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Thymic Epithelial Cells Are a Nonredundant Source of Wnt Ligands for Thymus Development

Fabian Brunk,* Iris Augustin,^{†,‡} Michael Meister,[§] Michael Boutros,^{†,‡} and Bruno Kyewski*

Wnt signaling has been implicated in T cell development. However, it remained unclear which cell type is the major source of Wnt ligands and to what extent thymic epithelial cell (TEC) development is dependent on Wnt signaling. In this study, we analyzed the role of Wnt ligands provided by TECs for the development of T cells and TECs without manipulating the intracellular Wnt signaling machinery in either cell type. To this end, we used conditional knockout mice (FoxN1-Gpr177) in which TECs are unable to secrete Wnt ligands. Gpr177 (Evi/Wls) is a Wnt-specific cargo receptor that is required for the secretion of Wnt ligands. We found that TECs are the main source of Wnt ligands in the thymus, which serves a nonredundant role, and lack of TEC-provided Wnt ligands led to thymic hypotrophy, as well as a reduced peripheral T cell pool. Despite being reduced in numbers, T cells that developed in the absence of TEC-secreted Wnt ligands were functionally competent, and the subset composition of the peripheral T cell pool was not affected. Thus, our data suggest that T cell development is not directly dependent on TEC-provided Wnt ligands. Rather, TEC-secreted Wnt ligands are essential for normal thymus development and normal peripheral T cell frequencies but are dispensable for T cell function in the periphery. *The Journal of Immunology*, 2015, 195: 5261–5271.

The Wnt signaling pathway has been implicated in the process of T cell development. T cell–specific deletion of the Wnt downstream effector β -catenin led to partially impaired thymic T cell development that was accompanied by a more substantial reduction in the frequency of peripheral T cells (1), whereas β -catenin was reported earlier to be dispensable for T cell development (2). T cell factor (Tcf)–1–deficient mice have severely impaired T cell development (3), marked by a developmental block at the CD4/CD8 double-negative (DN) stage, which was demonstrated to be T cell intrinsic. The transcriptional partner of Tcf-1 in β -catenin–dependent Wnt signaling, Lef-1, cooperates with Tcf-1 in supporting T cell development (4, 5). In a study using fetal thymic

organ culture experiments, only full-length Tcf could partially rescue T cell development in Tcf-1–deficient thymocytes, in contrast to a mutant form of Tcf lacking the β -catenin–binding domain (6). In addition to Tcf-1 and Lef-1, the Wnt downstream effector β -catenin or the Wnt antagonist adenomatous polyposis coli have been genetically targeted to study Wnt signaling in T cell development. Given the role of adenomatous polyposis coli as a Wnt antagonist, adenomatous polyposis coli loss-of-function experiments were conducted to elevate constitutive Wnt signaling levels. Lck-Cre– or CD4-Cre–mediated deletion of adenomatous polyposis coli was reported to impair T cell development in two studies (7, 8), whereas no drastic effect on T cell development was observed in a more recent report (9). Similarly, Lck-Cre–mediated stabilization of β -catenin by deletion of its destruction sequence (also referred to as Ctnb^{ex3}) enables thymocytes to bypass the β -selection checkpoint (10), but it reduces thymocyte numbers by increasing the apoptosis rate and reducing proliferation. Stabilization of β -catenin in thymocytes having passed the β -selection checkpoint by the use of CD4-Cre (CD4-Cre Ctnb^{ex3})–mediated deletion essentially gave the same results (8). When integrating these results, it is important to bear in mind that both β -catenin and adenomatous polyposis coli can also exert Wnt-independent functions (11–14).

Thymic epithelial cell (TEC)-specific overexpression of the secreted Wnt antagonist Dickkopf-1 (15) and loss of the Wnt antagonist Kremen 1 (16) on the one hand and TEC-specific constitutive activation of β -catenin (17) on the other hand cause thymic dystrophy and/or disorganization. Thus, dampening or enhancing Wnt signaling can affect TEC integrity, emphasizing the requirement for precise regulation of Wnt signaling levels in the thymus.

However, it is still unclear what role Wnt signaling plays in the interplay between the thymic stromal cell and the thymocyte compartments, because most studies investigating the role of Wnt signaling relied on T cell–intrinsic genetic ablation of Wnt signaling components, such as β -catenin or Tcf-1/Lef-1. Initial expression analysis suggested TECs as one source of Wnt ligands (18), and Wnt4 was shown to be particularly important for thymic cellularity and the structural integrity of the thymic epithelium

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F.B., B.K., I.A., and M.B. designed the experiments; I.A. generated the floxed Gpr177 mice; F.B. planned and performed experiments; M.M. performed the experimental autoimmune encephalomyelitis experiments and participated in the adoptive-transfer experiment; F.B. analyzed the data; and F.B. and B.K. wrote the manuscript.

The gene array data presented in this article have been submitted to the Gene Expression Omnibus database under accession number GSE69766.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CI, confidence interval; cTEC, cortical TEC; DC, dendritic cell; DKFZ, German Cancer Research Center; DN, double negative; DP, double positive; Fzd, Frizzled; LN, lymph node; mTEC, medullary TEC; P, postnatal day; SP, single positive; Tcf, T cell factor; TEC, thymic epithelial cell; wt, wild-type.

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(19–21). However, the role of TEC-secreted Wnt ligands in T cell development has not been tested directly.

To address this issue, we made use of a conditional knockout mouse line in which TECs are unable to secrete Wnt ligands, whereas thymocytes are genetically unaltered. The Wnt cargo receptor Evi/Wls/Gpr177 is required for the secretion of Wnt ligands (22, 23). We crossed *Gpr177^{lox/flox}* (24) mice to *FoxN1-Cre* mice to obtain a mouse line in which *Gpr177* knockout is targeted to thymic epithelial cells (termed *FoxN1-Gpr177*). We show in this article that TECs constitute a nonredundant source of Wnt ligands in the thymus, which plays an essential role in the production of T cells.

Materials and Methods

Animals

C57BL/6 mice were bred at the animal facility of the German Cancer Research Center (DKFZ). Mice with loxP-flanked alleles encoding *Gpr177* (*Gpr177^{lox/flox}*) were described earlier (24). We crossed these mice to *FoxN1^{Cre}*-transgenic mice (25) to obtain *FoxN1^{Cre} × Gpr177^{lox/flox}* mice (termed *FoxN1-Gpr177*). Unless otherwise indicated, mice were used at 4–6 wk of age, and both sexes were used at equal frequencies. All control mice were matched for sex and age in all experiments. Unless

otherwise indicated, C57BL/6 mice were used as control, because we did not observe any differences among C57BL/6 mice, *FoxN1^{Cre}* (*Gpr177^{+/+}*) mice, and (*FoxN1-Cre⁻*) *Gpr177^{lox/flox}* mice (Figs. 2, 3, data not shown).

All animal breeding and experiments were performed in agreement with the regional authorities and according to the guidelines of the DKFZ.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated using the TRI Reagent (Life Technologies) method. Equal amounts of total RNA were used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative real-time PCR was performed using the SYBR Green method (Power SYBR Green PCR Master Mix; Applied Biosystems) and the ABI 7300 Real-Time PCR System. For internal normalization, the geometric mean of the housekeeping genes *Gapdh* and *Hprt1* was used. All primers were tested for their specificity, as well as their dynamic range. Primer sequences are listed in Supplemental Table I. If primer sequences were retrieved through PrimerBank (<http://pga.mgh.harvard.edu/primerbank/index.html>), the respective PrimerBank ID is shown.

Flow cytometric analysis and cell sorting

Ab staining for flow cytometry was done following standard procedures.

FACS sorting was performed using a FACSaria I, II, or III (Becton Dickinson). Sorted cells were analyzed to assess purity, which was routinely > 95%. FlowJo 8.8.7 software (TreeStar) was used to analyze FACS data.

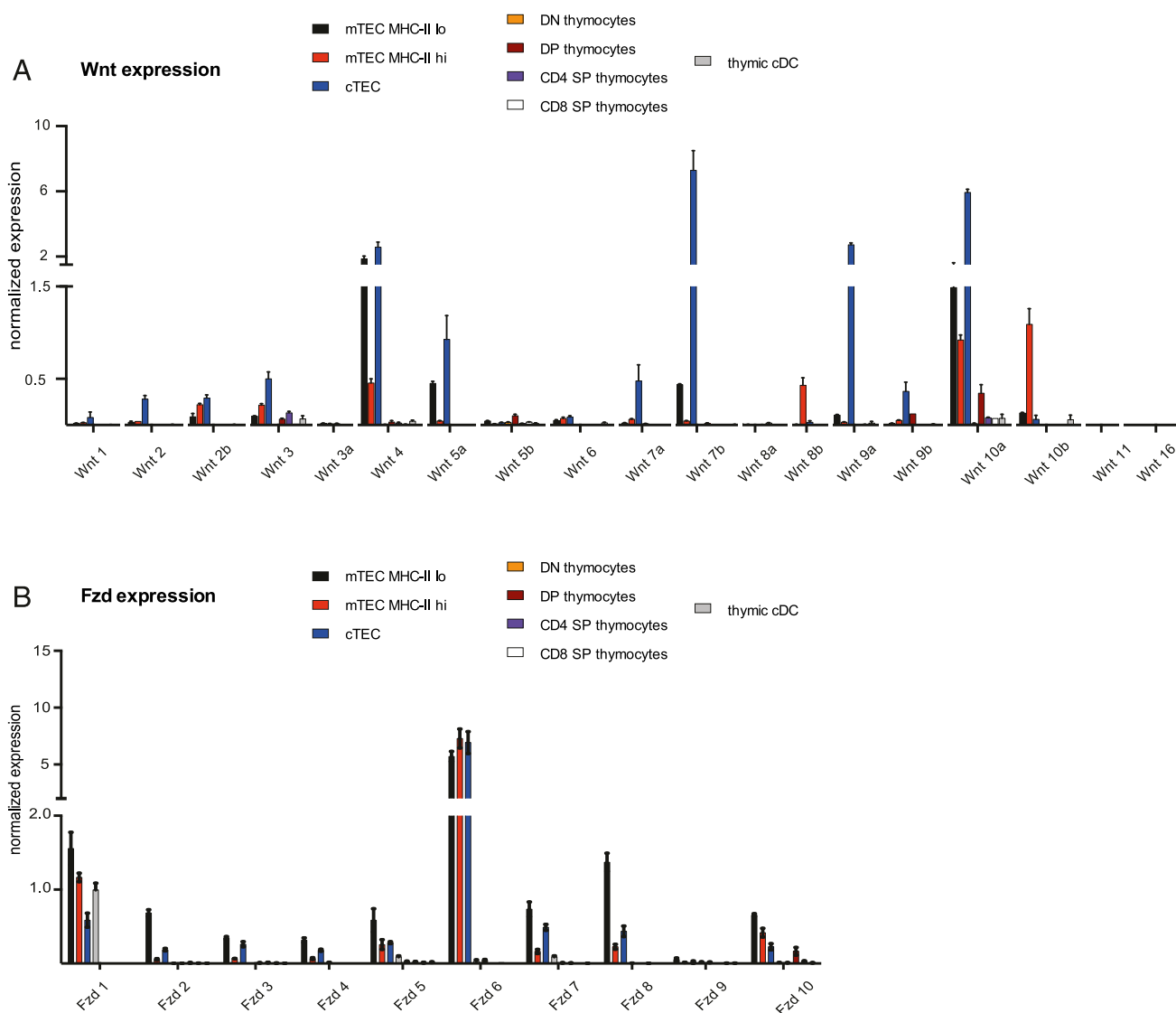


FIGURE 1. Wnt (A) and Fzd (B) receptor expression in different thymic cell populations. The indicated thymic cell populations were sorted by FACS, and Wnt/Fzd expression was assessed by quantitative real-time PCR. For internal normalization, gene-of-interest expression was normalized to the geometric mean of the housekeeping genes *Gapdh* and *Hprt1*. The $\Delta\Delta C_t$ method was applied to calculate the expression values. Data are representative of three independent experiments ($n = 3$).

Flow cytometric Abs

The following Abs were purchased from BD: CD3 (17A2), CD4 (H129.19), CD8 (53-6.7), CD11c (HL3), CD19 (1D3), CD45R/B220 (6B2), CD45 (30-F11), CD62L (Me1-14), CD69 (H1.2 F3), CD172/Sirpα (P84), Ly51 (6C3/BP-1), I-A/I-E (M5/114.15), Foxp3 (MF23), TCRβ (Vβ2-Vβ17a). The following Ab was purchased from eBioscience: Ki67 (SolA15). The following Abs were purchased from BioLegend: TCRβ (H57-597), CD3 (145-2C11), CD4 (H129.19), CD4 (GK1.5), CD5 (53-7.3), CD11b/Mac-1 (M1/70), CD25 (PC61), CD28 (27.51), CD44 (IM7), IFN γ (XMG1.2), IL-4 (11b11), IL-10 (JES5-16E3), IL-17a (TC11-18 H10.1).

The EpCAM (G8.8) Ab was produced and conjugated in-house. The TCRβ Ab was purchased from Jackson. The β -catenin (7F7.2) Ab was purchased from Millipore.

mRNA microarray

For mRNA microarrays, the respective cell populations were sorted by FACS and analyzed to ensure purity, and total RNA was isolated using the RNeasy Plus Micro Kit (QIAGEN). RNA quality was assessed at the DKFZ Genomics and Proteomics core facility prior to nonexponential RNA amplification (26). Subsequently, RNA was subjected to Illumina Sentrix microarrays analysis.

Isolation of TECs

Fat, connective tissue, and capsule tissue were removed from thymi. Thymi were cut into small pieces and stirred in RPMI 1640 (GE Healthcare) supplemented with Pen/Strep, HEPES, and 10% FCS for 10 min to liberate loose thymocytes. Thereafter, thymic tissue was digested for two rounds in Collagenase Type IV (0.2 mg/ml), followed by three to five rounds of Collagenase/Dispase (1.2 U/ml) plus DNase I (25 μ g/ml). Hematopoietic cells were depleted using CD45 MicroBeads (Miltenyi Biotec). The resulting CD45-depleted cell suspension was used for FACS staining. TECs were defined as CD45⁺EpCAM⁺. Cortical TECs (cTECs) were discriminated from medullary TECs (mTECs) by the expression of the Ly51/BP-1 Ag. mTECs were further subdivided into immature MHC-II^{low} and mature MHC-II^{high} subsets.

T cell in vivo competition assay

T cells were isolated by MACS depletion of non-T cells using biotinylated Abs to CD19, CD11b, CD11c, and Ter119 and Streptavidin beads (Miltenyi Biotec, no. 130-048-101) (purity > 95%) from total splenocytes and lymphocytes (pooled from inguinal, brachial, axillary, superficial cervical, and mesenteric lymph nodes [LNs]) taken from FoxN1-Gpr177 or C57BL/6 mice. Purified T cells were labeled with 2 μ M CFSE or 10 μ M

Cell Proliferation Dye eFluor 450 to distinguish between the two donors. Cells were mixed in a 1:1 ratio, and 2×10^6 cells were injected into the tail vein of Rag2-deficient mice. Aliquots from each batch were used to assess the starting distribution (ratio donor 1/donor 2). To exclude a potential influence of either of the dyes, a criss-cross labeling scheme was applied. As a control, one Rag2^{-/-} mouse, which did not receive any T cells, was included. On day 6, recipient mice were sacrificed, spleens and LNs were removed, and cells were analyzed by flow cytometry.

Cell cycle analysis of TECs

Following normal surface staining (including fixable viability dye), cells were fixed for 30 min at 4°C, washed with permeabilization buffer, and incubated overnight at 4°C. Permeabilized cells were stained for the proliferation marker Ki67 for 45 min. Cells were washed, resuspended with permeabilization buffer, incubated with DAPI for 15 min, and measured by flow cytometry without an additional washing step. Fixation and permeabilization were performed using the Foxp3 Staining Buffer Set (eBioscience).

Isolation of thymic dendritic cells

The isolation of thymic dendritic cells (DCs) was performed as described (27), with minor adjustments. Briefly, chopped thymus pieces were digested for two rounds with Collagenase. Subsequently, the cells were washed and incubated for 5 min with 10 mM EDTA to dissociate DCs from attached thymocytes. A Nycoprep (1.077 g/ml) gradient was used to enrich cells of low density. These cells, which included DCs, were pelleted in 2 mM EDTA and used for FACS sorting.

Statistical analysis

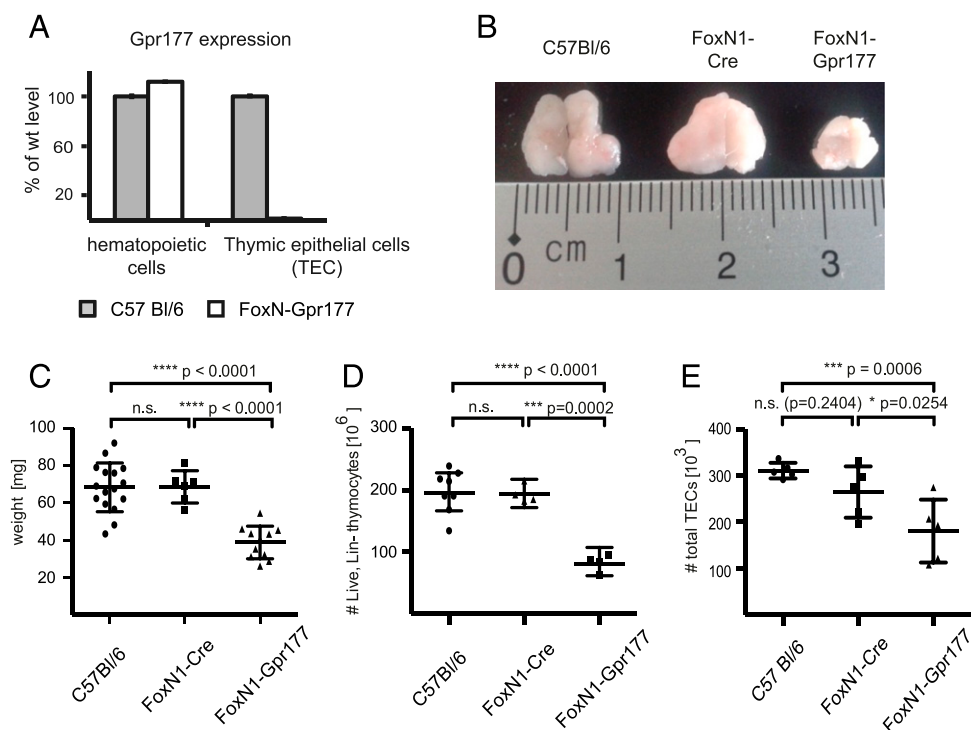
Unless otherwise indicated, data are presented as geometric mean with 95% confidence interval (CI). Statistical significance was tested using GraphPad Prism 6.0 software. A two-tailed unpaired *t* test, two-tailed Mann-Whitney *U* test, or ordinary one-way ANOVA (corrected for multiple comparison using the Tukey correction) was applied. Data sets were tested for normal distribution using the D'Agostino and Pearson omnibus test.

Results

TECs are the primary source of Wnt ligands in the thymus

Although Wnt expression in the thymus had been assessed previously (18, 19, 28), a comprehensive thymic expression analysis of all known Wnt ligands and Frizzled (Fzd) receptors has not been done. This precluded a direct comparison among different thymic

FIGURE 2. Thymus hypotrophy in FoxN1-Gpr177 mice. Thymi were prepared from young adult (4–6 wk) mice. (A) Confirmation of the specific knockout of Gpr177 in TECs. (B) Representative photograph of thymi from FoxN1-Gpr177 and C57BL/6 mice. (C) Scatterplot summarizing the weight of thymi prepared from FoxN1-Gpr177 mice and C57BL/6 or FoxN1-Cre mice as control. Symbols represent individual mice. (D) Quantification of total thymocytes. The lineage mixture contained biotinylated Abs to CD19 (B cells), CD11c (DCs), CD11b (monocytes/macrophages), and Ter119 (erythrocytes). (E) Scatterplot depicting the quantification of total TECs. Statistical significance was assessed by one-way ANOVA (corrected for multiple comparison) using GraphPad Prism 6 software. n.s., not significant.



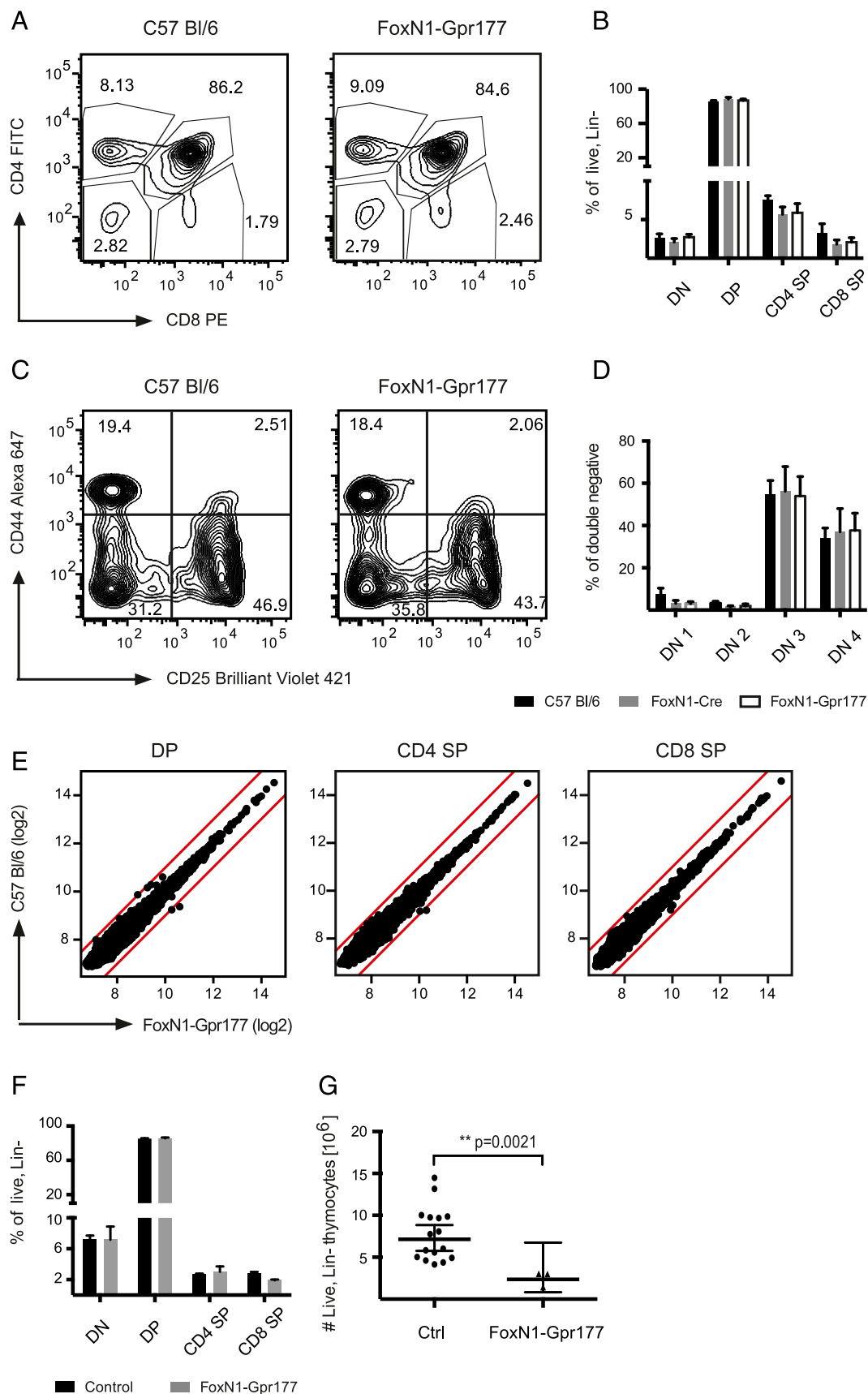


FIGURE 3. Thymocyte subset distribution is unaffected in FoxN1-Gpr177 mice. Thymocyte subset distributions were assessed by flow cytometry. **(A)** Representative FACS plots showing CD4 versus CD8 distribution of C57BL/6 and FoxN1-Gpr177 thymocytes (gated on live, Lin⁻, single cells). **(B)** Bar graph summarizing the geometric mean of DN, DP, and SP thymocyte subpopulation frequencies. **(C)** Representative FACS plots showing the CD4/CD8-DN population further subdivided (DN1–DN4) by the markers CD44 and CD25. **(D)** Bar graph summarizing the geometric mean of DN1–DN4 subpopulation frequencies. Error bars in **(B)** and **(D)** represent 95% CI. **(E)** Scatterplots summarizing the mRNA microarray results (*Figure legend continues*)

cell populations with regard to their respective contribution to Wnt ligand production in the thymus. To identify the thymic cell type(s) that serve(s) as the primary source of Wnt ligands, we isolated and sorted thymic DCs and different developmental stages of thymocytes and mTECs and cTECs by FACS, and assessed Wnt expression by quantitative real-time PCR (Fig. 1A).

We found that, among these cell types, TECs expressed multiple Wnt ligands and that the expression levels far exceeded those of other cell types, even though Wnt expression by thymocytes and DCs was detectable at low levels. Among TECs, cTECs expressed high levels of Wnt4, Wnt7b, Wnt9a, and Wnt10a, immature MHC-II^{low} mTECs primarily expressed Wnt4 and Wnt10a, and mature MHC-II^{high} mTECs predominantly expressed Wnt10a and Wnt10b. In line with previous studies (18), Wnt4 was among the most prominently expressed Wnt ligand.

At the same time, TECs expressed high levels of Fzd receptors (Fig. 1B), suggesting that TECs are the primary source of Wnts in the thymus and are likely to be Wnt responsive.

Loss of Wnt secretion by TECs impairs thymus development

It was suggested that Wnt ligands produced by TECs might foster T cell development (6, 18). Furthermore, the thymic stroma was demonstrated to be Wnt receptive (15, 17, 29). Gpr177 is a Wnt-specific cargo receptor that is required to transport Wnt ligands to the cell surface and, thus, for Wnt secretion (22, 23). We demonstrated that TECs express multiple Wnt ligands (Fig. 1A), and the secretion of all require Gpr177. To test how the loss of TECs as a thymic Wnt source might affect thymus development, we generated a conditional knockout mouse line. We crossed Gpr177 flox/flox mice (24) to FoxN1-Cre mice to obtain mice in which TECs are unable to secrete Wnt ligands. The deletion efficiency in TECs and the TEC specificity of the knockout are depicted in Fig. 2A. FoxN1-Gpr177 mice are viable and, with the exception of a mild hair loss (presumably due to FoxN1 promoter activity and concomitant loss of Gpr177 in keratinocytes), show no developmental defects or overt health problems (24). Unless otherwise stated, we used mice of both sexes at the age of 4–6 wk. The analysis of thymi from FoxN1-Gpr177 mice revealed thymic hypotrophy (Fig. 2B) that was associated with reduced thymus weight (Fig. 2C), whereas the thymus weight of FoxN1-Cre control mice was normal. We determined the absolute number of thymocytes and TECs and observed an ~2-fold reduction in the absolute number of thymocytes (Fig. 2D) and a reduction in the absolute number of TECs (Fig. 2E) in FoxN1-Gpr177 mice, whereas FoxN1-Cre mice had normal thymus cellularity.

Loss of Wnt secretion by TECs does not block T cell development

A reduction in thymocytes could reflect impaired T cell development. We tested this possibility by analyzing the distribution of the major developmental stages of thymocytes by flow cytometry. We analyzed the CD4 versus CD8 profile (CD4/CD8 DN, CD4/CD8 double positive [DP], and CD4 or CD8 single positive [SP]). However, we did not detect a block at any particular developmental stage (Fig. 3A, 3B), including the early developmental stages DN1 (CD44⁺CD25[−]), DN2 (CD44⁺CD25⁺), DN3 (CD44[−]CD25⁺), and DN4 (CD44[−]CD25[−]) (Fig. 3C, 3D). Additionally, we performed analysis using the markers CD5 and TCRβ,

which allows subdivision of the DP stage into pre- and post (positively)-selected thymocytes (30) (data not shown). This analysis confirmed normal thymocyte development. Further, we used flow cytometry to assess whether IL-7R expression was reduced in FoxN1-Gpr177 mice, because this might impair expansion of early thymocytes. However, we did not find evidence for altered IL-7R expression (data not shown), supporting the phenotype of unchanged thymocyte development. To test whether the transcriptome of thymocytes from FoxN1-Gpr177 mice might be altered as a consequence of missing Wnt ligands normally provided by TECs, we performed gene-expression profiling. To this end, the transcriptome of sorted CD4/CD8-DP, CD4-SP, and CD8-SP thymocytes from FoxN1-Gpr177 and C57BL/6 mice was analyzed by mRNA microarrays (Gene Expression Omnibus accession number GSE69766) (Fig. 3E). The expression profile of thymocytes from FoxN1-Gpr177 or C57BL/6 mice was virtually identical, arguing against a direct impact of the lack of TEC-supplied Wnts on thymocytes, at least on the transcriptional level.

Thymus hypotrophy in FoxN1-Gpr177 mice sets in around birth

FoxN1-Cre-mediated deletion of Gpr177 was initiated in the early embryonic thymus (embryonic day 12.5), as judged by β-Gal staining (data not shown); thus, the observed phenotype could already arise in the fetal thymus. To narrow down the developmental window in which thymus hypotrophy was established in FoxN1-Gpr177 mice, we analyzed thymi from newborn (postnatal day [P]1) mice, assessed their cellularity, and analyzed T cell subsets by flow cytometry. Equivalent to what we observed in adult mice, the composition of thymocytes subsets (DN, DP, CD4/CD8 SP) was unchanged in newborn FoxN1-Gpr177 mice (Fig. 3F, data not shown). In contrast, the absolute number of thymocytes was already reduced at this stage of development compared with Gpr177-sufficient littermates (control) (Fig. 3G). This indicates that the observed phenotype of thymus hypotrophy is already established perinatally.

FoxN1-Gpr177 mice have a reduced peripheral T cell pool

It was shown that a reduction in thymopoiesis can be compensated for by means of homeostatic proliferation (31) in the periphery. Hence, we analyzed the peripheral T cell pool from spleen and pooled LNs (inguinal, brachial, axillary, superficial cervical, and mesenteric) from FoxN1-Gpr177 and control mice by flow cytometry. We observed a modest, but consistent, reduction in the frequency of peripheral T cells (Fig. 4A, 4B). This reduction was also evident with regard to absolute T cell numbers (Fig. 4C). The lower frequency of peripheral T cells also persisted in mice up to 16 wk old (Fig. 4D).

Based on the markers CD44 and CD62L, we observed a tendency toward a memory-like phenotype (Supplemental Fig. 1) that also persisted with age. This might be due to lymphopenia-driven proliferation of T cells in the periphery to compensate for the lower thymocyte numbers. Further characterization of the peripheral T cell pool showed that the CD4/CD8 ratio (data not shown), Th subset distribution (Th1, Th2, Th17, regulatory T cell-like) (data not shown), and TCR repertoire (when analyzed for usage of TCRVβ-chains by flow cytometry) (Fig. 4E–G) were unaltered. Thus, our data show that peripheral T cells, similar to thymocytes, were reduced in number but otherwise were phenotypically normal.

of thymocytes (DP, CD4 SP, or CD8 SP) sorted by FACS from FoxN1-Gpr177 mice and C57BL/6 mice as control. (F) Thymocyte subset distribution in newborn FoxN1-Gpr177 and C57BL/6 mice. (G) Scatterplot summarizing the absolute numbers of thymocytes in newborn FoxN1-Gpr177 and C57BL/6 mice. Symbols represent individual mice.

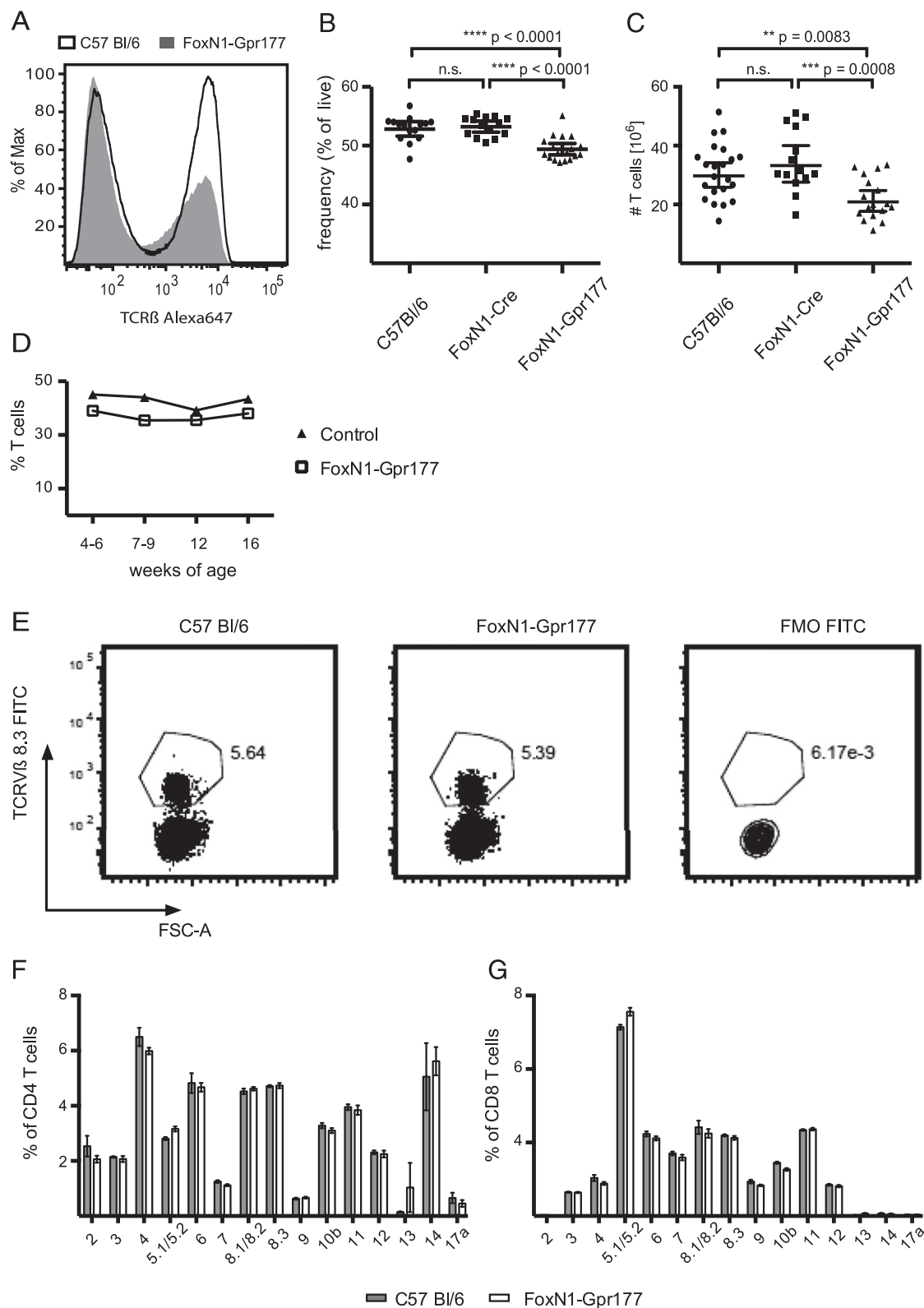
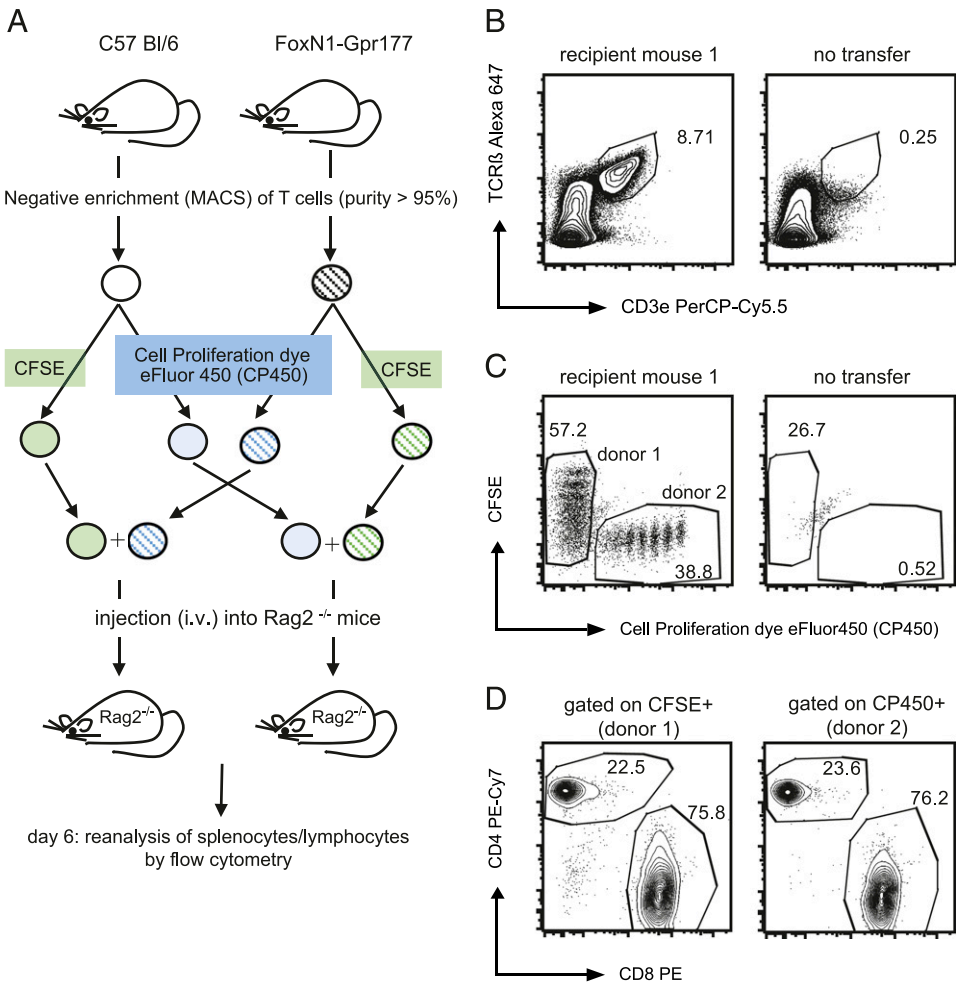


FIGURE 4. FoxN1-Gpr177 mice have a reduced peripheral T cell pool. Pooled splenocytes and lymphocytes (pooled from inguinal, brachial, axillary, superficial cervical, and mesenteric LNs) were analyzed by flow cytometry. **(A)** Representative FACS plot showing graphs of the frequency of T cells in FoxN1-Gpr177 mice and C57BL/6 mice. **(B)** Scatterplot showing the cumulated frequency of T cells. T cells were defined as live, low-scatter (lymphocyte gate), B220⁻, TCRβ⁺. **(C)** Cumulative data for the absolute numbers of T cells. Symbols represent individual mice. Statistical significance was assessed using one-way ANOVA (corrected for multiple comparison). **(D)** Frequency of peripheral T cells in FoxN1-Gpr177 mice with increasing age. **(E)** Representative FACS plots showing the frequency of TCRVβ8.3⁺ T cells (gated on CD4 T cells) in wt versus FoxN1-Gpr177 mice (and the fluorescence minus one for FITC). **(F)** Frequency of the indicated TCRVβ⁺ T cells in CD4 T cells. **(G)** Frequency of the indicated TCRVβ⁺ T cells in CD8 T cells. Data in (F) and (G) represent four mice/group. Error bars represent SEM. The experiment was performed twice with similar results. n.s., not significant.

FIGURE 5. T cells from FoxN1-Gpr177 mice show normal homeostatic proliferation in vivo. Assessment of the homeostatic potential of FoxN1-Gpr177-derived T cells under lymphopenic conditions. **(A)** Schematic representation of the experimental outline. Purified T cells from FoxN1-Gpr177 or C57BL/6 mice were labeled with 2 μ M CFSE or 10 μ M Cell Proliferation Dye eFluor 450 to distinguish between the two donors. Cells were then mixed in a 1:1 ratio, and 2×10^6 cells were injected into the tail vein of Rag2-deficient mice. One Rag2^{-/-} mouse, which did not receive any T cells, was included as a control. On day 6, recipient mice were sacrificed, spleens and LNs were removed, and cells were used for flow cytometry analysis. **(B)** Representative FACS plots showing the gating of T cells (CD3e⁺TCR β ⁺). **(C)** Representative FACS plots showing the respective donor contribution to the newly established T cell pool. **(D)** Representative FACS plot showing the CD4/CD8 ratio gated on donor 1 (CFSE⁺CP450⁻) and donor 2 (CFSE⁻CP450⁺).



Intact homeostatic proliferation potential of T cells from FoxN1-Gpr177 mice

Considering that T cells were reduced in FoxN1-Gpr177 mice (Fig. 4) without alterations of T cell subsets, we wondered whether the T cells in these mice might have an impaired ability to fill the T cell niche by means of homeostatic proliferation. To test this directly, we conducted an in vivo competition experiment in which we cotransferred an equal number of T cells from C57BL/6 and FoxN1-Gpr177 mice into B cell- and T cell-deficient Rag2^{-/-} mice. To distinguish between the two donors, we labeled the control T cells with CFSE and the FoxN1-Gpr177-derived T cells with (CFSE-equivalent) Cell Proliferation Dye eFluor 450 or vice versa (Fig. 5A).

On day 6 after T cell transfer, we isolated total splenocytes and lymphocytes and analyzed the cells by flow cytometry. Because we chose T cell-deficient Rag2^{-/-} mice as hosts, all T cells found on the day of analysis were considered donor T cells (Fig. 5B).

Control and FoxN1-Gpr177 T cells proliferated to a comparable degree (Fig. 5C, Table I); consequently, they contributed virtually equally to the newly established T cell pool in the Rag2^{-/-} host (Fig. 5C). As in the steady-state, there was no discernable difference in the CD4/CD8 ratio (Fig. 5D) between the wt and FoxN1-Gpr177 donors. These results argue against a defect in the potential of FoxN1-Gpr177 T cells to undergo homeostatic proliferation.

FoxN1-Gpr177 TECs show normal chemokine expression

Upon entry into the thymus, T cell progenitors migrate from the deep cortex to the outer cortex. cTECs provide signals to these progenitors, which are required for T lineage commitment (32, 33). To address the question of whether cTECs in FoxN1-Gpr177 mice might be defective for any of these early signals, we sorted cTECs by FACS and analyzed the expression of genes relevant to thymocyte progenitor attraction to the thymus or early T lineage-inductive

Table I. In vivo assessment of the homeostatic potential of FoxN1-Gpr177 T cells

	Recipient Mouse 1		Recipient Mouse 2		Recipient Mouse 3	
	C57BL/6	FoxN1-Gpr177	C57BL/6	FoxN1-Gpr177	C57BL/6	FoxN1-Gpr177
Before transfer (%)	55.7	43.7	50.7	47.4	52.7	45.5
After transfer (%)	57.2	38.7	56.3	39.7	44.1	49.7
CD4 ⁺	22.5	23.6	36.2	31.7	26.6	30.4
CD8 ⁺	75.9	76.2	62.1	68.2	73	66
Proliferation index CD4	1.26	1.17	1.36	1.16	1.15	1.47

A summary of the frequencies of respective donor contributions (percent, before and after transfer), the CD4/CD8 ratio, and the proliferation index of CD4 T cells.

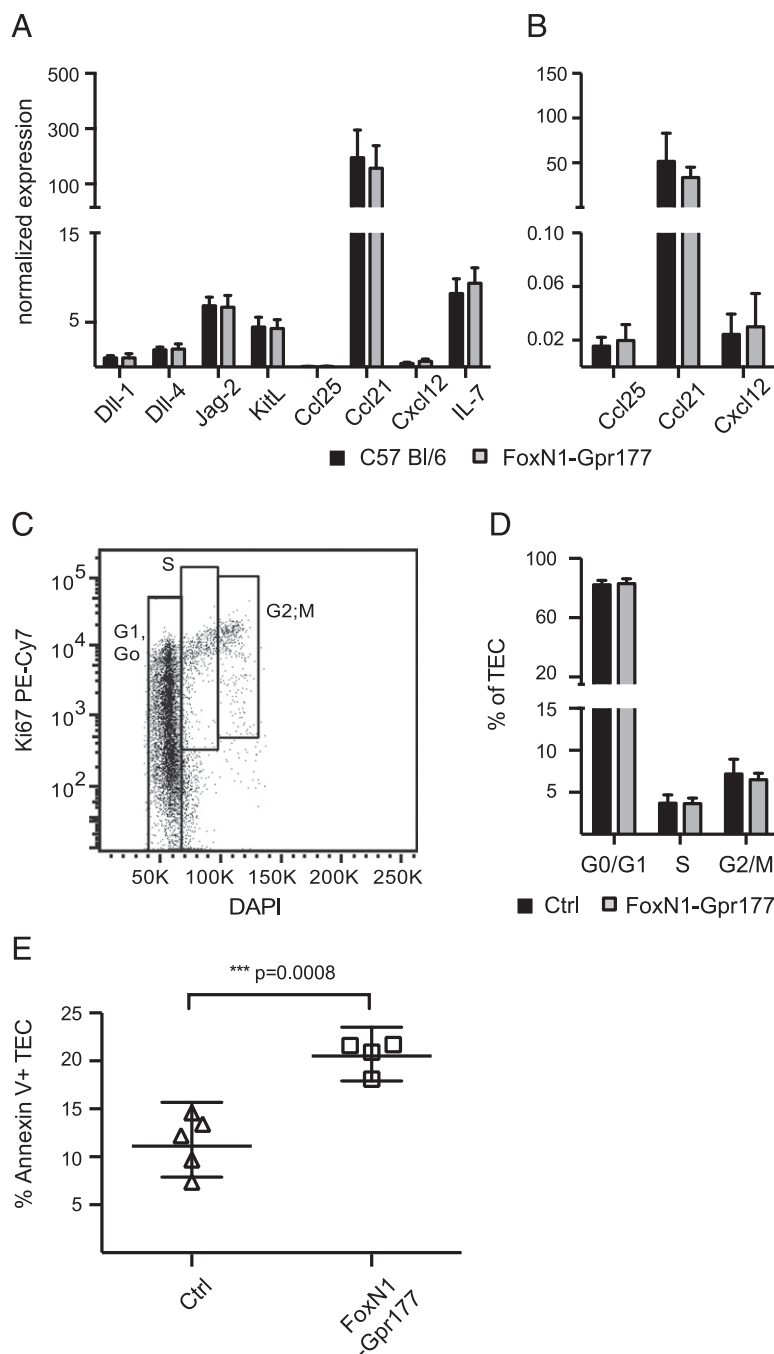


FIGURE 6. Chemokine expression is unaltered in FoxN1-Gpr177 TECs. cTECs (live, CD45⁻EpCAM⁺Ly51⁺; *n* = 4) (**A**) or total TECs (live, CD45⁻EpCAM⁺; *n* = 3) (**B**) were sorted by FACS, and gene expression was determined by quantitative real-time PCR. For internal normalization, the geometric mean of Gapdh and Hprt1 was used. Error bars represent the SD. (**C–E**) Thymi prepared from newborn (P3) mice. (**C**) Representative dot plot showing the gating for G₀/G₁, S, and G₂/M phases based on the marker Ki67 and the DNA content, as visualized by DAPI. (**D**) Bar graph summarizing the frequencies of TECs in G₀/G₁, S, and G₂/M phases from FoxN1-Gpr177 mice or control littermates. Data are geometric mean with 95% CI. (**E**) Scatterplot summarizing the frequencies of AnnexinV⁺ cells of total TECs from FoxN1-Gpr177 mice or control littermates. Statistical significance was assessed using the unpaired, two-tailed *t* test assuming unequal SDs.

signals usually provided by cTECs. The expression of Notch ligands (Dil-1, Dil-4, Jag-2), chemokines guiding T cell progenitors to and within the thymus (Ccl25, Ccl21, Cxcl12), and IL-7 (growth factor for early thymocytes) was unaltered in FoxN1-Gpr177 cTECs (Fig. 6A) and total TECs (Fig. 6B). Thus, our data indicate that the reduced number of thymocytes in FoxN1-Gpr177 mice is unlikely to be due to a diminished potential of TECs to attract thymocyte progenitors or provide Notch ligands to impose T lineage commitment.

FoxN1-Gpr177 TECs have a higher apoptosis rate but proliferate normally

In thymi from adult FoxN1-Gpr177 mice, we observed reduced absolute TEC numbers (Fig. 2E), as well as epithelial-free areas (Supplemental Fig. 2) within medullary regions. To assess whether the reduction in total TECs is caused by proliferation defects in

TECs, we analyzed the cell cycle status by flow cytometry. We expected potential proliferation defects to be more obvious in newborn mice (P3) because the thymus is still growing at this stage. We stained for the proliferation marker Ki67 and measured the DNA content by DAPI (Fig. 6C). However, we did not detect differences in the cell cycle status of TECs (Fig. 6D), including cTECs and mTECs (data not shown). We next addressed whether the reduction in TEC numbers might reflect a specific decline in the mTEC or cTEC compartment. The abundance of cTECs peaks around birth, whereas the frequency of cTECs in young adult mice is low (34). Therefore, we also analyzed the mTEC/cTEC ratio in newborn mice (P3) and found that it was not affected in FoxN1-Gpr177 mice (Supplemental Fig. 3A, 3B).

Finally we addressed whether survival of TECs from (newborn) FoxN1-Gpr177 mice is impaired. To test this, we made use of

annexin V staining to detect early apoptotic cells by flow cytometry. We observed a significantly increased apoptosis rate in FoxN1-Gpr177 TECs compared with control littermates (Fig. 6E). Collectively, based on the presented data, we propose the scenario depicted in Fig. 7.

Discussion

In the present study, we identified TECs as the primary source of Wnt ligands in the thymus by directly comparing Wnt expression of TECs with different thymocyte subsets (CD4/CD8 DN, CD4/CD8 DP, CD4 SP, and CD8 SP), as well thymic DCs. Although Wnt or Fzd receptor expression was reported in previous studies (18, 28, 35), it remained unknown which thymic cell population is the major producer of Wnts, because only the expression of selected Wnts was tested previously. By analyzing the most numerous and functionally relevant cell types and directly comparing the expression of all 19 known Wnt ligands, as well as all 10 known Fzd receptors, we provide a comprehensive overview of the extent to which the various

thymic cell populations produce Wnts and express the cognate receptors (Fzds) (Fig. 1).

To analyze the function of (TEC-) secreted Wnt ligands on T cell development, irrespective of whether these would induce canonical or noncanonical Wnt signaling, we made use of a conditional knockout mouse line (FoxN1-Gpr177) in which TECs are unable to secrete Wnt ligands. This allowed us to study the role of Wnt in T cell development without interfering with the intracellular signaling machinery of T cells. In light of the observation that TECs are the primary source of Wnt ligands in the thymus (Fig. 1), this model likely reflects a situation in which the thymus is largely depleted of Wnt ligands. We also verified that, in the absence of TEC-provided Wnt ligands, thymocytes do not compensate for this lack of Wnts by enhanced expression of Wnt ligands (data not shown).

In FoxN1-Gpr177 mice, the thymus was reduced in size and cellularity to ~60% of the wt level (Fig. 2C–E). This reduction was most apparent with regard to the absolute numbers of thymocytes (Fig. 2D). The degree of reduction in thymocytes was

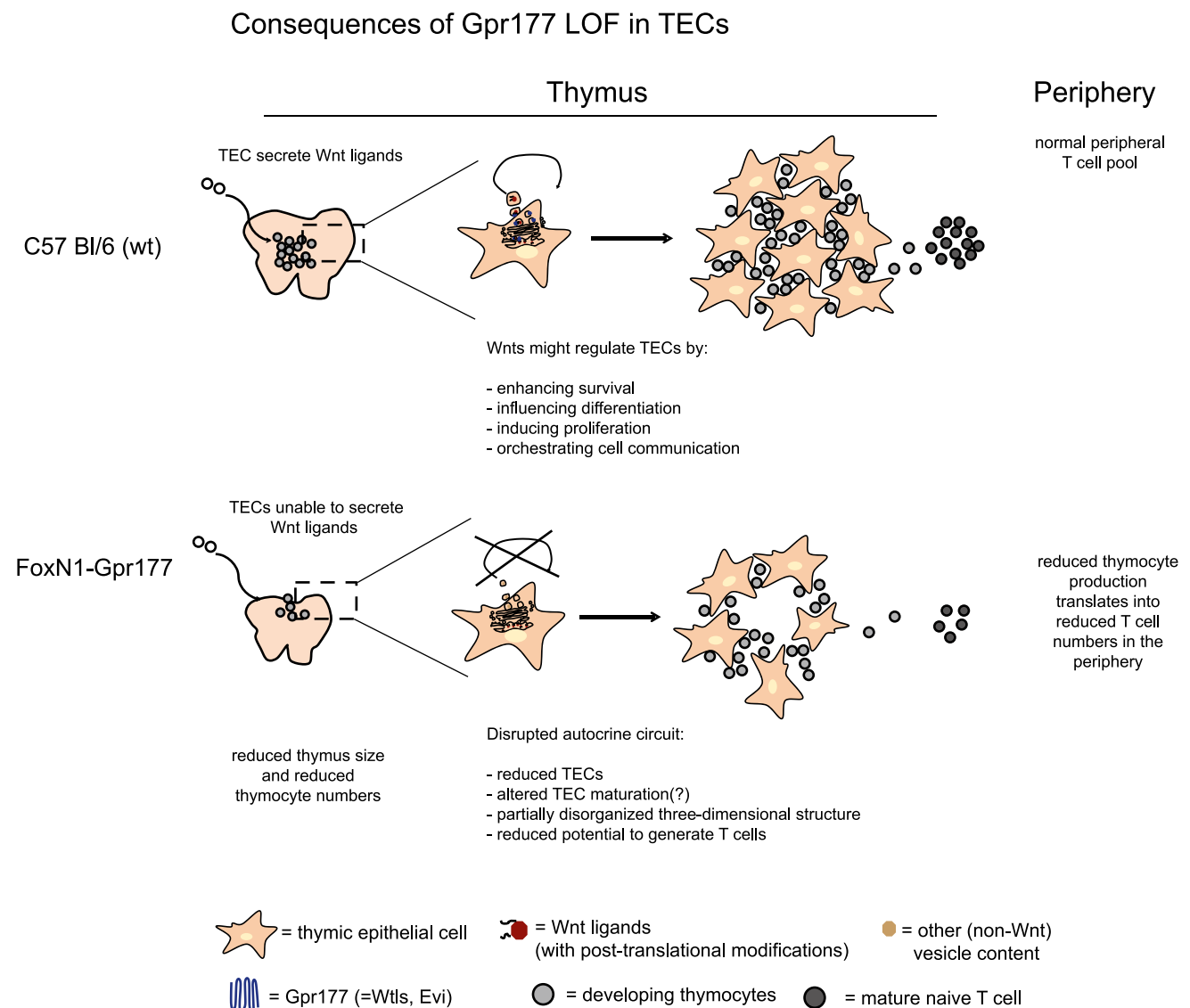


FIGURE 7. Model of how loss of Gpr177 in TECs affect the T cell pool. TECs normally secrete Wnt ligands, which promote normal thymus development (*upper panel*). In FoxN1-Gpr177 mice (*lower panel*), TECs are unable to secrete Wnt ligands because they lack the Wnt-specific cargo receptor Gpr177. This might disrupt the autocrine Wnt signaling required for normal TEC development and/or maintenance, causing a reduction in TEC numbers and three-dimensional architecture. As a consequence, fewer T cells can be produced, which, in turn, decreases thymic cross-talk between TECs and thymocytes. The resulting reduction in thymocytes also translates into fewer peripheral T cells.

more pronounced than what was reported for mice in which β -catenin was targeted in thymocytes by Lck-Cre [$\sim 15\%$ reduction (1)], but not as severe as the $\sim 90\%$ reduction reported for Tcf-1-knockout mice (3). We also found, albeit to a lesser degree, a reduction in the frequency (Fig. 4B) and absolute numbers (Fig. 4C) of peripheral T cells. The reduction in T cells was not due to an impaired potential of the remaining T cells to undergo homeostatic proliferation, because T cells from FoxN1-Gpr177 mice performed equally well in an in vivo competition experiment compared with their wt counterparts (Fig. 5B, Table I). To verify that FoxN1-Gpr177 T cells can be primed and exert complex effector functions in vivo, we also conducted experimental autoimmune encephalomyelitis experiments. Experimental autoimmune encephalomyelitis was equally well induced in FoxN1-Gpr177 and C57BL/6 mice (data not shown), confirming the functional competence of FoxN1-Gpr177 T cells.

We also assessed whether the reduced thymocyte numbers and the resulting reduced peripheral T cell frequencies in FoxN1-Gpr177 mice reflect impaired T cell development. The subset frequencies among all analyzed thymocyte populations were indistinguishable between FoxN1-Gpr177 and control mice (Fig. 3A–D). In line with this, mRNA microarray analysis of thymocytes revealed very similar gene-expression profiles between FoxN1-Gpr177 and control mice (Fig. 3E), indicating normal T cell development. An alternative explanation for the observed reduction in thymocyte numbers could be a skewed clonal composition. We tested this notion by analyzing TCR V β usage by flow cytometry (Fig. 4F, 4G). However, we did not detect evidence of a skewed TCR repertoire in CD4 or CD8 T cells. Our results argue against a direct involvement of (TEC-provided) Wnt ligands in T cell development. Therefore, the perturbed T cell development reported for adenomatous polyposis coli- or Tcf-1-knockout mice (3, 7) might reflect cross-talk of Wnt-downstream components with other signaling pathways during T cell development. In support of this scenario, it was shown previously that, in lymphatic cells, Tcf-1 and Lef-1 have the inherent potential to act independently of β -catenin by interacting with the Fos family member Atf2 instead (36, 37). We cannot formally rule out the possibility that the reduction in thymocytes in FoxN1-Gpr177 mice is caused by a decreased settling of the thymus with T cell progenitors; however, we consider this unlikely because the expression of the chemoattractants Ccl21, Cxcl12, and Ccl25 by cTECs (Fig. 6A) and by total TECs (Fig. 6B) was unaltered.

In addition to paracrine effects on T cells, the lack of TEC-provided Wnt ligands could affect the development/maintenance of TECs by abrogating autocrine Wnt signaling. This would be in line with a previous report demonstrating a direct role for Wnt4 in TEC development (19). Furthermore, the thymic hypotrophy observed upon inducible overexpression of the Wnt antagonist Dickkopf-1 was attributed to a direct effect on TECs (15). In FoxN1-Gpr177 mice, the absolute number of TECs was reduced, albeit not as severely as the absolute number of thymocytes (Fig. 2E). This reduction might be caused by a higher susceptibility of FoxN1-Gpr177 TECs to apoptotic cell death because the apoptosis rate was reduced perinatally (Fig. 6E). Using histology, we defined continuous cortical areas interspersed with clearly demarcated medullary islets (Supplemental Fig. 2), including autoimmune regulator-positive mTECs (data not shown), thus indicating that the overall thymic architecture was intact in FoxN1-Gpr177 mice. However, we detected small cytokeratin 14-negative patches within the medullary areas (Supplemental Fig. 2), which may be evidence of subtle abnormalities in thymus organization. Given the tight interaction between TECs and thymocytes,

commonly referred to as thymic cross-talk, these alterations might account, at least in part, for the observed reduction in thymocyte numbers. Additionally, FoxN1-Gpr177 mice seem to have relatively fewer MHC-II^{low} mTECs, which might indicate an impaired supply of immature mTECs (Supplemental Fig. 3D). A major autocrine Wnt signaling circuit (from and to TECs) would be in accordance with the high expression of Fzd receptors, which is most prominent in the immature mTEC MHC-II^{low} subset (Fig. 1B). Based on the planar cell polarity pathway phenotype of Fzd6-knockout mice (38), the prevalent expression of Fzd6 in TECs could indicate that the noncanonical Wnt signaling pathway contributes to thymus integrity.

Taken together, we propose a model that is summarized in Fig. 7. Under normal conditions, TECs produce and respond to Wnt ligands, resulting in normal TEC and thymocyte development. If this autocrine signaling circuit is disrupted (FoxN1-Gpr177), TECs do not develop and organize correctly; as a result, fewer thymocytes are produced. The conclusion that the reduced TEC compartment might be responsible for the decreased thymus size and cellularity in FoxN1-Gpr177 mice is in line with a previous study showing that the stromal niche (including TECs) is the limiting factor in regulating thymus size and that DN thymocytes compete for the availability of this niche (39).

Irrespective of the exact mechanism underlying our observations, the current data highlight a nonredundant role for TECs as a source of Wnt ligands that promote normal thymopoiesis.

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Disclosures

The authors have no financial conflicts of interest.

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