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DOI:  
[10.1084/jem.20122070](https://doi.org/10.1084/jem.20122070)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*  
Cowan, JE, Parnell, SM, Nakamura, K, Caamano, JH, Lane, PJL, Jenkinson, EJ, Jenkinson, WE & Anderson, G 2013, 'The thymic medulla is required for Foxp3+ regulatory but not conventional CD4+ thymocyte development', *The Journal of Experimental Medicine*, vol. 210, no. 4, pp. 675-81. <https://doi.org/10.1084/jem.20122070>

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# The thymic medulla is required for Foxp3<sup>+</sup> regulatory but not conventional CD4<sup>+</sup> thymocyte development

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**A key role of the thymic medulla is to negatively select autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes, a process important for T cell tolerance induction. However, the involvement of the thymic medulla in other aspects of  $\alpha\beta$  T cell development, including the generation of Foxp3<sup>+</sup> natural regulatory T cells (nT<sub>reg</sub> cells) and the continued maturation of positively selected conventional  $\alpha\beta$  T cells, is unclear. We show that newly generated conventional CD69<sup>+</sup>Qa2<sup>-</sup> CD4 single-positive thymocytes mature to the late CD69<sup>-</sup>Qa2<sup>+</sup> stage in the absence of RelB-dependent medullary thymic epithelial cells (mTECs). Furthermore, an increasing ability to continue maturation extrathymically is observed within the CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> subset of conventional SP4 thymocytes, providing evidence for an independence from medullary support by the earliest stages after positive selection. In contrast, Foxp3<sup>+</sup> nT<sub>reg</sub> cell development is medullary dependent, with mTECs fostering the generation of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors at the CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> stage. Our results demonstrate a differential requirement for the thymic medulla in relation to CD4 conventional and Foxp3<sup>+</sup> thymocyte lineages, in which an intact mTEC compartment is a prerequisite for Foxp3<sup>+</sup> nT<sub>reg</sub> cell development through the generation of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors.**

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Abbreviations used: dGuo, 2-deoxyguanosine; mTEC, medullary TEC; nT<sub>reg</sub> cell, natural regulatory T cell; qPCR, quantitative PCR; TEC, thymic epithelial cell.

In the thymus, positive selection of CD4<sup>+</sup>8<sup>+</sup> thymocytes recognizing self-peptide/MHC on cortical thymic epithelial cells (TECs) triggers the entry of CD4/CD8 single-positive (SP) T cells into the thymic medulla, a process essential for tolerance induction (Kurobe et al., 2006). Additionally, the medulla is also considered a key site of differentiation that supports thymocyte maturation after positive selection, including stages defined by loss of CD24/CD69 and acquisition of CD62L/Qa2 (McCaughy et al., 2007; Li et al., 2007).

Although the medulla also contains SP4 Foxp3<sup>+</sup> natural regulatory T cells (nT<sub>reg</sub> cells; Liston et al., 2008), its role in nT<sub>reg</sub> cell generation remains unclear, with both medullary TECs (mTECs) and DCs being implicated (Aschenbrenner et al., 2007; Proietto et al., 2008; Spence and Green, 2008; Wirnsberger et al., 2009; Hinterberger et al., 2010). Importantly, nT<sub>reg</sub> cell development is a multistage process, with TCR–MHC (Lio and Hsieh, 2008) and

CD28–CD80/86 interactions (Lio et al., 2010; Vang et al., 2010; Hinterberger et al., 2011) driving the generation of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors that give rise to Foxp3<sup>+</sup>CD25<sup>+</sup> nT<sub>reg</sub> cells (Lio and Hsieh, 2008). However, the role of mTECs during Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursor generation is unknown.

Here, we define steps in both conventional and nT<sub>reg</sub> SP4 thymocyte maturation, mapping their requirements for a RelB-dependent mTEC compartment (Burkly et al., 1995; Weih et al., 1995; Heino et al., 2000). We show that conventional SP4 thymocytes can complete their maturation in the absence of RelB-dependent mTECs, with evidence of thymic independence occurring by the CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 thymocyte stage. In contrast, Foxp3<sup>+</sup> nT<sub>reg</sub> cells

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require an intact thymic medulla, with a requirement for RelB-dependent mTEC mapping to the generation of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors at the CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> stage. Collectively, our data reveal the differential importance of the thymic medulla during SP4 thymocyte development and highlight a specific role for mTECs in Foxp3<sup>-</sup>CD25<sup>+</sup> precursor generation.

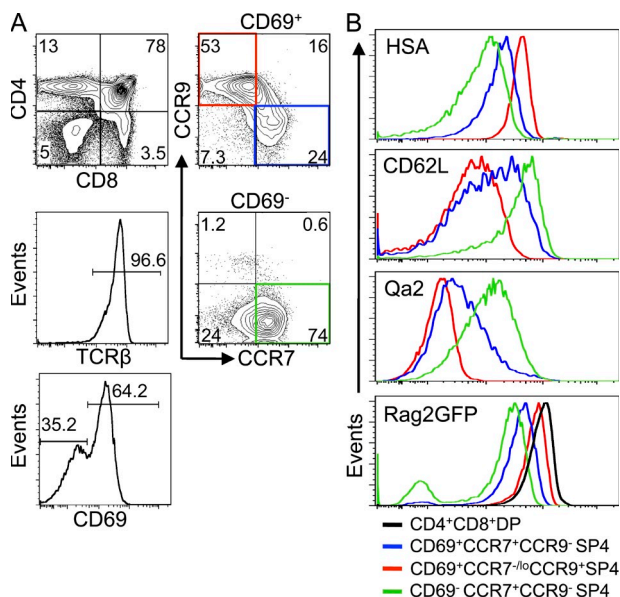
## RESULTS AND DISCUSSION

### Emergence of conventional and nT<sub>reg</sub> cell precursors in CD4 thymocytes

As positive selection involves changes in the chemokine receptors CCR7 and CCR9 (Choi et al., 2008), we used these and other (HSA/CD69/CD62L/Qa2) markers to define distinct subsets of SP4 thymocytes in Rag2GFP reporter mice, in which GFP levels are a measure of maturation status (Boursalian et al., 2004; McCaughy et al., 2007). Fig. 1 A shows that CD69<sup>+</sup>αβ TCR<sup>hi</sup> SP4 thymocytes contain CCR7<sup>+</sup>CCR9<sup>-</sup> and CCR7<sup>-/lo</sup>CCR9<sup>+</sup> subsets, whereas more mature CD69<sup>-</sup> cells are CCR7<sup>+</sup>CCR9<sup>-</sup>. Analysis of CD62L/Qa2 showed the CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> subset to be CD62L<sup>lo</sup>Qa2<sup>lo</sup> and to express the highest levels of Rag2GFP among SP4 thymocytes, whereas CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> cells were CD62L<sup>hi</sup>Qa2<sup>hi</sup> with the lowest levels of Rag2GFP (Fig. 1 B). CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> and CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> but not CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 thymocytes (Fig. 2 A) expressed mRNA for *Foxo1* and *Klf2*, known regulators of thymocyte

egress (Carlson et al., 2006; Gubbels Bupp et al., 2009), whereas mRNA encoding *S1pr1*, another regulator of thymocyte emigration (Matloubian et al., 2004), was limited to the CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> subset (Fig. 2 A). Importantly, when reaggregate thymus organ cultures (Rossi et al., 2007) were initiated with purified CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 thymocytes, their progeny had up-regulated CCR7 and down-regulated both CCR9 (Fig. 2 B) and CD69 (not depicted), providing evidence for a maturation sequence in which the CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> subset represents newly generated SP4 thymocytes after positive selection, followed by CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> and then CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> cells (Fig. 2 C).

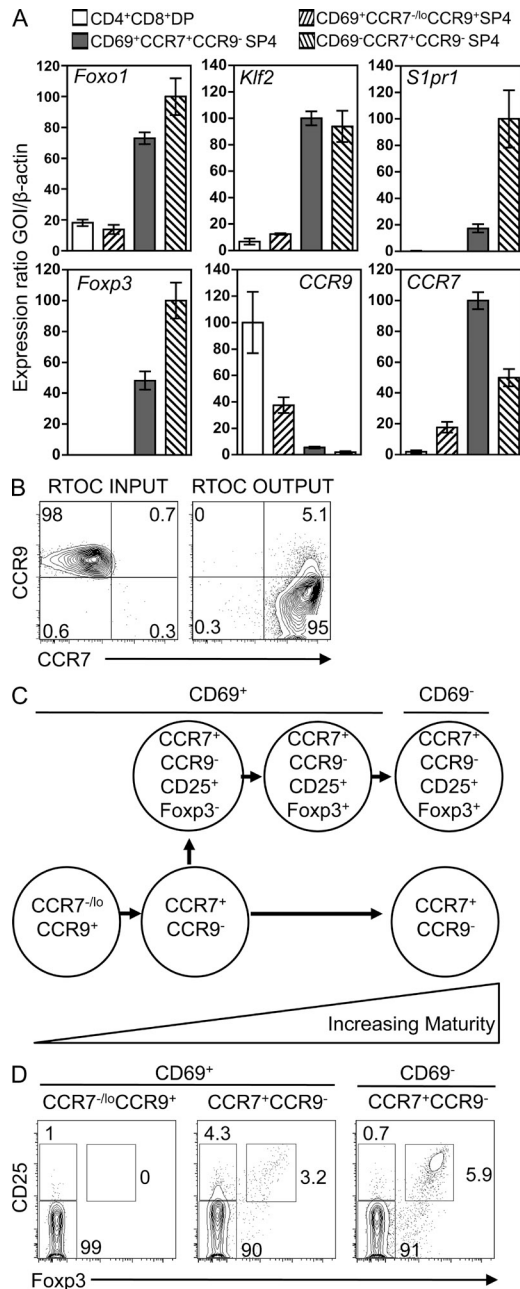
In relation to nT<sub>reg</sub> cell emergence, *Foxp3* mRNA was detectable in CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes, with higher levels noted in the most mature CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> subset (Fig. 2 A), indicating that Foxp3<sup>+</sup> nT<sub>reg</sub> cell development is first detectable within the CD69<sup>+</sup> stage of SP4 thymocyte maturation but after induction of CCR7 and loss of CCR9. To further relate changes in CCR7/CCR9 to distinct stages in nT<sub>reg</sub> cell development, we analyzed CD25 and Foxp3 by flow cytometry. Consistent with quantitative PCR (qPCR) data, the earliest CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> cells were Foxp3<sup>-</sup>, and Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors were barely detectable in this population (Fig. 2 D). In contrast, CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes contained Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors as well as their more mature CD25<sup>+</sup>Foxp3<sup>+</sup> progeny (Fig. 2 D), whereas the most mature CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes contained Foxp3<sup>+</sup> nT<sub>reg</sub> cells but lacked Foxp3<sup>-</sup>CD25<sup>+</sup> precursors (Fig. 2 D). Thus, Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursor appearance maps to the transition between CCR7<sup>-/lo</sup>CCR9<sup>+</sup> and CCR7<sup>+</sup>CCR9<sup>-</sup> stages in the CD69<sup>+</sup> phase of SP4 thymocyte development (Fig. 2 C).



**Figure 1. CCR7 and CCR9 define distinct subsets of SP4 thymocytes.** (A) CD69<sup>+</sup> and CD69<sup>-</sup> subsets of SP4 thymocytes from Rag2GFP mice analyzed for CCR7/CCR9 expression. Data are typical of four experiments. (B) Levels of HSA, CD62L, Qa2, and Rag2GFP in the following SP4 subsets: CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> (red), CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> (blue), and CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> (green). For comparison, Rag2GFP expression by CD69<sup>-</sup>CD4<sup>+</sup>8<sup>+</sup> thymocytes is shown (black). Data are typical of three separate experiments.

### Conventional SP4 thymocyte development occurs independently of RelB-dependent mTECs

A recent study has suggested a correlation between the thymic medulla and SP4 thymocyte development, most notably the absence of CD69<sup>-</sup>Qa2<sup>+</sup> SP4 thymocytes in *Relb*<sup>-/-</sup> mice displaying a severe block in mTEC development (Fig. 3 A; Li et al., 2007). However, detailed analysis of the role of mTECs in *Relb*<sup>-/-</sup> mice is confounded by their complex phenotype that includes DC deficiencies and multiorgan autoimmunity (Weih et al., 1995; Wu et al., 1998). We therefore investigated the mTEC requirements of both conventional and nT<sub>reg</sub> SP4 thymocytes by grafting alymphoid 2-deoxyguanosine (dGuo)-treated fetal thymus organ cultures (Rossi et al., 2007), from either *Relb*<sup>-/-</sup> or WT embryos, into unmanipulated WT mice. Importantly, any absence of Qa2<sup>+</sup> cells in our experiments is not caused by the genetic background of the *Relb*<sup>-/-</sup> mice used here, as *Relb*<sup>-/-</sup> fetal liver chimaeras with WT hosts generated Qa2<sup>+</sup> SP4 thymocytes (not depicted). Confocal analysis of WT and *Relb*<sup>-/-</sup> TEC grafts confirmed an mTEC defect in the latter (Fig. 3 B), consistent with a cell-intrinsic role for RelB in mTEC development. Importantly,



**Figure 2. Developmental emergence of conventional and Foxp3<sup>+</sup> T<sub>reg</sub> SP4 cells.** (A) qPCR in indicated thymocyte subsets. Error bars indicate SEM. mRNA levels were normalized to  $\beta$ -actin. Data are from at least two independently sorted biological samples, with each gene analyzed two times. (B) Phenotype of purified CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 thymocytes before (left) and after (right) incorporation into reaggregate thymus organ cultures (RTOCs). Data are typical of four experiments. (C) Developmental sequence of SP4/nT<sub>reg</sub> thymocyte maturation. (D) CCR7/CCR9 subsets of adult WT  $\alpha\beta$  TCR<sup>hi</sup> SP4 thymocytes for CD69, CD25, and Foxp3 expression. Data represent at least three separate experiments.

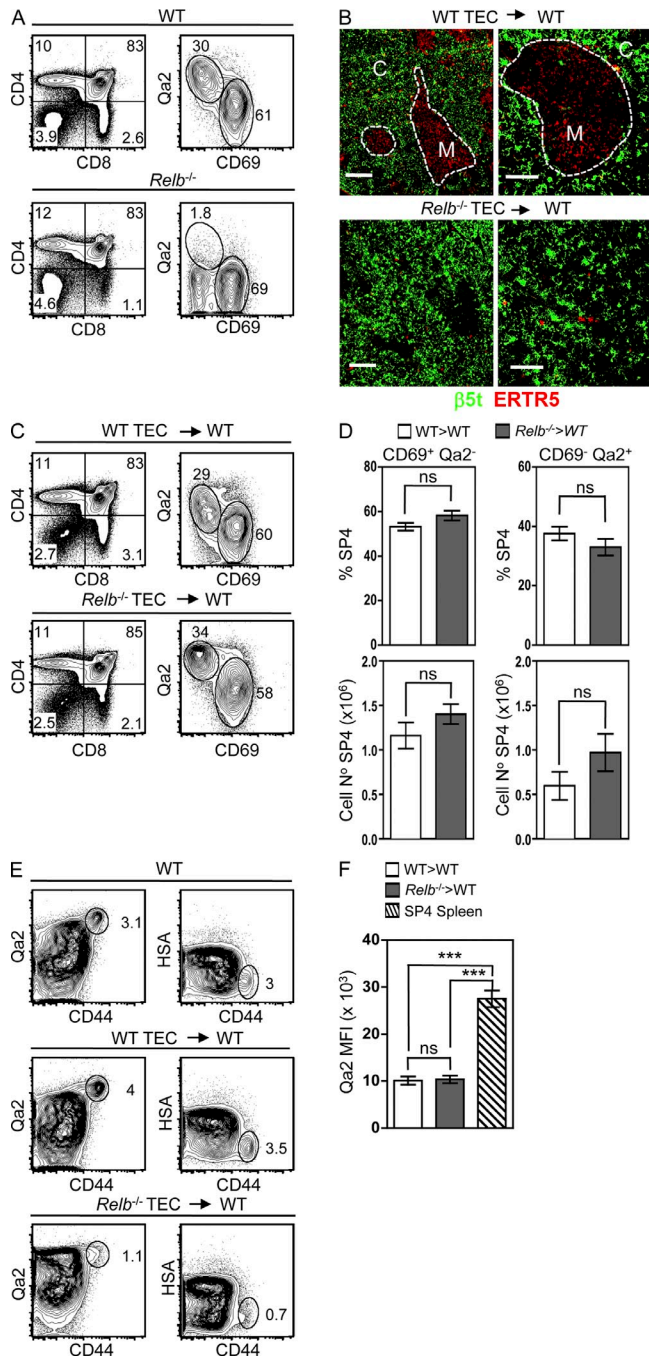
although grafting of *Relb*<sup>-/-</sup> TECs into nude hosts resulted in autoimmunity (Fig. 4, A and B), WT hosts grafted with *Relb*<sup>-/-</sup> TECs showed no signs of disease (not depicted), presumably

as a result of peripheral tolerance mechanisms involving T<sub>reg</sub> cells generated in the host WT thymus. Thus, grafting of unmanipulated WT hosts with thymuses harboring a cell-intrinsic RelB-dependent mTEC deficiency provides a model to study the role of mTECs in SP4 thymocyte development in the absence of autoimmunity.

Analysis of SP4 thymocytes in *Relb*<sup>-/-</sup> TEC grafts within WT hosts revealed both early CD69<sup>+</sup>Qa2<sup>-</sup> and late CD69<sup>-</sup>Qa2<sup>+</sup> subsets at proportions and numbers comparable with WT grafts (Fig. 3, C and D). Importantly, SP4 thymocytes in WT and *Relb*<sup>-/-</sup> grafts were predominantly CD44<sup>lo</sup>HSA<sup>int</sup> (Fig. 3 E) and expressed lower levels of Qa2 compared with peripheral CD4 T cells (Fig. 3 F), indicating that they were not peripheral T cells circulating back to the thymus (McCaughy et al., 2007; Hale and Fink, 2009). Our findings do not support previous suggestions from experiments involving in vitro TEC lines (Li et al., 2007), that mTECs provide essential support for SP4 thymocyte maturation. The reason for this difference is unknown, although it may relate to whether in vitro systems reflect the functional capacities of mTECs in vivo. Instead, our data show that conventional SP4 thymocyte development in vivo occurs in the absence of RelB-dependent mTECs. Interestingly, absence of Qa2<sup>+</sup>CD69<sup>-</sup>SP4 thymocytes was also reported in *Aire*<sup>-/-</sup> mice (Li et al., 2007). Given that *Aire*<sup>-/-</sup> mice show an increased mTEC compartment (Anderson et al., 2002), the impact made by Aire on thymocyte development is unclear.

We next analyzed the requirements for thymic support after positive selection by investigating the ability of CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 thymocytes to mature extrathymically. Although recent thymic emigrants undergo maturation outside the thymus (Boursalian et al., 2004), it is unclear how far back in development this window of thymic independence extends, particularly in relation to the post-positive selection stages described here. Initially, CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 thymocytes from adult CD45.2<sup>+</sup> WT mice, with an HSA<sup>hi</sup>CD62L<sup>lo</sup>Qa2<sup>-</sup>CD69<sup>+</sup> phenotype (Fig. 4 C), were i.v. injected into congenic CD45.1<sup>+</sup> WT mice. Analysis after 7 (Fig. 4 C) and 14 d (not depicted) showed that injected cells had acquired an HSA<sup>lo</sup>CD62L<sup>hi</sup>Qa2<sup>hi</sup>CD69<sup>lo</sup> phenotype comparable with host naive CD4<sup>+</sup> T cells. To provide a comparative analysis of extrathymic maturation, CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> thymocytes from CD45.2<sup>+</sup> mice were coinjected into CD45.1<sup>+</sup>CD45.2<sup>+</sup> hosts at a 1:1 ratio with either less mature CD45.1<sup>+</sup>CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> or more mature CD45.1<sup>+</sup>CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes. Analysis of spleen 7 d later revealed a ratio of 10:1 after coinjection of CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 and less mature CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes and a 0.2:1 ratio after coinjection of CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 and more mature CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes (Fig. 4 D). Thus, CD69<sup>+</sup>CCR7<sup>-/-</sup>CCR9<sup>+</sup> SP4 thymocytes show an emerging capacity for thymic independence, strengthening the notion that conventional SP4 thymocyte development can occur in the absence of thymic medullary support from the earliest SP4 thymocyte stages. Although mice receiving





**Figure 3. RelB-dependent mTECs are dispensable for conventional SP4 thymocyte development.** (A) Qa2/CD69 expression in WT (top) and *Relb*<sup>-/-</sup> (bottom) thymocytes after gating on  $\alpha\beta$  TCR<sup>hi</sup> SP4 thymocytes. Data are representative of three experimental replicates. (B) Immunofluorescent staining of WT and *Relb*<sup>-/-</sup> graft sections for ERTR5 and  $\beta$ 5t. C denotes cortex, and M denotes medulla. Bars: (left) 200  $\mu$ m; (right) 100  $\mu$ m. (C) Thymocytes from WT (top) and *Relb*<sup>-/-</sup> (bottom) thymus grafts, with Qa2/CD69 levels shown for  $\alpha\beta$  TCR<sup>hi</sup> SP4 cells. (D) Frequencies of Qa2/CD69 SP4 thymocyte subsets recovered from WT and *Relb*<sup>-/-</sup> grafts. (E) Qa2/HSA/CD44 expression in SP4 thymocytes recovered from WT thymus (top), WT TEC grafts (middle), and *Relb*<sup>-/-</sup> TEC grafts (bottom). (F) Mean fluorescence intensity (MFI) of Qa2 expression in CD69<sup>-</sup> SP4 T cells from WT spleen or CD69<sup>-</sup> SP4 thymocytes from WT

CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 thymocytes showed no signs of autoimmunity (not depicted), it is unclear whether this reflects the timing of negative selection in relation to this subset or the control of autoreactivity by T<sub>reg</sub> cells generated in the WT host thymus.

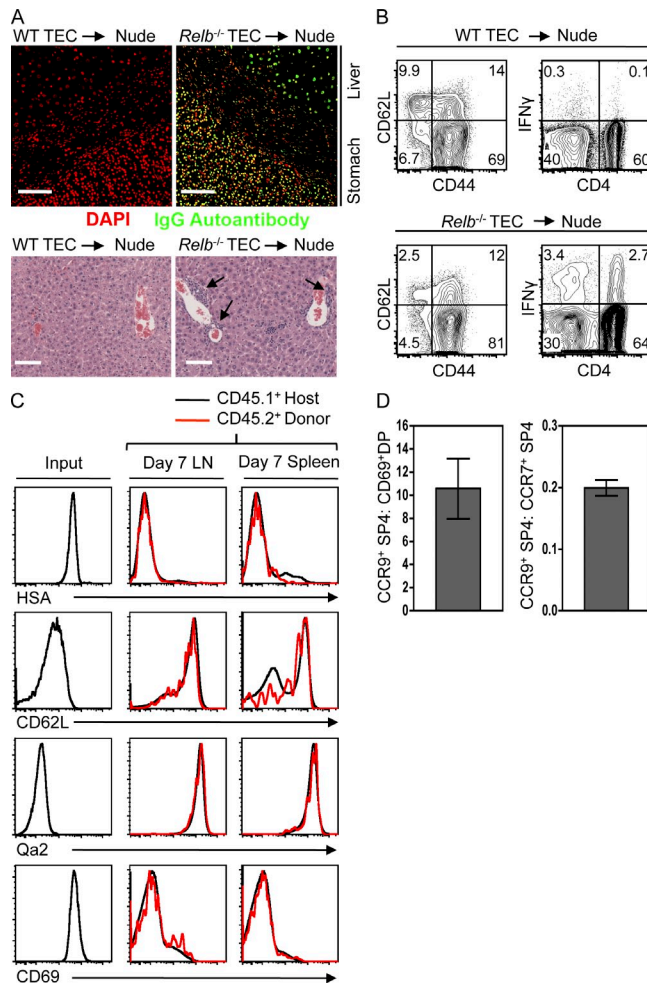
### RelB-dependent mTECs control Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursor generation

We next examined the role of RelB-dependent mTECs in the development of Foxp3<sup>+</sup> nT<sub>reg</sub> cells and Foxp3<sup>-</sup>CD25<sup>+</sup> precursors. CD69<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>+</sup> and CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> subsets of SP4 thymocytes, the former reflecting nT<sub>reg</sub> cell precursors (Lio and Hsieh, 2008) and the latter capable of extrathymic generation of conventional CD4 T cells (Fig. 4), were purified from CD45.2<sup>+</sup> Foxp3GFP reporter mice and transferred i.v. into WT CD45.1<sup>+</sup> hosts. CD69<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors, shown here to be at a CD69<sup>+</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> intermediate stage (Fig. 2 D), generated Foxp3<sup>+</sup>CD25<sup>+</sup> nT<sub>reg</sub> cells (Fig. 5 A). In contrast to their capacity to generate conventional CD4 T cells extrathymically (Fig. 4), CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 thymocytes failed to give rise to Foxp3<sup>+</sup>CD25<sup>+</sup> nT<sub>reg</sub> cells outside the thymus (Fig. 5 A). Thus, the CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> stage after positive selection marks a point where continued maturation of conventional but not Foxp3<sup>+</sup> regulatory SP4 thymocytes can occur independently of thymic support.

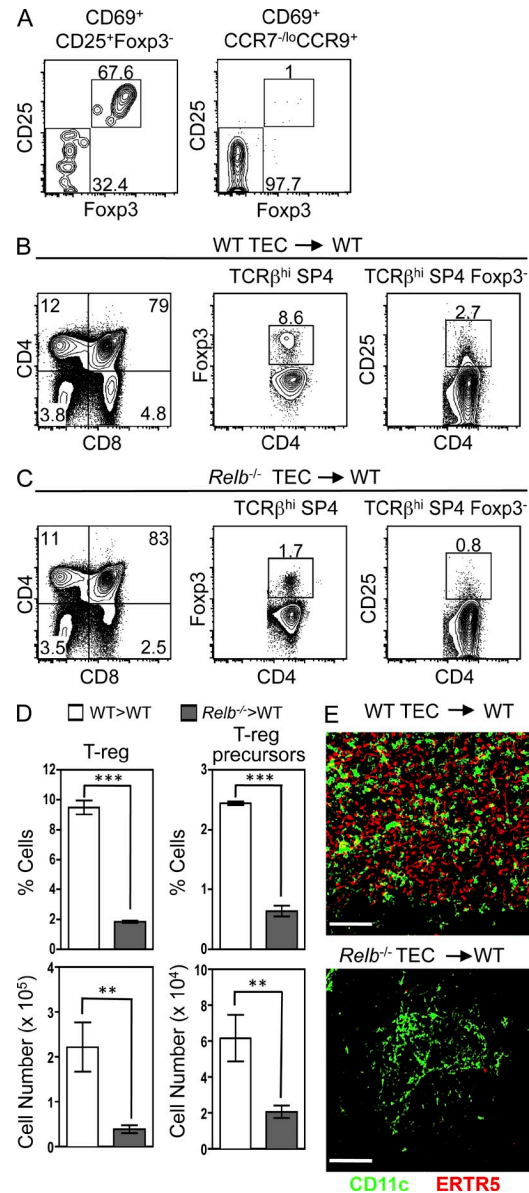
Importantly, and unlike conventional SP4 thymocyte development, *Relb*<sup>-/-</sup> TEC grafts showed a significant reduction in Foxp3<sup>+</sup> SP4 thymocytes compared with WT grafts (Fig. 5, B–D). Furthermore, nT<sub>reg</sub> cell precursors were also significantly reduced in mTEC-deficient *Relb*<sup>-/-</sup> TEC grafts (Fig. 5, B–D). Although the few nT<sub>reg</sub> cells in *Relb*<sup>-/-</sup> grafts do not rule out inefficient generation via residual RelB-independent mTECs, the large majority of nT<sub>reg</sub> cell development appears to be controlled by RelB-dependent mTECs. Moreover, although the presence of host-derived CD11c<sup>+</sup> DCs in both WT and *Relb*<sup>-/-</sup> grafts (Fig. 5 E) argues against the defect in nT<sub>reg</sub> cell production being solely caused by the absence of DCs, we cannot exclude impaired DC function in the absence of RelB-dependent mTECs.

In conclusion, we show that conventional and Foxp3<sup>+</sup> nT<sub>reg</sub> CD4 T cells demonstrate a differential requirement for RelB-dependent medullary thymic microenvironments during their development. In particular, mTECs support the generation of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursors and their Foxp3<sup>+</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell progeny from within the pool of medullary CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 thymocytes. The dependency of Foxp3<sup>-</sup>CD25<sup>+</sup> nT<sub>reg</sub> cell precursor generation on both CD28–CD80/CD86 and TCR–MHC interactions (Lio and Hsieh, 2008; Lio et al., 2010; Vang et al., 2010; Hinterberger

or *Relb*<sup>-/-</sup> TEC grafts. Error bars represent SEM; data in C–F are from at least three independent experiments, with a minimum of five of each graft type per experiment. In an unpaired Student's two-tailed *t* test, ns denotes a nonsignificant difference where *P* > 0.1; \*\*\*, *P* < 0.001.



et al., 2011) fits well with the expression of these co-stimulatory molecules and MHC class I/II by mTECs (Rossi et al., 2007) and suggests that provision of these molecules by mTECs is linked to their ability to support nT<sub>reg</sub> cell generation as shown here. That mTECs provide TCR ligands for nT<sub>reg</sub> cell development fits well with the generation of antigen-specific TCR transgenic T<sub>reg</sub> cells after the targeting of model antigens to mTECs (Aschenbrenner et al., 2007; Hinterberger



et al., 2010) and the normal numbers of nT<sub>reg</sub> cells generated when hematopoietic cells are selectively MHC class II deficient (Aschenbrenner et al., 2007; Liston et al., 2008). In contrast, the ability of conventional SP4 thymocytes to continue their maturation, either in the absence of RelB-dependent mTECs or extrathymically, reveals differences in the maturational requirements for nT<sub>reg</sub> cells and conventional T cells

and warrants a rethinking of the role of the medulla in T cell development. Thus, rather than representing a microenvironment fostering late-stage  $\alpha\beta$  T cell development per se, the primary role of the medulla is in the generation of self-tolerance via negative selection and the generation of Foxp3<sup>+</sup> CD4 nT<sub>reg</sub> cells, a scenario compatible with the T cell-mediated autoimmunity in *Ccr7*<sup>-/-</sup> mice that occurs after inefficient thymocyte access to the medulla (Kurobe et al., 2006).

## MATERIALS AND METHODS

**Mice.** WT CD45.2<sup>+</sup> C57BL/6, congenic CD45.1<sup>+</sup> C57BL/6 (Boyl), CD45.1<sup>+</sup>CD45.2<sup>+</sup> C57BL/6, nude C57BL/6, C57BL/6 *Relb*<sup>-/-</sup> (Weih et al., 1995) mice, FVB/N RAG2GFP (Yu et al., 1999), and C57BL/6 Foxp3GFP reporter mice (gift from T. Strom, Beth Israel Deaconess Medical Center, Boston, MA; Bettelli et al., 2006) were bred at the University of Birmingham in accordance with Home Office regulations. For timed matings, the day of vaginal plug detection was designated as day 0. All animal experiments were performed in accordance with University of Birmingham (Local Ethical Review Panel) and national UK Home Office regulations.

**Antibodies, flow cytometry, and cell sorting.** Thymocyte, splenocyte, and LN suspensions were stained with the following antibodies: PECy7/Alexa Fluor 700/PE anti-CD4 (clone GK1.5; eBioscience) or PerCP-Cy5.5/APC eFluor 780/V500 anti-CD4 (clone RM4-5; eBioscience/BD), eFluor 450/FITC/V500 anti-CD8 (clone 53-6.7; eBioscience/BD) or biotinylated anti-CD8 clone (YTS156.7.7; BioLegend), APC eFluor 780 anti-TCR $\beta$  (clone H57-597; eBioscience), FITC/APC/PerCP-Cy5.5 anti-CD69 (clone H1.2F3; eBioscience), APC anti-CD62L (clone MEL-14; BioLegend), FITC/Alexa Fluor 700 anti-CD44 (clone IM7; eBioscience), PE anti-CD3 $\epsilon$  (clone 145-2C11; eBioscience), APC eFluor 780/PE anti-HSA/CD24 (clone M1/69; BD/eBioscience), biotinylated/FITC anti-Qa2 (clone 695H1-9.9; BioLegend/eBioscience), eFluor 780/eFluor 450 anti-CD45.1 (clone A20; eBioscience), PE/Alexa Fluor 700 anti-CD45.2 (clone 104; eBioscience), PE anti-CCR9 (clone eBio CW-1.2; eBioscience), APC/PE anti-CD25 (clone PC61/PC61.5; BioLegend/eBioscience), and APC anti-IFN- $\gamma$  (clone XMG1.2; BD). For surface CCR7 expression, thymocytes were incubated in recombinant CCL19-Ig (eBioscience), followed by biotinylated goat anti-human Ig (eBioscience). All biotinylated antibodies were picked up with PECy7-conjugated streptavidin (eBioscience). For intracellular staining of Foxp3, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol and stained with PE anti-Foxp3 (clone FJK-16s; eBioscience). Flow cytometry was performed on a Fortessa analyzer using FACSDiva6.2 software (BD), with data subsequently analyzed with FlowJo 8.7 software (Tree Star). Purified populations of thymocytes were sorted from adult thymus suspensions using a MoFlo XDP cell sorter (Beckman Coulter).

**Fetal thymus organ culture and thymus grafting.** Embryonic day (E) 15 thymus lobes, cultured for 7 d in 1.35 mM dGuo were transplanted under the kidney capsule of 8-wk-old recipient mice as described previously (Rossi et al., 2007), with grafts in WT mice and nude mice harvested after 8–12 wk and 6 wk, respectively.

**i.v. cell transfer.** CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup>Foxp3<sup>-</sup> and CD69<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>+</sup> subsets of SP4  $\alpha\beta$  TCR<sup>hi</sup> thymocytes were MoFlo sorted from CD45.2<sup>+</sup> Foxp3GFP adult reporter mice and transferred i.v. in a 300- $\mu$ l volume of PBS into CD45.1<sup>+</sup> Boyl mice. LN and spleen were harvested after 7 d and analyzed by flow cytometry for CD45.1/CD45.2 together with maturation markers to study the persistence of donor-derived cells. For coinjection experiments, purified CD69<sup>+</sup>CCR7<sup>-/lo</sup>CCR9<sup>+</sup> SP4 from CD45.2<sup>+</sup> C57BL/6 mice and either CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> or CD69<sup>-</sup>CCR7<sup>+</sup>CCR9<sup>-</sup> SP4 from CD45.1<sup>+</sup> Boyl mice were mixed at a 1:1 ratio and injected i.v. into CD45.1<sup>+</sup>CD45.2<sup>+</sup>

hosts as described above. After 7 d, injected cells were identified in LN and spleen preparations on the basis of either a CD45.1<sup>+</sup>CD45.2<sup>-</sup> or CD45.1<sup>-</sup>CD45.2<sup>+</sup> phenotype by flow cytometry and expressed as a ratio.

**Immunohistochemistry.** Thymus grafts were recovered from host mice, and 7- $\mu$ m sections were cut, then fixed in acetone, and stained with the following antibodies: the mTEC marker ERTR5 (gift of W. van Ewijk, Riken Yokohama Institute, Yokohama City, Kanagawa, 230-0045), detected with Alexa Fluor 594 goat anti-rat IgM (Invitrogen), the cortical TEC marker rabbit anti- $\beta$ 5t (MBL), detected with donkey anti-rabbit 488 (Invitrogen), FITC-conjugated anti-CD11c (clone N418; eBioscience) followed by rabbit anti-FITC (Invitrogen), and donkey anti-rabbit 488 (Invitrogen). Images were obtained using an LSM 780 microscope and analyzed using LSM software (Carl Zeiss).

**Analysis of autoimmunity.** C57BL/6 nude mice, grafted with either WT or *Relb*<sup>-/-</sup> dGuo-treated thymus lobes, were sacrificed 6 wk after transplantation, and organs and sera were collected. LN cells from the indicated grafted mice were analyzed for intracellular IFN- $\gamma$  cytokine production and CD44/CD62L expression as described previously (Gaspal et al., 2011). To detect autoantibodies, sera (1:40 dilution) was added to sections of composite tissue blocks containing the indicated tissues and detected with goat anti-mouse IgG FITC (SouthernBiotech), counterstained with DAPI. Lymphocytic infiltrates were detected in paraffin wax-embedded sections of liver as described previously (Rossi et al., 2007). Images were acquired with a microscope (DM6000; Leica) using a 20 $\times$  objective.

**qPCR.** qPCR analysis of freshly sorted thymocyte populations was performed exactly as described previously (Roberts et al., 2012). Primer used are as follows:  $\beta$ -actin QuantiTect Mm *Actb* 1SG Primer Assay (QIAGEN QT00095242); *Foxo1* forward, 5'-TGTCAGGCTAAGAGTTAGTGAGCA-3'; and reverse, 5'-GGGTGAAGGGCATCTTTG-3'; *Klf2* forward, 5'-CTCA-CGGAGCCTATCTTGCC-3'; and reverse, 5'-CACGTTGTTTAGGT-CCTCATCC-3'; *S1pr1* forward, 5'-AAATGCCCAACGGAGACT-3'; and reverse, 5'-CTGATTTGCTGCGGCTAAATTC-3'; *Foxp3* forward, 5'-CCAGGAAAGACAGCAACCT-3'; and reverse, 5'-TTCTCAC-AACCAGGCCACTTG-3'; *Ccr9* forward, 5'-ACCATGATGCCACAGA-CAACT-3'; and reverse, 5'-GGGAAGAGTGGCAAGAAAGA-3'; and *Ccr7* forward, 5'-CTAGCTGGAGAGACAAGA-3'; and reverse, 5'-TATCCGTCATGGTCTTGAGC-3'.

We thank Dr. Terry Strom for Foxp3GFP mice, provided by Dr. Nick Jones.

This study was supported by a Medical Research Council (MRC) Programme Grant. J.E. Cowan is supported by a PhD Studentship from the MRC Centre for Immune Regulation.

The authors have no competing financial interests.

Submitted: 14 September 2012

Accepted: 11 February 2013

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