Rank Signaling Links the Development of Invariant γδ T Cell Progenitors and Aire+ Medullary Epithelium

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DOI 10.1016/j.immuni.2012.01.016

SUMMARY

The thymic medulla provides a specialized microenvironment for the negative selection of T cells, with the presence of autoimmune regulator (Aire)-expressing medullary thymic epithelial cells (mTECs) during the embryonic-neonatal period being both necessary and sufficient to establish long-lasting tolerance. Here we showed that emergence of the first cohorts of Aire+ mTECs at this key developmental stage, prior to αβ T cell repertoire selection, was jointly directed by Rank+ lymphoid tissue inducer cells and invariant Vγ5+ dendritic epidermal T cell (DETC) progenitors that are the first thymocytes to express the products of gene rearrangement. In turn, generation of Aire+ mTECs then fostered Skint-1-dependent, but Aire-independent, DETC progenitor maturation and the emergence of an invariant DETC repertoire. Hence, our data attributed a functional importance to the temporal development of Vγ5+ γδ T cells during thymus medulla formation for αβ T cell tolerance induction and demonstrated a Rank-mediated reciprocal link between DETC and Aire+ mTEC maturation.

INTRODUCTION

Shaping of the immature αβ TCR repertoire within the thymus is necessary to generate a naïve T cell pool biased toward the recognition of self MHC molecules (positive selection) but purged (by negative selection) of potentially autoreactive specificities (Boehm, 2011). These αβ T cell selection events appear to be anatomically compartmentalized in the thymus (Takahama, 2006), in keeping with the finding that intrathymic microenvironments contain distinct, functionally specialized epithelial cell types that regulate thymic selection (Jiang et al., 1995; Surh et al., 1992). Although the epithelial cells in the thymic cortex play a key role in the positive selection and continued maturation of CD4+CD8+ thymocytes able to interact with self-peptide-MHC complexes (Gommeaux et al., 2009; Honey et al., 2002; Murata et al., 2007; Nitta et al., 2010; Ripen et al., 2011), epithelial cells and dendritic cells (DCs) in the thymic medulla play a key role in negative selection, by which thymocytes bearing strongly self-reactive αβTCRs are eliminated from the developing αβ T cell repertoire (Kyewski and Klein, 2006). In particular, medullary thymic epithelial cells (mTECs), including those expressing the Aire gene (Björses et al., 1998; Heino et al., 1999, 2000), influence negative selection in several ways (Anderson et al., 2002; Derbinski et al., 2005; Liston et al., 2003), including expression of a wide array of tissue-restricted antigens for direct and indirect antigen presentation to newly selected thymocytes (Gallegos and Bevan, 2004), and the regulation of intrathymic DC positioning via Aire-dependent XCL1 expression (Lei et al., 2011).

Normal mTEC development depends on NF-κB signaling, as shown by medullary abnormalities and tolerance breakdown in mice deficient in RelB (Berkly et al., 1995; Naspetti et al., 1997), Traf6 (Akiyama et al., 2005), and Nik (Kajura et al., 2004). Moreover, mTEC maturation requires hematopoietic cell cross-talk (Shores et al., 1991), which involves signaling through various mTEC-expressed TNF receptor superfamily (TNFRSF) members (Boehm et al., 2003; Zhu and Fu, 2008). Regarding the Aire+ mTEC subset, which first emerges around embryonic day (E) 16 of gestation (Gäbler et al., 2007; White et al., 2008; Zuklys et al., 2000), Rank (TNFRSF11a, CD265, TRANCER) plays a key role (Rossi et al., 2007), whereas in the steady-state adult thymus, synergy between Rank and CD40 regulates Aire+ mTEC development (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008). Importantly, by controlling and limiting the temporal deletion of Aire+ mTECs to either neonatal or adult thymus,
a recent study showed that Aire+ mTECs in the embryonic and neonatal period are both essential and sufficient to establish long-term T cell tolerance (Guerau-de-Arellano et al., 2000). Thus, the development of the first cohorts of Aire+ mTECs from Rank-expressing mTEC progenitors is a key step in the avoidance of autoimmunity. Whereas Rank ligand (RankL)-expressing, positively selected thymocytes play a role in the development of Aire+ mTECs in the adult thymus (Hikosaka et al., 2008), we showed that RankL+ lymphoid tissue inducer (LTi) cells, master regulators of lymphoid tissue organogenesis (Eberl et al., 2004; Finke et al., 2002; Mebius et al., 1997; Sun et al., 2000), are a key determinant of Rank-dependent thymus medulla development in the embryo (Rossi et al., 2007). Taken together with the key role of the first Aire+ mTEC cohorts in tolerance induction (Guerau-de-Arellano et al., 2009), these findings support a preeminent role for innate LTi cells, in which Aire+ mTECs develop independently of and prior to αβ T cell-positive selection, ensuring that they are in place to induce tolerance in the nascent αβ T cell repertoire. However, although RankL+ LTi induce mTEC differentiation, the presence of Aire+ mTECs in the developing embryonic thymus of LTi-deficient Rorc-/- mice at a stage prior to αβ T cell selection (White et al., 2008) suggests that additional embryonic cell types play a distinct role in establishing the medullary microenvironments that ensure T cell tolerance induction.

Prior to αβ T cells, the embryonic thymus generates T cells that express the γδTCR (Havran and Allison, 1988, 1990; Pennington et al., 2003). Indeed, the first appearance of Aire+ mTECs (White et al., 2008; Zuklys et al., 2000) coincides with that of progenitors of Vδ5+ dendritic epidermal T cells (DETCs), a subset of invariant intraepithelial lymphocytes (IELs) whose development is uniquely linked to the embryonic thymus (Asarnow et al., 1988; Ikuta et al., 1990; Mallick-Wood et al., 1998). Although the invariant nature of the VγδV5Vδ1TCR repertoire expressed by DETC progenitors distinguishes them from diverse αβ T cell precursors, several studies demonstrate a role for selection events during intrathymic DETC development (Passoni et al., 1997; Xiong et al., 2004). In particular, Skint-1, an immunoglobulin superfamily member expressed by TECs, is essential for DETC progenitor maturation and DETC progenitor maturation. Confocal analysis of the anatomical distribution of LTi and Vδ5 T cells, the functional maturation of Vδ5+ DETCs proceeded normally in Aire-/- mice. Collectively, our data have identified the key cellular components that regulate the emergence of Aire+ mTECs at initial stages of thymus development and defined a critical role for Rank-RankL-mediated interactions between fetal γδ T cell progenitors and mTECs that reciprocally regulate their maturation.

RESULTS

Vδ5+ DETC Progenitors Associate with Fetal mTECs

We previously showed that generation of the first cohorts of Aire+ mTECs in the embryonic thymus involves signals from RankL+ LTi cells (Rossi et al., 2007), a population originally identified as a key player in the development of stromal microenvironments within secondary lymphoid tissues such as lymph node (Mebius, 2003). However, when we analyzed thymus medulla formation in Rorc-/- embryonic mice lacking LTi, we found Aire+ mTECs still present, albeit at reduced numbers (White et al., 2008). This nonessential role for LTi cannot be explained by RankL provision by positively selected αβTCR+ thymocytes, because Aire+ mTECs are present in Rorc-/-embryonic mice prior to αβ T cell selection (White et al., 2008).

To investigate the possibility that previously unidentified cell types influence initial thymus medulla formation in the developing embryonic thymus, we screened the cellular makeup of medullary areas in E17 thymus tissue sections. Of the cell types analyzed, and consistent with an earlier report (Farr et al., 1990), a pan-γδTCR antibody defined a striking concentration of γδTCR+ thymocytes within developing embryonic EpCAM1+ medullary areas (not shown). Moreover, by using a Vγ5STCR-specific antibody, we found that essentially all of the medullary-resident γδTCR+ thymocytes at this stage expressed the Vγ5STCR (Figures 1A and 1B), representing DETC progenitors. Further analysis showed that some medullary-resident Vδ5+ T cells expressed high levels of CD45RB (Figure 1C), a maturational marker of intrathymic DETC progenitors (Lewis et al., 2006), suggestive of a link between thymus medulla development and DETC progenitor maturation. Confocal analysis of embryonic thymus sections stained to reveal the localization and frequency of Vγ5+ thymocytes and RORγT+CD4+CD3-IL-7Rα+ LTi showed an abundance of Vγ5+ thymocytes relative to LTi cells, with quantitative analysis indicating an approximate 10:1 ratio for Vγ5+ thymocytes:LTi within medullary areas (Figure 1D).

Importantly, dual staining with antibodies to Aire and Vγ5STCR revealed individual medullary areas containing both Aire+ mTECs and Vγ5+ thymocytes (Figure 1E), whereas staining with Aire and pan-γδTCR antibodies together with IL-7Rα and RORγT antibodies to reveal IL-7Rα+RORγT+ LTi demonstrated the presence of both γδTCR+ thymocytes and LTi within individual Aire-expressing medullary areas (Figure 1F).

Analysis of the anatomical distribution of LTi and Vγ5 expression was found to be Aire independent, and intrathymic Vδ5+ thymocyte development and the emergence of invariant Vδ5+ DETCs proceeded normally in Aire-/- mice. Collectively, our data have identified the key cellular components that regulate the emergence of Aire+ mTECs at initial stages of thymus development and defined a critical role for Rank-RankL-mediated interactions between fetal γδ T cell progenitors and mTECs that reciprocally regulate their maturation.
thymocytes in relation to Aire+ mTECs in thymus tissue sections failed to reveal a defined pattern to the distribution of these cells within multiple medullary areas (not shown). However, although there may be no difference in the topological positioning of these cells in the thymic medulla, the combined presence of Vγ5+ thymocytes and LTi cells within individual medullary areas suggests that they act collectively to influence mTEC development. Taken together, these results indicate that in the fetal thymus, in addition to the presence of LTi cells, there is an anatomical association between fetal-specific Vγ5 thymocytes and thymic medullary epithelial cells.

Vγ5+ Thymocytes Express Rankl and Drive Aire+ mTEC Development

To investigate the possibility that Vγ5+ DETC thymocyte progenitors influence the formation of embryonic mTEC microenvironments, we first made reaggregate thymus organ cultures (RTOCs) by using 2 dGuo fetal thymus lobes, known to contain Rank-Rankl+ thymocytes that are known to lack Rankl expression (Hikosaka et al., 2008), purified Vγ5+ thymocytes and LTi cells were both found to be lower than that of LTi, it was comparable to that of positively selected CD4+CD8+ thymocytes. Thus, Rankl expression by Vγ5+ thymocytes was found to be sufficient to drive Aire+ mTEC development in the adult (Hikosaka et al., 2008). Although Rankl expression by LTi cells were both found to expressed readily detectable levels of Rankl (Figure 2B). Given that previous experiments highlighted the particular importance of Rank-Rankl signaling in embryonic Aire+ mTEC maturation, we next analyzed Rankl mRNA expression in a variety of thymic populations by qPCR. In contrast to CD4+CD8+ thymocytes, purified Vγ5+ thymocytes and LTi cells were both found to expressed readily detectable levels of Rankl (Figure 2B). Although Rankl expression by Vγ5+ thymocytes was found to be lower than that of LTi, it was comparable to that of positively selected CD4+CD8+ thymocytes. Thus, Rankl expression by LTi cells were both found to expressed readily detectable levels of Rankl (Figure 2B). Given that previous experiments highlighted the particular importance of Rank-Rankl signaling in embryonic Aire+ mTEC maturation, we next analyzed Rankl mRNA expression in a variety of thymic populations by qPCR. In contrast to CD4+CD8+ thymocytes, purified Vγ5+ thymocytes and LTi cells were both found to expressed readily detectable levels of Rankl (Figure 2B). Although Rankl expression by Vγ5+ thymocytes was found to be lower than that of LTi, it was comparable to that of positively selected CD4+CD8+ thymocytes. 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with functional data on the importance of Rankl in Aire+ mTEC development, this suggests that both immature and mature Vγ5+ thymocytes have the potential to influence mTEC development. Collectively, these findings demonstrate that intrathymic Vγ5+ DETC progenitors express Rankl, are accumulated in developing medullary areas of the fetal thymus, and can induce the maturation of mTEC progenitors into mature Aire+ mTECs.

We next analyzed the frequency of Aire+ mTECs within the fetal thymus at E17 of gestation in WT mice and in mice individually deficient in either LTi (Rorc<sup>−/−</sup>) or γδ T cells (Tcrd<sup>−/−</sup>) or both (Rorc<sup>−/−</sup> × Tcrd<sup>−/−</sup>). As expected, mice lacking the capacity to provide hematopoietic crosstalk signals via combined blockade of T cell development/LTi development (CD3<sup>ε</sup>tg26/Rorc<sup>−/−</sup> mice) displayed a complete absence of Aire+ mTECs (Figure 2D). By contrast, both Tcrd<sup>−/−</sup> and Rorc<sup>−/−</sup> single mutant mice showed a partial, but statistically significant, defect in the generation of Aire+ mTECs as compared to WT controls (Figure 2D). Interestingly, analysis of Rorc<sup>−/−</sup> × Tcrd<sup>−/−</sup> double-deficient mice revealed a further reduction in the frequency of Aire+ mTECs compared to Tcrd<sup>−/−</sup> and Rorc<sup>−/−</sup> single mutants. Thus, these findings indicate that although LTi and γδ T cells are required in order to generate Aire+ mTECs at a normal frequency, the presence of small numbers of Aire+ mTECs in their combined absence, which contrasts to the total absence of Aire+ mTECs in CD3<sup>ε</sup>tg26/Rorc<sup>−/−</sup> mice, suggests that additional hematopoietic cell types can also be involved.

**Skint-1 Is Expressed by Mature mTECs and Is Induced by Rank Signaling**

Intrathymic development of invariant Vγ5+ thymocyte progenitors and the generation of an invariant Vγ5+ DETC population in the epidermis depends upon thymic stromal cell expression of Skint-1, an Ig superfamily member expressed by mTECs (Lewis et al., 2006). To investigate the possible link between this thymic stromal cell expression of Skint-1, the medullary accumulation of Vγ5+ thymocytes, and Aire+ mTEC development, we further analyzed Skint<sup>1</sup> expression in embryonic cTEC and mTEC subsets (Shakib et al., 2009), including immature CD80<sup>+</sup> and mature CD80<sup>−</sup> mTEC populations previously to have a direct precursor-product relationship, with the latter containing Aire+ cells (Gäbler et al., 2007; Gray et al., 2007; Rossi et al., 2007). As expected, Aire expression was limited to mature CD80<sup>−</sup> mTECs (Figure 3A), but of note this restricted pattern mirrored that of Skint<sup>1</sup>, which was undetectable in immature and mature stages of the cTEC lineage and in immature CD80<sup>−</sup> mTECs (Figure 3A).

Given our report of a key role for Rank in the development of fetal mTECs, we analyzed its importance in the regulation of Skint<sup>1</sup>. When we stimulated the development of Aire+ mTECs in dGuo-treated FTOCs with agonistic Rank antibodies, the induction of Aire expression (Figure 3B) was accompanied by the induction of Skint<sup>1</sup> expression, with unstimulated dGuo-treated FTOCs showing an absence of Skint<sup>1</sup> expression (Figure 3B). In addition, in comparison to levels seen in WT littermate
controls, analysis of CD45− thymic stromal cell populations from fetal Tnfrsf11a−/− mice showed a dramatic reduction in expression of both Aire and Skint1 (Figure 3C). Thus, our findings that Skint1 expression in the mTEC lineage is restricted to more mature CD80+ mTECs and is linked to Rank-mediated signaling in mTECs further highlight a potential reciprocal link in the development of Aire+ mTECs and Vγ5+ T cells.

**Rank Regulates Vγ5+ Thymocyte Development and the Emergence of an Invariant DETC Repertoire**

To study the potential link between Aire+ mTECs and Vγ5+ DETC progenitors, we initially studied the embryonic thymus of Relb−/− mice, a transcription factor in the alternative NF-κB signaling pathway that is downstream of several TNFSF-R family members including CD40 and Rank, known regulators of mTEC maturation. Consistent with this, and as previously reported, Aire+ mTECs were absent from the thymus of E17 Relb−/− embryos (data not shown). When we analyzed Vγ5+ thymocyte development, with upregulation of CD45RB as a marker of maturation (Lewis et al., 2006), we found a statistically significant decrease in both CD45RBlo and CD45RBhi subsets in Relb−/− embryonic thymuses compared to WT controls (Figures 4A and 4B). Despite a reduction in both CD45RB subsets, a dramatic skewing in the ratio (approximately 6:1) of WT:Relb−/− CD45RBhi cells was observed, compared to a ratio of 1.6:1 for WT:Relb−/− CD45RBlo cells, suggesting that although RelB may play a subtle role in the initial emergence of Vγ5CD45RBlo cells, it appears critically important in the maturation to the Vγ5+CD45RBhi stage.

Given that RelB is downstream of multiple, TNFSF-R family members, we next analyzed by flow cytometry thymocyte suspensions obtained from neonatal Tnfrsf11a−/− mice. Again, we found a dramatic reduction in the frequency of cells expressing high levels of both the Vγ5TCR and CD45RB (Figures 5A–5C),...
which resulted in a skewing in the mature:immature DETC ratio in Tnfrsf11a−/− mice (1:100) as compared to WT littermate controls (1:3) (Figure 5D). In addition, analysis of neonatal epidermal preparations showed that Tnfrsf11a−/− mice have a dramatic reduction in the frequency of total epidermal CD3+ T cells (Figure 6A), as well as a disproportionate reduction in those expressing the Vγ5TCR (Figures 6B and 6C). Further flow cytometric analysis with an antibody that detects the Vγ5VδTCR (clone 17D1) showed that the small numbers of Vγ5+ DETCs present in Tnfrsf11a−/− mice epidermis were also Vδ1+, suggesting that residual levels of Skint1 detected in the absence of Rank may still support the generation of small numbers of invariant DETCs. Nevertheless, the combined data on Tnfrsf11a−/− mice, demonstrating a defect in intrathymic Vγ5+ thymocyte maturation, coupled to diminished numbers of Vγ5+ DETCs, demonstrate that efficient maturation of an invariant Vγ5TCR+ DETC repertoire depends upon expression of the TNFSF-Receptor Rank, just as it depends on Skint-1 (Barbee et al., 2011; Lewis et al., 2006).

Skint-1-Mediated Vγ5+ DETC Development Proceeds Normally in Aire-Deficient Mice

To investigate whether the importance of Rank-dependent Aire+ mTEC development for Skint-1-mediated DETC maturation is directly dependent upon Aire itself, we analyzed Skint1 expression and the emergence of epidermal Vγ5+ DETCs in neonatal Aire-deficient mice. We found that in contrast to the known Aire dependency of genes such as Spt1 (salivary protein 1) (Figure 7A), Skint1 expression was unaltered in Aire−/− neonatal thymus (Figure 7A). In line with this lack of requirement for Aire in the expression of Skint1, comparison of the neonatal epidermis of Aire−/− and WT mice showed no changes in the frequency of epidermal CD3+ cells (Figure 7B), including those expressing Vγ5+ (Figures 7C and 7D). Thus, despite the association between the maturation of Aire+ mTECs and invariant Vγ5TCR+ DETC maturation, Aire deficiency does not impair the emergence of an invariant Skint-1-dependent DETC pool.

DISCUSSION

Intrathymic medullary microenvironments are known to play an important role in establishing tolerance in newly generated αβ T cells (Anderson et al., 2007; Takahama, 2006). In particular, medullary epithelial cells that express the Aire gene have been shown to participate in T cell tolerance induction in several ways (Mathis and Benoist, 2009). For example Aire+ mTECs have been directly linked to the negative selection of single-positive thymocytes that bear αβTCRs recognizing tissue-restricted antigens (Liston et al., 2003), as well as the development of FoxP3+ natural regulatory T cells (Aschenbrenner et al., 2007), and most recently in the intrathymic positioning of XCR1+ populations are shown, and asterisks indicate a statistically significant difference where p < 0.01. n.s. is not significant.

Figure 5. Rank Regulates Intrathymic Maturation of Vγ5+ DETC Progenitors

(A) Neonatal thymocyte suspensions from WT and Tnfrsf11a−/− mice were stained with antibodies to the Vγ5TCR and CD3. Circles indicate immature CD3loVγ5lo and mature CD3hiVγ5hi subsets.

(B) Proportions of Vγ5lo (white bars) and Vγ5hi (black bars) thymocytes in WT and Tnfrsf11a−/− newborn mice is shown. Averages and standard errors of populations are shown, and asterisks indicate a statistically significant difference where p < 0.01. n.s. is not significant.

(C) FACS analysis of thymocytes from neonatal WT and Tnfrsf11a−/− mice for expression of CD45RB and Vγ5. Boxes indicate the percentages of Vγ5hiCD45RBhi cells.

(D) Ratio of Vγ5loVγ5hi cells in WT (white bars) and Tnfrsf11a−/− (black bars) neonatal thymus.
dendritic cells (Lei et al., 2011). Thus, the establishment of medullary thymic areas containing Aire-expressing epithelial cells represents an important and multifaceted component of intrathymic tolerance mechanisms. Of equal importance, recent data show that the nascent cohorts of Aire + mTECs that are generated in the fetal and neonatal periods are both essential and sufficient for tolerance induction (Guerau-de-Arellano et al., 2009). Moreover, although shaping of the $\alpha\beta$ T cell repertoire fosters Aire+ mTEC differentiation in the adult thymus.
the initial emergence of Aire+ mTECs during these key developmental stages occurs independently of, and prior to, the generation of mature thymocytes by positive selection (Derbinski et al., 2001; White et al., 2008; Zuklys et al., 2000). Collectively, these observations suggest the existence of an alternative, perhaps developmental stage-specific, mechanism that enables functionally competent intrathymic medullary microenvironments to be in place prior to γδ T cell selection events, where they impose tolerance induction on the first cohorts of newly generated T cells.

Here, we show that initial thymus medulla formation, involving generation of the first cohorts of Aire+ mTECs, occurs as a result of a cellular combination of innate LTI cells and progenitors of an invariant T cell (DETC) subset that is defined by invariant TCR expression of the Vγ5Vδ1TCR. Indeed, we found that Tcrdγδ− mice lack a significant fraction of the Aire+ mTEC compartment and that DETC progenitors express Rankl, a known regulator of Aire+ mTEC development. Our finding that both immature CD45RB− and mature CD45RB+ Vγ5+ thymocytes express Rankl suggests that the ability of Vγ5+ thymocytes to influence mTEC development is not limited to the mature stages of their development. This is in contrast to the impact made by γδ T cells, which require continued maturation from the CD4−CD8− to the CD4+ or CD8+ stage to express Rankl (Hikosaka et al., 2008). Rather, a scenario can be envisaged that during hematopoietic crosstalk for the initiation of mTEC development, and within individual medullary areas of embryonic thymus, simultaneous input from LTI and Vγ5+ thymocytes can occur, rather than a temporal sequence that first involves LTI cells and then mature Vγ5+ thymocytes, the latter generated as a result of Skint-1-mediated differentiation. Interestingly, although analysis of Tcrdγδ− × Rorcγδ− double-deficient mice showed a further reduction in the frequency of Aire+ mTECs as compared to single mutants, small numbers of Aire+ mTECs remain. Whether these cells arise as a result of the early emergence of mature RanklγδTcR+ thymocytes that are already known to influence mTEC development or through Rankl provision by an additional hematopoietic cell type is currently unknown. Whatever the case, our finding suggests that, at this early developmental stage, LTI and γδ T cells combine to influence thymus medulla formation.

That initial embryonic thymus medulla formation is influenced by invariant DETC progenitors appears relevant to their highly selective and timed appearance in thymus ontogeny at around E14–E16 (Havran and Allison, 1988), parallel to the emergence of the first Aire-expressing medullary environments. It is currently unclear whether the ability of cells of the γδ T cell lineage to direct Aire+ mTEC development is limited to those expressing the Vγ1VδTCR. However, it is interesting to note that γδTcR+ thymocytes in the adult also express Rankl (Hikosaka et al., 2008), and mTEC numbers are reduced in adult Tcrdγδ− mice (Hikosaka et al., 2008). Thus, in the adult thymus, γδTcR+ and γδTcR− thymocytes may synergize to regulate thymus medulla development.

Importantly, the present results also show that the development of Vγ5+ DETC progenitors and Aire+ mTECs in the fetal thymus are reciprocally linked, in that the intrathymic crosstalk of the two cell types is required to generate epithelial environments that support and select γδ T cells. Thus, we show that Rank-mediated stimulation of immature mTECs induces expression of Skint1, and that in the absence of Rank expression there is a block in the maturation of Vγ1VδTCR+ thymocytes and a corresponding increase in the frequency of CD3+ epidermal-resident DETC-bearing TCRs encoded by other Vγ gene segments. Collectively, these findings therefore establish a new role for intrathymic expression of Rank in the development of invariant γδ T cells, related to its capacity to promote maturation of mTECs, the intrathymic source of Skint-1. However, despite their similar expression patterns, Skint1 gene expression in mTECs is Aire independent and the invariant DETC T cell pool is intact in neonatal Aire−/− mice. Although γδ T cells are not grossly affected in the context of Aire deficiency in mouse and man (Tuovinen et al., 2009), the possibility that there are alterations in the fine specificities of diverse, adaptive γδ T cells merits further study. Thus, the induction of Aire+ mTECs by the first wave of γδ T cells may limit the emergence of potentially harmful, self-reactive γδTcR+ and γδTcR− cells.

**EXPERIMENTAL PROCEDURES**

**Mice**

The following mice were used in this study: C57BL/6, Tcrd−/− (Itohara et al., 1993), Rorc−/− (Sun et al., 2000), Tnfrsf1a−/− (Rossi et al., 2007), Rieb−/− (Weil et al., 1995), and Aire−/− (Ramsey et al., 2002). Tcrd−/− and Rorc−/− were intercrossed at The University of Birmingham to generate Tcrdγδ− × Rorcγδ− double knockout (DKO) mice. All mice were bred and maintained at the Biomedical Services Unit, University of Birmingham, except for Aire−/− mice, which were housed at Biomedical Services, John Radcliffe Hospital, University of Oxford. For the generation of timed pregnancies, day of detection of a vaginal plug was designated day zero. All experiments were performed under the authorization of the UK Home Office.

**Antibodies and Immunocojugates**

The following antibodies were used for flow cytometry: anti-TCRγδ+ (clone 536, BD PharMingen), anti-Vγ1Vδ1 (clone 17D1), anti-CD3 (clone 145.2C11, BD PharMingen), anti-CD205 (clone NLDC-145, Abcam), anti-CD40 (clone 3/23, BD PharMingen), anti-EpCAM (clone G8.8, kind gift of A. Farr, University of Washington), anti-CD45 (clone 30-F11, eBioscience), anti-CD45RB (clone H1S2, kind gift of H. Scott, Adelaide University), anti-Ly51 (clone 6C3, eBioscience), and anti-CD80 (clone 16-10A1, eBioscience). Biotinylated antibodies were revealed with streptavidin conjugated to Alexa Fluor 555. The following additional primary antibodies were used for confocal analysis: anti-medullary epithelium (clone ERTR5, kind gift of W. van Ewijk), anti-CD8 (clone CT-CD8b, eBioscience), anti-RORγT (clone AFKJS-9, eBioscience), anti-IL-7R (clone A7R34, eBioscience), pan-γδTCR (clone GL3, eBioscience), and goat anti-Aire (D-17, Santa Cruz Biotechnology). For detection of polyclonal goat anti-Aire, anti-goat Alexa Fluor 594 (Invitrogen) was used. For detection of ERTR5 antibody, anti-Rat IgG Alexa Fluor 594 (Invitrogen) was used. Detection of RORγT Abs was achieved with anti-rat IgG FITC (Jackson Immunoresearch), then anti-FITC-Alexa Fluor 488 (Invitrogen), and finally anti-rabbit IgG-Alexa Fluor 488 (Invitrogen).

**Real-Time PCR Analysis**

cDNA was obtained from purified mRNA with μMacs One-step cDNA synthesis kit, according to the manufacturer’s instructions (Milenyi Biotec). Real-time PCR was performed with SYBR Green with primers specific for ACTB (β-actin), Aire, Spft1 (salivary protein 1), and Skint1 on the Rotor-Gene 3000 PCR machine (Corbett Research, NSW, Australia). PCRs were conducted in 15 μl volumes in reaction buffer containing 1× SensiMix QPCR SYBR No ROX Mix (Quanta) and 200 nM of primers for Aire, Rankl, Spft1, and Skint1; ACTB primers were designed and synthesized as a Quantitect Primer Assay (QIAGEN). After an initial denaturation step (95º C for 10 min), cycling was performed at 95º C for 15 s, 60 ºC (ACTB, Spft1, and Skint1) or 62 ºC (Aire and Rankl) for 20 s, and 72 ºC for 15 s (40 cycles). Specific amplification
was verified by melt curve analysis. Reaction amplification efficiency and the Ct values were obtained from Rotor Gene 6.0 software (Corbett Research) with standard curves generated from Mouse Universal cDNA Reference-oligo dT primed (Biochain Institute). Calculation of the relative mRNA expression values for each sample normalized to Actb8 was performed as described with the Pfaffl quantitation method (Pfaffl, 2001). Fold levels represent the mean ±SEM of replicate reactions and data shown are representative of at least two independent experiments. Information on primers is summarized in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at doi:10.1016/j.immuni.2012.01.016.

ACKNOWLEDGMENTS

We thank R. Bird for assistance with cell sorting and BMSU staff for excellent animal husbandry. This work was supported by an MRC Programme grant to E.J.J., P.J.L.L., and G.A. and a Wellcome Trust Programme to P.J.L.L. and G.A.

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