data are representative of 3-5 experiments. *, P<0.05; **, P<0.01; ***, P<0.001.
Figure 2.6. Apoptosis and activation of thymocytes following peptide injection. (A and B) BDC2.5/TCRα⁺ mice were injected with PBS, 1 μg sBDC, or 10 μg sBDC and then rested for 16-18 hours prior to analysis of activated caspases/apoptosis (VAD-FMK⁺) and activation (CD69⁺) of thymocytes via FACS (n=3/group). (C and D) CL4.scid mice were injected with PBS, 1 μg HA, or 5 μg HA and analyzed as in Panels A and B (n=3/group). Note that no clear delineation of DP and CD8SP thymocytes was observed in these mice, thus all CD8⁺ thymocytes, which expressed high levels of TCR (unpublished observations), were analyzed as a single population. **, P<.01; ***, P<.001 (ANOVA with Bonferroni posttest). CaspACE FITC-VAD-FMK In Situ Marker was purchased from Promega and used at a 1:500 dilution.
Figure 2.7. Activation of peripheral T cells alone does not activate thymic DC.  (A) Experimental design to determine if peripheral T cell activation leads to thymic DC activation.  (B) Detection of transferred T cells by characteristic TCR Vβ staining.  Thymus panels were gated on total, live thymocytes; spleen panels were gated on live, CD3ε+Thy1.2hi cells.  AT, Adoptive Transfer.  (C-D) Analysis of thymic DC phenotype in NOD.TCRα−/− mice that received (C) BDC2.5 CD4+ T cells and 5 μg sBDC or (D) CL4 CD8+ T cells and 5 μg HA as described in panel A.  (E) IFNγ concentration in the serum of T cell-injected NOD.TCRα−/− recipients or BDC2.5/TCRα−/− and CL4.scid mice injected with peptide (n=3-6/group). * P<.05 by two-way repeated measures ANOVA with Bonferroni posttest.
Figure 2.8. CD40/CD40L partially regulates thymic DC phenotype. (A) NOD and NOD.TCRα−/− mice were injected i.p. with 200 µg agonist αCD40 or isotype control (Ctrl) mAb, and MHC and costimulatory molecule expression by thymic DC assessed 16-18 h later. Inset asterisks represent analysis of Ctrl vs. αCD40. (B) BDC2.5/TCRα−/− or (C) CL4.scid mice were treated daily i.p. for 3 d with 250 µg blocking αCD40L mAb or PBS then, at the time of the final αCD40L treatment, injected i.v. with 5 µg sBDC (B) or 5 µg HA (C), and thymic DC expression of MHC and costimulatory molecules measured 16-18 h later. Asterisks within histograms represent analysis of PBS + peptide vs. αCD40L + peptide. Data are representative of 3-5 experiments. *, P<0.05; **, P<0.01; ***, P<0.001.
Figure 2.9. Thymic DC turnover is not dramatically different in NOD versus NOD.TCRα⁻/⁻ mice. A) NOD and NOD.TCRα⁻/⁻ mice were administered BrdU for 2, 5, or 8 days and then BrdU incorporation by thymic DC subsets was assessed via FACS. Gating is based on cells stained with αBrdU from mice not administered BrdU (No BrdU). B) Summary of data compiled from 3 independent experiments as in (A) showing mean %BrdU⁺ ± SEM. n=6/time point and genotype. *, P<.05; ***, P<.001.
Figure 2.10. Thymic DC phenotype is unaffected in AIRE-deficient mice. Thymic DC were isolated from 4-wk-old wild-type NOD and disease-free NOD.AIRE⁻/⁻ mice and expression of MHC II (IA^β^), CD40, CD80, and CD86 assessed via FACS. Graphs show mean ± SEM of 3 mice examined. ***, P<.001; **, P<.01 (two-way ANOVA with Bonferroni posttest).
CHAPTER 3: Dendritic cell nibbling may preferentially target surface molecules clustered into lipid rafts

3.1 Summary

Dendritic cells (DC) are important antigen presenting cells (APC) in the thymus for the maintenance of central tolerance. Unlike mTEC, however, DC are not known to intrinsically express significant amounts of PTA. One way in which DC acquire relevant antigen (Ag) for presentation to developing thymocytes is via intracellular Ag transfer, or nibbling, from mTEC. Despite the significance of this process in central tolerance, the factors regulating thymic DC nibbling are poorly understood. Here, we demonstrate that thymic DC may preferentially acquire surface molecules densely clustered into lipid raft membrane microdomains. Using an MHC-mismatch-based in vitro system, thymic DC were found to nibble MHC from TEC and other DC but not B cells. Notably, B cell MHC remained inefficiently acquired even when surface expression was increased over 5-fold, indicating that acquisition is independent of absolute surface levels. Interestingly, lipid rafts were acquired from both TEC and B cells. When a B cell surface molecule, namely IgM, was actively clustered into lipid rafts, thymic DC acquired IgM, which was otherwise poorly nibbled from resting B cells. These data suggest that thymic DC may preferentially nibble densely clustered molecules in lipid rafts.
3.2 Introduction

The generation of a self-MHC restricted peripheral T cell population purged of autoreactivity is a critical step in the development of the immune system. T cell development in the thymus thus has several mechanisms in place to ensure that both of these goals are met. Thymocytes that have assembled a functional TCR must first be “positively” selected by self-MHC presented by cTEC (16). However, this process alone is insufficient to generate an appropriate peripheral T cell population. For example, T cells that develop in an environment where cTEC are the only thymic source of self-MHC are not tolerized to self-MHC (62). Thus, antigen presentation by additional thymic APC, namely mTEC and DC, is critical for purging the T cell repertoire of autoreactivity and fostering “normal” T cell development.

mTEC are a heterogeneous group of cells that can be divided into multiple subpopulations, including those expressing relatively low levels of CD80 and MHC II and those expressing high levels of CD80 and MHC II (so-called mTEC\textsuperscript{hi}) (73). The latter mTEC\textsuperscript{hi} population is often ascribed the most significance due to expression of the AIRE transcription factor. The AIRE protein is noteworthy for its ability to drive expression of many peripheral tissue Ag (PTA), which leads to central tolerance induction to these Ag (79, 120). Interestingly, AIRE also has additional described functions outside of driving PTA expression, including potentially promoting mTEC apoptosis (121) and inducing the production of a range of chemokines (78). The latter observation has obvious implications for driving thymocyte migration into the medulla, but is also important for promoting thymic DC localization to the medulla (118), where these cells can interact with and delete self-Ag-specific thymocytes. Notably, AIRE-dependent chemokines may not simply recruit thymic DC into the medulla but also facilitate interactions between mTEC and thymic DC (78).
Thus, mTEC make crucial contributions to T cell central tolerance via both direct Ag presentation and via driving important thymic cell populations into the medulla.

Thymic DC are also a critical medullary APC population that are known to promote negative selection of thymocytes (19). However, as thymic DC are not known to express biologically-relevant levels of AIRE (18, 122), thymic DC have developed other means of acquiring relevant self-Ag for presentation in the thymus. Some thymic DC, namely the SIRPα+ cDC and pDC subsets, can acquire Ag in the periphery and then migrate to the thymus to enforce central tolerance (69, 70, 83). In addition the CD8α+ cDC and SIRPα+ cDC subsets can acquire blood-borne Ag for subsequent processing and presentation to thymocytes (80, 82); though it has been argued that SIRPα+ cDC preferentially acquire blood-borne Ag due to ideal intrathymic positioning (81). Interestingly, thymic DC also acquire mTEC-derived Ag via intercellular Ag transfer, which may occur consequent to mTEC apoptosis or by acquisition of molecules from live mTEC via “nibbling” (75–78). While this process has been shown to influence the efficacy of negative selection, little is known about the mechanisms governing thymic DC Ag acquisition from mTEC. Moreover, it remains unclear whether surface proteins are acquired indiscriminately, or whether there are factors that promote selective acquisition of proteins. In this study, we investigated Ag acquisition by thymic DC via nibbling using an in vitro system of MHC mismatched TEC and thymic DC. In this setting, acquisition of MHC from live TEC by thymic DC was readily observed, as was acquisition of MHC from other thymic DC and splenic DC. Uniquely, MHC could not be acquired from B cells, and this was found to be independent of the relative levels of MHC surface expression. Notably, lipid raft membrane microdomains (also known as detergent-resistant membrane domains) could be acquired from TEC and B cells,
and may play a role in governing nibbling by thymic DC. While surface IgM was not acquired from resting B cells, in which IgM is mostly excluded from lipid rafts, active clustering of B cell surface IgM to lipid rafts induced nibbling of IgM by thymic DC. Thus, lipid rafts may represent a distinct plasma membrane locale targeted by thymic DC for surface Ag acquisition via live cell nibbling.
3.3 Materials and Methods

3.3.1 Mice

NOD/LtJ (NOD), BALB/cJ (BALB/c), NOD.Cg-Tg(TcraBDC2.5,
TcrbBDC2.5)1Doi/DoiJ x NOD.129P2(C)-Tcra\textsuperscript{tm1Mjo}/DoiJ (BDC2.5/C\(\alpha\)\(0\)), and
NOD.129S2(B6)-Aire\textsuperscript{tm1.1Doi}/DoiJ (NOD.AIRE\(+/-\)) were originally purchased from the
Jackson Laboratories and housed under specific pathogen-free conditions at the University of
North Carolina at Chapel Hill (UNC-CH). All animal procedures were approved by the
UNC-CH Institutional Animal Care and Use Committee. Mice were used between 4 and 8
weeks of age. Both male and female mice were used but sex-matched within an experiment.
No differences between cells isolated from male or female mice were observed for any assay
(unpublished observations).

3.3.2 Cell isolation

For DC isolation, thymi or spleens were harvested and finely minced with a razor
blade. Following a 30 min room temperature digestion with 1 mg/ml Collagenase D and 20
\(\mu\)g/ml DNase I (Roche) in R2 (RPMI 1640/2% fetal calf serum [FCS]/10 mM HEPES pH
7.4) and 5 min incubation with ~10 mM EDTA, dispersed thymic cells were centrifuged
through an OptiPrep (60% w/v iodixanol; Axis-Shield) gradient (123). Cells were suspended
in 4 ml of a 15% w/v iodixanol solution, then overlaid with 5 ml of a 12% w/v iodixanol
solution, then overlaid with magnetic-activated cell sorting (MACS) buffer (PBS/0.5%
bovine serum albumin/2 mM EDTA). Gradients were centrifuged for 15 min at 600g at room
temperature, and then low density cells banding at the interface between the 12% w/v
iodixanol and MACS buffer were used for purification of DC with CD11c microbeads and an AutoMACS separator (Miltenyi Biotec).

For isolation of TEC, thymi were digested with 1 mg/ml Collagenase D and 20 µg/ml DNAse I in R2 at 37°C (3 rounds, 15 min each), then for an additional 15 min with 1.25 mg/ml Collagenase/Dispase (Roche) and DNAse I, followed by incubation with 10 mM EDTA for 5 min. Cells were suspended in 4 ml of a 1.115 specific gravity Percoll (GE Healthcare) solution, overlaid with 2 ml of a 1.065 specific gravity Percoll solution, then overlaid with 2 ml of PBS (specific gravity ~1.0). Gradients were centrifuged for 30 min at 2700 revolutions per minute at 4°C, and TEC-enriched light density cells banding at the interface of the 1.065 Percoll solution and PBS were used for further enrichment of TEC via CD45⁺ cell depletion on an AutoMACS separator.

B cells and CD4⁺ T cells were isolated following mechanical dispersion of spleens using the EasySep Mouse B Cell Isolation Kit (Stem Cell Technologies) or CD4+ T cell isolation kit II (Miltenyi Biotec), respectively.

3.3.3 FACS

The following mAb and other reagents were purchased from BD Biosciences, BioLegend, eBioscience, or Life Technologies and used for FACS: αCD11c (N418), αCD8α (53-6.7), αSIRPα (P84), αCD45RA (14.8), αB220 (RA3-6B2), αCD3ε (145-2C11), αCD49b (DX5), αCD19 (1D3), erythroid cells (Ter-119), αIAk/g7 (10-3.6), αIEd (14-4-4S), αH2Dd (34-2-12), αEpithelial cell adhesion molecule (EpCAM, G8.8), αCD45.1 (A20), αCD45.2 (104), αIgMa (MA-69), and streptavidin. To label lipid rafts, TEC or B cells were incubated with 1 µg/ml Cholera Toxin B subunit (CTxB, Life Technologies) in PBS for 15
min, then washed extensively. Cells were Fc receptor blocked (2.4G2) prior to mAb staining. Dead cells were excluded from all analyses using propidium iodide or DAPI (Life Technologies). Data were acquired on an LSR II (BD Biosciences) and analyzed using FlowJo (TreeStar Inc.). Sorting was performed on a MoFlo XDP (Beckman Coulter) and population purity was >95%. To analyze proliferation, purified BDC2.5 CD4+ T cells were labeled with 5 μM CellTrace Violet (Life Technologies), then cultured as indicated in the text.

3.3.4 Nibbling Assay

To examine nibbling in vitro, cells were isolated from MHC-mismatched mice (e.g. thymic DC from H2g7 NOD and “donor” TEC, DC, or B cells from H2d BALB/c). In some experiments, the donor BALB/c population was labeled with CTxB as described above to examine acquisition of lipid rafts. DC and donor cells were then co-cultured for 3-4 h in R10 (RPMI 1640/10% FCS/4 mM L-glutamine/1x non-essential amino acids/1 mM sodium pyruvate/100 units/ml penicillin/100 μg/ml streptomycin/55 μM β-mercaptoethanol/10 mM HEPES pH7.4) and analyzed via FACS. In some experiments, a 0.4 μm TransWell apparatus (Corning) was used wherein DC were seeded in the well and the donor population was seeded in the well with the DC or into the TransWell and thus separated from the DC. In some experiments, DC were pre-treated with a blocking mAb for scavenger receptor type A (SR-A, clone 2F8, AbD Serotec) for 20-30 min, then TEC were added and the cells were co-cultured as above.
3.3.5 Cell stimulation

To assess acquisition of IE\textsuperscript{d} and CTxB from activated B cells, BALB/c splenic B cells were purified as above and then cultured for 16-20 h with the following stimuli in R10: 10 \(\mu\)g/ml \(\alpha\)CD40 (clone 1C10, produced in-house), 10 \(\mu\)g/ml \textit{E. coli} lipopolysaccharide (LPS, Sigma-Aldrich L2630), 25 \(\mu\)g/ml goat anti-mouse IgM F(ab’)\textsubscript{2} (Jackson ImmunoResearch), or LPS + \(\alpha\)IgM. Cells were washed extensively then labeled with CTxB and cultured with thymic DC as above.

To examine the acquisition of IgM during \(\alpha\)IgM stimulation, Alexa Fluor 647-conjugated goat anti-mouse IgM F(ab’)\textsubscript{2} (\(\alpha\)IgM.Ax647, Jackson ImmunoResearch) was used because unconjugated \(\alpha\)IgM rendered IgM undetectable using other \(\alpha\)IgM Ab (unpublished observations). Prior to use, \(\alpha\)IgM.Ax647 was buffer-exchanged against sterile PBS using a 10,000 Dalton molecular-weight cutoff spin column (Millipore) to remove preservatives. Buffer-exchanged \(\alpha\)IgM.Ax647 was confirmed to retain B cell stimulatory potential as assessed by upregulation of MHC II (unpublished observations). Purified B cells were coated with titrated concentrations of \(\alpha\)IgM.Ax647 at 4\(^\circ\)C for 20-30 min, washed of excess \(\alpha\)IgM.Ax647, then mixed with purified thymic DC and cultured at 37\(^\circ\)C for 3-4 h.

3.3.6 qPCR

Thymic DC were purified via FACS and RNA isolated using TriZol (Life Technologies). DNAse digestion was performed using TURBO DNase (Ambion), and then cDNA was generated using Maxima H Minus Reverse Transcriptase (Thermo Scientific) with oligo(dT)\textsubscript{18} priming. Quantitative polymerase chain reaction (qPCR) analysis was performed on 20 nanograms of cDNA using the Maxima SYBR Green Master Mix (Thermo
Scientific) and a MyIQ iCycler (Bio-Rad). Cycling conditions were as follows: 95°C, 10 min followed by 40 cycles of 95°C, 15 s; 60°C, 30 s; 72°C, 30 s followed by dissociation curve analysis. No template and no reverse transcriptase controls consistently yielded no amplification, and dissociation curves confirmed only 1 amplicon/primer set. Relative expression of *Epcam* was assessed by the comparative Ct (ΔΔCt) method (124) using *Hprt* as the endogenous reference and EpCAM− cDC as the calibrator sample. The following primers were produced by the UNC-CH Nucleic Acids Core Facility and used for qPCR: *Epcam* Forward, 5’ – GCGGCTCAGAGAGACTGTG – 3’; *Epcam* Reverse, 5’ – CCAAGCATTTAGACGCCAGTTT – 3’; *Hprt* Forward, 5’ – GCTATAAAATTCTTTGCTGACCTGCTG – 3’; *Hprt* Reverse, 5’ – AATTACTTTATGTCCCCTGTGACTGG – 3’.
3.4 Results

3.4.1 Thymic DC produce EpCAM intrinsically

A recent investigation into acquisition of TEC Ag by thymic DC observed a strong association between EpCAM positivity on DC and acquisition of a TEC-specific fluorescent reporter (77). However, whether the capacity to acquire TEC Ag is restricted to one or more specific DC subsets was not addressed. Analysis of EpCAM on thymic DC subsets revealed a significant portion of EpCAM+ cells within both the CD8α+ and SIRPα+ cDC populations but no EpCAM+ pDC (Fig. 3.1A-B). Interestingly, some of the EpCAM+ cDC showed particularly high levels of EpCAM labeling—levels comparable to those observed on TEC (Fig. 3.1A). It was surprising that such a marked amount of EpCAM could be acquired by DC. Moreover, whether EpCAM can be expressed by thymic DC per se has not been examined. To address this possibility, thymic cDC were FACS-sorted into EpCAM+ and EpCAM− populations (Fig. 3.1C) for analysis of Epcam mRNA via RT-qPCR. Strikingly, EpCAM+ cDC produced roughly 70-fold more Epcam transcript compared to EpCAM− cDC (Fig. 3.1D). Moreover, Epcam transcript levels correlated with surface EpCAM staining—the intensity of EpCAM staining on EpCAM+ cDC was at least an order of magnitude higher than on EpCAM− cDC (Fig. 3.1A,B). By comparison, TEC themselves expressed >200-fold more Epcam than EpCAM+ cDC and thus approximately three times the amount as EpCAM+ cDC. To extend these findings, EpCAM levels on thymic DC isolated from Aire-deficient NOD mice (NOD.AIRE−/−) were examined. AIRE-expression in mTEC has been previously shown to regulate transfer of Ag from mTEC to DC in vivo, possibly through regulation of chemokine expression that promotes mTEC-DC cognate interactions (78). Comparison of NOD and NOD.AIRE−/− thymic DC showed comparable frequencies of EpCAM+ thymic DC
subsets (Fig. 3.1E). Together, these data suggest that EpCAM+ thymic DC produce EpCAM intrinsically; therefore it is difficult to determine what proportion of EpCAM, if any, is acquired from mTEC versus produced intrinsically by thymic DC.

3.4.2 DC can acquire MHC I and MHC II from TEC and DC but not B cells in vitro

To establish a more controlled system to test the nibbling ability of thymic DC, we used an in vitro co-culture system relying on the MHC-mismatch of cells isolated from different mouse strains (Fig. 3.2A). In a typical assay, thymic DC were isolated from NOD mice (IA\(^{g7}\)/IE\(^{null}\)/H2D\(^{b}\)) then co-cultured with “donor” TEC, thymic or splenic DC, or B cells isolated from BALB/c mice (IA\(^{d}\)/IE\(^{d}\)/H2D\(^{d}\)). Cells were then labeled with antibodies detecting IE\(^{d}\) and H2D\(^{d}\), and acquisition of these molecules by NOD thymic DC was examined via FACS. First, the antibodies used to detect IE\(^{d}\) and H2D\(^{d}\) showed no background staining of NOD DC (Fig. 3.2B-E, upper panels), indicating minimal cross-reactivity with NOD MHC II or MHC I, respectively. When NOD thymic DC were co-cultured with BALB/c TEC, significant amounts of both MHC I and MHC II were acquired (Fig. 3.2B), with most DC acquiring either both MHC I and MHC II or neither MHC I nor MHC II. A similar pattern of acquisition was observed with BALB/c thymic DC as the MHC donors, with a trend towards MHC II versus MHC I acquisition (Fig. 3.2C). However, this “preference” may be indicative of the relative detection intensity on the donor cell population rather than true preference of acquisition, i.e. the MFI of IE\(^{d}\) on BALB/c thymic DC is greater than the MFI of H2D\(^{d}\) on BALB/c thymic DC under our analysis conditions (Fig. 3.2F). When BALB/c splenic DC were used as MHC donors, the overall frequency of BALB/c MHC\(^{+}\) NOD DC was lower relative to cells cultured with TEC or thymic DC (Fig.
3.2D), but acquisition was nonetheless readily detectable. Again, a preference for acquisition of MHC II versus MHC I was observed. Strikingly, when BALB/c splenic B cells were used as the MHC donors, no acquisition of MHC I or MHC II by thymic DC was observed (Fig. 3.2E). Similar results were obtained if the acquiring and donor cell MHC haplotypes were switched (data not shown). These data indicate that thymic DC can readily acquire both MHC I and MHC II from TEC, thymic DC, and splenic DC but, interestingly, not splenic B cells. Subsequently, we also compared the relative efficiency of thymic and splenic DC nibbling TEC. Splenic DC also significantly nibbled TEC MHC I and MHC II (Fig. 3.3A). While the frequency of BALB/c MHC+ DC was similar between thymic and splenic DC, (Fig. 3.3B), significantly more MHC I and MHC I was acquired by thymic DC as assessed by MFI (Fig 3.3C). This indicates that the ability to nibble MHC I and MHC II is not unique to steady-state thymic DC, although thymic DC may be uniquely efficient nibblers compared to splenic DC.

To further validate this assay design, it was important to determine whether acquired MHC molecules were functional for T cell stimulation. To address this, TEC were isolated from NOD mice and pulsed with titrated concentrations of sBDC peptide, while thymic DC were isolated from BALB/c mice. Thymic DC were co-cultured with peptide-pulsed TEC as above, and then thymic DC and TEC were separated via FACS (Fig. 3.4A). Following separation, thymic DC and TEC were cultured with responder CellTrace Violet-labeled BDC2.5 CD4+ T cells for 3 d, and then proliferation assessed via FACS. As expected, robust proliferation of BDC2.5 cells was observed when peptide-pulsed TEC were used as the stimulators (Fig. 3.4B). When post-nibbling thymic DC were used, some proliferation above background was observed when BALB/c thymic DC that were cultured with unpulsed TEC
were cultured with BDC2.5 cells, suggesting a small degree of allogeneic responsiveness. However, detectable proliferation was observed in a manner dependent on the peptide concentration with which the donor TEC were pulsed (Fig. 3.4B-C). Based on the proliferation observed when peptide-pulsed TEC versus post-nibbling thymic DC were used as stimulators, we estimate that the stimulatory capacity of post-nibbling DC was approximately 10- to 50-fold lower than that of peptide-pulsed TEC. Together, these data indicate that acquired peptide-MHC II molecules can be used directly for T cell stimulation.

We subsequently investigated the factors that may regulate preferential acquisition of MHC II from TEC but not B cells by thymic DC.

3.4.3 Thymic DC acquire lipid rafts from TEC and B cells

Little is known about the factors that regulate the cell-to-cell transfer of MHC molecules. In the case of T cell trogocytosis, a process analogous to nibbling whereby T cells can acquire peptide-MHC and other molecules from APC, a cognate Ag-specific interaction and the formation of an immunological synapse is necessary (125, 126). However, such an interaction does not occur between thymic DC and TEC. We hypothesized that other factors regulating cell membrane organization may facilitate DC nibbling. To this end, we investigated the role of lipid rafts in DC nibbling. Lipid rafts are membrane microdomains enriched in certain classes of molecules, including cholesterol, sphingolipids, and other glycolipoproteins (127). Interestingly, lipid rafts are critical for various immunologically important phenomena, including TCR-MHC interactions, BCR signaling, and Fc receptor signaling (128). To investigate lipid rafts, we took advantage of the binding of CTxB to the lipid raft ganglioside G\textsubscript{M1} (129). BALB/c TEC and B cells were labeled with fluorochrome-
conjugated CTxB, then co-cultured with NOD thymic DC as above to examine DC acquisition of lipid rafts (CTxB) and IE\textsuperscript{d}. Robust acquisition of CTxB was observed by virtually all DC that were co-cultured with TEC (Fig. 3.5A). As before, a significant portion of DC also acquired TEC-derived IE\textsuperscript{d}. Interestingly, whereas no IE\textsuperscript{d} was acquired from BALB/c B cells, essentially all DC could acquire CTxB from B cells (Fig. 3.5B). Thus, lipid rafts can be readily acquired from both TEC and B cells.

3.4.4 Thymic DC nibbling of MHC II/lipid rafts is contact-dependent

To begin to investigate the factors that regulate thymic DC nibbling of MHC II and lipid rafts, we analyzed the requirement for cell-cell contact in the acquisition of IE\textsuperscript{d} and CTxB. When DC and TEC (Fig. 3.5C) or DC and B cells (Fig. 3.5D) were physically separated in a TransWell (TW) apparatus, no acquisition of IE\textsuperscript{d} and an 80-90% reduction in the acquisition of CTxB was observed. These data indicate that DC acquisition of MHC II and lipid rafts is dependent on cell-cell contact. In this context, it has been previously shown that class A scavenger receptor (SR-A) plays a role in regulating Ag acquisition from live cells via nibbling by non-human primate DC (130). To address whether SR-A plays a similar role in murine thymic DC nibbling of MHC II, a nibbling assay was performed as above with or without a blocking \(\alpha\)SR-A mAb, which completely inhibits macrophage binding of known SR-A ligands (131). However, \(\alpha\)SR-A did not affect thymic DC nibbling of mTEC MHC II or lipid rafts (Fig. 3.5E). Therefore, murine thymic DC nibbling of MHC II occurs independently of SR-A.
3.4.5 Inefficient nibbling of B cell MHC II is independent of absolute level of B cell MHC II expression

The fact that DC can acquire CTxB but not IE\textsuperscript{d} from B cells suggests that B cells are not completely resistant to DC nibbling, but rather that there may be something unique about B cell IE\textsuperscript{d} that causes it to be inefficiently acquired by DC. One possibility is the absolute level of IE\textsuperscript{d} expression, which is about 7-fold lower on splenic B cells than on TEC (Fig. 3.2F). To address this, we used various B cell activating stimuli, all of which increase IE\textsuperscript{d} expression >5-fold (Fig. 3.6A). When B cells were activated via αIgM stimulus, a small amount of IE\textsuperscript{d} was observed on nibbling DC, while CTxB was acquired to similar levels as from Ctrl B cells (Fig. 3.6B). Less IE\textsuperscript{d} was observed on DC cultured with αCD40- or LPS-activated B cells. Interestingly, when B cells were stimulated with αIgM and LPS together, less acquisition of both IE\textsuperscript{d} and CTxB was observed (Fig. 3.6B), suggesting that LPS stimulation is somewhat “inhibitory” when combined with αIgM versus αIgM alone. Nonetheless, the intensity of MHC acquisition by thymic DC cultured with activated B cells was far lower than when thymic DC were cultured with TEC (Fig. 3.2B, 3.5A). Overall, these data suggest that factors other than the absolute level of MHC II expression regulate the ability of MHC II to be acquired by thymic DC.

3.4.6 Strong clustering of IgM to lipid rafts promotes nibbling of IgM by DC

Based on the above data, and on other work showing the significance of lipid rafts in various immunological processes (128), we hypothesized that lipid rafts may play a role in mediating thymic DC nibbling of MHC II. However, considering that a significant portion of MHC II is constitutively localized to lipid rafts, including on B cells (132, 133), it is
reasonable to conclude that localization of a given molecule to lipid rafts is not sufficient to permit subsequent nibbling by thymic DC. To directly examine this question, acquisition of another B cell-restricted molecule, surface IgM was examined. In resting B cells, surface IgM is minimally associated with lipid rafts; however upon cross-linking with an αIgM Ab, IgM is rapidly and robustly clustered into lipid rafts (134). First, nibbling of IgM from resting B cells was determined using an αIgM\textsuperscript{a} specific mAb (recognizing BALB/c-derived IgM\textsuperscript{a} but not NOD-derived IgM\textsuperscript{b}). Similar to experiments examining acquisition of IE\textsuperscript{d} from BALB/c B cells, minimal IgM\textsuperscript{a} was acquired by thymic DC despite high expression of IgM\textsuperscript{a} by B cells (Fig. 3.6C). To address whether actively clustering IgM to lipid rafts could promote acquisition by thymic DC, splenic B cells were bound with fluorescent αIgM Ab (αIgM.Ax647) at 4°C to prevent premature IgM internalization, then αIgM.Ax647-coated B cells were cultured with thymic DC at 37°C to induce IgM clustering to lipid rafts. Under these conditions, IgM was acquired by thymic DC in an antibody dose-dependent manner—under stronger stimulation conditions, more IgM was acquired (Fig. 3.6D). Under the conditions used, B cells were strongly labeled with αIgM.Ax647 (Fig. 3.6E). These data suggest that extensive localization of a surface molecule to lipid rafts may be sufficient to promote acquisition by DC via nibbling, though other changes in B cell surface protein expression/organization could also be responsible for enhanced nibbling observed.
3.5 Discussion

While thymocyte development has been extensively studied, many of the mechanisms of thymic Ag presentation remain ill-defined. With this study we investigated one mechanism regulating thymic Ag presentation—the process of intercellular Ag transfer of MHC molecules from mTEC to DC. Initially, we attempted to garner insight into the \textit{in vivo} nibbling of mTEC molecules by thymic DC using EpCAM as a readout. Strikingly, however, EpCAM\textsuperscript{+} thymic DC were found to produce 70-fold more \textit{Epcam} transcript compared to EpCAM\textsuperscript{-} thymic cDC (Fig. 3.1D). In addition, the frequency of EpCAM\textsuperscript{+} thymic cDC was comparable in WT NOD and NOD.AIRE\textsuperscript{-/-} mice (Fig. 3.1E), the latter of which show reduced mTEC-to-DC Ag transfer (78). Collectively, these data suggest that thymic cDC produce EpCAM intrinsically, though we cannot rule out the possibility that some EpCAM is also acquired from TEC. It is notable that surface EpCAM has also been observed on DC in peripheral lymphoid organs, such as skin-draining lymph nodes and the spleen (135), neither of which possesses many EpCAM\textsuperscript{+} stromal cells (136). It is interesting to speculate on the reason thymic DC produce EpCAM intrinsically. EpCAM is a cell surface glycoprotein that mediates homophilic adhesion (137), therefore EpCAM expression by thymic DC could facilitate interaction with EpCAM-expressing TEC (or other EpCAM-expressing DC). This may explain why high levels of EpCAM were observed on DC that acquired a TEC-specific reporter \textit{in vivo} (77). Whether EpCAM facilitates Ag transfer from mTEC to thymic DC could be the subject of future studies.

Using an \textit{in vitro} system to test nibbling, it was found that thymic DC robustly acquire MHC from mTEC (Fig. 3.2B), which can be directly used for T cell stimulation (Fig. 3.4). Based on these experiments, we have estimated that nibbling thymic DC are roughly
10- to 50-fold less efficient T cell stimulators than directly peptide-pulsed TEC. This efficiency is higher than was estimated in a previous study analyzing peptide-MHC I acquisition during bone-marrow derived DC nibbling of other DC (138). There are many differences between this and our own study, including analysis of MHC I versus MHC II, the identity of both the donor and the nibbling populations, and likely the affinity with which the TCR transgenic T cells used as responders recognize the cognate peptide-MHC. Whether the identity of the donor population regulates the efficiency with which nibbling DC can stimulate T cells or thymocytes is unknown. Given identical levels of Ag, our data would suggest that DC that have nibbled mTEC would be more efficient stimulators than DC that have nibbled other DC, based on the relative acquisition of MHC I and MHC II when these cells are cultured together in vitro (Fig. 3.2B-D). Moreover, it is currently unknown whether Ag transfer occurs between thymic DC subsets in vivo. Since two of the three thymic DC subsets are migratory (69, 70, 83), this raises the possibility that Ag acquired in the periphery may then be transferred to intrathymic DC for presentation to thymocytes. This is a particularly attractive possibility in the case of thymic pDC; cognate thymocyte deletion was observed when peptide-pulsed pDC were transferred i.v. (83), yet thymic pDC are poor T cell stimulators compared to thymic cDC ex vivo (139). Formal examination of this possibility warrants future investigation.

Are there signals or factors that govern nibbling of specific molecules, while leaving others unaffected? Our data suggest that this may indeed be the case, and that the specific “signal” may involve membrane organization in the form of lipid rafts, which are readily transferred from both TEC and B cells to thymic DC (Fig. 3.5A-B). Notably, this is the first investigation to consider the role of membrane organization; previous investigations have
relied on general lipophilic dyes that label the entire membrane (130, 140). It is currently unclear, however, whether lipid rafts are “preferentially” transferred versus non-raft membrane microdomains. It is also possible that, while lipid rafts are certainly acquired by nibbling DC, localization of proteins to lipid rafts is not a prerequisite for acquisition. This idea is inferred from experiments in which both MHC I and MHC II are acquired from TEC but not B cells (Fig. 3.2), as MHC II is proposed to constitutively localize to lipid rafts to some degree (132, 141) while MHC I is excluded from lipid rafts (133). Yet, induced clustering of IgM to lipid rafts on B cells via αIgM Ab crosslinking promoted acquisition of IgM by nibbling DC (Fig. 3.6D). Thus, these data suggest that there may be a highly dynamic relationship between cell surface Ag organization and DC nibbling.

It is interesting to speculate on the advantage gained by the immune system via cell-to-cell Ag transfer, particularly in the thymus. Through the production of various PTA driven by AIRE, mTEC promote tolerance to a range of Ag that would not otherwise be available during thymocyte development (18, 73). While some studies have indicated that mTEC possess robust T cell stimulatory capacity ex vivo (142) and contribute in a direct, Ag presentation-mediated manner to the deletion of thymocytes specific for AIRE-driven neo-Ag (79), other studies have documented a necessary role for Ag-transfer to bone marrow-derived cells—most likely DC—for full tolerance induction to mTEC-expressed Ag (75, 77, 78). Perhaps one main reason for the necessity of Ag transfer, especially in the context of MHC II Ag presentation, reflects classical Ag processing and presentation pathways. Ag acquired by DC via nibbling could more efficiently enter MHC II processing pathways as exogenous Ag, whereas these Ag would most likely enter MHC I processing pathways as endogenous Ag in mTEC. Interestingly, mTEC have recently been characterized as having a
high level of constitutive macroautophagy (143), which promotes presentation of endogenous Ag via MHC II (144). Thus, it is most likely that direct Ag presentation by mTEC and indirect Ag presentation consequent to transfer of Ag to DC operate concurrently to enforce central tolerance to mTEC-expressed Ag. Further understanding of the mechanisms by which thymic DC nibbling of mTEC is regulated will help to refine our understanding of the relative role of DC versus mTEC in central tolerance.
Figure 3.1 EpCAM⁺ thymic cDC produce Epcam intrinsically. A) Thymic DC were enriched and surface EpCAM levels were assessed via FACS. Thymic DC are gated from Live/Lin⁻/CD11c⁺ (cDC) or Live/Lin⁻/CD11c⁺/CD45RA⁺ (pDC). Gate shown on Lin⁻/CD11c⁻ cells represent a small number of TEC that are also enriched during DC isolation. Lin = CD3ε/CD19/DX5/Ter-119. The EpCAM⁺ gates were set based on EpCAM staining of Lin⁻ cells, which include TEC (right-most panel). B) Data compiled from a representative analysis as in (A). n=3. Representative of at least 3 independent experiments. C) Representative pre-sort and post-sort analysis of EpCAM⁻ and EpCAM⁺ cDC used for subsequent RT-qPCR analysis of Epcam transcript. Cells are gated on Live/CD45⁺/CD11c⁺. D) Relative expression of Epcam by thymic DC (normalized to EpCAM⁻ cDC = 1). Data are pooled from 3 independent experiments. E) Thymic DC were enriched from NOD and NOD.AIRE⁻/⁻ mice and analyzed as in (A). n=3. All error bars represent SEM.
Figure 3.2 Thymic DC can nibble MHC I and II from TEC and DC but not B cells. A) Cartoon of Nibbling Assay design. Thymic DC are isolated from NOD mice while TEC are isolated from BALB/c mice. The cells are co-cultured for 3-4 h and then DC acquisition of IE\textsuperscript{d} and H2D\textsuperscript{d} is assessed. B-E) Staining for IE\textsuperscript{d} and H2D\textsuperscript{d} on NOD thymic DC after co-culture with BALB/c B) TEC, C) thymic DC, D) splenic DC, or E) splenic B cells. All cells were cultured at 1:1 ratios except for TEC (1:2 TEC:DC). Data are representative of 2-3 experiments. F) Level of IE\textsuperscript{d} and H2D\textsuperscript{d} expression by donor cell populations used in (B-E). Donor cells were not analyzed side-by-side, but each graph pairs with representative nibbling analysis shown in (B-E).
Figure 3.3 Splenic DC also nibble MHC I and II, but less efficiently than thymic DC. A) NOD thymic and splenic DC were isolated and then co-cultured with BALB/c TEC as in Fig. 3.2A for analysis of DC nibbling of IE^d and H2D^d. Representative graphs show culture at 1:2 (TEC:DC) ratio. B) Percent IE^d+ and H2D^d+ of NOD thymic and splenic DC after co-culture with BALB/c TEC. C) MFI of IE^d and H2D^d on nibbling thymic and splenic DC. DC were held at a constant 250 x 10^3/well. Data are representative of 2-3 similar analyses.
Figure 3.4 Peptide-MHC II nibbled from TEC are functional. A) Representative pre-sort and post-sort analysis of BALB/c thymic DC after 4 h culture with peptide-pulsed NOD TEC (1:1 TEC:DC). Cells are gated on Live cells. All CD11c+ DC sorted were also CD45.2+. B) Thymic DC and TEC were separated via FACS after co-culture for 4 h, then cultured for 3 d with CellTrace Violet-labeled BDC2.5 CD4+ T cells. TEC were used at a 1:10 (TEC:T cell) ratio, and DC were used at a 1:2 (DC:T cell) ratio. BDC2.5 cells were held at a constant 1x10^5/well. Cells are gated on Live/Thy1.2+/CD4+ cells. C) Data pooled from 3 independent experiments as in (B) showing mean ± SEM percent Proliferated.
Figure 3.5 DC nibble lipid rafts from TEC and B cells in a contact-dependent manner.

A-B) NOD thymic DC were purified and co-cultured with CTxB-labeled A) TEC (1:2 TEC:DC) or B) splenic B cells (1:1 B:DC) from BALB/c mice, and DC acquisition of IE$^d$ and CTxB was assessed via FACS. Data are representative of 3 experiments. C) BALB/c TEC were isolated and CTxB labeled as in (A), then co-cultured with DC (1:2 TEC:DC) in a 0.4 μm TransWell (TW) apparatus as indicated. D) BALB/c B cells were isolated, labeled with CTxB, and then co-cultured with NOD DC (1:1 B:DC) in a TW apparatus as indicated in (C). Data are representative of 2-3 experiments. E) Thymic DC were pre-incubated with or without a blocking αSR-A mAb, then CTxB-labeled TEC were added (1:2 TEC:DC) as in (A).
Figure 3.6 Acquisition of MHC II and IgM from activated B cells. A) BALB/c B cells were purified and stimulated as indicated for 16-20 h, and then expression of IE\textsuperscript{d} analyzed via FACS. Histogram is gated on Live/CD19\textsuperscript{+} B cells. Inset values represent IE\textsuperscript{d} MFI. B) BALB/c B cells stimulated as in (A) were co-cultured with NOD thymic DC (1:1 B:DC), and DC acquisition of IE\textsuperscript{d} and CTxB examined. Data are representative of 2-3 experiments. C) Nibbling of BALB/c-derived IgM\textsuperscript{a} from resting splenic B cells by NOD thymic DC. Bottom left panel indicates level of IgM\textsuperscript{a} on BALB/c B cells used in the assay. D) B cells were purified, coated with the indicated concentration of \(\alpha\text{IgM.Ax647}\), washed, and then co-cultured with NOD thymic DC. DC acquisition of IgM was then assessed via FACS. E) Level of IgM.Ax647 on B cells used in (D). B cells are gated on Live/CD45.2\textsuperscript{+}/CD45RA\textsuperscript{+}.
CHAPTER 4: Discussion

DC contribute to both immunity and tolerance in many critical ways. Original studies did not identify DC in the thymus (145) on the basis of adherence properties and cytosolic organelle composition as assessed via phase contrast microscopy. As techniques for identifying cells have progressed, we now know that DC do inhabit the thymus and contribute to both thymocyte clonal deletion (19) and tTreg induction (94). The studies presented herein further enhance our understanding of thymic DC by illuminating the factors regulating thymic DC homeostasis and antigen acquisition.

In the first part of our study, the factors regulating thymic DC homeostasis were studied. It was found that feedback during Ag-specific interactions with SP thymocytes was critical for fostering normal thymic DC numbers, phenotype, and function. In this context, we employed two model systems: 1) NOD.TCRα−/− mice, in which thymocyte development is blocked at the immature TCR−DP stage, and 2) TCR transgenic mice into which cognate Ag was injected. The second model system used provides many interesting opportunities for future studies. While our data show that TCR-MHC interaction is necessary for feedback to occur, we subsequently identify CD40L as the primary molecular mechanism during CD4SP-mediated feedback. The role of affinity/avidity of the cognate TCR-MHC interaction during DC:thymocyte crosstalk was not examined. This could be addressed by using the same TCR transgenic mice and injecting peptides of differing affinities, or by employing additional TCR transgenic models in which the affinity of the cognate TCR-MHC interaction varies. Such studies may determine an “affinity threshold” at which Ag-specific interactions provide sufficient feedback to promote thymic DC maturation. Also in this context, it is worthwhile to note that the effects of ligation of CD40 by an agonist mAb and the feedback provided
during cognate TCR-MHC interaction were not identical (Fig. 2.5E-F, 2.8A). Thus, while CD40L is necessary during Ag-specific interactions with CD4SP thymocytes (Fig. 2.8B), additional factors are also likely involved. Further addressing the relationship between TCR-MHC affinity and DC:thymocyte crosstalk could help to identify additional factors involved in thymocyte feedback to DC.

In our studies of cognate Ag administration to TCR transgenic mice, we note significant activation and apoptosis of thymocytes under the conditions used (Fig. 2.6). Thus, our data support a model in which feedback to DC is provided specifically during interactions most consistent with negative selection. However, as thymic DC also induce tTreg induction (94), it is possible that feedback could be provided during interactions resulting in tTreg formation. To our knowledge, it is currently unknown whether thymocytes undergoing tTreg induction upregulate/express a similar array of surface molecules as thymocytes undergoing negative selection, though both outcomes are dependent on cognate Ag recognition by thymocytes. Notably, however, CD40-CD40L interactions are critical for normal development of tTreg cells (96, 146). Thus, it seems likely that DC:thymocyte interactions resulting in either negative selection or tTreg induction would provide feedback to promote thymic DC maturation.

One highly novel aspect of our studies is the identification of a role for CD8SP-derived feedback in regulating the homeostasis of thymic DC (Fig. 2.5F); by comparison, mTEC only accept feedback signals from CD4SP (103). Not surprisingly, CD8SP feedback was found to be independent of CD40L (Fig. 2.8C) (101, 104). Thus, the identification of the molecular mechanism of CD8SP-derived feedback to thymic DC is of great interest and significance. Due to the utility of mAb blockade in identifying CD40L as a molecular
mechanism of CD4SP-derived feedback (Fig. 2.8B), a similar approach in CL4.scid mice could be used to identify the molecular mechanism of CD8SP-derived feedback. Interestingly, our findings with CD4SP and CD40L identify an analogy between peripheral CD4+ T cell and CD4SP feedback during cognate interactions with DC. Could a similar relationship between CD8+ T cell and CD8SP feedback exist? In this scenario, granulocyte-macrophage colony-stimulating factor (GM-CSF) may represent an interesting candidate for investigation during CD8SP feedback to DC. GM-CSF has recently been shown to promote CD40-independent DC activation during cognate CD8+ T cell:DC interactions (147), and a neutralizing αGM-CSF mAb is readily available and efficacious in vivo (148). Further candidates could be identified by transcriptional or proteomic profiling of CD8SP thymocytes, or by use of existing resources such as the Immunological Genome Project (www.immgen.org) (149, 150). These approaches represent promising means of further understanding the molecular mechanisms of DC:thymocyte crosstalk.

In the second part of our study, the factors regulating thymic DC nibbling of mTEC MHC were investigated. We found that mTEC serve as uniquely efficient MHC donors for nibbling DC, whereas B cells are inefficient MHC donors. It seems likely that this mechanism has developed purposefully, possibly to overcome or work in concert with the relatively poor inherent stimulatory capacity of mTEC via MHC II-mediated Ag presentation (75, 77, 144). In fact, the thymic medulla has specific mechanisms in place to foster such interactions between mTEC and DC. Certain chemokines are produced by mTEC to promote thymic DC migration into the medulla (78, 118), many in an AIRE-dependent manner. This could further promote preferential interaction with AIRE-expressing mTEC, which produce many critical PTA to which central tolerance must be enforced (18, 151, 152). Furthermore,
DC in the medulla are typically observed tethered to thymic stromal cells (153), which may facilitate extensive Ag exchange with mTEC in vivo. As thymocytes also utilize stromal cells as “highways” for migration throughout the thymus (153, 154), DC are also optimally positioned for presentation of Ag—whether produced intrinsically or acquired from mTEC—to medullary thymocytes. Thus, the thymic medulla represents a unique environment designed to facilitate extensive interaction between mTEC, DC, and developing thymocytes and promote central tolerance.

As mentioned above, resting B cells served as poor donors during nibbling as assessed by the acquisition, or lack thereof, of both surface IgM and MHC (Fig. 3.2, 3.6). On the other hand, when IgM was actively clustered to lipid rafts via Ab crosslinking, acquisition of IgM was observed (Fig. 3.6D). These results raise interesting questions as to how DC define which molecules should be acquired versus ignored during nibbling. Could part of this preference be for densely clustered surface molecules? Dense clustering of surface receptors is typically induced by extensive interaction with a cognate ligand (132), thus heavily clustered molecules could represent molecules of the greatest utility in a given environment. Actively ignoring non-clustered surface molecules could help prevent acquisition of irrelevant molecules by DC. Interestingly, DC have also been shown to acquire intracellular Ag (130, 140), including nuclear self-Ag (77). It is tempting to speculate that heavy clustering of intracellular Ag could also facilitate acquisition by DC. For example, prior to export to the cell surface, peptide-MHC II complexes accumulate in discrete intracellular vesicles (155). Investigating whether distinct signals regulate acquisition of surface versus intracellular Ag will help to further understand the regulation of thymic DC Ag acquisition and presentation during central tolerance.
When taken as a whole, the topics studied herein illustrate the complexity of APC biology in the thymus. Historically, much of the discussion of thymic Ag presentation has focused on whether a “division of labor” exists among cTEC, mTEC, DC, or macrophages (156). While it is clear that this may be the case when comparing cTEC to medullary APC such as mTEC and DC, it also now appears evident that this is most likely not the case when comparing mTEC and DC (157). Furthermore, it is also clear that there is an inextricable linkage between thymocyte negative selection and the maintenance of thymic APC homeostasis: in the absence of signals provided by negatively selected thymocytes, thymic APC (both mTEC and DC) are significantly perturbed, and in the absence of “normal” thymic APC, negative selection is less efficient. The level of complexity of thymic Ag presentation and the regulation of thymic APC homeostasis ensures that additional secrets of central tolerance are yet to be uncovered. Continued exploration of these topics is crucial to our understanding of the means by which T cell tolerance is maintained.
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