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How thymic antigen presenting cells sample the body's self-antigens

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Our perception of the scope self-antigen availability for tolerance induction in the thymus has profoundly changed over the recent years following new insights into the cellular and molecular complexity of intrathymic antigen presentation. The diversity of self-peptide display is on the one hand afforded by the remarkable heterogeneity of thymic antigen presenting cells (APCs) and on the other hand by the endowment of these cells with unconventional molecular pathways. Recent studies show that each APC subset appears to carry its specific antigen cargo as a result of cell-type specific features: firstly, transcriptional control (i.e. promiscuous gene expression in medullary thymic epithelial cells); secondly, antigen processing (i.e. proteasome composition and protease sets); thirdly, intracellular antigen sampling (i.e. autophagy in thymic epithelial cells) and fourthly, extracellular antigen sampling (i.e. immigrating dendritic cells sampling extrathymic milieus). The combinatorial expression patterns of these attributes in distinct APC subsets result in a self-peptide display partly unique to the cortex mediating positive selection and to the medulla mediating tolerance induction.

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Introduction

A strict quality control of developing T cells in the thymus is essential for the prevention of autoimmune diseases. The stochastic assembly of the T cell receptor during the development of thymocytes results in the generation of numerous T cells, which can recognize MHC complexes loaded with peptides generated from self-proteins of the host. These potentially autoreactive thymocytes are either deleted, anergized or converted into regulatory T cells after cognate peptide/MHC recognition on the various thymic antigen presenting cells (APCs) subsets foremost in the medulla. Hence the scope of central tolerance is dictated by the available repertoire of self-peptides displayed by thymic APCs, which for a long time have been thought to exclude peripheral tissue-specific antigens.

Here we will address recent developments adding to our current understanding of the generation of the proteome and MHC/peptidome by thymic stromal cells, a process which has turned out to be much more intricate than previously assumed. We will point out that despite the large number of self-peptides covering each peripheral tissue, several recent experimental studies document an essential role of certain 'dominating' self-antigens in safeguarding organ-specific tolerance. Furthermore we will discuss how an efficient and rapid scanning process by medullary thymocytes ensures the sampling of those antigens, which are only expressed by infrequent scattered APCs.

The emerging picture is that the medullary APCs comprehensively mirror the self-peptide repertoire encountered by mature T cells in the periphery by exploiting partly unique and partly pathways of antigen sampling and processing similar to peripheral APCs. In contrast, cTECs employ different means for peptide generation, which are thought to be uniquely apt to mediate positive selection of T cells.

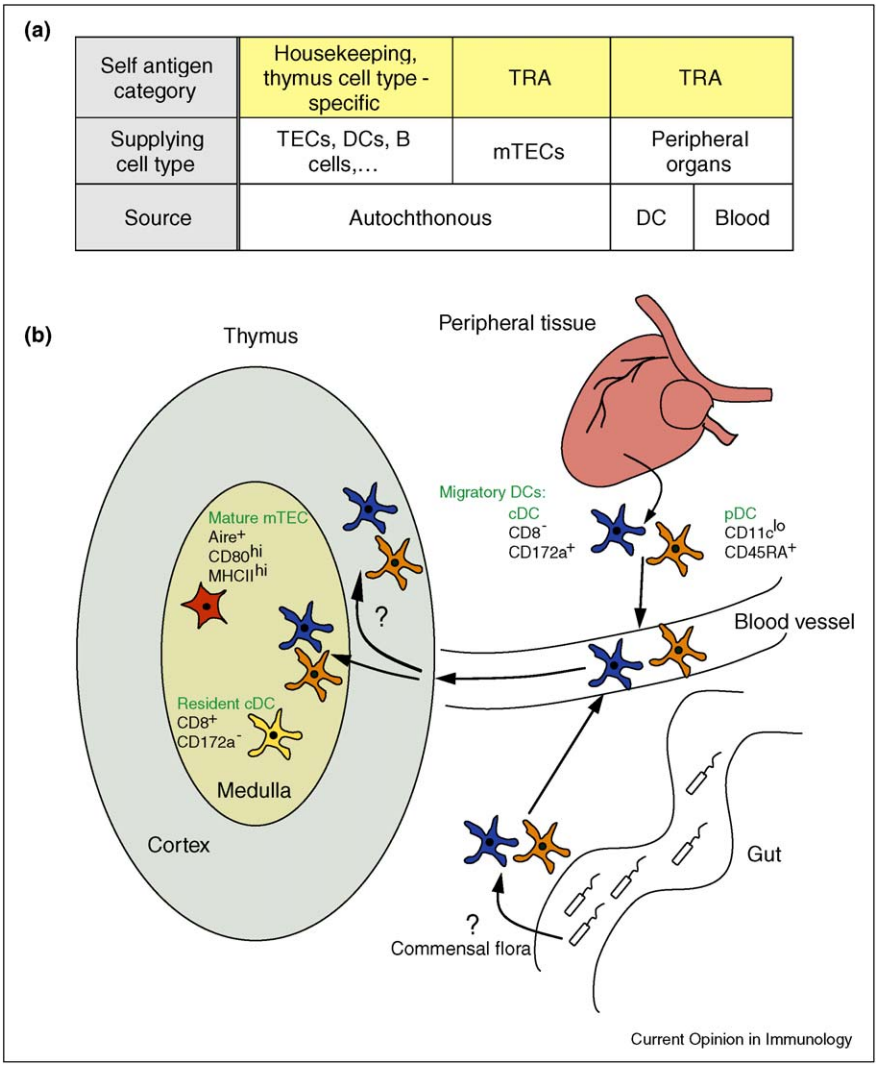
The intrathymic proteome available for tolerance induction

The complement of intrathymic self-proteins is supplied by various complementing sources thus ensuring a maximal representation of the 'immunological self' (Figure 1a). In addition to ubiquitously expressed housekeeping antigens, the various cell types of the thymus supply their cell-type-specific constituents. Furthermore, it has been known for a long time that blood-borne antigens access the medulla, and induce deletional tolerance mediated by DCs [1,2]. Recently, two additional routes of antigen supply have been added, which will be discussed here in more detail, promiscuous gene expression (pGE) by mTECs and import of organ-specific antigens of extrathymic origin via a steady influx of peripheral DCs.

Promiscuous gene expression

Medullary thymic epithelial cells (mTECs) specialize in so-called pGE of hundreds of self-antigens, which

Figure 1



Different sources and cell types supply the thymus with self-antigens. **(a)** Principal self-antigen categories that are available for central tolerance induction and are supplied by different cell types. Tissue-restricted antigens (TRA) are: firstly, either imported from the periphery in soluble form by the blood stream or in cell-bound form by the steady influx of peripheral DCs or secondly, are generated *in situ* by promiscuous gene expression in mTECs. **(b)** Migratory DC subsets make up about 50% of all thymic DCs, they continuously enter the thymus under non-inflammatory conditions and encompass plasmacytoid (pDC) and conventional (cDC) subsets. Thymus seeking DCs patrol peripheral organs including the gut. Their antigen cargo might also include foreign constituents like commensal gut flora. The precise destination of the DC subsets within the thymus has not been fully resolved.

otherwise are expressed in a strictly temporally and spatially restricted fashion in particular cell lineages [3,4]. The essential contribution of this particular antigen pool to self-tolerance has been amply documented [5•,6•]. Promiscuously expressed antigens either presented by mTECs themselves or cross-presented by DCs mediate tolerance both via deletion and induction of Tregs [7•,8••]. How a terminally differentiated epithelial cell is able to transcribe and translate all these genes, which serve no specific function in these cells other than being degraded and loaded onto MHC molecules, is still poorly understood. The only factor identified to date driving the expression of many of these TRAs is the Autoimmune regulator (Aire), a transcriptional regulator [5•,6•,9]. Recent studies show that Aire partakes in multiple steps of the gene transcription process. Aire preferentially recognizes unmethylated histone H3 lysine 4 by its plant homeodomain (PHD) 1 domain [10•,11•]. Since promoters of tissue-restricted antigens (TRA) usually do not carry the activating histone mark trimethylated H3K4 outside of their respective tissue, it is assumed that the binding of the non-methylated H3K4 promoters represents the initial targeting step for TRA expression in mTECs. Moreover, Aire associates with a multi-protein complex, which promotes transcriptional elongation by the induction and religation of single- and double-strand

DNA breaks [12••]. This probably serves to resolve supercoiling of DNA thus facilitating access of the transcription machinery to chromatin and the reassembly of histones around elongating RNA Pol II. The second complex, with which Aire is associated, enhances pre-mRNA processing leading to elevated levels of mature transcripts of TRAs in TECs. This multitasking of Aire is thought to act in synergy to induce and/or augment transcription in the apparent absence of tissue-specific transcription factors. Despite these significant new insights many issues remain to be explained within the conceptual framework of this model. Are the findings, which were mostly obtained in cell lines, fully valid in mTECs *in vivo*? Currently, the precise conditions for Aire to promote gene expression are unknown. Thus, Aire is necessary for transcription of certain target genes like insulin at the single cell level, but it is not sufficient [13•]. In this context it is important to mention that promiscuously expressed TRAs exhibit stochastic expression patterns at the single cell level [13•,14•]. There is also high variability of Aire-controlled gene expression at the population level [15•] among different mouse strains and even different thymic lobes of the same individual pointing to nongenetic 'noise' influencing the action of Aire. Since Aire targets different sets of genes in different cell types [16•], it will be important to know whether in mTECs Aire specifically selects genes representing the 'peripheral self', for example due to a unique complement of modifying factors or chromatin configuration? Importantly, we have no clue as to the regulation of TRAs that are independent of Aire, which are often dispersed among Aire-dependent genes in the genome.

Most studies addressing the regulation of pGE focus on the transcriptional level, while still little is known about the corresponding protein expression. How well does mRNA expression correlate with protein expression considering that the amount of mRNA of certain TRAs at the single cell level is 50- to 170-fold lower than in the corresponding tissue [13•]? Given the highly inefficient processing of protein antigens for presentation by MHC molecules [17], it is all the more surprising that these low mRNA levels still suffice to generate sufficient peptide display to mediate a tolerogenic TCR signal.

Another poorly understood issue concerns an apparent hierarchy among self-antigens in safe guarding tissue-specific tolerance given the fact that each tissue is usually represented by more than one antigen in mTECs. Thus it was reported that the intrathymic lack of IRBP or insulin leads to the breakdown of organ-specific tolerance, that is uveitis or diabetes, respectively [18••,19••]. This finding begs the question, why bystander suppression does not prevent these autoimmune reactions, since several retina- and beta cell-specific antigens are expressed by mTECs. In this context, the identification of novel target antigens in autoimmune diseases has been greatly aided by the judicious exploration of the Aire knock-out mouse model. A series of recently described Aire-dependent and -independent self-antigens, which are targeted in organ-specific autoimmunity not only in mice but also in humans, are listed in Table 1.

Self-antigens available to thymic DCs

Resident thymic DCs have been known for a while to present blood-borne antigens to developing thymocytes and in addition to cross-present mTEC-derived antigens (for details see [7•]). Only recently however has it been fully appreciated that about 50% of thymic DCs are immigrants, which continuously replenish the pool of thymic DCs. It is presumed that they display antigen cargo picked up in extrathymic sites, thus representing an

Table 1

Self-antigens expressed in mTECs and targeted in organ-specific autoimmunity in mice or man. Further examples can be found in Ref. [54].

Identified autoantigen	Disease	Aire-dependent expression	Thymic loss sufficient for development of autoimmunity	Reference
seminal vesicle secretory protein 2 (SVS2) and *semenogelin*	Prostatitis	+	Unknown	[55•]
Vomeromodulin (VM) and *LPLUNC1*	Interstitial lung disease (ILD)	+	Unknown	[56•]
Odorant binding protein 1a (OBP1a)	Sjögren's syndrome	+	Unknown	[57•]
Mucin 6	Autoimmune gastritis	+	Unknown	[58]
Insulin	Diabetes mellitus type 1	+	Yes	[19••]
IRBP	Uveitis	+	Yes	[18••]
a-fodrin	Sjögren's syndrome-like	– (but Aire required for protection)	Unknown	[59]
PDIp	Pancreatitis	– (but Aire required for protection)	Unknown	[60]
NALP5	Hypoparathyroidism	+[a]	Unknown	[61•]
KCNRG	Pulmonary autoimmunity	+[a]	Unknown	[62•]

Human autoantigens are denoted in italics.
[a] Inferred from APS1 patients but not formally proven.

additional mode by which DCs diversify the pool of self-antigens imprinting tolerance [20•,21•,22]. Currently, thymic DCs are subdivided into three subpopulations [23]: two conventional CD11c^{hi} subtypes (cDCs) that are either CD11b^{-}CD8a^{hi}CD172a^{-} or CD11b^{+}CD8a$^{-/lo}$CD172a^{hi} and CD11c^{int}CD45RA^{+} plasmacytoid DCs (pDCs). The 'resident' CD11b^{-}CD8a^{hi}CD172a^{-} subset differentiates terminally within the thymus from a DC-committed pre-thymic precursor [24•], whereas mature pDC and CD8a$^{-/lo}$CD172a^{hi} cDCs continuously home to the thymus under steady-state conditions [20•]. In one model system it was demonstrated that these migratory DCs pick up antigen in the periphery followed by antigen-specific central tolerance induction. Thus, OT-II T cells recognizing ovalbumin in the context of MHC class II were deleted in the thymus, when at the same time membrane-bound ovalbumin was specifically expressed in cardiac myocytes [22]. Recently, Proietto *et al.* described both negative selection and induction of antigen-specific Tregs in thymi from OT-II TCR-transgenic mice that had been grafted into recipient mice that expressed ovalbumin under control of the CD11c promoter [21•]. Again this approach showed that extrathymic DCs home to the graft, where they present cell-bound antigen in a tolerogenic fashion. Published data on the *in situ* localization of the different thymic DC subsets, which contributes to the peptide repertoire available for thymocytes at a particular stage of their development, is not entirely consistent. The majority of DCs have been allocated to the medulla and the cortico-medullary junction (CMJ) [23]. Studies using fluorescence-tagged DCs or DCs derived from parabiotic partner animals show that migratory DCs, that is pDCs and CD8a$^{-/lo}$CD172a^{hi} cDCs also home to the medulla and the CMJ [20•]. Yet, staining thymic sections for CD172a expression in non-manipulated mice revealed CD172a positive cells predominantly in the cortex, some of which were found in peri-vascular regions and in close vicinity of small vessels [25]. Consistent with this distribution, uptake of soluble OVA by CD172a^{+} cells was observed in the cortex next to peri-vascular regions. In contrast, uptake of soluble HEL was only detected in the medulla, but not the cortex [26].

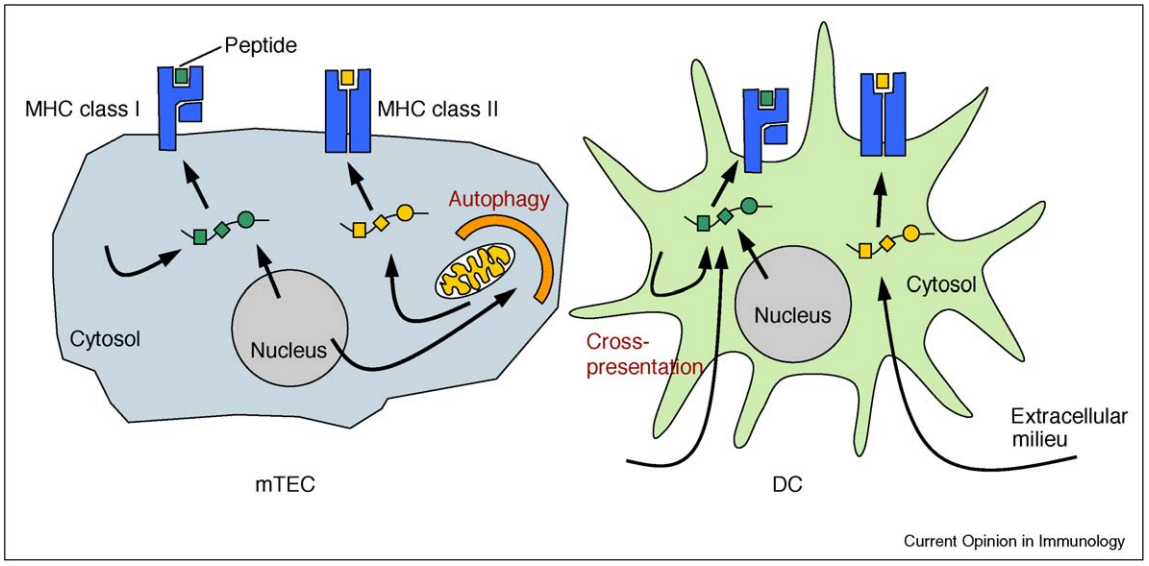
Notwithstanding these contradictory findings, the overall physiological contribution of antigen-laden migratory DCs or other myeloid cells to central tolerance remains ill defined [27,28]. In particular, we do not know to which extent these DCs complement the set of TRAs generated by pGE. Intriguingly, DCs patrolling the gastrointestinal tract could also sample the commensal gut flora and food antigens and thus would link central and oral tolerance (Figure 1b). The observation that activation of DCs by LPS before their injection impairs their thymus homing ability [22] suggest that DCs residing in inflammatory sites of ongoing immune responses, would be prevented from importing foreign pathogens into the thymus and thus exempt immunogenic antigens from central tolerance induction.

Sub-cellular antigen sampling and peptide generation

The self-proteins available to the thymus localize to different sub-cellular compartments (nucleus, organelles, cytoplasm, surface membrane) and the extracellular space. Thymic APCs seem particularly well equipped to *sample* these different pools, a prerequisite for their presentation to T cells. Thus DCs exhibit a high rate of endocytosis and possibly special means to take up antigens from mTECs [29•,30•], while TECs adopted constitutive macro-autophagy to target their intracellular antigen cargo to MHC class II [31••] (Figure 2). Macro-autophagy enables a fraction of terminally differentiated mTECs, which exhibit notoriously low endocytic activity, to autonomously present a fraction of promiscuously expressed self-antigen residing in the nucleus, cytoplasm, and organelles. The essential contribution of this pathway to central tolerance has been inferred from the observation that transplantation of macro-autophagy-deficient thymi into nude mice resulted in severe colitis and multi-organ inflammation [31••]. Since the majority of cTECs exhibit constitutive macro-autophagy under non-starved conditions, the specific contribution of mTECs to this autoimmune phenotype still needs to be formally proven. Constitutive macro-autophagy by cTECs is likely to enlarge the repertoire of MHC class II-restricted self-peptides involved in the process of positive selection. This aspect will not be further discussed here.

The intracellular antigen sampling pathways are inextricably linked with the *processing* of proteins into MHC-binding peptides. Again we might ask whether antigen processing in medullary APC is optimized to serve the purpose of tolerance induction, that is do they faithfully generate the self-peptide repertoire potentially encountered on peripheral APCs? The proteasome, a multi-protein complex, which initiates the proteolytic degradation of ubiquitylated intracellular proteins, generates peptides for loading onto MHC class I. There are two major forms, the housekeeping and the immunoproteasome, both of which generate partly non-overlapping peptides [32,33]. Medullary APCs, that is DCs and mTECs express a mixture of both forms (with a preponderance of the immunoproteasome) [34] thus potentially covering peptides generated in peripheral tissues under non-inflammatory conditions by the housekeeping proteasome and under inflammatory conditions (e.g. IFNg) by the immunoproteasome [32] (Table 2). MHC class II-binding peptides are generated in the endosomal/lysosomal compartment by a mixture of proteases including cathepsin L and S, which similar to the two major proteasome forms, generate different sets of peptides [35]. Cathepsin S expression and enzymatic

Figure 2



Antigen sampling strategies of thymic DCs and mTECs. Medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs) use various routes to sample intracellular compartments and the extracellular space for self-antigen display. MTECs focus on the degradation of their intracellular contents for presentation by MHC class I and class II molecules. By way of macro-autophagy a fraction of mTECs channels peptides derived from self-antigens located in the cytoplasm, nucleus and organelles into the MHC class II pathway. In contrast, DCs exhibit a high rate of endocytosis to sample the extracellular space, cross-present extracellular antigens on MHC class I molecules and possibly employ special means to take up antigen from mTECs (not depicted here).

activity are found both in peripheral APCs and in BM-derived APCs of the thymus and in mTECs (though enzyme activity has not yet been documented in mTECs). In contrast, cathepsin L expression is restricted to cTECs. Thus BM-derived thymic APCs and mTECs involved in negative selection again mirror peripheral APCs with regard to differential expression of components of the antigen processing machinery.

The close functional correspondence between the thymic medullary and the peripheral APC compartments is underscored, when compared to cTECs, the third major APC type of the thymus exhibiting constitutive MHC class I and class II expression. CTECS express: firstly, a unique form of the proteasome by incorporating the proteasome subunit beta5t instead of beta5 or beta5i [36]; secondly, cathepsin L instead of cathepsin S and thirdly, the thymus-specific-serine-protease (TSSP) in their endosomal/lysosomal compartments [37•]. These features result in the generation of a cTEC-specific peptide repertoire both on MHC class I (proteasome) and MHC class II (specific protease set). The functional importance of these cTEC-specific peptide pools has been clearly documented by the strong reduction of the CD8 compartment (in case of beta5t k.o. mice), the CD4 compartment (in case of cathepsin L k.o. mice) [38] and the impediment of positive selection of certain MHC class II-restricted TCRs (in case of TSSP k.o. mice) [37•]. The strong reduction of the CD4SP compartment in cathepsin L k.o. mice was not solely due to a block in invariant chain cleavage, but also to a defect in the generation of positively selecting peptides [39]. Remarkably, the loss of beta5t not only had a quantitative but also a qualitative effect on the mature CD8 T cell repertoire as reflected by reduced allogeneic and antiviral immune responses [40••]. Two scenarios have been put forward to explain why there is a requirement for a cTEC-specific MHC/peptidome: firstly, unique peptide MHC complexes on cTECs may be necessary to counteract excessive loss of positively selected T cells. This loss would incur when thymocytes encounter the same peptide on cTECs during positive selection and with higher avidity on mTECs/DCs resulting in negative selection [7•]. Secondly, the thymoproteasome is assumed to generate preferentially peptides with low MHC class I binding affinities (i.e. high off rates) [36,41•]. It has been argued that such unstable peptides are required for the positive selection step, which involves low avidity interactions. The same argument might also apply to peptides generated by cathepsin L and TSSP for MHC class II-restricted selection, though there is no experimental evidence for this.

Scanning self-antigens: a hurdle race in time and space

Tolerance will only be effective and 'tight', if the plethora of displayed self-peptides is faithfully scanned by most if not all post-selection thymocytes. The stringency and efficacy of this process is set by certain parameters: firstly, the residence time of thymocytes in the medulla; sec-

Table 2

Differential distribution of components of the antigen processing machinery under non-inflammatory conditions in different APC compartments.

	Thymus		Periphery				Reference
cTEC	mTEC	BM-derived APC	BM-derived APC	Kidney	Liver	Intestinal epithelial cells	
Thymoproteasome + housekeeping	Immunoproteasome + housekeeping	Immunoproteasome + housekeeping	Immunoproteasome + housekeeping	Housekeeping	Housekeeping	Housekeeping	[34,36, 63,64]
TSSP	–	–	–	–	–	–	[65,66]
–	CatS[a]	CatS	CatS	–	–	CatS	[3,35, 38,47]
CatL	CatL[a]	–	– (CatL in macrophages)	CatL	CatL	–	
Autophagy	(partially)	–	–	–	–	–	[31••]

Note the similarities between thymic and peripheral APCs highlighted in grey.
[a] Only mRNA data, no enzymatic activity investigated.

ondly, the frequency and distribution of APCs expressing a given antigen; thirdly, the *in situ* half-life of mTECs and DCs and fourthly, the stability of gene expression during the lifetime of individual APCs. Recent studies show that each of these parameters imposes tight restrictions on this process which make it all the more astounding that central tolerance as a whole works so efficiently [42]. Thus the residency time of thymocytes in the medulla is estimated to be in the range of 4–5 days and thus shorter than previously reported [43]. Each TRA is only expressed by 1–3% of mTECs, which are scattered throughout the medulla [4]. MTECs and most DCs rapidly turn over within about 2 weeks [44,45] and pGE appears to be transient/fluctuating in individual mTECs (A Sinemus *et al.*, unpublished), altogether resulting in a highly dynamic and unstable pattern of antigen display in time and space within medullary micro-domains. How do thymocytes cope with these apparent hurdles? A recent study by Borgne *et al.* addressed this issue in an experimental model. They simultaneously tracked fluorescence-tagged thymocytes and DCs in slice cultures of explanted thymic lobes via intra-vital two-photon laser scanning microscopy. Medullary thymocytes moved much faster than cortical thymocytes (e.g. 12 versus 6 μm/min) making sequential, brief contacts with DCs (5.5 contacts/hour lasting for 1.2 min each) [46••]. It was estimated that medullary thymocytes could contact in this way about 500 DCs during their sojourn in the medulla. Surprisingly, their movement was confined to certain micro-domains with an approximate diameter of 30 μm during the observation time of 20 min. It remains currently unknown, how long medullary thymocytes will stay in such a confinement zone and whether they eventually will set out to enter another zone. Interestingly, in the presence of their cognate antigen (i.e. OT-I T cells in RIP-mOVA thymi) medullary thymocytes slowed down, became more restricted in their migratory range and engaged in prolonged and repetitive contacts with local DCs. These data document the rapid and highly dynamic scanning of DCs in the medulla and show how this process is affected by self-antigen recognition. It will be interesting to know how mTECs tie into this picture (they had not been labelled in this study), since OVA expression in RIP-OVA mice is actually restricted to mTECs [47]. The presumed *in situ* recognition of OVA on DCs is likely to be due to cross presentation. Despite the remarkable methodological sophistication of this and related studies [48], we are still far a way from understanding how the scanning behaviour of thymocytes is fine tuned to accommodate the spatial and temporal dynamics of self-antigen display. Several observations however indicate that this interplay operates at its limits. Thus moderate reductions in the level of self-antigen expression [49], the number of mature mTECs and, importantly, disturbances of their three- dimensional array subvert the tolerance process [50,51]. This speaks for a finely tuned balance between these parameters, any one of which may not be well buffered.

Conclusions and perspectives

Recent studies underpin the emerging concept that the APC compartment of the thymic medulla (re)presents a mirror image of peripheral self-antigen display. This is accomplished by complementing features of the two main APC types — mTECs and DCs — which pertain to their intra- or extrathymic origin and antigen sampling territories, their migratory or sessile nature, their adoption of constitutive macro-autophagy, specialization in intercellular antigen transfer and their versatile antigen processing faculties. Altogether this results in maximizing the availability of self-antigens in the thymus, which impart self-tolerance on the nascent T cell repertoire already before mature T cells seed the peripheral lymphoid organs. Interestingly, this close relationship between the medulla and peripheral lymphoid organs is also reflected in their dependence on the NFkB pathway for their organogenesis and homeostasis [52]. In contrast, the thymic cortex stands apart, when considering the same parameters involved in the generation of the self-peptide repertoire. The cortex probably represents the 'proto thymus' primarily responsible for the generation of a diverse T cell repertoire, whereas the medulla as a later addition mainly serves the quality control of the nascent T cell repertoire. The close functional interdependence between intrathymic self-antigen display and the regulation of thymocyte mobility would argue for co-evolution of both processes. Currently, the only piece of experimental data tracing the evolutionary origin of pGE is the structural conservation of the Aire gene 'down to' teleosts [53].

Obviously, we still need to fill many gaps regarding the function of the various APC subsets.

Which is the specific part of each subset in covering a particular section of the entire self-antigen pool? Is oral tolerance to commensal gut bacteria or innocuous food antigens in part mediated via central rather than peripheral tolerance? These questions will in the near future be addressed and hopefully answered with the help of targeted genetic ablation of components of the antigen processing machinery or lineage-specific ablation of the various APC subsets.

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