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Positive and negative selection of the T cell repertoire: what thymocytes see and don't see

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Abstract

The fate of developing T cells is specified by interactions of their antigen receptor with self-peptide/MHC complexes displayed by thymic antigen presenting cells (APCs). Various thymic APCs subsets are strategically positioned in particular thymic microenvironments and orchestrate the selection of a functional and self-tolerant T cell repertoire. Here, we will review the different strategies that these APCs employ to sample and process self-antigens and thereby generate partly unique, 'idiosyncratic' peptide/MHC ligandomes. We will discuss how the particular composition of these APC-subset-specific peptide/MHC ligandomes not only shapes the T cell repertoire in the thymus, but may also indelibly imprint the behavior of mature T cells in the periphery.

The recognition of self-peptides that are embedded in major histocompatibility complex (MHC) molecules on thymic antigen-presenting cells (APCs) is critical for determining the fate of developing αβ T cells. Somewhat paradoxically, recognition of self can elicit diametrically opposed outcomes. On one hand, it is essential for thymocyte survival and commitment to either the $CD4^+$ or $CD8^+$ T cell lineage (that is, for **positive selection** of thymocytes). On the other hand, recognition of self can be a death verdict for thymocytes, mediating the **negative selection** of these cells, or it can skew cells to alternative fates, such as regulatory T (T_{Reg}) cell differentiation. The classical affinity model of thymocyte selection offers an attractive conceptual framework to resolve this apparent contradiction (**Box 1**). However, it does not take into account the fact that positive and negative selection largely occur in discrete thymic microenvironments, namely the cortex and the medulla, respectively. Both compartments contain selection niches composed of different types of APCs (**Figure 1**), thereby providing microenvironments that orchestrate a spatial and temporal segregation of thymocyte selection. In this Review, we will focus on recent advances in our understanding of key features of individual thymic APC subsets and discuss how these relate to the generation of a functional and self-tolerant αβ T cell repertoire.

Antigen presentation in the cortex

At the peak of its productivity, the mouse thymus each day generates around fifty million $CD4^+CD8^+$ double positive (DP) thymocytes that audition for selection[1]. More than 90% of these precursors are subject to **death by neglect**, as they express 'useless' T cell receptors (TCRs) that do not mediate positive selection. Positive selection of 'mainstream' αβ T cells is contingent upon permissive interactions with a single APC type, namely cortical thymic epithelial cells (cTECs). For conceptual clarity, we will therefore restrict a more detailed discussion of antigen presentation in the cortex to cTECs and their role in positive selection, and will only briefly touch upon negative selection in the cortex at the end of this section.

Cortical epithelial cells

cTECs are arranged in a three dimensional scaffold that supports intimate interactions with double negative (DN) and DP thymocytes. In addition, individual cTECs can form multi-cellular complexes that encompass up to 20 thymocytes and are referred to as thymic nurse cells (TNCs). TNC numbers are decreased in TCR-transgenic mice, possibly as a consequence of 'facilitated' transit of thymocytes through **β-selection** and positive selection [2]. Thus, it seems that TNC formation is not essential for T cell development *per se*, but may result from lengthy 'audition' events that occur when only a small subset of DP thymocytes meets the positive selection criteria. Consistent with this, in non-TCR transgenic mice, TNCs were enriched in thymocytes harbouring secondary TCRα rearrangements[2]. Whether such unusual selection niches are indeed required to promote thymocyte survival and/ or continued TCR rearrangements remains to be shown.

Why is positive selection crucially dependent on a single stromal cell type, when tolerance, as discussed further below, can be mediated by a variety of cell types? One might assume that the essential function of cTECs simply depends upon their location and abundant surface expression of MHC molecules. However, this is not the case. Instead, it is becoming increasingly clear that the crucial role of cTECs is, at least in part, a result of the unique machineries that these cells use to process antigens. It is likely that these proteolytic pathways (**Figure 2**) – discussed in detail in a previous review [3] – endow cTECs with a largely unique **peptide–MHC (pMHC) ligandome** that is distinct from that displayed by any other thymic or peripheral APC.

Antigen processing in cTECs

In terms of MHC class I antigen presentation, cTECs express a unique catalytic subunit of the **proteasome** referred to as β5t. Proteasomes that incorporate β5t are referred to as 'thymoproteasomes'. They have a substrate preference that is distinct from proteasomes containing the β5 or β5i subunits [4] (termed 'housekeeping proteasomes' and 'immunoproteasomes', respectively). Mice lacking thymoproteasomes show a substantial defect in positive selection of $CD8^+$ T cells [5].

In terms of MHC class II antigen presentation, cTECs express the unique lysosomal proteases cathepsin L and thymus-specific serine protease (TSSP). Deficiency in these proteases results in impaired selection of $CD4^+$ T cells. Cathepsin L-deficient mice show a

strongly decreased polyclonal CD4$^+$ T cell repertoire in the thymus [6], whereas TSSP deficient mice have normal polyclonal CD4$^+$ T cell numbers, yet display defective positive selection of certain MHC class II-restricted transgenic TCRs as well as altered antigen-specific CD4$^+$ T cell responses [7]. Moreover, cTECs display an unusually high rate of constitutive **macroautophagy**, a mechanism that can support the 'unconventional' loading of peptides onto MHC class II molecules via an endogenous route [8]. Positive selection of several MHC class II-restricted transgenic TCRs was altered upon interference with macroautophagy in thymic epithelium, consistent with the idea that autophagy shapes the MHC class II ligandome of cTECs [9].

Bearing in mind that the avidity/affinity model of thymocyte selection does not envisage any need for unique positively selecting peptides, why may these distinct processing pathways have evolved? Do they generate 'private' peptides that are exclusively displayed by cTECs and that have unique properties required for positive selection? Or do these peptides simply dilute ubiquitous 'public' peptides, which are nonetheless the major mediators of positive selection? Alternatively, do peptides on cTECs merely have to be different from those presented by other thymic APCs? The latter proposition is supported by the finding that the reconstitution of cathepin L–deficient mice with MHC class II$^{-/-}$ bone marrow, which abrogates negative selection of CD4$^+$ T cells by hematopoietic APCs, largely rescued their CD4$^+$ T cell compartment [10]. This indicates that positive selection of CD4$^+$ T cells by Cathepsin L-deficient cTECs is not per se inefficient; however, an unusually large fraction of cells selected in this way are subject to negative selection. Hence, positive selection on different (but not functionally unique) ligands might be necessary to prevent a disproportionate loss of T cells due to subsequent re-encounter of the very same peptides that mediated positive selection in a 'negatively selecting setting', that is, on medullary APCs that express abundant co-stimulatory molecules [3]. Nevertheless, several observations concerning the role of the thymoproteasome for the selection of CD8$^+$ T cells suggest a different scenario. Thus, neither the reconstitution with MHC class I-deficient bone marrow cells nor the inactivation of *Bim* rescued the CD8$^+$ T cell compartment of thymoproteasome-deficient mice [11, 12]. Therefore, the role of thymoproteasome-dependent peptides cannot be to avert excessive thymocyte deletion. Gene-replacement experiments provide further evidence for the notion that it is the actual nature of the peptides generated by the thymoproteasome, rather than a mere difference between the pMHC repertoires of cTECs and other APCs, that matters. By inserting *β5i* into the *β5t* gene locus in *β5i*$^{-/-}$ mice, animals were engineered in which, independent of β5t, the MHC class I ligandomes differed between cTECs and other APCs (in this case shaped by the immunoproteasome versus the housekeeping proteasome, respectively) [12]. This difference alone did not restore positive selection in these animals; by inference, peptides generated by β5t-containing thymoproteasomes are not only different, but may somehow bear unique biophysical features related to positive selection.

The putative significance of 'private' peptides

How could 'private' peptides on cTECs be specialized for positive selection? They might bind MHC molecules more weakly, as suggested by the observation that β5t-containing proteasomes, in contrast to those harbouring β5 or β5i, inefficiently cleave substrates

adjacent to hydrophobic amino acids [5, 13]. MHC class I molecules preferentially bind peptides with hydrophobic C-termini. Therefore, wobbly binding of β5t-derived peptides might result in a faster TCR off-rate and thereby promote positive selection, a scenario similar to the generation of partial agonists by altering the **MHC anchor residues** of immunogenic peptides [14]. Although attempts to compare the stability of pMHC complexes on cTECs with that on other APCs have so far failed to disclose such differences [11, 12], there is independent evidence that β5t engenders a bias towards 'weak' interactions for positive selection. **CD5** expression-levels on SP thymocytes are thought to reflect the signalling intensity of the positively selecting TCR–pMHC interaction, and 'tuned' CD5 levels persist on mature peripheral T cells as a footprint of thymic selection [15]. Intriguingly, the diminished $CD8^+$ SP compartment found in $\beta5t^{-/-}$ mice is mostly composed of cells expressing elevated levels of CD5 and also **Nr4a1**, suggesting that positive selection in the absence of β5t mostly entails interactions of relatively higher affinity [12]. In the same vein, TCR transgenic studies showed that selection of 'natural' $CD5^{low}$ clones, such as $CD8^+$ T cells expressing the **HY TCR**, is highly dependent on β5t, whereas selection of $CD5^{hi}$ clones, such as those expressing the **OT-I TCR,** is not, although amongst five different TCR transgenics the extent of β5t dependency did not show a perfect inverse correlation with CD5 expression levels [11]. Thus, thymoproteasome-derived peptides, and possibly private peptides generated through other cTEC-specific pathways in general, might favour selection of $CD5^{lo}$ T cell clones.

Selection of TCRs across an affinity range for self

It has been suggested that the re-exposure of mature T cells to their positively selecting peptide(s) is necessary for homeostasis through continual **tonic TCR stimulation**[16]. According to this scenario, T cells selected on 'private' pMHC ligands that are not re-encountered outside the thymus are predicted to have a competitive disadvantage during steady state homeostasis. Consistent with this idea, mature $CD5^{low}$ T cells in secondary lymphoid tissues are indeed less responsive to homeostatic cytokines when compared to their $CD5^{hi}$ counterparts[17, 18]. In further support of such a link between thymic pMHC-experience and mature T cell homeostasis, $CD5^{low}$ T cells expressing the β5t-dependent HY TCR are notoriously poor at homeostatic proliferation, whereas $CD5^{hi}$ cells expressing the OT-I TCR, which is selected fairly efficiently in the absence of β5t, show robust homeostatic expansion [11]. Also, TCRs of $CD5^{low}$ cells, in distinction from those of $CD5^{hi}$ cells, are less 'pre-loaded' with basal phosphorylation of TCRç, which might put them at a competitive disadvantage in responding to foreign antigens[16, 19]. Indeed, in several infection models in which polyclonal $CD4^+$ T cell responses to pathogens were examined, $CD5^{hi}$ T cells out-competed $CD5^{low}$ T cells. This observation lead to the suggestion that the *raison d'etre* of positive selection, rather than imprinting self-MHC restriction, is to bias T cell selection towards strongly self-reactive clones endowed with a homeostatic advantage and a head start in anti-pathogen responses [19]. Hence, the idea that private peptides serve the purpose of skewing positive selection towards $CD5^{low}$ T cells that weakly respond to self may appear counter-intuitive.

$CD5^{low}$ cells nonetheless do constitute a considerable fraction of the *steady state* T cell repertoire [19]. So why would this be beneficial or even necessary for a functional immune

system? First, a repertoire solely composed of clones with high self-reactivity might be prone to incite autoimmunity. However, there is as yet no evidence to support this notion. For instance, β5t$^{-/-}$ mice display intact negative selection [11] and do not exhibit any signs of autoimmunity. Second, the presumed competitive disadvantage of CD5^{low} clones selected through low-affinity interactions may in fact not be a general rule. Persaud *et al.* compared two CD4^{+} T cell clones with identical affinity for a *Listeria* antigen, one presumably selected and maintained by weak interactions with *self*, as inferred from its CD5^{lo} phenotype, the other by stronger interactions, i.e. being CD5^{hi} and displaying higher TCRç phosphorylation [20]. This alleged differential signalling strength during positive selection correlated with differences in basal TCR signalling in mature peripheral cells. Thus, the CD5^{hi} clone was poised to produce more IL-2, whereby this property, consistent with the 'tonic signalling' hypothesis, was actively maintained in the periphery through recognition of unknown self-pMHC ligands. Regardless of this difference, in the course of a *Listeria* infection, CD5^{low} and CD5^{hi} T cells showed comparable proliferative responses, and the CD5^{hi} clone eventually underwent more extensive cell death, so that in this case the CD5^{low} clone ultimately dominated the immune response.

Taken together, unusual antigen processing in cTECs seems to diversify the T cell repertoire for maximal versatility, as best exemplified by the thymoproteasome and CD8^{+} T cell selection. Interference with this cTEC-specific pathway of pMHC generation results in a 'crippled' CD8^{+} T cell repertoire that seems dominated by T cells with higher affinity for self antigens. Corresponding consequences of Cathepsin L- or TSSP-deficiency for the peripheral CD4^{+} T cell repertoire have yet to be described.

Negative selection in the cortex

As pointed out in the beginning, the vast majority of thymocyte death in the cortex can be attributed to failure of a large fraction of DP cells to undergo positive selection [21]. Nonetheless, there is also a substantial loss of DP thymocytes through negative selection. Recent data show that the number of thymocytes dying through negative selection in the cortex is in fact much higher than previously appreciated and may even exceed the number of cells that pass through positive selection [22, 23]. Using a TCR signalling reporter to identify thymocytes that were rescued from deletion in mice lacking **Bim**, it was estimated that 5×10^5 cells per day undergo negative selection in the cortex [23]. This figure not only exceeds the estimated number of positively selected cells, but is also around two-fold higher than the number of cells believed to undergo negative selection in the medulla.

Intriguingly, cortical negative selection of thymocytes specific for 'ubiquitous' self-antigens was shown to depend on a crucial contribution of dendritic cells (DCs). The heterogeneity and functional attributes of thymic DCs will be discussed in the section on medullary APCs. At this point, it may suffice to highlight that the critical role of DCs in cortical negative selection is all the more remarkable considering that there are very few DCs in the cortex compared with the medulla and because 'ubiquitous' antigens are predicted to also be displayed by cTECs [24]. Possibly, these observations reflect an inherent inefficacy of cTECs to support negative selection. Consistent with this, imaging analyses of cortical negative selection *in situ* revealed that thymocytes arrest and signal adjacent to DCs, even when

antigen is also displayed by cTECs [25]. Because these experiments involved exogenous delivery of agonist peptide, cTEC-specific pathways of antigen processing are unlikely to be the sole determinant of this impaired capacity of cTECs to induce clonal deletion. Future experimentation is needed to assess the contribution of other candidate parameters such as co-stimulation, cell-adhesion and MHC-turnover.

Antigen presentation in the medulla

The medulla serves a crucial function for T cell tolerance induction, as a disarrayed 3D architecture of the medulla, disturbed development of its stromal components, impaired transit of positively selected thymocytes into or premature egress from the medulla all result in spontaneous manifestations of autoimmunity (reviewed in [3, 26]). Central hallmarks of the thymic medulla that specify this critical tolerogenic role are on the one hand the 'ectopic' expression of a myriad of otherwise strictly tissue-restricted antigens (TRAs) by medullary thymic epitelial cells (mTECs) and on the other hand the unique ensemble of hematopoietic APCs that seed this microenvironment.

Medullary thymic epithelial cells

The phenomenon of promiscuous gene expression in mTECs has been reviewed in detail elsewhere [27, 28]. Some salient features of promiscuous gene expression and novel insights are highlighted in **Box 2**. Although the entire mTEC population collectively expresses almost all 'peripheral' transcripts, each TRA is only expressed by a minor fraction (1–3%) of mTECs at any given time **(Figure 3)**. How this mosaic expression pattern ultimately translates into faithful presentation of thousands of self-antigens in a way that ensures efficient tolerance remains puzzling.

Self antigens expressed by mTECs may be seen by T cells in two ways **(Figure 3)**: first, through 'autonomous' presentation by mTECs themselves or, second, through antigen hand-over and presentation by neighbouring APCs. Direct presentation of endogenously expressed antigens by mTECs can not only induce negative selection of $CD8^+$ T cells [29, 30] but also efficiently elicits $CD4^+$ T cell tolerance [31-34]. At the same time, mTECs are conspicuously inefficient in 'conventional' MHC class II presentation of extracellular substrates [35, 36]. Hence, mTECs apparently evolved strategies to bypass the classical exogenous pathways of MHC class II loading in order to focus their MHC class II-ligandome on endogenous self-antigens.

Endogenous MHC class II loading in mTECs

How do mTECs load MHC class II molecules with intracellular antigens? Candidate pathways fall into two categories (reviewed in [8]). The first comprises proteasome- and TAP-dependent mechanisms, implying a leakage of the ER-content into MHC class II loading compartments. The second category comprises processes collectively known as autophagy ('*self eating*'): microautophagy, chaperone-mediated autophagy and macroautophagy. Their common principle is the delivery of cytoplasmic constituents to lysosomes, which presumably intersect with the MHC class II loading pathway [37]. So far, only the role of macroautophagy has been examined in the context of thymocyte selection. Athymic nude

mice grafted with macroautophagy-deficient thymi displayed various manifestations of immune-mediated tissue-damage, consistent with a crucial function of macroautophagy in TECs for loading peptides onto MHC class II molecules for T cell repertoire selection [9]. However, these studies left open whether the observed symptoms actually reflected a failure of negative selection by mTECs or were driven by impaired positive selection by autophagy-deficient cTECs, two not mutually exclusive possibilities.

More recent work provided compelling evidence that macroautophagy indeed supports tolerogenic endogenous MHC class II loading in mTECs. When two closely related model antigens were targeted to the cytosol of mTECs, a variant that was earmarked for autophagosomal degradation was presented with much higher efficacy and displayed a superior capacity to induce negative selection of $CD4^+$ T cells [38]. The same study also showed that a mitochondrial version of a model-antigen did require macroautophagy for tolerogenic presentation by TECs, whereas direct presentation of a membrane-bound form of the same antigen was macroautophagy-independent [38]. Possibly, endogenous access to MHC class II of substrates residing in the cytoplasm or within organelles, such as mitochondria, peroxisomes or the nucleus, may generally require macroautophagy, consistent with the role of autophagy in sampling these sub-cellular compartments [39]. By contrast, membrane proteins seem to be inherently prone to access MHC class II loading compartments independently of macroautophagy [40].

Direct versus indirect presentation of self antigens by mTECs

A clear delineation of the quantitative or qualitative impact of direct versus indirect presentation of TRAs by mTECs or DCs (or any other thymic APC for that matter), respectively, is only slowly emerging, partly due to potential redundancies between the two mechanisms. Relying on transgenic neo-self antigens, there is a wealth of information supporting the idea that direct presentation by mTECs is an exquisitely efficient tolerance mechanism (reviewed in [41]). At the same time, there is accruing evidence that the medulla provides a specialized micro-milieu conducive to intercellular antigen transfer [42]. Yet, few experimental models document an essential requirement for such antigen hand-over, and some of these findings remain controversial [29, 43]. In a recent study, MHC class II-tetramers were employed to monitor steady state negative selection of polyclonal $CD4^+$ T cells reactive to interphotoreceptor retinoid-binding protein (IRBP), an AIRE-dependent TRA exclusively expressed by mTECs. Ablating MHC class II expression in hematopoietic cells abolished negative selection of T cells specific for this physiologically expressed self antigen, indicating an essential requirement for intercellular transfer between antigen-expressing mTECs and antigen-presenting hematopoietic APCs, at least for certain epitopes of IRBP [44].

A conclusive dissection of the dual role of mTECs (as antigen providers and presenters) in tolerizing the polyclonal T cell repertoire remains experimentally challenging. Selective ablation of either MHC class I or MHC class II expression on mTECs by conditional gene targeting has been surprisingly difficult to achieve. A further caveat of such an approach is that MHC class II-dependent **‘thymic crosstalk’** between thymocytes and mTECs organizes mTEC differentiation [45], so that abolition of MHC class II on mTECs will most likely affect

promiscuous gene expression in qualitative or quantitative terms. In order to avoid such confounding effects an experimental strategy of tissue-specific knockdown of MHC class II molecules in transgenic mice (termed ***C2TAkd* mice**) has been devised [31]. The selective attenuation of antigen presentation by mTECs in these mice resulted in sporadic bouts of mild tissue infiltrations, yet did not elicit overt autoimmunity. These findings contrast with the spontaneous autoimmunity ensuing from AIRE-deficiency or even from selectively abrogating the expression of single TRAs in mTECs [46, 47]. At first glance, this could be interpreted to indicate that direct antigen presentation to $CD4^+$ T cells by mTECs, in contrast to TRA expression, is not essential to prevent autoimmunity; however, it is equally possible that the residual MHC class II expression on mTECs in *C2TAkd* mice might still suffice to censor auto-reactive $CD4^+$ T cells at the high affinity-end of the TCR spectrum.

In further support of a substantial autonomous contribution of mTECs as APCs for negative selection of polyclonal $CD4^+$ T cells, the $CD4^+$ SP thymocyte compartment in *C2TAkd* mice was markedly enlarged. In fact, when compared, the decreased expression of MHC class II molecules on mTECs in these mice and the complete ablation MHC class II expression on DCs in *MHC class* $II^{-/-} \rightarrow$ *WT* BM chimeras had a similar impact on the degree of negative selection within the $CD4^+$ SP thymocyte compartment [31]. Moreover, combining hematopoietic MHC class II deficiency with MHC class II reduction on mTECs had an additive effect, suggesting a non-redundant contribution of both DCs and mTECs to negative selection.

Given the low frequency of mTECs that express and hence potentially present a given TRA [1], 'saturating' tolerance induction through direct presentation would require exceedingly efficient scanning of numerous mTECs by thymocytes. Real-time imaging of thymocyte motility showed that this is indeed the case: SP thymocytes 'randomly walk' within medullary areas at a velocity of 10 μm/min, allowing them to engage in multiple contacts with APCs[48-50]. Estimates of the number of APCs that can be scanned within the 4-5 day sojourn of SP cells in the medulla vary from a few hundred to several thousand [1, 49, 51]. Bio-informatic modelling based on available TRA (co-)expression data at the single-cell level [52-54] predicts that 200 to 500 mTECs will be sufficient to cover the full TRA repertoire at a given point in time (B.K., H. Mayer and S. Pinto). Shifting TRA expression patterns over time and corresponding fluctuations in the pMHC ligandome of individual mTECs would further reduce the minimal number of cells that need to be scanned, provided that T cells re-encounter the same mTEC over time [49, 53]. Notwithstanding a considerable error margin in these calculations, it seems that T cells may not even need to roam through large volumes of the medulla in order to saturate TRA encounters resulting from autonomous presentation by mTECs.

Thymic dendritic cells

The overall contribution of DCs to the total thymic cellularity is in the order of 0.5%. Thymic DCs can be subdivided into three major subsets [55], two of which belong to the conventional (also known as classical) DC (cDC) lineage, whereas the remaining third of thymic DCs belongs to the plasmacytoid DC (pDC) lineage. The heterogeneity of DCs in the thymus raises obvious issues as to a possible functional specialization of individual

subtypes. Determinants of such a division-of-labour could be cell-biological features pertaining to APC function (antigen uptake and processing), intra- versus extra-thymic origin and the positioning within distinct thymic microenvironments. All these features will eventually define the sampling territories of each subset and hence its self peptide–MHC ligandome.

Resident versus migratory cDCs

Around two thirds of thymic DCs can be classified as CD11c^{hi}CD45RA^{-} cDCs. These can be further subdivided according to differential co-expression of CD8α and SIRPα, with roughly two thirds of thymic cDCs displaying a CD8α^{+}SIRPα^{-} and one third a reciprocal CD8α^{-}SIRPα^{+} surface phenotype [55]. The major CD8α^{+}SIRPα^{-} cDC subset originates from an intrathymic differentiation pathway, and hence these cells are commonly referred to as ‘resident’ cDCs, whereas the minor CD8α^{-}Sirpα^{+} cDC subset is maintained by steady state immigration from the periphery, and these cells are therefore referred to as migratory cDCs [56].

Resident cDCs in the thymus bear obvious phenotypic resemblance to CD8α^{+} cDCs in the periphery. The latter are known to be particularly efficient in cross-presentation, that is, the presentation of exogenous antigens in the context of MHC class I [57]. Thymic CD8α^{+}SIRPα^{-} cDCs indeed also showed a superior cross-presentation capacity *in vitro* when compared to the migratory subset [58]. *In vivo*, intrathymic cross-presentation was found to contribute to CD8 T cell tolerance towards a model-antigen mimicking a TRA-like expression pattern in mTECs [29]; since these studies did not address the identity of the cross-presenting cell type, it remains to be established whether there is a differential contribution of resident versus migratory cDCs in this context.

Although, on the whole, DCs are markedly more abundant in the medulla than in the cortex, it is unclear whether this applies in equal terms to both migratory and resident cDCs. Recent work has identified the chemokine XCL1 (also known as lymphotactin) as a crucial determinant of the medullary localization of cDCs [59], as *Xcl1*-deficient mice have fewer medullary cDCs. Although not directly addressed in this study, the fact that only CD8α^{+} cDCs express the receptor for XCL1 (XCR1) suggests that this mis-localization primarily affects resident, but not migratory, cDCs. As mTECs are the only thymic stromal cells producing XCL1 (notably in an AIRE-dependent manner), the XCL1–XCR1 chemokine axis may orchestrate the localisation of resident cDCs next to mTECs. Such a close apposition should facilitate the transfer of mTEC-derived TRAs to DCs, although this scenario still awaits experimental proof.

The migratory CD8α^{-}Sirpα^{+} cDC subset seems to be guided by different cues. Thus, CCR2-deficient mice showed a selective diminution of migratory DCs in the thymus[60], whereby CCR2 signalling seems crucial for the mobilization of peripheral SIRPα^{+} cDCs rather than their final intrathymic positioning. The same report showed that migratory cDCs can accumulate in the cortex in the vicinity of small vessels and inside perivascular regions, whereas other investigators found that SIRPα^{+} cDCs preferentially localized near blood vessels at the cortico-medullary junction and within deeper regions of the medulla (D. Atibalentja and E. Unanue, personal communication). Notwithstanding these apparent

discrepancies, there is some consensus that SIRPα$^+$ migratory cDCs more efficiently sample intravenously injected model antigens from the bloodstream *in vivo* when compared with resident cDCs [35, 60-62].

Taken together, the differences between resident and migratory cDCs in their origin, responsiveness to chemokines and selective occupancy of microenvironmental niches imply a degree of functional specialization. Resident cDCs preferentially seed the medulla and seem best equipped to focus their antigen display on self antigens captured within the thymic microenvironment. By contrast, migratory cDCs are found both in the medulla and the cortex and may serve a dual role: first, to transport peripherally acquired self antigens into the thymus and, second, to capture and present blood-borne self antigens. It is unlikely, however, that there is a strict demarcation between these functions.

Plasmacytoid dendritic cells

Roughly one third of all thymic DCs are plasmacytoid Dendritic Cells (pDCs). pDCs enter the thymus as a migratory population from peripheral sites [56], indicating a close lineage relationship between peripheral and thymic pDCs. As peripheral pDCs serve a crucial function in the protection against viral infections through their production of type I interferons[63], the presence of a pDC population in the thymus was suggested to reflect a similar innate immune function in a primary lymphoid organ [55].

Given the poor antigen presentation capacity of pDCs [64], a role in central tolerance had been considered rather unlikely; however, recent data suggest that this view may need to be revised. First, in a reductionist *in vitro* setting, thymic pDCs were capable of presenting peptide antigen to specific thymocytes and promoting their differentiation into T_{Reg} cells[65]. Second, pDCs were reported to be surprisingly efficient in picking up soluble or particulate model-antigens at peripheral sites *in vivo* and transporting them into the thymus[56, 66]. Interestingly, the chemokine receptor **CCR9**, which controls T progenitor homing to the thymus, is also crucial for pDC recruitment to the thymus [66]. As a subset of CCR9$^+$ immature pDCs in peripheral lymphoid tissues was able to promote conversion of naive T cells into T_{Reg} cells[67], this ‘tolerogenic’ pDC subset may also exert tolerogenic functions upon recruitment into the thymus. Indeed, adoptively transferred OVA-loaded wild-type pDCs, but not *Ccr9*$^{-/-}$ pDCs, migrated to the thymus, where they specifically located to the medulla and promoted clonal deletion of OVA-specific OT-II thymocytes [66].

Altogether, these new data present a strong case for a contribution of pDCs to central tolerance. Of note, pDCs, in contrast to both subsets of cDCs, do not pick up mTEC-derived antigens (J. Nedjic, T. Yamano, J. Derbinski, L.K. and B.K., unpublished observations), indicating that they may sample self antigens in the periphery and then ‘freeze’ their antigen cargo. Moreover, activation of TLRs prevents both cDCs and pDCs from migrating to the thymus[6866], thereby conceivably preventing central tolerance to pathogens under inflammatory conditions. Finally, considering that CCR9 also promotes migration to the intestine, CCR9$^+$ pDCs may not only sample *bona fide* self antigens but also innocuous foreign antigens, such as food components or constituents of the commensal microflora. However, there is as yet no experimental data to support this intriguing scenario.

Thymic B cells

Approximately 0.3% of thymic cells are B cells, a figure similar to that seen for DCs. The origin of thymic B cells is still a matter of debate; it is unclear whether they derive from intrathymic B lymphopoiesis and/or immigration of peripheral B cells [69, 70]. The phenotypic and functional attributes of thymic B cells closely resemble those of conventional B cells (that is, B-2 cells) found in the periphery [71, 72]. However, compared with splenic B cells, thymic B cells show elevated expression of MHC class II and co-stimulatory molecules, indicative of potent antigen presentation capacity. Indeed, thymic B cells have repeatedly been found to be capable of inducing negative selection. Most convincingly, myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^+$ thymocytes were negatively selected when an epitope of MOG was exclusively presented by B cells [73].

However, it is presently unclear how thymic B cells fare in their overall contribution to tolerance when compared with DCs and mTECs. For instance, deletion of superantigen-reactive $CD4^+$ T cells was more efficiently induced by DCs, and in the same context B cells entirely failed to negatively select $CD8^+$ thymocyte [74]. Consistent with these *in vivo* data, selective supplementation of reaggregate thymus organ cultures (RTOCs) with different thymic APCs in the presence of soluble OVA peptide resulted in negative selection of OVA-specific $CD4^+$ thymocytes when resident or migratory cDCs were used as APCs, but not when B cells were the APC [75].

Relatively little is known about the parameters that may shape the pMHC-ligandome of thymic B cells. Traditionally, peripheral B cells are considered poor presenters of exogenous antigens. This may also be the case for thymic B cells, explaining their poor performance in the aforementioned studies on negative selection in RTOCs. However, in contrast to the poor efficacy with which B cells present soluble antigens, B cell receptor (BCR)-mediated cognate interactions lead to exceptionally efficient antigen presentation [76]. Given the copious amounts of MHC class II expressed by thymic B cells, it is conceivable that B cells may not only present BCR-captured external antigens, but in manner similar to mTECs, may focus their pMHC class II ligandome towards endogenously expressed proteins. This intracellular antigen pool is likely to include germline-encoded or even clonotype-specific regions of the BCR [77]. Lack of tolerance towards variable (V)-regions of the BCR caused T cells to provide ‘chronic’ inappropriate help to B cells expressing the respective BCR in a double-transgenic model. This ultimately resulted in systemic autoimmunity[78], indicating that robust tolerance towards this special class of self antigens is indispensable.

Cognate T-B interactions are central for germinal centre formation during immune responses to foreign antigens, but may have an intriguing counterpart in the central tolerance process. Thus, BCR-transgenic B cells efficiently mediated negative selection of $CD4^+$ thymocytes expressing a transgenic TCR specific for the same cognate self antigen [72]. Although it may be difficult to envision how rare cognate self-antigen-specific B cells within a polyclonal repertoire may be sufficient to impose tolerance, the same study reported that even a polyclonal B cell population mediated a degree of deletion of TCR transgenic $CD4^+$ T cells.

In sum, these intriguing new data should reignite interest in the role of B cells in central T cell tolerance. Thymic B cells may afford another layer of tolerance towards BCR-derived self-constituents, thus pre-emptying the previously described peripheral checkpoints of T cell tolerance to this unique class of self antigens [79]. Other relevant issues in this regard are whether the scope of B cell-mediated central T cell tolerance is indeed dictated by the BCR repertoire of thymic B cells, how diverse the thymic BCR repertoire is, and whether auto-reactive B cells may be enriched in the thymus to allow for efficient presentation of soluble self-antigens.

Perspectives

Following our review of key cell biological attributes of the different thymic APC populations in the context of T cell repertoire selection, we will close with some speculative thoughts on how the intrathymic encounter of self (or the lack thereof) may imprint peripheral T cell behaviour, orchestrate dominant versus recessive mechanisms of tolerance and specify targets of autoimmunity.

Positive selection, homeostatic fitness and immunity to pathogens

Our discussion of antigen presentation for positive selection converged on the view that cTECs generate and display functionally and possibly structurally distinct private self peptides that may sustain the selection of T cell clones displaying weak tonic self-reactivity in the periphery. This notion is at odds with the proposition that the very same self peptides that mediate positive selection are also essential for naïve T cell homeostasis in the periphery and act as co-agonists when T cells respond to foreign antigens [16, 80, 81].

How can this apparent discrepancy be reconciled? First, it is possible that the peripheral self peptides supporting homeostasis and co-activation are not identical, but instead functionally equivalent to those supporting positive selection. Second, one may argue that the functional competence of the peripheral T cell repertoire requires a balanced distribution of clones covering a relatively wide range of tonic self-reactivity, as represented by $CD5^{low}$ and $CD5^{hi}$ T cells. Possibly, a corresponding mix of private and public MHC ligands on cTECs is a prerequisite to select such a composite of T cell clones with low or high tonic affinity, respectively.

One can envisage a potential benefit of having T cells with a wide range of affinities for self antigens (**Figure 4**). Following infection with pathogens, T cells with high affinity for self could provide a rapid, yet relatively short-lived initial immune response that is then followed by a sustained response by T cells with lower self affinity. The latter are presumably not only less prone to burn out, but also less likely to cause bystander damage to self tissues. This scenario would fit with the observation that $\beta5t^{-/-}$ mice, which have a numerically smaller but presumably more strongly self-reactive $CD8^+$ T cell repertoire, die in response to infection with influenza virus[11]. Yet, since the flu-specific response was not tracked in that study, it remains to be determined whether these $CD5^{hi}$-skewed $CD8^+$ T cells indeed either collapsed faster, made an over-shooting pathogenic response, or failed to respond to antigen at all. Against this background, it will also be interesting to see whether the duration of infections (chronic versus acute) or the spread of pathogens (systemic versus local) are

crucial determinants of the relative contribution of $CD5^{low}$ and $CD5^{hi}$ T cell clones to the immune response to foreign antigens, and how these parameters affect their partitioning into the memory pool.

Notwithstanding these considerations, we still lack experimental data to directly link the selection of a given 'low self-affinity' TCR-specificity to a specific private peptide the processing of which would be dependent on any of the cTEC-specific pathways of antigen processing. Solving this issue has been hampered by our current ignorance of the identity of the peptides bound to MHC on cTECs. The scarcity of cTECs ($1 – 3 \times 10^4$ per thymus) renders this a daunting task (**Box 3**). In this context, the fundamental issue of whether selection of a given TCR specificity actually requires a single, specific self peptide has not been resolved. Likewise, we do not know whether private peptides on cTECs are equally important for $CD4^+$ and $CD8^+$ T cell repertoire selection. This question is all the more interesting since high tonic self-responders among naïve $CD4^+$ T cells seem inherently more prone to undergo peripheral conversion into induced $FOXP3^+$ T_{Reg} cells [82].

Modes of central tolerance: deletion versus T_{Reg} cell differentiation

A detailed discussion of how antigen presentation in the thymus may intercalate with factors such as TCR affinity, access to cytokines or developmental tuning of thymocyte responsiveness to specify clonal deletion versus clonal deviation into the $FOXP3^+$ T_{Reg} cell lineage exceeds the scope of this article (for recent reviews see [83, 84]). Here, it may suffice to highlight two pertinent issues.

First, there is no evidence to indicate that any thymic APC subset is specialized to exclusively promote clonal deletion or T_{Reg} cell differentiation. Although efficient T_{Reg} cell differentiation apparently requires a properly formed medullary microenvironment [85], the overall size of the polyclonal T_{Reg} cell pool is barely affected by the specific lack of DCs or subsets of mTECs, or by aberrant MHC class II expression on these cells (reviewed in [86]). Thus, thymic APC subsets obviously can compensate for each other as far as filling the physiological T_{Reg} niche is concerned. However, considering the evidence for partly non-overlapping pMHC ligandomes on different thymic APCs, we deem it unlikely that this reflects a true redundancy with respect to the level of TCR specificities that are recruited into the T_{Reg} cell repertoire. Large scale TCR sequencing of T_{Reg} cell repertoires in the above mentioned settings is expected to resolve this issue.

Second, it seems unlikely that any particular modalities of antigen expression, for instance a 'promiscuous gene expression-like' mosaic pattern versus ubiquitous expression, will invariably result in either clonal deletion or clonal deviation. The view that the essential role of *Aire* in mTECs can be solely ascribed to recessive tolerance through negative selection [87] is difficult to reconcile with the ability of mTECs to autonomously induce antigen-specific T_{Reg} cells in neo-self antigen transgenic settings [34]. A recent study extended the latter finding to a naturally expressed protein by showing that an *Aire*-dependent self-antigen induced thymic differentiation of cognate T_{Reg} cells [88]. Intriguingly, T_{Reg} cell progenitors of unknown specificity were found to compete for recognition of rare stimulatory TCR self ligands in the thymus [89-91], which is also suggestive of a link between promiscuous gene expression in rare mTECs and T_{Reg} cell differentiation. The possibility that expression and

presentation of TRAs by very few mTECs may concomitantly induce negative selection and T_{Reg} cell induction has obvious implications regarding the issue that interactions of thymocytes with rare APCs presenting a given cognate self antigen perhaps need not be saturating after all, because the occasional escape of autoreactive T cells would be counterbalanced by dominant regulatory mechanisms.

Autoimmunity: what thymocytes may not see

With increasing knowledge of the multi-layered cellular and molecular mechanisms of central tolerance one is bound to ask which parameters are most limiting and pose the highest risk for autoimmunity. Deep sequencing data of the mRNA signature of mTECs indicate that the scope of promiscuous gene expression is even broader than previously revealed by microarray analyses [92]. Given this apparently comprehensive intrathymic representation of otherwise tissue-specific transcriptomes, it becomes even more intriguing and informative to know which fraction of self is actually not covered by central tolerance. Any advance in understanding the molecular regulation of promiscous gene expression by AIRE and beyond would help us to understand how the tolerizing MHC-ligandome in the thymus is generated in the first place and, importantly, how dys-regulation at the genetic, epigenetic and biochemical level could undermine central tolerance and thus possibly explain immune pathologies. In fact, there is an increasing number of examples of how ‘holes’ in central tolerance might specify dominant T cell targets in organ-specific autoimmune diseases.

First, very low expression levels of particular self antigens in human mTECs correlate with a high frequency of cognate, auto-reactive T cells and/or autoantibodies in the case of the myosin heavy chain 6 [93, 94], an autoantigen in autoimmune myocarditis, or GAD65 [95] and ZnT8, both targets in type 1 diabetes (B.K., S. Pinto, R. Mallone, unpublished observations). Genetic polymorphisms in regulatory regions of some autoantigens, which exert subtle effects on the expression level of self antigens in mTECs but not peripheral tissues correlate with susceptibility to organ-specific auto-immunity as shown for insulin and type 1 diabetes [96-98], the alpha-chain of the acetylcholine receptor and myasthenia gravis [99] and the thyroid stimulating hormone receptor and Graves’ disease [100]. Second, similar effects are observed when dominant T cell epitopes are only present in peripheral tissues but not in the thymus either due to differential splicing [101, 102] or due to mis-initiation of mRNA transcription in mTECs (S. Pinto, B.K., unpublished observations). Mis-initiated transcription is possibly a more general feature and thus a risk factor of promiscuous gene expression[54]. Finally, post-translational modifications of auto-antigens affecting T cell epitopes by cell-type specific expression of modifying enzymes in peripheral target tissues but not mTECs may work to the same effect. Examples in this regard are glycosylation of collagen type II and citrullination of aggrecan and vimentin in the case of rheumatoid arthritis [103] or deamidation of insulin in the case of type I diabetes [104].

Conclusions

The affinity model remains a useful framework to conceptualize thymic selection events, and there has been considerable progress in understanding how the TCR relays quantitative

differences in signal strength into qualitatively different cell fates (reviewed in [105]). Yet, as reviewed here, a coherent model of thymocyte selection will in addition need to consider temporal, spatial and qualitative aspects of self recognition in distinct thymic microenvironments. In terms of positive selection, it remains tantalizing why 'monogamous' interactions of developing T cells with a single 'dedicated' stromal cell type are so critical, and we have discussed how this crucial role of cTECs may be specified by their ability to generate 'private' pMHC ligands. With regard to central tolerance induction, it is intuitively obvious why 'promiscuity', not only at the level of TRA expression in mTECs, but similarly in terms of the diverse characteristics and sheer number of contributing APC subsets, is advantageous. Unravelling functional non-redundancies between tolerogenic APCs, at the level of the self antigen spectra that are presented and regarding tolerance mechanisms (deletion versus T_{Reg} cell induction), remains a major challenge for future investigations.

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Biography

Ludger Klein is Professor at the Institute for Immunology of the University of Munich. His group is interested in functional adaptations of thymic stromal cells for T cell repertoire selection. A second research focus is unravelling the parameters that discriminate clonal deletion and T_{Reg} differentiation as alternative cell fate choices of autoreactive thymocytes.

Bruno Kyewski is head of the Division of Developmental Immunology at the German Cancer Research Center. His laboratory studies the structure–function relationship of the thymic microenvironment. Current research in his laboratory focuses on three areas: the cellular and molecular mechanisms underlying the phenomenon of promiscuous gene expression by thymic epithelial cells, the developmental biology of thymic epithelial cells and the role of central tolerance in human autoimmune diseases.

Paul Allen is the Robert L. Kroc Professor of Pathology and Immunology at Washington University. His laboratory has had a long-standing interest in the processing and presentation of self-proteins and how self-peptide/MHC complexes play a critical role in positive selection and peripheral T cell function.

Kris Hogquist is Professor and Associate Director of the Center for Immunology at the University of Minnesota. Her group studies how thymic selection processes shape the T cell repertoire. Their current research is focused in three areas: positive and negative selection in the thymus, iNKT cell development, and the human T cell response to EBV.

Glossary terms

Positive selection	The process by which immature double-positive thymocytes expressing T cell receptors with intermediate affinity and/or avidity for self-peptide–MHC complexes are induced to differentiate into mature single-positive thymocytes.
Negative selection	(Also known as clonal deletion). The intrathymic elimination of double-positive or single-positive thymocytes that express T cell receptors with high affinity for self antigens.
Peptide–MHC ligandome	The repertoire of peptides that are bound by MHC molecules.
Death by neglect	Double-positive thymocytes have a finite life-span of 3 – 4 days. Failure to engage in positively selecting interactions with self pMHC complexes on cTECs within this period result in programmed cell death.
Bim	BCL-2-interacting mediator of cell death is a pro-apoptotic molecule that is crucial for negative selection.
β-selection	The pre-TCR-driven process by which double-negative thymocytes that carry a productively rearranged TCR β-chain undergo proliferative expansion and developmental progression.
Proteasome	The standard proteasome is composed of 14 α and 14 β subunits, of which three, β1, β2 and β5, are involved in peptide-bond cleavage. Interferon-γ induces the expression of the immunosubunits β1i, β2i and β5i that can replace the catalytic subunits of the standard proteasome to generate the immunoproteasome, which has distinct cleavage-site preferences.
Macroautophagy	The generally nonspecific sequestration of cytoplasm into a double- or multiple-membrane-delimited compartment (autophagosome) of non-lysosomal origin. Certain proteins, organelles and pathogens may be selectively degraded by macroautophagy.
MHC anchor residues	Amino-acid residues of an antigenic peptide that bind in pockets in the peptide-binding groove of a major histocompatibility molecule and account for much of the binding energy and specificity of binding.
CD5	A membrane protein that associates with the TCR complex. It modulates the TCR signal transduction cascade through interactions with various kinases and phosphatases.
Nr4a1	Also known as Nur77. Nr4a1 encodes an orphan nuclear receptor whose expression is up-regulated by TCR signalling in thymocytes and mature T cells.

HY TCR	An MHC class I-restricted transgenic TCR recognizing a self-antigen encoded on the Y-chromosome.
OT-I TCR	An MHC class I-restricted transgenic TCR recognizing an Ovalbumin epitope.
Tonic TCR stimulation	Continuous ‘sub-threshold’ recognition of self-peptide/MHC complexes by mature T cells, resulting in a basal activation state that enables T cells to rapidly respond to foreign antigen.
Tissue-restricted antigens (TRAs)	Self-constituents encoded by genes which are expressed by only one or few tissue-specific cell lineages as opposed to housekeeping genes. The term TRA is an operational definition based on available expression catalogues, according to which TRAs are expressed in less than 5 tissues of 60 tested.
Thymic cross-talk	The mutual developmental dependence of the T cell and the stromal cell (i.e. non-T cell) compartments of the thymus, specified by complex receptor-ligand interactions.
***C2TAkd* mice**	A mouse strain that expresses a designer microRNA targeting the class 2 trans-activator (C2TA) specifically in mTECs. This leads to a reduction of MHC class II expression to about 10% of its physiological levels, while preserving intact mTEC differentiation and TRA expression.
CCR9	C-C chemokine receptor type 9 is a G protein coupled receptor recognizing the chemokine CCL25 (TECK; thymus expressed chemokine).

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Box 1 | The affinity model of thymocyte selection (with 'Figure Box 1')

This model centres on the strength of the TCR's interaction with self peptide-MHC complexes as a critical cell fate determinant. Weak interactions are required to protect thymocytes from 'death-by-neglect' and to promote the positive selection of naïve T cells. Strong interactions cause negative selection by apoptosis. TCR transgenic studies showed that negative selecting peptides are primarily high affinity agonists (including cognate ligands, i.e. peptides that lead to activation of mature T cells), while positive selecting peptides are often low affinity antagonists or weak agonists (often called altered peptide ligands). Interestingly, in TCR transgenic systems, high affinity agonists were also shown to cause clonal deviation, that is, the re-direction of autoreactive T cells into to the T_{Reg} cell lineage. A surprisingly broad range of affinities seem to be permissive for T_{Reg} cell differentiation, and as affinity rises, the propensity to generate T_{Reg} cells increased. Thus, a modified version of the affinity model, where T_{Reg} cell differentiation occurs optimally within a window between positive and negative selection, is currently favored. Of note, the demarcation between clonal deletion and clonal deviation is remarkably plastic and seems to be subject to stochastic influences. For example, in TCR transgenic systems, intrathymic expression of an agonist ligand can concomitantly promote either T_{Reg} cell or naïve cell fates in some cases, or both T_{Reg} cell differentiation and deletion in other cases. Not surprisingly then, the natural repertoire of TCRs expressed by naïve CD4 T cells and T_{Reg} cells shows some overlap. Mathematical modelling suggests that distinct fates can arise from one receptor's interactions with the same ligand by iterative summing of TCR signals during multiple interactions, or by changes ('developmental tuning') of thymocyte sensitivity over time [106].

Box 2 | Promiscuous gene expression

The term promiscuous gene expression refers to the ectopic expression of otherwise tissue-restricted antigens (TRAs) by medullary thymic epithelial cells (mTECs). Remarkably, besides tissue-specificity, promiscuous gene expression violates other fundamental rules of tightly controlled lineage-specific gene expression such as developmental switches and sex-specificity, thus comprehensively representing the immunological self of peripheral tissues.

The Autoimmune Regulator (AIRE) protein remains the only identified molecular regulator dedicated to controlling a sizeable fraction of the promiscuously expressed gene pool [27]. It does not seem to act as a classical sequence-specific DNA-binding transcription factor. Instead, AIRE targets and turns on silent gene-loci by binding to non-methylated H3K4, which marks promoters in closed chromatin regions [107, 108]. It apparently acts as a docking platform for different multi-protein complexes known to facilitate transcription by generating local double-strand breaks, promoting mRNA processing and relieving stalled RNA polymerase [109, 110]. Yet, none of the presently discussed models of AIRE's function [111] satisfactorily accounts for all the intricacies of promiscuous gene expression, most notably the observation that a given TRA is only expressed by a minor subset of 1-3 % of mTECs at any point in time (see **Figure 3**). Understanding how this mosaic pattern of promiscuous gene expression is generated and whether it is maintained in time and space at the single cell level will be key to eventually comprehend how central tolerance can be so efficient, sensitive and stringent.

Although it was initially thought that promiscuous gene expression in single mTECs is stochastic [52, 54], a recent study revealed distinct and fluctuating patterns of gene co-expression in subsets of human mTECs, implying that mTECs transiently display TRAs in certain linkage groups [53]. Co-expression groups ranged between approximately 100 and 300 TRAs, preferentially mapping to particular chromosomes whereby co-expressed gene loci co-localized within nuclear subdomains, pointing to a level of regulation at the higher genome order.

Box 3 | Towards directly addressing the peptide-MHC ligandome of thymic stromal cells

Remarkably, one of the first attempts in the early 1990's to assess the peptide (p)MHC ligandome by peptide-elution and -sequencing reported a comparison between cTECs and splenic APCs [112]. At the time, only 17 of the most abundant self peptides were identified, from an estimated 2,000 to 10,000 distinct peptides presented by MHC class II or I, respectively. With technology improving, the field has seen increasingly detailed assessments of cell-type specific pMHC ligandomes, particularly of tumor cells. However, rare *ex vivo* isolated populations such as thymic stromal cells remain a major technical challenge.

In general, naturally processed peptides have been characterized from acid extractions of affinity-purified MHC molecules, which are then sequenced typically by mass spectrometry. This approach was used to describe peptides bound to MHC class I and II molecules in the human thymus [113, 114]. However, since whole tissue was used, these studies fell short of assigning the identified peptides to particular stromal cell types. Another recent study identified 50-100 peptides from HLA class II or I molecules from human thymic DCs and compared these to peptides from thymic APCs depleted of DCs [115], giving initial insight into differences in the ligandomes of thymic APCs. The amount of starting material required with this approach (at least 10^8 cells) so far precludes an informative analysis of MHC bound peptides from rare populations like cTECs. Another complication arises from the possibility that the elusive *private* self-peptides presumed to be generated by β5t may more weakly bind to MHC Class I molecules and hence might be lost during the immunoprecipitation step. An alternative approach has been reported that characterized peptides directly extracted with mild acid elution from the surface of intact cells. Since this procedure generates a large number of peptides, not just those bound by MHC, the authors employed a bioinformatics comparison to peptides extracted from MHC-deficient cells to assign them as 'MHC-bound'[116]. Chemical and metabolic labelling have also been employed to provide quantitative comparisons between two populations, although this has not been applied to the thymus [117].

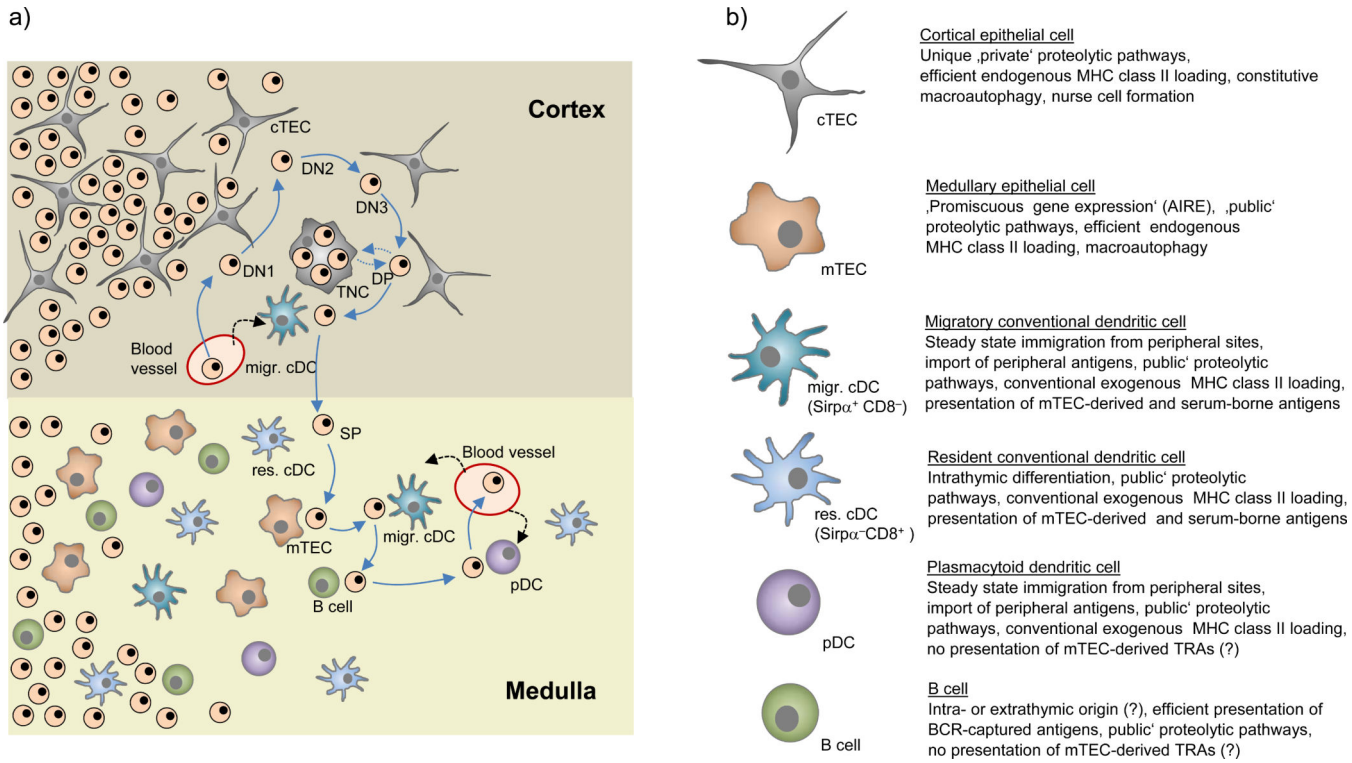

Figure 1. Stromal cell interactions during T cell development

(a) Successive stages of double-negative (DN) T cell development are accompanied by an outward movement of thymocytes towards the sub-capsular zone. Subsequent to β-selection at the DN3 stage, double-positive (DP) cells 'randomly walk' through the outer cortex, which possibly facilitates the 'scanning' of cortical thymic epithelial cells (cTECs) for positively selecting ligands. At this stage, DP thymocytes may be engulfed by cTECs and form so-called thymic nurse cells (TNCs), whereby the molecular control and physiological relevance of this process remains to be established. Interactions of DP cells with cortical conventional dendritic cells (cDCs) may lead to negative selection. It remains open whether these cortical cDCs exclusively belong to the migratory Sirpα^{+} subset. Positively selected, CD4 or CD8 lineage-committed thymocytes relocate into the medulla by directed migration. Upon reaching the medulla, single-positive (SP) cells again assume a 'random walk' motion pattern. Through this random migration, SP cells may now 'scan' resident (res.) and migratory (migr.) cDCs, medullary thymic epithelial cells (mTECs), plasmacytoid dendritic cells (pDCs) and B cells. It is estimated that SP cells engage in around five contacts with antigen presenting cells (APCs) per hour, so that over their 4-5 days residency in the medulla, T cells may serially interact with several hundred APCs. **(b)** Key functional properties of thymic APCs discussed in this Review.

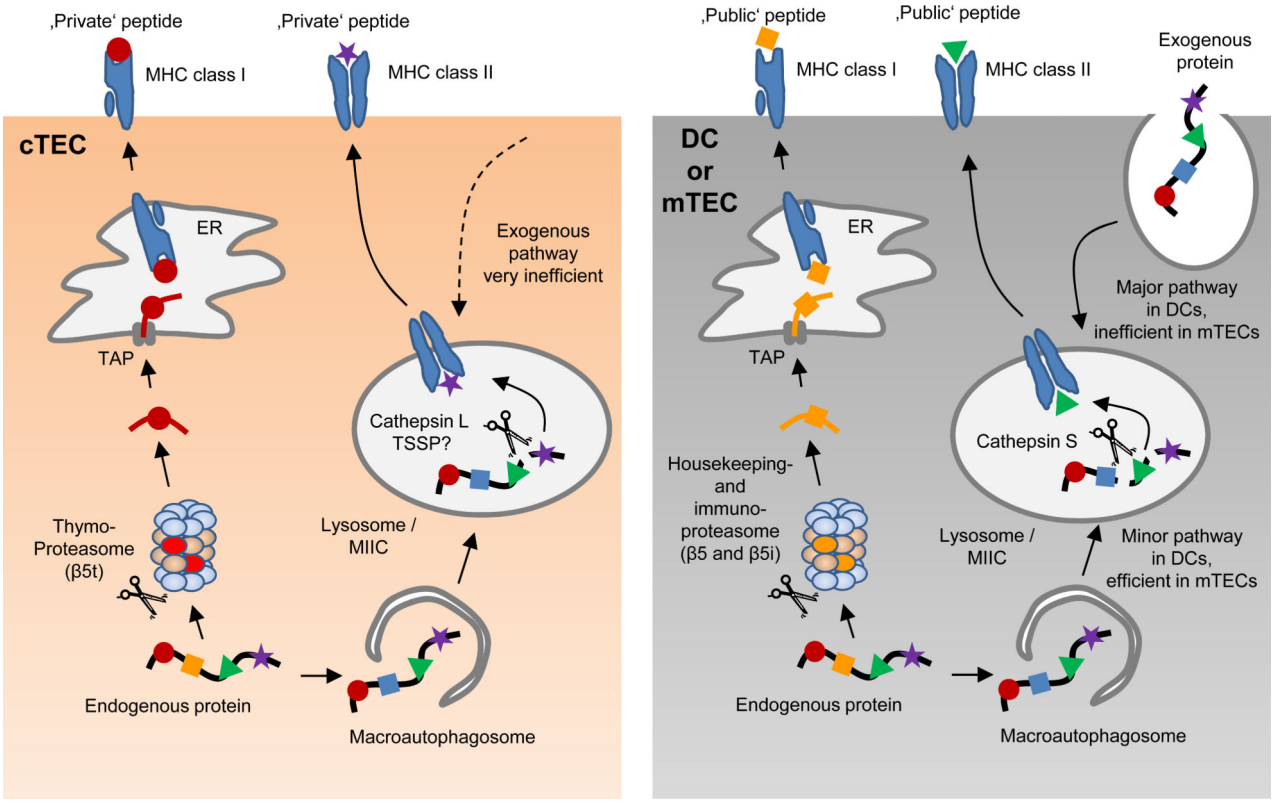

Figure 2. Unique proteolytic pathways generate 'private' MHC-bound peptides in cTECs

Processing of a given endogenous protein substrate by cTECs may give rise to unique, 'private' peptides, which differ from 'public' peptides generated by mTECs and DCs. MHC class I-bound peptides on the surface of cortical thymic epithelial cells (cTECs) are predominantly processed by proteasomes containing the catalytic subunit β5t (so called thymoproteasomes). Due to a distinct proteolytic activity of the thymoproteasome, this is likely to lead to the generation of cTEC-specific, 'private' peptide epitopes that differ from 'public' epitopes generated by mTECs or DCs through the housekeeping proteasome or the immuno-proteasome. MHC class II-bound peptides on cTECs seem to be mostly derived from an unconventional, endogenous MHC class II-loading pathway that involves the macroautophagy-mediated shuttling of cytoplasmic proteins into lysosomes. In this proteolytic compartment, processing by the proteases cathepsin L and thymus-specific serin protease (TSSP) may generate unique 'private' peptides. MHC class II-bound peptides on mTECs may likewise be mostly derived from macroautophagy–mediated endogenous MHC class II-loading; however, the lysosomal proteases that generate MHC class II-bound peptides in mTECs differ from those in cTECs, being essentially identical to those used by DCs for the processing of exogenously-derived substrates along the 'conventional', exogenous MHC class II pathway. Of note, it is likely that the pMHC ligandome of cTECs represents a mixture of 'private' and 'public' peptides that are uniquely present on cTECs or shared with other APCs, respectively (see **Figure 4**).

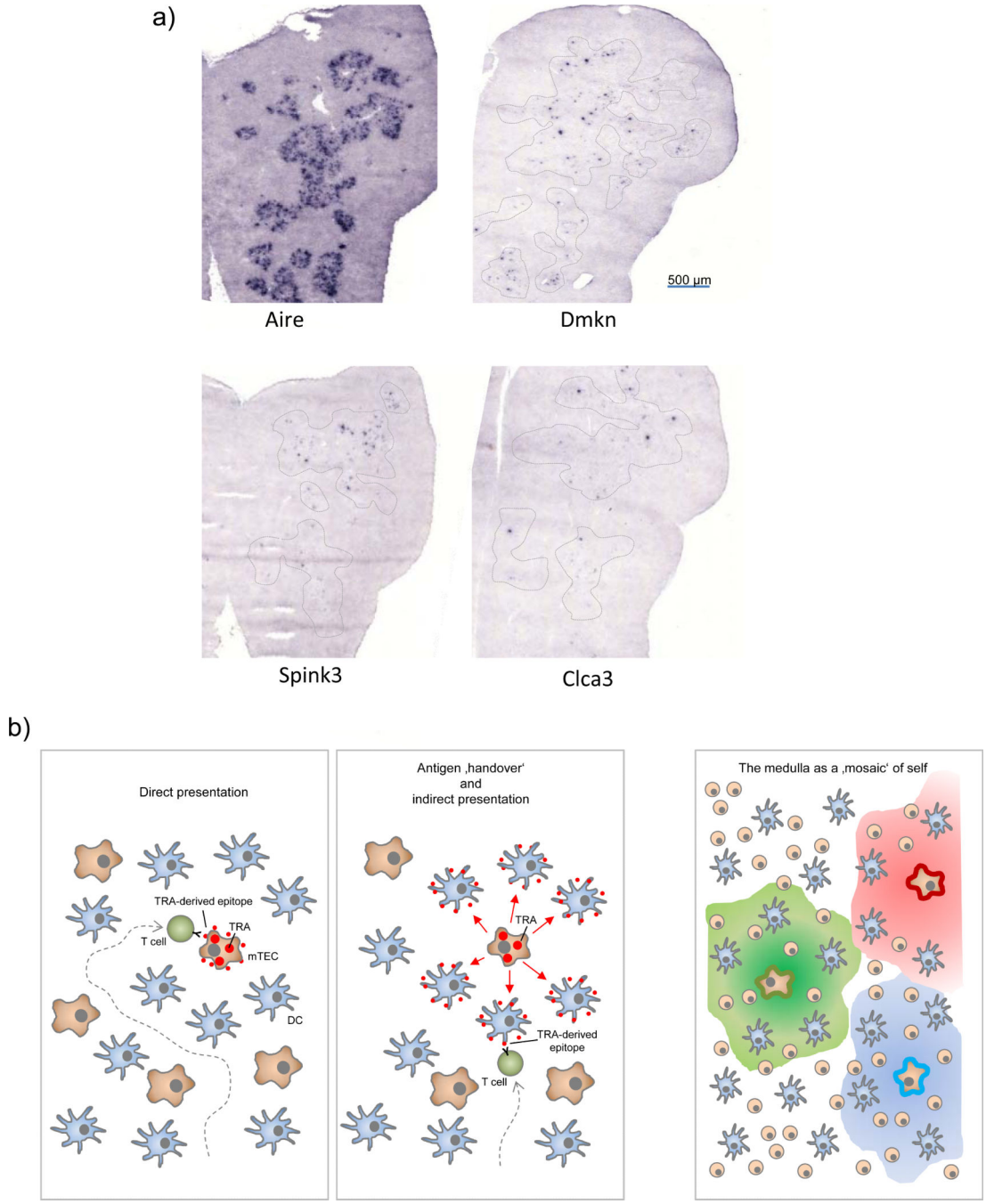

Figure 3. Topological aspects of 'promiscuous gene expression' and direct versus indirect presentation of TRAs

(a) Ttissue-restricted antigens (TRAs) are expressed by only a small subset of mTECs at any given point in time: In-situ hybridization of a section through an entire thymic lobe for Aire mRNA expression (left) shows that Aire-expressing cells are densely packed in medullary regions. By contrast, transcripts of three representative TRAs (dermakine, Dmkn; Serin protease inhibitor kazal type 3, Spink3; chloride channel calcium activated 3; Clca3) are only detectable in very few cells that are scattered throughout medullary areas (dotted lines). **(b)** Tolerogenic presentation of TRAs that are expressed by few mTECs may occur in two

not mutually exclusive ways: (left) direct presentation by TRA-expressing mTECs themselves, whereby efficient endogenous MHC class II-loading by mTECs in conjunction with serial ‘scanning’ of multiple medullary APCs by thymocytes increases the likelihood of cognate self-antigen interactions. (middle) ‘Antigen handover’ to neighbouring cDCs may extend the area of tolerogenic presentation in a mosaic fashion beyond the topologically restricted expression pattern (right). The mechanistic details of this ‘directional antigen transfer’ transfer remain to be established. It is conceivable that TRAs are released or shed in soluble form to be subsequently captured and processed by cDCs for presentation on MHC class I or II. Apoptosis of terminally differentiated mTECs may lead to the release of apoptotic fragments that can also transfer mTEC-derived self-antigens to cDCs. In addition, functional peptide-MHC ligands are unidirectionally translocated from mTECs to DCs [42, 118].

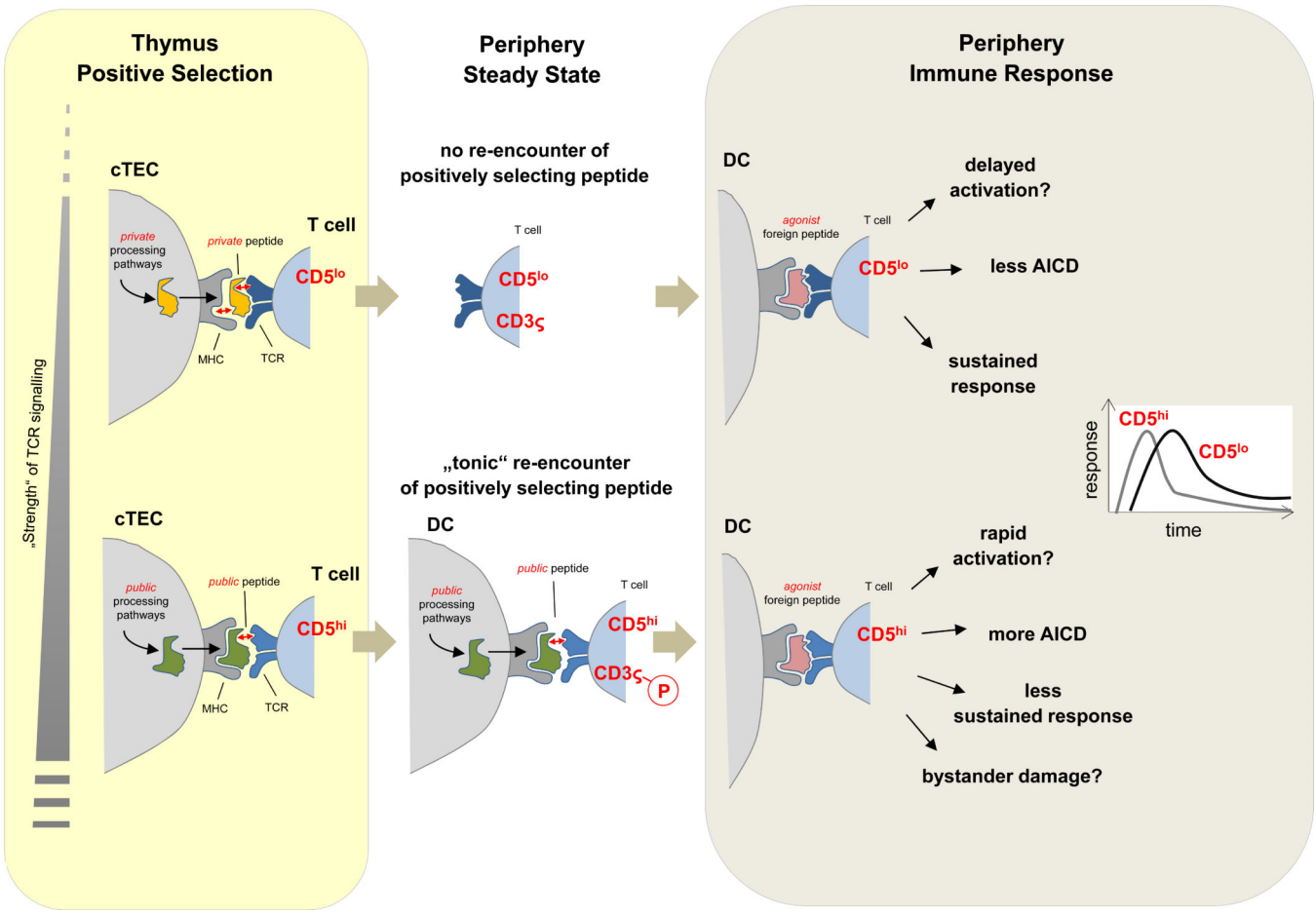

Figure 4. Consequences of positive selection by 'private' versus 'public' peptides: a hypothesis

(Upper panel) 'Private' peptides generated through unique proteolytic pathways in cortical thymic epithelial cells (cTECs) may preferentially support selection of CD5^{low} T cell clones via interactions at the lower end of the affinity range that is permissive for positive selection. One determinant of these 'low strength' interactions could be that private peptides are weak MHC binders, indicated here by the loose fit between peptide and MHC (red arrow). In the periphery, T cells selected in this way do not re-encounter the positively selecting peptides and hence do not receive tonic signals. As a consequence, their CD3ς chains are not pre-loaded with basal phosphorylation. Yet, it remains possible that CD5^{low} clones receive a degree of tonic input through exposure to cross-reactive 'public' peptides in the periphery. (Lower panel) Public peptides may preferentially support selection of CD5^{hi} clones via positively selecting interactions at the relatively higher end of the affinity range. Public peptides might be good MHC binders that generate 'low strength' interactions by loosely binding to the TCR (red arrow). In the periphery, continual interactions with the very same peptides support T cell homeostasis and mediate partial CD3ς chain phosphorylation. During an immune response to foreign antigens, CD5^{low} and CD5^{hi} T cell clones of identical specificity may differentially respond with respect to timing and magnitude of

clonal expansion and contraction. The dominance of either type of responder might vary with parameters such as duration and anatomical distribution of the infection.