BRIEF DEFINITIVE REPORT

The thymic medulla: a unique microenvironment for intercellular self-antigen transfer

Christian Koble and Bruno Kyewski

Division of Developmental Immunology, Tumor Immunology Program, German Cancer Research Center, 69120 Heidelberg, Germany

Central tolerance is shaped by the array of self-antigens expressed and presented by various types of thymic antigen-presenting cells (APCs). Depending on the overall signal quality and/or quantity delivered in these interactions, self-reactive thymocytes either apoptose or commit to the T regulatory cell lineage. The cellular and molecular complexity underlying these events has only recently been appreciated. We analyzed the ex vivo presentation of ubiquitous or tissue-restricted self-antigens by medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs), the two major APC types present in the medulla. We found that the ubiquitously expressed nuclear neo–self-antigen ovalbumin (OVA) was efficiently presented via major histocompatibility complex class II by mTECs and thymic DCs. However, presentation by DCs was highly dependent on antigen expression by TECs, and hemopoietic cells did not substitute for this antigen source. Accordingly, efficient deletion of OVA–specific T cells correlated with OVA expression by TECs. Notably, OVA was only presented by thymic but not peripheral DCs. We further demonstrate that thymic DCs are constitutively provided in situ with cytosolic as well as membrane–bound mTEC-derived proteins. The subset of DCs displaying transferred proteins was enriched in activated DCs, with these cells being most efficient in presenting TEC-derived antigens. These data provide evidence for a unique, constitutive, and unidirectional transfer of self-antigens within the thymic microenvironment, thus broadening the cellular base for tolerance induction toward promiscuously expressed tissue antigens.

The scope of central tolerance is determined by the recognition of self-antigens presented by various APCs in the thymus. The thymic medulla is the major but not exclusive site of central tolerance induction (McCaughtry et al., 2008). The complexity of this process with regard to the composition and role of distinct APC types, and the sets of self-antigens displayed has only recently become apparent. Self-antigens can be supplied by the proteome of epithelial cells (i.e., cortical and medullary thymic epithelial cells [mTECs]) and BM-derived APCs (i.e., DCs, macrophages [MΦ], and B cells). In addition, self-antigens from the periphery can reach the thymus medulla via the blood stream or be imported via immigrating antigen-laden DCs (Klein et al., 2001; Bonasio et al., 2006; Li et al., 2009). Among these different self-antigen pools, the array of promiscuously expressed tissue-restricted self-antigens by mTECs is of particular interest, because it preempts the peripheral self. Qualitative or quantitative alterations of this antigen pool predispose to organ-specific autoimmunity. How central tolerance to these antigens is achieved is closely connected to the questions of which APCs present these antigens and which processing pathways are involved (Klein and Kyewski, 2000).

Previous studies addressing these issues have been limited to broadly expressed self-antigens (i.e., Eκ; Humblet et al., 1994), or neo–self-antigens driven by promoters resulting in expression in a major subset of mTECs (i.e., the Aire promoter; Aschenbrenner et al., 2007) or in more restricted expression, e.g., the rat insulin promoter (Zhang et al., 2003; Gallegos and Bevan, 2004; Zehn and Bevan, 2006). In case of the latter transgenes, it remained however unclear...
whether the frequency of antigen-positive mTECs and the expression levels per cell faithfully reflected the pattern of promiscuously expressed tissue-restricted antigens (TRAs). Both parameters are critical for T cell (TC) selection.

In this report, we analyzed ex vivo presentation of the two native self-antigens, P1A and proteolipid protein (PLP). The tumor rejection antigen P1A is a prototypic cytosolic TRA whose expression is tightly regulated and only detectable in male germ cells and rare mTECs (Derbinski et al., 2001). PLP, an oligodendrocyte-specific myelin protein, is strongly expressed in mTECs and weakly expressed in thymic DCs (Derbinski et al., 2001). Moreover, we wanted to delineate the relative contribution of thymic APC types to tolerance when antigen expression is not confined to a particular cell type. To this end, we chose the neo–self-antigen OVA, expressed ubiquitously and targeted specifically to the nucleus (Kawahata et al., 2002).

We found both thymic DCs and mTECs to present these self-antigens ex vivo and in vivo. Efficient presentation by DCs, however, was contingent on antigen provision by TECs irrespective of their subcellular localization or expression pattern. This efficient self-antigen transfer, which apparently is unique to the thymus, broadens the cellular base of self-tolerance and thus enhances its efficacy.

RESULTS AND DISCUSSION
Ex vivo presentation of an ubiquitous, nuclear self-antigen by thymic APCs

We chose to analyze presentation of the neo–self-antigen OVA driven by the ubiquitous MHC class I (Lδ) promotor. In contrast to previous studies that mainly focused on soluble (Klein et al., 2001) or membrane-bound neo–self-antigens (Zhang et al., 2003; Gallegos and Bevan, 2004; Zehn and Bevan, 2006; Aschenbrenner et al., 2007), in this case a nuclear localization sequence targets OVA protein to the nucleus, thus representing self-antigens of the nuclear compartment (Kawahata et al., 2002).

Ex vivo OVA presentation was assayed by co-culture of purified APCs from the thymus, spleen, and mesenteric lymph nodes of Lδ-nOVA mice together with naive DO11.10 CD4+ Tcs expressing a TCR specific for the OVA peptide 323–339 in conjunction with I-Aδ (Murphy et al., 1990; Kawahata et al., 2002). Thymic DCs displayed the most efficient presentation followed by mTECs, whereas splenic and lymph node–derived DCs induced a 10– to 40-fold lower proliferation (Fig. 1 A). At the same time, all four APC populations showed comparable antigen presentation on a per cell basis when loaded with exogenous peptide, excluding overall presentation competence to account for the observed differences (Fig. S1 A). Moreover, thymic and splenic DCs displayed similar OVA expression at the RNA level (Fig. 1 B). One reason for the remarkable difference in efficacy of OVA presentation between thymic and peripheral DCs could reside in their respective composition. 60–90% of thymic DCs belong to the CD8αα+ subset, which was shown to be most efficient in cross-presentation, whereas among splenic DCs, only 10–20% express CD8αα (Vremec et al., 1992; den Haan et al., 2000). Hence, we separately assessed CD8αα+ and CD8αα− DCs from the thymus and spleen. Enrichment of either subset rescued splenic OVA presentation, arguing against variable DC subset composition to account for the observed interorgan differences (Fig. S2).

These results suggest that the efficient presentation of the ubiquitously expressed self-antigen OVA by thymic DCs either reflects a property of a specialized thymic DC subset (if presented autonomously), or that antigen is provided specifically within the medullary microenvironment, i.e., by transfer from hemopoietic cells or TECs. To distinguish between these alternatives, we generated BM chimeras in which either BM-derived APCs or TECs expressed OVA exclusively. Remarkably, efficient OVA presentation by thymic DCs was observed only when OVA was expressed by radio-resistant host cells. In contrast, OVA presentation by thymic DCs was inefficient when DCs themselves (and other hemopoietic cells), but not radioresistant stromal cells, were transgenic for Lδ-nOVA (Fig. 2 A). At the same time, OVA presentation by mTECs was strictly dependent on endogenous expression (Fig. 2 A). However, antigen presentation was lower in mTECs isolated from chimeras as compared with WT, possibly as a consequence of radiation damage. Conversely, DCs isolated from BALB/c → BALB/c chimeras induced higher TC activation than those from nonmanipulated mice. A possible explanation, not further explored in this report, is the generation of cross-reactive epitopes by radiation, which is not without precedent (Reits et al., 2006).

Presentation by splenic DCs remained low, irrespective of the type of chimera (Fig. 2 A). These data show that nuclear OVA is autonomously presented to CD4+ Tcs by mTECs, whereas its presentation by thymic DCs mostly relies on antigen transfer from radio-resistant TECs, but not from hemopoietic cells. A similar efficient transfer mechanism obviously does not exist in the microenvironment of the spleen and lymph nodes, at least at the level of resolution attained here. The differences in ex vivo presentation of OVA were also reflected at the level of TC selection. Deletion of OVA-specific DO11.10 Tcs was most efficient when OVA was expressed by radio-resistant TECs irrespective of additional OVA expression in BM-derived cells (Fig. 2, B and C). The lower but perceptible degree of deletion by OVA-expressing BM cells indicates that OVA does access MHC class II on DCs, possibly via the secretory pathway followed by endocytosis, a route that has not been strictly excluded by the study of Kawahata et al. (2002).

Ex vivo presentation of ectopically expressed endogenous TRAs by thymic APCs

To verify whether presentation of promiscuously expressed endogenous antigens follows the same rules as deduced from transgenic models (Zhang et al., 2003; Gallegos and Bevan, 2004; Zehn and Bevan, 2006), we assessed the ex vivo presentation of the TRAs P1A and PLP via MHC class I and II, respectively.
The tumor antigen P1A is only expressed in mTECs but not in thymic or splenic DCs (Fig. 1 D), whereby 1–3% of mTECs express P1A at the protein level (Derbinski et al., 2001). Ex vivo presentation of P1A was assayed by proliferation of freshly isolated naive CD8+ TCs expressing a transgenic TCR specific for the P1A epitope 35–43. Again, thymic DCs proved to be most efficient (Fig. 1 C), pointing toward cross-presentation of mTEC-derived P1A, whereas direct presentation by mTECs was clearly weaker and only revealed by isolating the mature mTEC subset (Fig. S3). No significant proliferation was induced by splenic DCs. Again, this pattern could not be explained by differences in the presentation competence of the various APC populations (Fig. S1 B).

A previous report on in vivo deletion of autoreactive OVA-specific CD8+ and CD4+ TCs showed differences in the requirement for cross-presentation (Gallegos and Bevan, 2004). Although both mTECs and DCs were able to delete MHC class I–restricted OT-1 cells, deletion of MHC class II–restricted OT-2 cells required OVA cross-presentation by thymic DCs in the RIP-mOVA mouse model. In contrast, TECs were sufficient to delete CD4+ TCs specific for soluble human C-reactive protein (Klein et al., 1998, 2001), and mTECs were capable of deleting CD4+ Tcs specific for membrane-bound hemagglutinin (Aschenbrenner et al., 2007).

We chose PLP, a myelin sheath component, to assess MHC class II–restricted ex vivo presentation. PLP is promiscuously expressed by both cortical thymic epithelial cells and mTECs and at marginal levels in DCs (Fig. 1 F), with 1–3% of all mTECs expressing the PLP protein (Derbinski et al., 2001). Presentation of PLP was assayed using a CD4+ Tc line specific for the epitope 239–250 in the context of I-Aβ. Importantly, this epitope is present in the DM20 isoform of PLP, which is predominantly expressed in the thymus (Klein et al., 2000; Derbinski et al., 2001). In line with previous results, thymic DCs efficiently presented PLP (Fig. 1 E). In contrast, neither mTECs, including the mature subset, nor splenic DCs induced proliferation above background levels. The lack of presentation by mature mTECs was unexpected.

Figure 1. Efficient presentation of TEC-derived self-antigens by thymic DCs. mTECs and DCs from the thymus (Thy-DC), spleen (Sp-DC), and lymph nodes (LN-DC) of (A) Ld-nOVA, (C) BALB/c, or (E) C57BL/6 mice, respectively, were co-cultured for 3 d with antigen-specific Tcs. Presentation of OVA (A), P1A (C), and PLP (E) was assessed by antigen-specific proliferation of naive DO11.10 CD4+ Tcs, P1A-TCR transgenic CD8+ Tcs, or the PLP-specific Tc line TPLP-15-5-2. Shown are the triplicate mean values ± SD of one representative of at least two experiments performed. Transcription of (B) OVA, (D) P1A, and (F) DM20 by the indicated cell populations was evaluated by quantitative (B and F) or semiquantitative (D) PCR analysis. Expression levels were normalized to HPR7/Ubiquitin (B) or β-actin (D and F). Error bars in B and F indicate the SD of triplicates of the same cDNA preparation.
given the strong mRNA signal and detectable protein expression (Derbinski et al., 2001). Possible explanations are a lower PLP protein expression and stability and/or inefficient processing by mTECs when compared with P1A. Irrespective of these considerations, PLP expression and presentation by mTECs and DCs are inversely correlated, which is indicative of antigen transfer from TECs to DCs rather than autonomous presentation by DCs. This interpretation is indirectly supported by the lack of PLP presentation by splenic DCs.

Thus, presentation of these two TRAs, P1A and PLP, follows similar rules as shown for L^d-nOVA. Which features of the medullary microenvironment might account for the efficient transfer of self-antigens?

Transfer routes from mTECs to DCs
At least four mechanisms might contribute to the intercellular transfer of proteins of different subcellular compartments: exosome transfer, uptake of apoptotic bodies, membrane exchange ("nibbling"), and gap junctions (Albert et al., 1998; Harshyne et al., 2001; Thery et al., 2002; Neijssen et al., 2005). The most direct way to confer MHC-restricted antigen presentation onto another cell is the exchange of surface membrane–bound peptide–MHC complexes. We addressed this option by analyzing the transfer of MHC class II molecules in P→F1 type chimeras, i.e., BALB/c → (BALB/c × C57BL/6)F1 and C57BL/6 → (BALB/c × C57BL/6)F1 mice. 6–8 wk after reconstitution, thymic DCs were isolated and analyzed for expression of host-specific MHC haplotypes. Clearly, DCs stained for MHC class II molecules transcribed by radio-resistant host cells, with the density of expression of these “acquired” MHC molecules being lower than on DCs of heterozygous animals (Fig. 3, A and B). These results concur with a recent report describing a unidirectional transport of MHC class II molecules between thymic APCs in vitro and in vivo (Millet et al., 2008).

We next explored a second route, namely the transfer of cytoplasmic proteins, that either could occur via uptake of apoptotic bodies or directed exosome traffic. To this end, we analyzed thymi of FoxN1-eGFP reporter mice. These mice express eGFP intrathymically exclusively in TECs under the control of the epithelium-specific FoxN1 promoter, but not in hemopoietic cells (Terszowski et al., 2006). Thymic DCs from this strain displayed a green fluorescence signal clearly above background level but weaker than that of TECs (Fig. 3, C and D). Interestingly, the degree of eGFP staining of thymic DCs correlated with surface staining for epithelial cell adhesion molecule (EpCAM), which presumably, like MHC class II molecules, was acquired from TECs (Fig. 3, D and E). Expression of EpCAM on hemopoietic cells had previously been observed but not further.

Figure 2. Ex vivo presentation of the nuclear neo–self-antigen OVA by thymic DCs and efficient deletion of DO11.10 TCs in vivo depend on antigen transfer from radio-resistant stromal cells. (A) Lethally irradiated BALB/c WT or L^d-nOVA transgenic mice were reconstituted with TC-depleted BM cells of the indicated donor strains. After 5–8 wk, CD11c^+ DCs of the thymus (Thy-DC) and spleen (Sp-DC) and mTECs, respectively, were isolated and co-cultured with DO11.10 CD4^+ TCs. OVA presentation was assessed by antigen-specific proliferation of TCs. Bars represent threefold titration steps of the indicated APC population ranging from 3 × 10^5 to 10^7 cells/well. Shown are the triplicate mean values ± SD of one representative of two or more experiments performed. (B) 5–6 wk after reconstitution, the number of total CD4^+ single-positive and DO11.10 clonotype-specific CD4^+ single-positive thymocytes was assessed. Symbols represent values of 7–10 individual thymi in each group. * P < 0.005. (C) FACS analysis of clonotypic DO11.10 CD4^+ TCs of each chimera type. Shown are overlay histograms of three individuals. Arrows indicate DO11.10 clonotype-specific CD4^+ TCs.
were enriched in cells expressing CD80, CD86, CD103, and MHC class II, indicative of a mature phenotype (Fig. 4 and Fig. S4). At the same time, this subset was devoid of CD11b- and F4/80-positive cells, i.e., monocytes/MΦ. Most markers showed a monophasic distribution with the exception of CD103, CD24, and mPDCA1, indicating further heterogeneity among this DC subset.

Next we asked whether TEC-tainted DCs were also enriched in DCs cross-presenting TEC-derived self-antigens, explored (Borkowski et al., 1996). The co-staining of eGFP and EpCAM by a subset of DCs implies that the same cells acquire TEC-derived membrane receptors and cytoplasmic proteins.

**Phenotype and function of “TEC-tainted” DCs**

Given the correlation between EpCAM and eGFP expression, we used these surrogate markers to further characterize this DC subset with respect to phenotype and function. Assessing a panel of markers, eGFP/EpCAM-positive DCs

---

**Figure 3.** Cotransfer of membrane-bound and cytoplasmic proteins from epithelial cells marks DCs with efficient cross-presentation. (A and B) Lethally irradiated CB6/F1 mice were reconstituted with either BALB/c (A) or C57BL/6 (B) TC-depleted BM. After 6 wk, low density TSCs were enriched by Nycodenz density gradient centrifugation. Cells were stained with mAb against CD11c, EpCAM, and the parental MHC class II haplotypes I-A<sup>b</sup> and I-A<sup>d</sup>. Thymic DCs were defined as CD11c<sup>high</sup> EpCAM<sup>intermediate</sup> cells. Expression of the respective host-specific MHC haplotype by thymic DCs is displayed as shaded histograms, whereas lines represent expression of the indicated haplotypes by C57BL/6 (solid), CB6/F1 (dashed), or BALB/c (dotted) thymic DCs. (C) EGFP-containing cells are detectable within the CD11c<sup>+</sup> thymic DC population of FoxN1-eGFP mice. Thymic CD11c<sup>+</sup> cells were preenriched using MACS technology and analyzed by flow cytometry, excluding autofluorescent cells. (D) EGFP-expressing TECs are distinguishable from eGFP-containing thymic DCs on the basis of differential CD11c expression, as shown by flow-cytometric analysis of single-cell suspensions. Cells were isolated and pooled from five FoxN1-eGFP thymi. (E) EpCAM expression of FoxN1-eGFP CD11c<sup>+</sup> thymic DCs correlates with eGFP fluorescence intensity, as shown for CD45<sup>+</sup>, non-autofluorescent cells. (F) EpCAM-expressing CD11c<sup>+</sup> thymic DCs (Thy-DC) present TEC-derived OVA most efficiently. CD11c<sup>+</sup> splenic DCs (Sp-DC) were enriched according to differential CD11c and EpCAM coexpression, resulting in the indicated subpopulations of ≥99% purity. CD11c<sup>+</sup> splenic DCs (Sp-DC) were enriched by magnetic separation (purity ≥ 90%). OVA presentation was assessed by proliferation of OVA-specific D011.10 CD4<sup>+</sup> Tcs after a 3-d co-culture period. Shown are the means of triplicates ± SD. Each figure (C–F) is representative of two to six independent experiments.
We consider this autonomous presentation of OVA by mTECs to be most likely caused by constitutive macroautophagy in TECs (Nedjic et al., 2008), which, in addition to cytosolic proteins, also includes nuclear self-antigens (Riedel et al., 2008). Indeed, peptides derived from nuclear proteins can be eluted from MHC class II of mTECs (unpublished data). Several explanations could account for the fact that OVA-expressing hemopoietic cells (including TCs, DCs, MΦ, and B cells) are poor in providing antigenic material for cross-presentation, depending on the mechanism of self-antigen transfer. If processed antigens were transferred, as recently shown in vitro (Millet et al., 2008), hemopoietic cells might lack the ability to generate the specific peptide-MHC class II complex to be transferred onto DCs (i.e., they lack constitutive autophagy). If antigen were transferred in its native form and processing occurred in the target cell, generation of transfer vehicles by hemopoietic cells might be less efficient than by TECs. Irrespective of these considerations, the observed antigen transfer could occur via membrane transfer of peptide-MHC complexes, exosomes, or apoptotic bodies. At present we cannot distinguish between these different possibilities.
modes. The fact that DCs expressing the highest amount of EpCAM also show the strongest GFP staining speaks in favor of cotransfer of cytosolic and membrane constituents i.e., via directed exosome trafficking or apoptotic bodies. However, we consider uptake of apoptotic bodies as the main mechanism less likely, because negative selection of autoreactive TCs directly interacting with mTECs (Irla et al., 2008) should provide plenty of antigenic material, yet such a transfer is not observed. Moreover, the extensive transfer of membrane complexes between mTECs and DCs would comply with the similar in vivo half-life of peptide–MHC complexes on both cell types, which is in the range of 25 h (unpublished data).

Our results imply that the self-antigen repertoire specifically generated by mTECs is also available to DCs for presentation. This concurrent presentation ensures that any particular tolerance mode linked to a special APC type will cover the array of promiscuously expressed genes. Thus, mTECs have been shown to be more efficient in selecting T reg cells, whereas DCs are the better deleters (Apostolou et al., 2002; Aschenbrenner et al., 2007). This preference, however, is relative rather than absolute, because bone marrow-derived APCs can also generate T reg cells directly interacting with mTECs (Irla et al., 2008), implying that this critical set of self-antigens is “double checked” for tolerance induction.

MATERIALS AND METHODS

Animals

C57BL/6, BALB/c, and CBA/F1 mice were obtained from Charles River Laboratories. Lα-nOVA mice (Kawahata et al., 2002) on the BALB/c background were provided by K. Yamamoto (University of Tokyo, Tokyo, Japan) and were further interbred. FoxN1-eGFP mice (Terszowski et al., 2006) were provided by A.-M. Schmitt-Verhulst (University of Marseille, France), and were generated on a Rag-1<sup>-/-</sup> background (Murphy et al., 1990) and were kept as an inbred strain. TCRP1A transgenic mice were obtained from C. Terszowski et al. (unpublished data).

Our data characterize a constitutive and unidirectional transfer route for self-antigens between mTECs and DCs, which is possibly unique to the microenvironment of the thymic medulla. By this way, the set of tissue-restricted self-antigens exclusively expressed by mTECs also becomes available to the second major APC type involved in TC repertoire selection, thus ensuring that this critical set of self-antigens is available to the thymic medulla.

Cell preparation and analysis

Isolation of mouse thymic stromal cells (TSCs). TSCs were purified as described previously (Derbinski et al., 2001) or with the following modifications: after three rounds of collagenase digestion and one round of collagenase/ dispase digestion, thymic rosettes were pooled, washed in PBS containing 0.5% BSA and 5 µM EDTA, and stained with anti-CD11c microbeads (Miltenyi Biotec). Subsequently, CD11c<sup>+</sup> thymic DCs were isolated using MACS technology according to the manufacturer’s protocol (Miltenyi Biotec), resulting in a suspension containing 90–95% CD11c<sup>+</sup> cells. For subfraction of thymic DCs, CD11c<sup>+</sup>-enriched TSCs were stained with various combinations of biotinylated anti-CD11c (HL3) and streptavidin-PE or streptavidin-allophycocyanin-Cy7 (BD), anti–EpCAM–Alexa Fluor 647 (G8.8), anti-CD4–PE or CD4–PerCP, respectively (both 30-F11; BD), and anti–CD8α–PE (53-6.7; BD). The TEC-containing fraction (collagenase/dispase digestion rounds two to five) was further enriched by MACS depletion of CD4<sup>+</sup> hematopoietic cells using anti-CD4 microbeads (Miltenyi Biotec), followed by staining with either anti–CDR1–Alexa Fluor 488 or anti–Ly51–FITC (6C3; BD), anti–EpCAM–Alexa Fluor 647 (G8.8), and anti–CD4–PE (30-F11). After staining, cells were resuspended in FACS buffer containing 50 µg/ml propidium iodide to exclude dead cells. For subfraction of mTECs, the following combinations were used: anti–CD80–PE (16-10A1) or anti–KLH–PE (Ha 4/8; isotype control), anti–CD4–PE (30-F11), anti–Ly51–FITC (6C3; all were obtained from BD), and anti–EpCAM–Alexa Fluor 647 (G8.8). FcR blocking with the anti–FcR mAb 2.4G2 preceded all stainings. Cell sorting was performed with a cell sorter (FACSDiVa; BD). Autofluorescent cells were excluded from the sorted populations.

For phenotype analysis of DC subpopulations, in addition to the aforementioned reagents the following antibodies were used: anti–CD86–PE (GL1; BD), anti–MHC class II–PE I-A<sup>+</sup> (AMS–32.1; BD), anti–CD24–PE (16-10A1; BD), and anti–CD8–PE (53-6.7; BD), and biotinylated anti–DO11.10 cloneotype (KJ1–26; eBioscience) antibodies, followed by streptavidin–allophycocyanin-Cy7 (BD).

Flow cytometric measurements were performed using a FACSCalibur or FACSCanto II (BD). Data were collected from viable lymphocytes by appropriate forward and side scatter gating. Data analysis was performed with FlowJo software (version 6.4.1; Tree Star, Inc.).

Isolation of lymphoid cells from the spleen. Myeloid and lymphoid DCs from the spleen were isolated as previously described (den Haan et al., 2000), with minor modifications. In brief, a collagenase-digested suspension of four to eight spleens was subjected to erythrocyte lysis and filtered through a 70-µm cell strainer. Subsequently, CD11c<sup>+</sup> DCs were isolated using MACS technology according to the manufacturer’s protocol (Miltenyi Biotec), resulting in a suspension containing 90–95% CD11c<sup>+</sup> cells.

For isolation of TCs, spleens of one to two mice were minced into small pieces and passed through a metal sieve. After erythrocyte lysis, the cell suspension was washed in PBS containing 0.5% BSA and 5 µM EDTA and stained with anti–CD11c microbeads (Miltenyi Biotec). Subsequently, CD11c<sup>+</sup> DCs were isolated using MACS technology according to the manufacturer’s protocol (Miltenyi Biotec), resulting in a suspension containing 90–95% CD11c<sup>+</sup> cells.

For isolation of TSCs, spleens of one to two mice were minced into small pieces and passed through a metal sieve. After erythrocyte lysis, the cell suspension was washed in PBS containing 0.5% BSA and 5 µM EDTA and stained with anti–CD11c microbeads (Miltenyi Biotec). Subsequently, CD11c<sup>+</sup> DCs were isolated using MACS technology according to the manufacturer’s protocol (Miltenyi Biotec), resulting in a suspension containing 90–95% CD11c<sup>+</sup> cells.
PCR analysis

PCRs were performed as previously described (Derbinski et al., 2001). Primer pairs were synthesized by the oligonucleotide synthesis facility of the German Cancer Research Center and, when possible, were designed to span at least one intron.

Amplification was performed in a DNA Engine Dyad ThermoCycler (Biozym) under standard thermal cycling conditions. Reaction products were separated on 1 × TAE (40 mM Tris-acetate, 1 mM EDTA) 1.5% agarose gels that contained 30 mg/ml ethidium bromide (Roth). PCR products were revealed with the Lumi-Imager F1 Workstation (Roche) or a video documentation system (BioVision; Peqlab), and bands were quantified with LumiAnalyst software (version 3.0; Roche) or Vision-Capture software (version 12.9; Peqlab). For semiquantitative PCR, cDNA preparations were normalized to β-actin expression before testing expression of the gene of interest.

Quantitative PCR

Real-time PCR reactions were performed in a final volume of 25 µl with optimal concentrations of forward and reverse primers (50–900 nM) using the qPCR core kit for SybrGreen I (Eurogentec) containing Hot GoldStar polymerase and Uracil-N-Glycosylase. Probes were used at a concentration of 200 nM in combination with the qPCR core kit. Reactions were run on a sequence detection system (GeneAmp 7300; Applied Biosystems) in triplicates, and expression values were normalized to β-actin or HPRT/ubiquitin expression using the comparative C method. Primers were purchased from Eurofins MWG Operon and, whenever possible, were designed to span at least one intron. Probes were purchased from Eurogentec.

Generation of radiation BM chimeras

4–5-wk-old recipients were lethally irradiated (8.5–9.5 Gy) and reconstituted with 2 × 106 BM (5 × 106 cells/animal). Functional analysis of antigen presentation and phenotype analysis of grafted animals was performed after another 5–8 wk.

Ex vivo proliferation assay

Purified TSCs were co-cultured with naive transgenic OVA-specific (DO11.10; Murphy et al., 1990) or P1A-specific (TCR–P1A; Shanker et al., 2004) DCs, or PLP-specific TC lines in flat-bottom 96-well plates in 200 µl of culture medium in the presence of 1 µg/ml of the respective antigen, as indicated in the figures. The following antigen-specific peptides have been used: OVA 

\[ \text{OVA}_{239-250} \]

(derived from the OVA protein expressed in thymic epithelial cells), and CD80

\[ \text{CD80}_{323-339} \]

mTEC subsets in comparison to thymic and splenic DCs.

Autoantigen-specific interactions with CD4+ thymocytes control ma

E. Rezavandy for expert technical assistance, and the staff of our animal facilities for and EpCAM expression. Online supplemental material is available at http:// JEM.1512.200802449. Submitted: 29 October 2008 Accepted: 3 June 2009

This work was supported by the German Cancer Research Center and the European Union–funded consortia Thymaide (grant 14362), Immunotherapy (grant 14363), and Tolerance (grant 14364). The authors declare no conflicting financial interests.

REFERENCES

Alpert, M.L., S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via αβ5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J. Exp. Med. 188:1359–1368.

Apostolou, I., A. Sanukhan, L. Klein, and H. von Boehm. 2002. Origin of regulatory T cells with known specificity for antigen. Nat. Immunol. 3:756–763.

Aschenbrenner, K., L.M. D’Cruz, E.H. Vollmann, M. Hinterberger, J. Emmrich, L.K. Swee, A. Kollek, and L. Klein. 2007. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary epithelial cells. Nat. Immunol. 8:351–358.

Borkowska, T., A.J. Nelson, A.G. Farr, and M.C. Uede. 1996. Expression of gp40, the murine homologue of human epithelial cell adhesion molecule (Ep-CAM), by murine dendritic cells. Eur. J. Immunol. 26:110–114.

Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powere. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β– and retinoic acid–dependent mechanism. J. Exp. Med. 204:1757–1764.

den Ham, J.M., S.M. Lehar, and M.J. Bevan. 2000. CD8+ but not CD4+ dendritic cells cross-prime cytotoxic T cells in vivo. J. Exp. Med. 192:1685–1696.

Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat. Immunol. 2:1032–1039.

Gallegos, A.M., and M.J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J. Exp. Med. 200:1039–1049.

Hardysh, L.A., S.C. Atkins, A. Gambotto, and S.M. Barratt-Boyes. 2001. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J. Immunol. 166:3717–3723.

Humblet, C., A. Rudensky, and B. Kyewski. 1994. Presentation and intercelular transfer of self antigen within the thymic microenvironment: expression of the E alpha peptide-I-Ab complex by isolated thymic stromal cells. Int. Immunol. 6:1949–1958.

Irla, M., S. Hugues, J. Gill, T. Nitta, Y. Hikosaka, I.R. Williams, F.X. Hubert, H.S. Scott, Y. Takahama, G.A. Holland, and W. Reith. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell class. Immunology. 29:451–463.

Ito, T., S. Hanabuchi, Y.H. Wang, W.R. Park, K. Arima, L. Bover, F.X. Qin, M. Gillett, and Y.J. Liu. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity. 28:870–880.

Kawahata, K., Y. Misaki, M. Yamashita, S. Tsunekawa, K. Setoguchi, I. Miyazaki, and K. Yamamoto. 2002. Peripheral tolerance to a nuclear autoantigen: dendritic cells expressing a nuclear autoantigen lead to persistent anergic state of CD4+ autoreactive T cells after proliferation. J. Immunol. 168:1103–1112.

Klein, L., and B. Kyewski. 2000. Self-antigen presentation by thymic stromal cells: a subtle division of labor. Curr. Opin. Immunol. 12:179–186.

Klein, L., T. Klein, U. Ruther, and B. Kyewski. 1998. CD4 T cell tolerance to human C-reactive protein, an inducible serum protein, is mediated by medullary thymic epithelial cells. J. Exp. Med. 188:5–16.

Klein, L., M. Klugmann, K.A. Nave, V.K. Tsooby, and B. Kyewski. 2000. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. Nat. Med. 6:56–61.

Klein, L., B. Roettinger, and B. Kyewski. 2001. Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. Eur. J. Immunol. 31:2476–2486.
Li, J., J. Park, D. Foss, and I. Goldschneider. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J. Exp. Med.* 206:607–622.

McCaughtry, T.M., T.A. Baldwin, M.S. Wilken, and K.A. Hogquist. 2008. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J. Exp. Med.* 205:2575–2584.

Millet, V., P. Naquet, and R.R. Guinamard. 2008. Intercellular MHC transfer between thymic epithelial and dendritic cells. *Eur. J. Immunol.* 38:1257–1263.

Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* 250:1720–1723.

Nedjic, J., M. Aichinger, J. Emmerich, N. Mizushima, and L. Klein. 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455:396–400.

Neijssen, J., C. Herberts, J.W. Drijfhout, E. Reits, L. Janssen, and J. Neefjes. 2005. Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* 434:83–88.

Oukka, M., E. Colucci-Guyon, P.L. Tran, M. Cohen-Tannoudji, C. Babinet, V. Lotteau, and K. Kosmatopoulos. 1996. CD4 T cell tolerance to nuclear proteins induced by medullary thymic epithelium. *Immunity* 4:545–553.

Reits, E.A., J.W. Hodge, C.A. Herberts, T.A. Groothuis, M. Chakraborty, E.K. Wansley, K. Luiten, A.H. de Ru, J. Neijssen, et al. 2006. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J. Exp. Med.* 203:1259–1271.

Riedel, A., F. Nimmerjahn, S. Burdach, U. Behrends, G.W. Bornkamm, and J. Mautner. 2008. Endogenous presentation of a nuclear antigen on MHC class II by autophagy in the absence of CRM1-mediated nuclear export. *Eur. J. Immunol.* 38:2090–2095.

Shanker, A., N. Auphan-Anezin, P. Chomez, L. Giraudo, B. Van den Eynde, and A.M. Schnitt-Verhulst. 2004. Thymocyte-intrinsic genetic factors influence CD8 T cell lineage commitment and affect selection of a tumor-reactive TCR. *J. Immunol.* 172:5069–5077.

Spence, P.J., and E.A. Green. 2008. Foxp3+ regulatory T cells promiscuously accept thymic signals critical for their development. *Proc. Natl. Acad. Sci. USA* 105:973–978.

Terszowski, G., S.M. Muller, C.C. Bleul, C. Blum, R. Schirmbeck, J. Reimann, L.D. Pasquier, T. Aragany, T. Boehm, and H.R. Rodevald. 2006. Evidence for a functional second thymus in mice. *Science* 312:284–287.

Thery, C., L. Duban, E. Segura, P. Veron, O. Lantz, and S. Amigorena. 2002. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* 3:1156–1162.

Vremec, D., M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *Proc. Natl. Acad. Sci. USA* 89:4882–4886.

Vremsen, L., T. Amagai, T. Boehm, and H.R. Rodevald. 2006. Evidence for a functional second thymus in mice. *Science* 312:284–287.