In the thymus, positive selection of CD4⁺8⁺ 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 (McCaughtry et al., 2007; Li et al., 2007).

Although the medulla also contains SP4 Foxp3⁺ natural regulatory T cells (nT_{reg} cells; Liston et al., 2008), its role in nT_{reg} 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_{reg} 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⁺CD25⁺ nT_{reg} cell precursors that give rise to Foxp3⁺CD25⁺ nT_{reg} cells (Lio and Hsieh, 2008). However, the role of mTECs during Foxp3⁺CD25⁺ nT_{reg} cell precursor generation is unknown.

Here, we define steps in both conventional and nT_{reg} 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 newly generated conventional CD69⁺Qa2⁻ CD4 single-positive thymocytes mature to the late CD69⁻Qa2⁺ 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⁺CCR7⁻/loCCR9⁺ subset of conventional SP4 thymocytes, providing evidence for an independence from medullary support by the earliest stages after positive selection. In contrast, Foxp3⁺ nT_{reg} cell development is medullary dependent, with mTECs fostering the generation of Foxp3⁺CD25⁺ nT_{reg} cell precursors at the CD69⁺CCR7⁺CCR9⁻ stage. Our results demonstrate a differential requirement for the thymic medulla in relation to CD4 conventional and Foxp3⁺ thymocyte lineages, in which an intact mTEC compartment is a prerequisite for Foxp3⁺ nT_{reg} cell development through the generation of Foxp3⁺CD25⁺ nT_{reg} cell precursors.

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require an intact thymic medulla, with a requirement for RelB-dependent mTEC mapping to the generation of Foxp3+CD25+ nTreg cell precursors at the CD69+CCR7+CCR9− 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+CD25+ precursor generation.

RESULTS AND DISCUSSION
Emergence of conventional and nTreg cell precursors in CD4 thymocytes
As positive selection involves changes in the chemokine receptors CCR7 and CCR9 (Choi et al., 2004; McCaughtry et al., 2007). Fig. 1 A shows that CD69+αβ TCR hi SP4 thymocytes contain CCR7+CCR9− and CCR7−/loCCR9+ subsets, whereas more mature CD69− cells are CCR7+CCR9+. Analysis of CD62L/Qa2 showed the CD69+CCR7−/loCCR9− subset to be CD62L/Qa2lo and to express the highest levels of Rag2GFP among SP4 thymocytes, whereas CD69+CCR7+CCR9+ cells were CD62L/Qa2hi with the lowest levels of Rag2GFP (Fig. 1 B). Importantly, when reaggregated thymus organ cultures (Rossi et al., 2007) were initiated with purified CD69+CCR7−/loCCR9− 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+CCR7−/loCCR9− subset represents newly generated SP4 thymocytes after positive selection, followed by CD69+CCR7+CCR9− and then CD69+CCR7+CCR9+ cells (Fig. 2 C).

In relation to nTreg cell emergence, Foxp3 mRNA was detectable in CD69+CCR7+CCR9− SP4 thymocytes, with higher levels noted in the most mature CD69+CCR7+CCR9− subset (Fig. 2 A), indicating that Foxp3+ nTreg cell development is first detectable within the CD69+ 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 nTreg cell development, we analyzed CD25 and Foxp3 by flow cytometry. Consistent with quantitative PCR (qPCR) data, the earliest CD69+CCR7−/loCCR9− cells were Foxp3−, and Foxp3+CD25− nTreg cell precursors were barely detectable in this population (Fig. 2 D). In contrast, CD69+CCR7+CCR9− SP4 thymocytes contained Foxp3−CD25+ nTreg cell precursors as well as their more mature CD25+Foxp3+ progeny (Fig. 2 D), whereas the most mature CD69−CCR7+CCR9− SP4 thymocytes contained Foxp3+ nTreg cells but lacked Foxp3−CD25+ precursors (Fig. 2 D). Thus, Foxp3−CD25+ nTreg cell precursor appearance maps to the transition between CCR7−/loCCR9+ and CCR7+CCR9− stages in the CD69+ phase of SP4 thymocyte development (Fig. 2 C).

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−Qa2+ SP4 thymocytes in Relb−/− 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−/− 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 nTreg SP4 thymocytes by grafting allogeneic 2-deoxyguanosine (dGuo)–treated fetal liver chimaeras with WT embryos, into unmanipulated WT mice. Importantly, any absence of Qa2+ cells in our experiments is not caused by the genetic background of the Relb−/− mice used here, as Relb−/− fetal liver chimaeras with WT hosts generated Qa2+ SP4 thymocytes (not depicted). Confoveal analysis of WT and Relb−/− 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 1. CCR7 and CCR9 define distinct subsets of SP4 thymocytes. (A) CD69+ and CD69− 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+CCR7−/loCCR9− (red), CD69−CCR7+CCR9− (blue), and CD69−CCR7+CCR9− (green). For comparison, Rag2GFP expression by CD69−CD4+8+ thymocytes is shown (black). Data are typical of three separate experiments.
as a result of peripheral tolerance mechanisms involving T_reg 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−/− TEC grafts within WT hosts revealed both early CD69+Qa2− and late CD69−Qa2+ subsets at proportions and numbers comparable with WT grafts (Fig. 3, C and D). Importantly, SP4 thymocytes in WT and Relb−/− grafts were predominantly CD44loHSAint (Fig. 3 E), indicating that they were not peripheral T cells circulating back to the thymus (McCaughtry 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+CD69− SP4 thymocytes was also reported in Aire−/− mice (Li et al., 2007). Given that Aire−/− 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−/CCR7lo/CCR9lo 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−CCR7−/loCCR9− SP4 thymocytes from adult CD45.2+ WT mice, with an HSAhiCD62LloQa2−CD69+ phenotype (Fig. 4 C), were i.v. injected into congenic CD45.1+ WT mice. Analysis after 7 (Fig. 4 C) and 14 d (not depicted) showed that injected cells had acquired an HSAloCD62LhiQa2hiCD69lo phenotype comparable with host naive CD4+ T cells. To provide a comparative analysis of extrathymic maturation, CD69−CCR7−/CCR9− SP4 thymocytes from CD45.2+ mice were coinfected into CD45.1+CD45.2+ hosts at a 1:1 ratio with either less mature CD45.1+CD4+8+69+ or more mature CD45.1+CD69−CCR7−CCR9− SP4 thymocytes. Analysis of spleen 7 d later revealed a ratio of 10:1 after coinjection of CD69−CCR7−/loCCR9− SP4 and less mature CD4+869+ thymocytes and a 0:2:1 ratio after coinjection of CD69−CCR7−/loCCR9− SP4 and more mature CD69−CCR7−CCR9− SP4 thymocytes (Fig. 4 D). Thus, CD69−CCR7−/CCR9− 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...
CD69+CCR7−/loCCR9+ 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 Treg cells generated in the WT host thymus.

RelB-dependent mTECs control Foxp3−CD25+ nTreg cell precursor generation

We next examined the role of RelB-dependent mTECs in the development of Foxp3+ nTreg cells and Foxp3−CD25+ precursors. CD69+Foxp3−CD25+ and CD69−CCR7−/lo CCR9+ subsets of SP4 thymocytes, the former reflecting nTreg 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+ Foxp3GFP reporter mice and transferred i.v. into WT CD45.1+ hosts. CD69+Foxp3−CD25+ nTreg cell precursors, shown here to be at a CD69+CCR7+CCR9− intermediate stage (Fig. 2 D), generated Foxp3+CD25+ nTreg cells (Fig. 5 A). In contrast to their capacity to generate conventional CD4 T cells extrathymically (Fig. 4), CD69−CCR7−/loCCR9+ SP4 thymocytes failed to give rise to Foxp3+CD25+ nTreg cells outside the thymus (Fig. 5 A). Thus, the CD69−CCR7−/loCCR9− stage after positive selection marks a point where continued maturation of conventional but not Foxp3+ regulatory SP4 thymocytes can occur independently of thymic support.

Importantly, and unlike conventional SP4 thymocyte development, Relb−/− TEC grafts showed a significant reduction in Foxp3+ SP4 thymocytes compared with WT grafts (Fig. 5, B–D). Furthermore, nTreg cell precursors were also significantly reduced in mTEC-deficient Relb−/− TEC grafts (Fig. 5, B–D). Although the few nTreg cells in Relb−/− grafts do not rule out inefficient generation via residual RelB-independent mTECs, the large majority of nTreg cell development appears to be controlled by RelB-dependent mTECs. Moreover, although the presence of host-derived CD11c+ DCs in both WT and Relb−/− grafts (Fig. 5 E) argues against the defect in nTreg 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+ nTreg CD4 T cells demonstrate a differential requirement for RelB-dependent medullary thymic microenvironments during their development. In particular, mTECs support the generation of Foxp3+CD25+ nTreg cell precursors and their Foxp3+CD25+ nTreg cell progeny from within the pool of medullary CCR7+CCR9− SP4 thymocytes. The dependency of Foxp3+CD25− nTreg 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...
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 nTreg cell generation as shown here. That mTECs provide TCR ligands for nTreg cell development fits well with the generation of antigen-specific TCR transgenic Treg cells after the targeting of model antigens to mTECs (Aschenbrenner et al., 2007; Hinterberger et al., 2010) and the normal numbers of nTreg 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 maturation requirements for nTreg cells and conventional T cells.

Figure 4. Extrathymic development of CCR7−/loCCR9+/CD69+ SP4 thymocytes. (A) Autoantibodies in serum from nude mice receiving WT or Relb−/− TEC grafts (top) and histological analysis (bottom) of lymphocytic infiltrates (arrows) in liver of the same mice. Bars, 100 µm. (B) CD44/CD62L expression in SP4 LN T cells from nude mice receiving either WT or Relb−/− TEC grafts. Right panels show intracellular IFN-γ in CD3+ LN T cells of the same mice. Data in A and B are typical of three experimental replicates. (C) HSA/CD62L/Qa2/CD69 expression in CD69−CCR7−/loCCR9+ adult αβ TCRhi SP4 thymocytes before transfer (input) and their SP4 T cell progeny (CD45.2+ cells) recovered from both spleen and LN after 7 d (red lines). Black lines show host CD45.1+ SP4 LN and splenic T cells for comparison. (D) Ratio of recovered SP4 progeny in spleen 7 d after coinjection of equal numbers of either CD69−CCR7−/loCCR9+ SP4 thymocytes and CD4−8−69+ thymocytes (left) or CD69−CCR7−/loCCR9+ SP4 thymocytes and CD69−CCR7+ SP4 thymocytes (right). Error bars represent SEM, and data in C and D are typical of four separate experiments.

Figure 5. RelB-dependent mTECs control Foxp3−CD25+ nTreg cell precursor generation. (A) Expression of Foxp3GFP/CD25 in the LN progeny of i.v. injected CD69−Foxp3−CD25+ (left) and CD69−CCR7−/loCCR9+ (right) T cells after gating on CD45.2+ injected cells. Data are typical of two experimental replicates. (B and C) WT (B) and Relb−/− (C) thymus grafts analyzed for Foxp3−CD25+ nTreg cell precursors and Foxp3+ nTreg cells within the SP4 subset. (D) Quantitation of these populations; error bars represent SEM. An unpaired Student’s two-tailed t test was performed: ***, P < 0.001; **, P < 0.01. Data in B–D represent four separate experiments. (E) Confocal analysis of WT and Relb−/− grafts. Bars, 100 µm.
and warrants a rethinking of the role of the medulla in T cell development. Thus, rather than representing a microenvironment fostering late-stage αβ 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+ CD4+ nTreg cells, a scenario compatible with the T cell–mediated autoimmune in Ccr7−/− mice that occurs after inefficient thymocyte access to the medulla (Kurobe et al., 2006).

**MATERIALS AND METHODS**

**Mice.** WT CD45.2+ C57BL/6, congenic CD45.1+ C57BL/6 (Bowy), CD45.1+CD45.2+ C57BL/6, nude C57BL/6, C57BL/6 Relr−/− (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, thymocyte, and LN suspensions were stained with the following antibodies: PE/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; BioLegend), APC/CD45.2 clone (clone MEL-14; BioLegend), FITC/Alexa Fluor 700 anti-CD44 (clone IM7; eBioscience), PE anti-CD3ε (clone 145-2C11; eBioscience), APC eFluor 780/PE anti-HSA/CD24 (clone M1/69; BD/eBioscience), biotinylated/FITC anti-Q2 (clone 609H1-9.9; 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-γ (clone XM1G2.2; BD). For surface CCR7 expression, thymocytes were incubated in recombiant CCL19-1gG (eBioscience), followed by biotinylated goat anti-human Ig (eBioscience). All biotinylated antibodies were picked up with PE/Cy7-conjugated streptavidin (eBioscience). For intracellular staining of Foxp3, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (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), followed by biotinylated goat anti–human Ig (eBioscience). All biotinylated antibodies were picked up with PECy7-conjugated streptavidin (eBioscience). Flow cytometry was performed on a Fortessa analyzer using FACSDiva6.2 software (BD), with data subsequently analyzed with FlowJo 8.7 software (Treestar). Purified PE anti-Foxp3 (clone FJK-16s; eBioscience). Flow cytometry was performed on 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 Fetal thymus organ culture and thymus grafting. Embryonic day (E) using a MoFlo XDP cell sorter (Beckman Coulter). Data subsequently analyzed with FlowJo 8.7 software (TreeStar). Purified PE anti-Foxp3 (clone FJK-16s; eBioscience). Flow cytometry was performed on a MoFlo XDP cell sorter (Beckman Coulter).

**qPCR.** qPCR analysis of freshly sorted thymocyte populations was performed exactly as described previously (Roberts et al., 2012). Primer used are as follows:

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