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Atypical Memory Phenotype T Cells with Low Homeostatic Potential and Impaired TCR Signaling and Regulatory T Cell Function in Foxn1Δ/Δ Mutant Mice

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Foxn1Δ/Δ mutants have a block in thymic epithelial cell differentiation at an intermediate progenitor stage, resulting in reduced thymocyte cellularity and blocks at the double-negative and double-positive stages. Whereas naive single-positive thymocytes were reduced >500-fold in the adult Foxn1Δ/Δ thymus, peripheral T cell numbers were reduced only 10-fold. The current data shows that Foxn1Δ/Δ peripheral T cells had increased expression of activation markers and the ability to produce IL-2 and IFN-γ. These cells acquired this profile immediately after leaving the thymus as early as the newborn stage and maintained high steady-state proliferation in vivo but decreased proliferation in response to TCR stimulation in vitro. Single-positive thymocytes and naive T cells also had constitutively low αβ TCR and IL7R expression. These cells also displayed reduced ability to undergo homeostatic proliferation and increased rates of apoptosis. Although the frequency of Fosp3+CD4+CD25+ T cells was normal in Foxn1Δ/Δ mutant mice, these cells failed to have suppressor function, resulting in reduced regulatory T cell activity. Recent data from our laboratory suggest that T cells in the Foxn1Δ/Δ thymus develop from atypical progenitor cells via a noncanonical pathway. Our results suggest that the phenotype of peripheral T cells in Foxn1Δ/Δ mutant mice is the result of atypical progenitor cells developing in an abnormal thymic microenvironment with a deficient TCR and IL7 signaling system. The Journal of Immunology, 2007, 179: 8153–8163.

Thymic epithelial cells (TECs) play many roles in thymocyte development, including the presentation of self-peptide-MHC complexes to the TCR for positive selection, the participation of medullary TECs in negative selection, and the elaboration of cytokines and chemokines that affect thymocyte migration and development (1, 2). Interactions between thymic stromal cells and thymocytes and factors secreted by both types of cells control T cell development and maturation in the thymus, which also directly affects T cell function and the size of the naive T cell pool in the periphery (3–5). Thus, the efficient production of a large, diverse, and effective peripheral T cell pool originates with and depends on thymic epithelial cells organized into a normal thymic architecture.

Once T cells leave the thymus, the number of peripheral T cells in a normal individual is always regulated by homeostatic proliferation and remains relatively constant in the absence of infection (6, 7). Many factors that result in the depletion of lymphocytes (or lymphopenia) such as irradiation, chemotherapy, or infection may initiate homeostatic proliferation (8–11). Under homeostatic proliferation, total lymphocyte number will compensate to normal level by an increase in phenotypic and functional memory T cells. However, the composition of the T cell pool may be anomalous, especially if thymic function is compromised, because the naive T cell pool depends on thymic output for long-term maintenance (12–14).

Most homeostatic proliferation studies are based on transferring T cells into lymphopenic recipients. The process of homeostatic proliferation is similar to Ag-driven activation and proliferation in that the interaction of TCR/MHC and cytokines is also required for homeostatic expansion. After homeostatic proliferation, naive cells acquire memory T cell markers and functional properties like those of Ag-experienced memory cells (9, 15, 16). Unlike the process of Ag-driven proliferation, homeostatic proliferation displays slower progression through the cell cycle, does not up-regulate activation markers such as CD25 and CD69, and increases CD44 expression progressively (17). Recent studies indicate that competition for limited resources such as TCR signals, IL-7, thymic stromal lymphopoietin, and CD4+CD25+ regulatory T (Treg) cells is involved in homeostatic regulation (18–22).

Neonatal mice are also physiologically lymphopenic (23), but the processes driving this proliferation are distinct in some ways from lymphopenia-induced or Ag-driven expansion. For example, CD4+ neonatal expansion is IL7 independent (10). This neonatal expansion has been termed “spontaneous proliferation,” distinct from slow homeostatic proliferation, and leads to the generation of a memory cell repertoire of broad specificity (15). This neonatal expansion has similar characteristics to the “fast” expansion that occurs after the transfer of cells to adult constitutively immunodeficient hosts, which is distinct from slow homeostatic proliferation, is IL7 independent, and may be driven primarily by foreign Ags rather than self-Ags (24).

We recently described a hypomorphic allele of Foxn1, Foxn1Δ/Δ, in which TEC differentiation is initiated but does not...
progress (25), resulting in an adult thymus consisting primarily of TECs with an immature progenitor phenotype and no mature thymic cortical or medullary regions. Overall thymic cellularity in these mutants is dramatically reduced, and few CD4+ and CD8+ single-positive (SP) thymocytes are produced. In this report, we characterized the phenotype and function of peripheral CD4+ and CD8+ T cells in Foxn1Δ/Δ mice. These cells had an activated and memory-like phenotype and function that differ from those in cells stimulated by exogenous Ag or derived from homeostatic proliferation because of their short lifespan, persistently low cell numbers, larger cell size, and expression of CD25 and CD69. Naive T cells in Foxn1Δ/Δ mice also had constitutively low surface TCR and IL7R expression and were hyperresponsive in both allo- and auto-MLR assays. In addition, Foxp3+ CD4+ CD25+ Treg cells did not have normal suppressive function. Foxn1Δ/Δ T cells display these phenotypes as early as the newborn stage. Our data indicate that these phenotypes may arise from atypical progenitors developing in the abnormal thymic microenvironment in Foxn1Δ/Δ mice.

Materials and Methods

Mice

Foxn1Δ/Δ mutant mice generated in our laboratory were described previously (25). The mice were of a mixed C57BL/6 and 129Sv background. Adult male mice were 6–10 wk old. Timed pregnancies were generated by mating homzygous mutant mice with heterozygous mice. The day a visible plug was detected was counted as embryonic day (E) 0.5 (E0.5). Adult and embryonic mice were genotyped by PCR analysis of tail DNA. Rag1−/− (BL6-Ly5.1) mice (18), a generous gift from Dr. E. V. Rothenberg (Division of Biology, California Institute of Technology, Pasadena, CA) were used in adoptive transfer experiments.

Flow cytometry analysis and Abs

Splenocytes, lymph node, and thymocyte cell suspensions were prepared and counted for total cell numbers. Cells (0.5×106) were stained with the following mAbs conjugated to PE, FITC, or allophycocyanin directly or with mAbs labeled with biotin followed by streptavidin-PerCP: anti-CD4 (RM4-5), anti-CD8a (clone 53-6.7), anti-CD44 (clone IM7), and anti-TCRβ (H57-597) (BD Pharmingen). Anti-CD16/32 (clone 2.4G2) (BD Pharmingen) and rat serum were used to block Fc receptors before staining. Rat anti-IL7Ra (A3734), a generous gift from Dr. P. Marrack (University of Colorado Health Sciences Center, Denver, CO), and a cell supernatant from clone A3734 were used for IL-7Ra staining followed by secondary donkey anti-rat IgG-FITC Ab (The Jackson Laboratory). Biotin-anti-Ly5.1 or Ly5.2 (BD Pharmingen) was used for the adaptive transfer assay. Three- or four-color immunofluorescence analysis was performed using a FACScalibur system. The data were analyzed using CellQuest software (BD Biosciences).

BrdU incorporation

Each mouse was injected with 1 mg of BrdU (Sigma-Aldrich) once i.p. and then fed with BrdU-containing water (0.8 mg/ml). Peripheral lymphocytes were harvested from the spleen and lymph nodes (LNs) after 7 days. For BrdU incorporation, CD4+ and CD8+ surface-stained cells were fixed and permeabilized in PBS containing 1% paraformaldehyde plus 0.01% Tween 20 for 48 h at 4°C and then submitted to the BrdU DNase detection technique. FITC-anti-BrdU Ab (clone 3D4; BD Pharmingen) was used for BrdU staining.

Intracellular IL-2, IFN-γ, and Foxp3 staining

Peripheral lymphocytes (2×106) prepared from spleen and LNs were plated in 24-well plates previously coated with 10 μg/ml anti-CD3ε Ab (clone 145-2C11) and cultured with or without an anti-CD28 Ab or PMA (Sigma-Aldrich) at 25 ng/ml plus ionomycin at 500 ng/ml for 5 or 24 h. As a control, cells were cultured in medium alone. To inhibit the secretion of newly synthesized IL-2 and IFN-γ, stimulations were conducted in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich). After stimulation, cells were harvested and stained with anti-CD4, anti-CD8, and anti-CD44 for cell surface markers, fixed in 4% paraformaldehyde at 4°C overnight, washed, and permeabilized with 0.5% saponin (Sigma-Aldrich) in Dulbecco’s PBS, 1% BSA, and 0.05% Na3N. Cells were then stained with anti-IL-2 FITC or anti-IFN-γ PE Ab (BD Pharmingen), washed twice with 0.5% saponin buffer, and kept in FACS store solution for flow cytometry analysis. Foxp3 staining was performed according to the protocol of the Treg staining kit from eBioscience.

Apoptosis analysis

Dead cells were removed from freshly isolated spleen cell suspensions and all LNs (mesentry, axillary, inguinal, superficial cervical, and mandibular) from Ly5.2 or Ly5.1 genotype donor mice. Ly5.2 T cells from Foxn1Δ/Δ and Foxn1Δ/Δ mice were sorted by a MoFlo FACS (DakoCytomation) and Ly5.1 CD4 T cells from B6 mice were purified by anti-CD4 beads (Miltenyi Biotech). Purified cells were labeled by incubation with 2 μm of CFSE (Molecular Probes) for 10 min at 37°C in a solution of PBS plus 0.1% BSA, and then unlabeled CFSE was quenched by adding 10% FCS medium and washing. Depending on the experiment, sublethal irradiated B6 Ly5.1 and RAG-1−/− Ly5.1 mice or C57BL6 and nude (Ly5.2) mice were injected retro-orbitally with CFSE-labeled cells. Five or 8 days after the transfer, peripheral lymphocytes collected from all LNs and spleen were analyzed by flow cytometry.

Proliferation and cell culture

Peripheral CD4+ T cells were purified by incubation with anti-CD4 beads and passage through a column (Miltenyi Biotech); the purity of cells from heterozygous mice was ~98% and that from homzygous mice was ~92% as determined by flow cytometry. Purified CD4+ T cells were then plated in 96-well plates coated with 10 μg/ml anti-CD3ε Ab (clone 145-2C11) (BD Pharmingen) in the presence or absence of an anti-CD28 Ab (clone 37.51) (BD Pharmingen) for 48 h. For Treg cell coculture, CD25+ CD4+ and CD25+ CD8+ T cells were sorted by a MoFlo FACS (DakoCytomation). T-depleted splenocytes from Foxn1Δ/Δ control mice were irradiated (3000 rad) as accessory cells (ACs). CD25+ CD4+ cells (5×104) were cultured in 96-well round-bottom plates in a total volume 200 μl/well with 5×104 ACs and 1 μg/ml soluble anti-CD3 and 2 μg/ml CD28 in the presence or absence of 2.5×104 CD25+ CD4+ cells at 37°C in 5% CO2 for 72 h. For MLR, 5×104 Foxn1Δ/Δ and T enriched Foxn1Δ/Δ cells from LNs (both ~40–50% of T cells in total peripheral mononuclear cells) were cultured in 96-well round-bottom plates with the same number of irradiated ACs from BL6 (auto-MLR) or BALB/c (allo-MLR) mice at 37°C in 5% CO2 for 72 h. Cultures were pulsed with CellTiter 96 AQueous One Solution Cell Proliferation Reagent (Promega) at 30 h of culture. As a control, cells were cultured in medium alone. We measured and recorded absorbance at 490 nm using a 96-well plate reader.

RT-PCR and real-time PCR

To delete thymocytes, F14.5 and F17.5 fetal thymus were individually treated with 1.35 mM 2dGlu in a high-oxygen fetal thymic organ culture for 5 days. Total RNA from treated thymus was then extracted using a Micro RNA isolation kit (Stratagene). Total RNA was extracted from freshly isolated adult thymus and mesenteric LNs using TRIzol reagent. (Invitrogen Life Technologies). RT-PCR for total RNA analysis was performed using reverse transcriptase (Invitrogen Life Technologies) and primer random P (P(dN)7; Roche Diagnostics). For real-time PCR, primers and probes were designed and synthesized by Applied Biosystems). The IL7 primers were 5′-CATATGAGAGTGTACTGATGATCAGCA-3′ (forward) and 5′-TTGG TTCAATTGCCTGGCATTAAC-3′ (reverse) and the IL7 probe was 6-FAM-TCAGTCTGCTATTTTGCTAAATCATTAGC-TAMRA. Primers and probes were tested for the optimal dose. The 18S RNA VIC-TAMRA primer-probe kit from Applied Biosciences was used as an endogenous control. All data were normalized to constitutive RNA values. An Applied Biosystems 7700 Sequence Detector was used to amplify target mRNA and quantify the difference between samples as calculated according to the manufacturer’s instructions.

Results

Peripheral T cell populations are severely reduced in Foxn1Δ/Δ mutant mice

We have previously reported that Foxn1Δ/Δ mice have a 50–100-fold decrease in the number of CD4+ and CD8+ SP thymocytes (25). To assess the effect of this decreased thymic output on peripheral T cell populations, we measured the numbers and...
composition of peripheral lymphocytes from adult spleen and mesenteric LNs in control and Foxn1Δ/Δ mutant mice. Although the total number of peripheral lymphocytes from Foxn1Δ/Δ mutant mice was not significantly different from that of control mice, the percentages of CD4⁺ and CD8⁺ T cells were reduced ~10-fold, from 18.2 and 8.4% in control mice to 2.0 and 1.1% in Foxn1Δ/Δ mutants mice, respectively (Fig. 1, a and b). In addition to the drop in total T cell number, the mean fluorescence intensity of CD4 and CD8 expression on the surface of Foxn1Δ/Δ T cells was reduced, indicating that the expression of both molecules on the T cell surface was down-regulated. This result was consistent with our previous data showing that surface TCRβ was reduced on adult SP thymocytes (25). These data showed that the decreased thymic output was not overcome in the periphery, resulting in a reduced peripheral T cell pool.

We also examined other peripheral lymphoid populations. The total number of peripheral B cells increased significantly (Fig. 1b), but NK cells were not significantly affected (data not shown). Furthermore, the number of TCRγδ T cells was not significantly different between Foxn1Δ/Δ mutants and control littermate mice (data not shown), indicating that the defect primarily affects αβTCR⁺ T cells.

Peripheral T cells from Foxn1Δ/Δ mutant mice display an activated/memory-like phenotype

Although the peripheral T cell population in Foxn1Δ/Δ mice was reduced, it was not as severe as the reduction in SP thymocytes.
(10-fold vs 100-fold), suggesting that some expansion of the peripheral cell population had occurred although not enough to reach wild-type levels. To investigate the mechanism underlying this apparent expansion of CD4+ and CD8+ T cells in Foxn1−/− mice, we analyzed markers of homeostatic proliferation in adult peripheral T cells, including CD44, CD69, and CD25. Ninety percent of CD4+ and 80% of CD8+ Foxn1−/− T cells were CD44high, whereas 27% of CD4+ and 26% of CD8+ cells were CD44high in control mice (Fig. 2a). The expression of CD69 and CD25 on Foxn1−/− T cells was also increased significantly compared with control littermate mice (Fig. 2a and b). In addition, T cells from Foxn1−/− mutant mice were on average larger than those in control mice as measured by forward scatter (Fig. 2c). In vivo BrdU incorporation also confirmed that T cells in Foxn1−/− mice had a higher proportion of proliferating cells (Fig. 2d). These profiles differ from those of cells derived from homeostasis-driven proliferation, which are typically CD44high but do not up-regulate CD69 and CD25.

To determine whether these cells represent memory cells, we analyzed the expression of CD62L (Fig. 3a). Most of the T cells from Foxn1−/− adult mice showed a CD44highCD62Llow/− effector memory (TEM) phenotype, (83.7% of CD4+ and 61.4% of CD8+ T cells, compared with only 19.8 and 15.3% in control mice). In the periphery, even at the newborn stage the majority of both CD4+ and CD8+ peripheral T cells from Foxn1−/− mice are CD44highCD62Llow/− and the percentage of cells with this phenotype does not significantly change with increasing age, although the cell numbers do increase (Fig. 3a). In contrast, in control mice the percentage drops over time. Thus this phenotype was acquired very rapidly after cells leave the thymus. In the thymus, the percentages of CD44highCD62Llow/− for both genotypes is essentially zero at newborn stage, then tracks behind the appearance of cells in the periphery. These results indicated that the memory-like phenotype is a post-thymus event and that T cells acquire the memory phenotype very soon after they emigrate from the thymus into the periphery. Because most SP thymocytes in Foxn1−/− adult mice were also CD44highCD62Llow/− (Fig. 3b), the actual number of SP thymocytes in the adult thymus is 5–10-fold lower than what we previously reported, as many of the SP cells in the adult thymus are likely recirculated CD44highCD62Llow/− peripheral T cells.

This high proportion of memory-like T cells in Foxn1−/− newborn mice results in an absolute number of effector T memory type cells (CD44highCD62Llow/−) that is similar to the number in control mice (Fig. 3c), followed by a 2-fold reduction in 14-day-old mice
and a 3-fold reduction in adult mice. Thus, the major cell population that was lost in Foxn1Δ/Δ mutants mice was naive T cells, which were decreased 100-fold in the adult periphery.

Foxn1Δ/Δ peripheral T cells have characteristics of functional memory-like T cells

CD44high expression is a general marker for memory T cells in mice, and most peripheral T cells in Foxn1Δ/Δ mutant mice have this phenotype. However, it is not clear whether these cells are functional T memory cells that have expanded because of Ag recognition or if they have arisen because of homeostatic expansion. One of the characteristics of the functional phenotype of memory T cells is the ability to rapidly produce IL-2 or IFN-γ following activation (26). Therefore, we monitored the production of intracellular IL-2 and IFN-γ after 5 and 24 h of stimulation in culture (Fig. 4). The ability to produce IL-2 is shown for the CD4+ subset. About 40% of total T cells (39.9%) and CD44highCD4+ T cells (43.5%) from Foxn1Δ/Δ mice produced IL-2 under costimulation with anti-CD3 and anti-CD28 for 5 hours. In contrast, only 8.7% of total T cells from control mice and 15% of CD44highCD4+ T cells produced IL-2 at 5 h. Similar results were obtained with stimulation by using PMA plus ionomycin. After activation for 24 h, the production of IL-2 was reduced greatly in CD44highCD4+ cells derived from Foxn1Δ/Δ mice; only 4.7% of total T cells and 5.2% of CD44highCD4+ cells produced IL-2, even with activation by PMA. In contrast, 25% of total T cells and 33% of CD44highCD4+ T cells from control mice produced IL-2 under stimulation with PMA for 24 h. The CD8+ subset had similar results (data not shown).

The ability to produce IFN-γ was also measured and is shown for CD8+ cells. Thirty seven point five percent (37.5%) of total T cells and 47.5% of CD44highCD8+ T cells from Foxn1Δ/Δ mice produced IFN-γ under stimulation with anti-CD3 and anti-CD28 for 5 h, and the percentages increased to 63.1 and 69.5%, respectively, after activation for 24 h. In control cells, 5.4% of total T cells and 17.5% of CD44highCD8+ T cells produced IFN-γ at 5 h, increasing to 38.5% of total T cells and 49.1% of CD44highCD8+ T cells at 24 h. (Fig. 4, a and b) The results were similar for CD4+ cells. These results indicate that both CD4+ and CD8+ T cells from Foxn1Δ/Δ mice have the ability to produce cytokines even more rapidly than cells from control mice, which is consistent with a memory phenotype.

We then analyzed cytokine production by cells from young mice. Although there was a high proportion of CD44high cells in the periphery of both normal and Foxn1Δ/Δ mice, the early CD44high memory-like cells from young control mice were unable to rapidly produce cytokines in response to stimulation. The cells

**FIGURE 4.** Peripheral T cells from Foxn1Δ/Δ mice displayed characteristics of functional memory T cells. Whole lymphocytes were cultured for 5 (left panels) or 24 h (right panels) under the conditions shown to the left of the figure. The cells were stained for intracellular IL-2 or IFN-γ. a, Expression of CD44 and IL-2 on gated CD4+ cells. b, Expression of CD44 and IFN-γ on gated CD8+ cells.
from Foxn1^−/− mice acquired the ability to produce IFN-γ 7 days after birth, and had a high proportion of cells producing IFN-γ 14 days after birth (Fig. 4b and data not shown). Low levels of IL-2 were first produced 14 days after birth (Fig. 4a). In contrast, the cells from control mice could not produce IL-2 or IFN-γ even 14 days after birth. These results paralleled the more rapid appearance of CD44^high memory-like cells in Foxn1^−/− neonates (Fig. 3c) and further indicated that the Foxn1^−/− T cells are functionally distinct from T cells that have undergone normal neonatal expansion.

**FIGURE 5.** T cells from Foxn1^−/− mice have reduced capability for homeostasis-driven proliferation. a and b, Sorted T cells (2–4 × 10^6 per mouse) from Foxn1^−/− and control mice (Ly5.2) were CFSE labeled and injected into Bl6-Ly5.1 Rag1^−/− (a) or sublethally irradiated Bl6 wild-type (b) mice. CFSE levels on gated donor cells were analyzed 5 days later. c, Percentage of recovery of Foxn1^+/− (open bars) and Foxn1^−/− (filled bars) donor cells (Ly5.2^+^) from Rag^−/− Ly5.1 hosts. Values shown are the average of three individual experiments. d, Transferred Ly5.2^+^ cells were gated from irradiated Bl6 mice. e, Anti-CD4 bead-purified cells from Bl6-Ly5.1 mice (2 × 10^6 per mouse) were labeled and injected into Foxn1^+/−, Foxn1^−/−, or nude mice (Ly5.2). CFSE levels and expressions of CD25, CD69, and CD44 on gated donor cells were analyzed 8 days later.
cell numbers. This result suggested that the T cells produced in the Foxn1Δ/Δ thymus were either unable to undergo homeostatic expansion efficiently or underwent increased cell death. To assess the capacity for homeostatic proliferation of Foxn1Δ/Δ T cells, we transferred CFSE-labeled T cells from control or Foxn1Δ/Δ mice into sublethally irradiated Rag−/− and Bl6Ly5.1mice. Five days after transfer, 38.8% of CD4+ and 30% of CD8+ cells transferred from control mice had undergone more than seven generations in Rag−/− mice and showed multiple rounds of slow homeostatic expansion on the right side of graph (Fig. 5a). As previously reported, the rapid expansion subset was significantly reduced after transfer into an irradiated wild-type host, whereas slow homeotic expansion was retained in control mice (Fig. 5b). In contrast, transferred cells from Foxn1Δ/Δ mice displayed a low percentage of rapidly proliferating cells in both Rag−/− and wild-type hosts (Fig. 5, a and b). Whereas CD8+ cells were able to undergo some level of slow homeostatic expansion in both hosts (although still reduced relative to controls), homeostasis-driven proliferation was nearly undetectable in CD4+ cells. Consistent with this result, a 6-fold lower percentage of Foxn1Δ/Δ T cells were recovered after transfer into Rag−/− hosts than that of T cells taken from control mice (Fig. 5c). These results indicated that, despite their memory phenotype, most T cells derived from Foxn1Δ/Δ adult mice had a severely reduced ability to undergo homeostatic proliferation in response to lymphopenia.

Because the percentage of CD4+ CD25+ cells was increased in Foxn1Δ/Δ mice (Fig. 2b), it is possible that these cells represented Treg cells that suppressed homeostatic proliferation in Foxn1Δ/Δ T cells, both in situ and after cotransfer into lymphopenic hosts. To test this possibility, we transferred purified CD4+ wild-type Ly5.1 cells into Foxn1Δ/Δ mice. Because peripheral T cells are reduced ~90% in nonirradiated Foxn1Δ/Δ mutant mice, transferred T cells should proliferate if they were not suppressed by endogenous CD25+CD4+ cells. Transferred cells proliferated in Foxn1Δ/Δ mice as well as they did in nude mice (Fig. 5e), indicating that the peripheral T cells present in the Foxn1Δ/Δ mice neither suppressed nor competed efficiently with the introduced cells. Furthermore, transferred wild-type cells in the Foxn1Δ/Δ mice up-regulated the expression of CD44, but not that of CD25 or CD69 (Fig. 5e), indicating that the peripheral environment in the Foxn1Δ/Δ mice can support normal homeostatic proliferation. This result confirmed that the relative increase in CD4+CD25+ cells was not responsible for the decreased homeostatic potential of T cells from Foxn1Δ/Δ mice.

Peripheral T cells derived from Foxn1Δ/Δ mutants display a reduced response to stimulation through TCR

Signaling through the TCR is critical for normal homeostatic expansion, as well as for T cell development in the thymus and activation, proliferation, and survival in the periphery. CD4 and CD8 molecules provide critical costimulation in these processes. Therefore, reduced cell surface expression of TCR, CD4, and CD8 molecules might directly affect TCR signal and T cell function. The reduced CD4 and CD8 expression on peripheral T cells in Foxn1Δ/Δ mice suggested that the previously reported reduction in surface TCRβ on SP thymocytes persisted in the periphery. Consistent with this expectation, the expression of αβTCR was reduced to 86 and 80% on peripheral CD4+ and CD8+ cells, respectively, in Foxn1Δ/Δ mice and mean fluorescence intensity on the surface of Foxn1Δ/Δ T cells was reduced as well (Fig. 6a). Consistent with the reduced expression of TCR, CD4, and CD8, the CD4+ T cells derived from Foxn1Δ/Δ mice are less able to respond to stimulation than those in control mice, especially under stimulation from both anti-CD3 and anti-CD28 (Fig. 6b). Because there was no significant difference between Foxn1Δ/Δ and control mice in the expression of CD28 (data not shown), the lack of an effect upon the addition of anti-CD28 indicated that the proliferation of T cells from Foxn1Δ/Δ mice is costimulatory molecule independent.

Because the data shown in Fig. 2 suggested that the majority of SP thymocytes cells in the adult Foxn1Δ/Δ thymus were actually recirculated peripheral T cells, it was possible that our previous analysis had failed to detect high level TCRβ expression on a
much smaller number of true SP thymocytes (25). However, after excluding the CD44high recirculated peripheral cells from our analysis, the remaining SP thymocytes were still TCRlow (data not shown). These data, combined with our previously published analysis of thymocytes, suggested that T cells in Foxn1ΔΔ mice never have high levels of αβTCR.

Reduced Treg function in Foxn1ΔΔ mice

Treg cells are also very important for regulating the activation and proliferation of T cells in the periphery. Because the percentage of CD4+CD25+ cells was increased in Foxn1ΔΔ mice, we analyzed the expression of Foxp3 and the function of CD4+CD25+ Treg cells. We found that the percentage of Foxp3-expressing cells in Foxn1ΔΔ CD4+CD25+ T cells was ~20% lower than that in Treg cells from control mice (70 vs >90%; Fig. 6c). Combined with the increased percentage of CD4+CD25+ cells, this resulted in a normal frequency of Foxp3+ putative Treg cells in Foxn1ΔΔ mice (~10% of CD4+ cells in both controls and mutants). Surprisingly, CD4+CD25+ T cells from Foxn1ΔΔ mice were unable to suppress the proliferation of control T cells in an in vitro activation assay (Fig. 6d). This failure to display Treg function was consistent with the normal homeostatic proliferation of wild-type T cells transferred into a Foxn1ΔΔ host.

We further tested T cell function in an MLR. T cells from Foxn1ΔΔ mice also showed both autoreactivity and autoreactivity in MLR (Fig. 6e). These autoreactive T cells could be due to failure of thymic selection in these mice, resulting in reduced MHC restriction. These results further reflect reduced Treg activity that fails to suppress autoreactive T cells present in the periphery in these mice.

T cells from Foxn1ΔΔ mice have higher rates of apoptosis

To investigate whether T cells from Foxn1ΔΔ adult mice also have a higher propensity for apoptosis, we measured the cell surface expression of annexin V on freshly isolated peripheral lymphocytes. Whereas only 6.8% of CD4+ and 3.2% of CD8+ cells in control mice were annexin V−, 21.5% of CD4+ and 13.2% of CD8+ fresh isolated cells from Foxn1ΔΔ mice were annexin V− (Fig. 7a). Foxn1ΔΔ T cells were also more likely to undergo apoptosis in response to the activation of TCR in vitro than T cells from control mice (Fig. 7b). It is worth noting that the T cells from Foxn1ΔΔ mice also had more annexin V+ cells under medium-only culture conditions in vitro, which indicated that they have a short lifespan both in vivo and in vitro.

IL-7 and IL-7Ra are reduced on Foxn1ΔΔ thymic epithelial cells and naive T cells

Both memory and homeostatic T cells require IL-7 for proliferation and survival. To test whether there was a defect in IL-7/IL-7R signaling in Foxn1ΔΔ mice, we assessed the expression of IL-7Ra and IL-7 by FACS analysis on T cells and the mRNA level in the thymus. Overall, the expression of IL-7Ra on adult peripheral CD4+ and CD8+ cells was reduced in Foxn1ΔΔ mice relative to control mice (Fig. 8a). In cells from control mice, IL-7Ra levels

FIGURE 7. Peripheral T cells from Foxn1ΔΔ mutant mice are highly sensitive to the induction of apoptosis. a, Percentage of annexin V+ cells among freshly isolated, gated CD4+ (open bars) and CD8+ (filled bars) lymphocytes. Wt, Wild type. b, Percentage of annexin V+ cells among gated CD4+ cells after activation. Whole lymphocytes were cultured under stimulation as indicated above the histograms. Cells were recovered and stained after activation for 5 or 24 h as indicated above the histograms.

FIGURE 8. IL-7/IL-7R signal pathway was reduced in Foxn1ΔΔ mutant mice. a, Upper panels show the expression of IL-7Ra on gated total CD4+ or CD8+ cells from control and Foxn1ΔΔ mutant mice. Lower panels show IL-7Ra expression on CD44high and CD44low subsets from control and Foxn1ΔΔ mice. b, IL-7 mRNA levels as determined by real-time PCR. Values shown are for total RNA from dGuo-treated E14.5 fetal thymi, freshly isolated adult thymi, and whole adult LNs. Open bars, Foxn1ΔΔ; filled bars, Foxn1ΔΔ.
were high regardless of the CD44 expression level (Fig. 8b). When examined relative to CD44 levels, it was apparent that in the Foxn1<sup>Δα</sup> mice CD44<sup>high</sup> cells had normal, high levels of IL7Rα, with a subpopulation even exceeding normal levels (Fig. 8b). In contrast, CD44<sup>low</sup> cells had reduced levels of IL-7Rα (Fig. 8b). This result was particularly striking for CD4<sup>+</sup> T cells. CD4<sup>+</sup> SP thymocytes expressing high levels of heat-stable Ag (HSA<sup>high</sup>) showed a similarly lower expression of IL7Rα than control cells (data not shown). In CD8<sup>+</sup> cells, CD44<sup>low</sup> cells were divided evenly into two distinct subpopulations, one similar to controls and the other one low or negative for IL7R expression. These results suggest that most T cells from Foxn1<sup>Δα</sup> mice were initially IL-7Rα<sup>low</sup> and then up-regulated IL-7Rα expression with CD44 up-regulation in the periphery. Thus, although the average IL-7Rα expression was lower than that in controls, there was a significant population of peripheral T cells with normal levels of IL-7Rα.

Because the primary defect in these mice is in TEC differentiation, it is possible that the low IL-7R expression on CD44<sup>low</sup> T cells was related to low IL-7 in the thymic microenvironment. We assayed IL-7 expression by RT-PCR in purified TECs from control and Foxn1<sup>Δα</sup> mice at fetal and postnatal stages. Compared with control mice, the expression of IL-7 mRNA in Foxn1<sup>Δα</sup> fetal TECs was moderately reduced by ~3-fold at E14.5 (Fig. 8c) and 1.3-fold at E17.5 (data not shown). In the adult thymus, IL-7 mRNA levels were further reduced (Fig. 8c). This reduction is likely to be an underestimate, as the T cell number is reduced dramatically in the Foxn1<sup>Δα</sup> thymus, resulting in a relatively greater proportion of TECs in the mutant thymus. This lower level of IL-7 in the thymic microenvironment may contribute both to defects in intrathymic proliferation and differentiation of thymocytes, as well as to reduced IL-7R levels on naïve T cells. IL7 mRNA levels were also reduced in adult Foxn1<sup>Δα</sup> peripheral LNs to ~50% of controls. Despite this reduction in peripheral IL7 mRNA, the mRNA levels of Jak3 and Bcl2 were not significantly different in peripheral T cells from mutant and control mice (data not shown), indicating that IL7 signaling in the periphery may not be significantly impaired.

**Discussion**

The primary defect in Foxn1<sup>Δα</sup> mice is a cell-autonomous effect on TEC differentiation, resulting in a highly abnormal microenvironment with no identifiable cortical or medullary regions. Although this mutation does not directly affect T cells, the abnormal thymic microenvironment in Foxn1<sup>Δα</sup> mice not only results in a reduction of the peripheral T cell pool but also causes changes of the peripheral T cell profiles and impacts the normal function of T cell. Other data from our laboratory has shown that T cells made in the Foxn1<sup>Δα</sup> thymus develop from an atypical CD11<sup>low</sup>-TCR<sup>-</sup> progenitor population via an unusual differentiation pathway that does not appear to involve transit through the double-negative (DN2) or DN3 stages (25, 27)(S. Xiao, D.-M. Su, and N. R. Manley, unpublished data). Thus, peripheral T cells in Foxn1<sup>Δα</sup> mice appear to arise from atypical progenitors in an abnormal environment, and peripheral T cell defects could be due to interactions with abnormal TECs and/or to differing intrinsic capabilities of the atypical progenitors. The characteristics of both CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cell phenotypes are likely due to a combination of reduced thymic output, abnormal thymic selection, and the intrinsic properties of these cells.

One of the most striking aspects of the phenotype is the constitutively low αβ TCR levels, which are present even on SP thymocytes. This reduced expression is not likely to be a result of low MHC expression in the thymus for two reasons. First, although MHC class II levels are significantly reduced in the Foxn1<sup>Δα</sup> thymus, class I levels are relatively normal (our unpublished data) and both CD4<sup>+</sup> and CD8<sup>+</sup> cells have low TCR levels. Secondly, even very low MHC levels should not result in this phenotype, because in H<sup>2</sup>-<sup>−</sup> mice the few CD4<sup>+</sup> T cells that are made are TCR<sup>high</sup> (28). The T cells are also not anergic because even thymocytes and newly made SP T cells are TCR<sup>low</sup>, can readily make cytokines after stimulation, and have high proliferation rates. We interpret this phenotype as evidence for an intrinsic defect in the T cells themselves due to development from atypical progenitors; i.e., these cells are unable to up-regulate TCR in response to positive selection or other inducements.

Another striking aspect of the phenotype is the apparent failure of Foxp3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells to have the suppressive activity normally associated with Treg cells. This is particularly surprising given that Foxp3 expression in CD4<sup>+</sup> CD25<sup>+</sup> T cells is sufficient to confer Treg function (29–32). Although the mechanism underlying this failure in regulatory activity is unclear, the most likely conclusion is that this phenotype also represents an intrinsic inability of these T cells to develop normally. Alternatively, there could be a previously unappreciated required contribution from the microenvironment during thymocyte development that makes CD4<sup>+</sup> T cells competent to become Treg cells in response to the cell-autonomous expression of Foxp3 that is absent in these mice.

The reduced ability of both CD4<sup>+</sup> and CD8<sup>+</sup> cells from Foxn1<sup>Δα</sup> mice to undergo slow homeostatic proliferation, even after transfer to a lymphopenic host and in the absence of Treg function, suggests that these cells are intrinsically unable to undergo this type of proliferation, which may in turn be due to their low TCR levels. Such homeostatic dysregulations have also been described in mice with TCR/MHC or IL-7R/Jak3 signaling abnormalities, both of which are required for T cell proliferation and survival in the periphery (8, 33–36). In the thymus, TCR/MHC signals determine selection; thymocytes with high affinity TCR/MHC interaction are deleted by negative selection, and thymocytes with relatively low affinity TCR/MHC interaction are positively selected and maintain the peripheral T cell pool (37). In Foxn1<sup>Δα</sup> mutant mice the expression of both MHC II and TCR are comparatively low on TECs and T cells, respectively (Ref. 25, this report, and our unpublished data). Thus, thymocytes with high affinity TCR/MHC interaction might be positively selected and might not be efficiently deleted by negative selection in the Foxn1<sup>Δα</sup> thymus because of the absence of mature medulla (25) (negative selection for CD4 T cells is reduced in Foxn1<sup>Δα</sup> mutant mice; our unpublished data). Consequently, these CD4<sup>+</sup> T cells with high affinity for TCR/MHC interaction would be highly sensitive to the same peptides presented by normal MHC-II molecules in the periphery. Thus, Foxn1<sup>Δα</sup> CD4<sup>+</sup> T cells are easily triggered and activated in the periphery, resulting in an activated/memory phenotype. This failure of selection could also lead to an increased incidence of autoimmune T cells as evidenced by the autoreactivity in the MLR assay. In contrast, these activated effector cells do not develop into true memory T cells and only survive a short time. This could explain both why peripheral T cell levels never reach wild-type levels and why few Foxn1<sup>Δα</sup> CD4<sup>+</sup> T cells are collected after transfer into a lymphopenic host. Consistent with these results, we found that both freshly isolated and activated Foxn1<sup>Δα</sup> T cells easily undergo apoptosis in vitro.

In contrast, CD8<sup>+</sup> peripheral T cells from Foxn1<sup>Δα</sup> mice are able to undergo at least some slow homeostatic proliferation when introduced into a lymphopenic host. A contributing factor may be that the thymic selection of CD8<sup>+</sup> T cells, which is dependent on MHC-I, might be more normal than Foxn1<sup>Δα</sup> CD4<sup>+</sup> T cells because the expression of MHC-I is not reduced in Foxn1<sup>Δα</sup> mice.
(our unpublished data). The low capability of homeostatic proliferation for CD8<sup>+</sup> cells is likely due to the low expression of TCR, which, in turn, diminishes signaling from the TCR-peptide/MHC-I complexes necessary for proliferation and survival. Thus, in the case of CD4<sup>+</sup> cells, the primary effect is on selection and is therefore intrathymic, whereas for CD8<sup>+</sup> cells the primary effect may be on their behavior in the periphery. The transfer experiment results are consistent with these conclusions. Further study of thymic selection in Foxn1<sup>Δ/Δ</sup> mice is ongoing in our laboratory.

The low levels of IL7R expression on both SP thymocytes and naive peripheral T cells may reflect their development in an IL7-poor thymic environment at both the fetal and adult stages. This situation could contribute to the reduced proliferation of thymocytes in these mutants. Unlike the constitutively low TCR expression, once these T cells encounter elevated IL7 in the periphery a substantial fraction of them readily up-regulate IL7R, suggesting that the level of receptors on naive cells may be an environmental rather than an intrinsic defect in these cells. However, the fact that a significant percentage of naive T cells does not up-regulate IL7R in the periphery may be due in part to these lower IL7 levels in the lymph nodes. Because Foxn1 is not expressed in the LN we cannot explain this phenotype directly, although it raises the intriguing possibility that peripheral IL7 levels may be influenced by T cells themselves. Interestingly, γ<sup>δ</sup> TCR cells are present in relatively normal numbers, even though their development depends on IL7 (38–41). We have previously reported a similar phenotype for Hoxa3<sup>−/−</sup>/?ax<sup>−/−</sup> mice (38–43). The current results further suggest either that low levels of IL7 are sufficient for γ<sup>δ</sup> TCR cell development or that the high levels of IL7 encountered in a small microenvironment in these mutants are sufficient for their development.

Other aspects of the peripheral T cells in these mice are likely to be secondary to the reduced thymic output in the Foxn1<sup>Δ/Δ</sup> mutant mice since the neonatal stage. Neonates regardless of genotype have a functionally lymphopenic environment that allows homeostatic expansion, wherein naive cells produced from the neonatal thymus proliferate and acquire the phenotype and function of activated or memory T cells (17, 23, 44). In wild-type mice this proliferation is balanced by a continuous thymic output of new naive cells that constitute at least a third of peripheral T cells even in newborns and, by 2 wk postnatally, represent the vast majority of peripheral T cells. In contrast, nearly all peripheral T cells (but not thymocytes) in Foxn1<sup>Δ/Δ</sup> newborns have the CD4<sup>+</sup>CD8<sup>+</sup> phenotype and maintain this phenotype to adulthood. This is true for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, although the effect is more pronounced for CD4<sup>+</sup> cells. This result suggests that the few naive cells produced by the Foxn1<sup>Δ/Δ</sup> thymus have an enhanced ability to immediately acquire a memory phenotype after thymic exit. In addition, the further production of naive cells is significantly reduced in Foxn1<sup>Δ/Δ</sup> mutant mice. Thus, the peripheral environment remains functionally lymphopenic in Foxn1<sup>Δ/Δ</sup> mutant mice. Ordinarily, this should trigger homeostatic expansion to restore the total T cell number, resulting in the acquisition of memory T cell markers and functional properties (9, 12, 16, 18, 36, 45). However, naive T cells in the adult Foxn1<sup>Δ/Δ</sup> mutant mice (both CD4<sup>+</sup> and CD8<sup>+</sup> subsets) may have not undergone correct positive and negative selection due to low TCR levels and an abnormal thymic environment. Thus, when they encounter the peripheral environment they respond by becoming similar to activated effector memory T cells, arising following exposure to foreign or endogenous Ags (24, 46) rather than by homeostasis-driven proliferation.

The question remains, how does the defect in TEC differentiation and the resulting abnormal thymic microenvironment result in these peripheral T cell phenotypes? Data from our laboratory indicate that the failure of TEC differentiation results in a complete block to the commonly known thymocyte development pathway, from c-kit<sup>+</sup> progenitors via the DN3 stage to double-positive and SP cells, at least in the postnatal thymus (S. Xiao, D.-M. Su, and N. R. Manley, unpublished data). These studies suggest that any T cells produced from the Foxn1<sup>Δ/Δ</sup> adult thymus likely arise from progenitors that are normally present in the wild-type thymus but do not normally make T cells, or at least not efficiently. It is unclear whether the production of T cells from this atypical development pathway is dependent on the Foxn1<sup>Δ/Δ</sup> microenvironment or can occur with low efficiency even in wild-type mice. These cells, when transferred to a wild-type thymus, are not recovered, presumably because of the huge competitive advantage of the canonical pathway (27). However, it is possible that a small number of T cells come from this pathway even in wild-type mice. Foxn1<sup>Δ/Δ</sup> mice may therefore be an experimental model in which the T cell-generating potential of these atypical progenitors is revealed. In this case, peripheral T cells generated in the adult Foxn1<sup>Δ/Δ</sup> mice may represent a cohort of T cells that are normally produced in small numbers but have distinct properties from those normal T cells and may therefore represent a novel class of peripheral T cells.

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