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Egress of Mature Murine Regulatory T Cells from the Thymus Requires RelA

Taro Fukazawa,*,† Noriko Hiraiwa,‡ Takeshi Umemura,§ Setsuko Mise-Omata,* Yuichi Obata,* and Takahiro Doi*

The mechanism of egress of mature regulatory T cells (Tregs) from the thymus to the periphery remains enigmatic, as does the nature of those factors expressed in the thymic environment. In this study, we examined the fate of thymic Tregs in TNF-α/RelA double-knockout (TA-KO) mice, because TA-KO mice retain a Treg population in the thymus but have only a small Treg population at the periphery. Transplantation of whole TA-KO thymus to under the kidney capsule of Rag1-null mice failed to induce the production of donor-derived splenic Tregs expressing neuropilin-1, which is reported to be a marker of naturally occurring Tregs, indicating that TA-KO thymic Tregs either do not leave the thymus or are lost at the periphery. We next transplanted enriched TA-KO thymic Tregs to the peripheries of TA-KO mice and traced mouse survival. Transplantation of TA-KO thymic Tregs rescued the lethality in TA-KO mice, demonstrating that TA-KO thymic Tregs remained functional at the periphery. The TA-KO thymic Treg population had highly demethylated CpG motifs in the foxp3 locus, indicating that the cells were arrested at a late mature stage. Also, the population included a large subpopulation of Tregs expressing IL-7Rα, which is a possible marker of late-stage mature Tregs. Finally, TA-KO fetal liver chimeric mice developed a neuropilin-1+ splenic Treg population from TA-KO cells, suggesting that Treg arrest was caused by a lack of RelA in the thymic environment. Taken together, these results suggest that egress of mature Tregs from the thymus depends on RelA in the thymic environment. The Journal of Immunology, 2015, 194: 3020–3028.

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egulatory T cells (Tregs) play key roles in maintaining immune homeostasis and self-tolerance via the dominant control of self-reactive T cells (1–4). Many Treg subsets have been reported (4); one of the best described and characterized is the CD4+CD25+Foxp3+ Treg subset, because the Foxp3 protein is the most distinct marker of Tregs. Scurfy mice, which have a mutation in the foxp3 gene, develop autoimmune diseases characterized by lymphohistocytic proliferation (5). In humans, mutation in the FOXP3 gene results in immunodeficiency polyendocrinopathy enteropathy X-linked syndrome, a fatal autoimmune disorder (6). Furthermore, in mice, overexpression of foxp3 results in naïve T cells converting toward a Treg phenotype (7). Hence, foxp3 is a key regulator of Tregs.

Tregs are generated both inside and outside the thymus, but Treg development occurs predominantly within the thymus (1, 8, 9). Thymectomy in neonatal mice up to day 3 after birth, but not after, results in autoimmunity (10), showing that after day 3 Tregs migrate from the thymus to the periphery and begin to suppress autoimmunity. Maturing thymic Tregs express molecules that are associated with their egress from the thymus and trafficking to secondary lymph tissue. CCR7 controls Treg homing to T cell zones in lymph nodes (11), and L-selectin (CD62L) is associated with Treg localization in the lymph nodes (12). Additionally, sphingosine-1-phosphate receptor-1 (SIPRI) regulates T cell egress from the thymus and several other peripheral lymphoid tissues by inducing chemotaxis toward the natural ligand SIP (13); it is assumed that this factor also affects Treg egress (14). Expression of CD62L and SIPRI is regulated by Kruppel-like factor 2 (15). Despite these recent studies uncovering the trafficking factors expressed by T cells or Tregs, the extent to which signaling in the thymic environment controls the egress of mature Tregs from the thymus remains unclear.

To address this issue, we used TNF-α/RelA double-knockout (TA-KO) mice (16) that were generated for the functional analysis of RelA, which is a member of NF-κB family. NF-κB is a transcriptional factor that plays pivotal roles in both the development and the responses of the immune system (17–19). RelA-KO mice show increased apoptosis of cells in the fetal liver and death by embryonic day 14, but this lethality can be rescued by knocking out TNF-α to produce TA-KO mice.

We previously reported that TA-KO mice show autoimmune disorders such as inflammatory cell infiltration in several organs (16) and die within 3 wk after birth. In this study, we show that TA-KO mice also have abnormal Treg localization, with a definite population of Tregs being located in the thymus, but few at the periphery. To determine the fate of TA-KO thymic Tregs, we transplanted whole TA-KO thymus under the kidney capsules of Rag1-null mice and found that the Rag1-null mouse splenocytes

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Abbreviations used in this article: dGuo, deoxyguanosine; KO, knockout; Nrp1, neuropilin-1; nTreg, naturally occurring regulatory T cell; SP, single-positive; S1P, sphingosine-1-phosphate; SIPRI, sphingosine-1-phosphate receptor-1; TA-KO, TNF-α/RelA double-knockout; TEC, thymic epithelial cell; Treg, regulatory T cell; TSDR, regulatory T cell-specific demethylation region; WT, wild-type.

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contained a smaller percentage of Tregs derived from donor thymocytes than did those of mice that received TNF-α-KO thymus. Additionally, most of these Tregs did not express neuropilin-1 (Nrp1), which is reported to be a marker of thymus-derived Tregs (nTregs) (20, 21). This implies that TA-KO Tregs either never leave the thymus or are lost at the periphery. To examine this, we transplanted enriched TA-KO thymic Tregs to the periphery in other TA-KO mice and traced mouse survival. TA-KO thymic Treg transplantation rescued the lethality in TA-KO mice, demonstrating that TA-KO thymic Tregs are fully functional at the periphery and indicating that TA-KO thymic Tregs are not lost at the periphery but in fact do not leave the thymus. We next analyzed at which stage of maturation the Tregs were arrested in TA-KO mice by staining for the maturation markers CD24, CD62L, and CD69. The TA-KO thymic Treg population showed slightly more mature phenotypes than did Treg populations from wild-type (WT) or TNF-α-KO mice. To further assess Treg maturation, we analyzed methylation of the CpG motif in the genomic foxp3 locus of thymic Tregs. We found that the TA-KO thymic Treg population was composed of late-stage mature Tregs, in contrast with the WT or TNF-α-KO thymic Treg populations, which also included immature Tregs. We also found that many of the TA-KO thymic Tregs expressed IL-7Rα, a possible marker of late-stage mature Tregs. Finally, we generated chimeric mice with TA-KO fetal liver cells and found that they had a splenic Treg population derived from TA-KO cells, and that the Nrp1 expression pattern of these TA-KO–derived Tregs was similar to that of control (TNF-α-KO–derived) Tregs; thus, thymic TA-KO Tregs are able to emigrate from the thymus when the thymic environment is WT and contains functional RelA. These results suggest that the thymic environment regulates Treg egress in a RelA-dependent manner.

Materials and Methods

Mice

We used CD45.2+ C57BL/6 (Ly5.2+) mice as WT controls; the TNF-α-KO and TA-KO mice have been described previously (16). Animals were bred and housed in a specific pathogen-free animal facility at the RIKEN BioResource Center and the Institute for Laboratory Animal Research at Kochi University. They were used in accordance with the protocols of the Animal Experiments Committee of RIKEN Tsukuba Institute and the Animal Experiments Committee of Kochi University.

For thymus transplantation, intact thymi were harvested from 1.5- to 2.5-wk-old TA-KO or TNF-α-KO mice and transplanted under the kidney capsules of Rag1-null mice (B6.129ST-Rag1tmMom; from the RIKEN Center for Integrative Medical Sciences) or BALB/c nude mice (purchased from RIKEN BioResource Center). One to 2 mo after the transplantation, recipient mice were dissected for flow cytometric analysis.

For deoxyguanosine (dGuo)-treated fetal thymus transplantation, fetal thymus from embryonic day 14.5 to embryonic day 15.5 embryos were treated for 5–7 d in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 50 μM 2-ME, 1x MEM nonessential amino acids solution (Life Technologies, Carlsbad, CA), 10 mM HEPES, 1 mM sodium pyruvate, and 1.2 mM dGuo (Sigma-Aldrich, St. Louis, MO) at 37°C under an atmosphere of 5% CO2. They were then transplanted under the kidney capsule of BALB/c nude mice (CLEA Japan, Tokyo, Japan). Two months after the transplantation, recipient mice were dissected for flow cytometric analysis.

To generate fetal liver chimeric mice, fetal liver cells were isolated from TA-KO mice or their littermate embryonic day 13.5 embryos (typically 2 or 3 × 10⁶ cells/recipient) and then i.v. transplanted into lethally irradiated (8 Gy, x-ray) CD45.1+ C57BL/6 adult mice (CD45.1+ C57BL/6 mice were purchased from RIKEN BioResource Center). Two to 3 mo after the transplantation, the mice were sacrificed and analyzed.

Treg transplantation into neonatal TA-KO mice

Thymic and splenic CD4+CD25+ Tregs were enriched from the thymocytes and splenocytes of TNF-α-KO mice by using a CD4+ CD25+ Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched Tregs from one thymus or spleen were then i.v. transplanted into a 2-d-old TA-KO mouse, and mouse survival was traced.

Flow cytometry

Anti-Nrp1 Ab and anti-SIPRI Ab were purchased from R&D Systems (Minneapolis, MN). All other Abs used were purchased from eBioscience (San Diego, CA) and BioLegend (San Diego, CA). Samples were analyzed with a BD LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). Doublet or multiplet events were gated out by using forward scatter height and width parameters.

For splenocyte or peripheral blood cell staining, erythrocytes were depleted by treating the cells with a buffer containing 155 mM ammonium chloride, 0.5 mM potassium bicarbonate, and 0.1 mM EDTA disodium salts.

For intracellular staining, cells were fixed with Fxsp3 fixation/permeabilization concentrate and diluted (eBioscience) and treated with the relevant Abs as per the manufacturer’s protocols.

Analysis of CpG motif methylation at the foxp3 locus

We isolated CD4+CD8a− CD25th thymocytes from 1.5- to 2.5-wk-old WT, TNF-α-KO, or TA-KO male mice by using a FACSaria cell sorter (BD Biosciences). Doublet or multiplet events were gated out by using forward scatter height, forward scatter width, and side scatter width and height parameters. The purity of the sorted populations was >95%. Genomic DNA was isolated from the sorted cells by using a DNeasy blood and tissue kit (Qiagen, Germantown, MD) in accordance with the manufacturer’s recommendations. Sodium bisulfite treatment of genomic DNA was performed with a MethylEasy Xceed rapid DNA bisulfite modification kit (eDNAgent, Genetic Signatures, Randwick, NSW, Australia) in accordance with the manufacturer’s recommendations. PCRs for bisulfite-converted genomic DNA were performed by using TaKaRa Taq Hot Start version (http://quma.cdb.riken.jp/index.html).

Quantitative real-time PCR

Cells were isolated by using a FACSaria cell sorter, and total RNA extraction and reverse transcription were performed with TRIZol reagent (Life Technologies, Carlsbad, CA) and a SuperScript VILO cDNA synthesis kit (Life Technologies), respectively. Quantitative real-time PCR was performed with SYBR Premix Ex Taq II (TaKaRa Bio, Otsu, Shiga, Japan) and the primer pairs (sense/antisense): 5'−GGGAACTTTGCGAGTG−AAACTAACATTCCAAAACCA−3' and 5'−ACATCGCTCAGATCATCCAC−3' (described as (22)). PCR products were subcloned (pGEM-T Easy vector, Promega, Madison, WI) and sequenced, and the sequence data were analyzed by using the Web-based quantification tool for methylation analysis (http://quma.cdb.riken.jp/index.html).

Transwell migration assay

We isolated CD4+CD8a− CD25th thymocytes from 1.5- to 2-wk-old TNF-α−/− RelA−/− or TA-KO mice by using a FACSaria cell sorter. Sorted cells were suspended in RPMI 1640 supplemented with 0.5% fatty acid–free BSA (Wako, Osaka, Japan). One hundred microrter cells suspension (0.6–1.2 × 10⁶ sorted cells) was placed in a Transwell chamber polycarbonate membrane insert (diameter, 6.5 mm; pore size, 5 μm; Costar, Cambridge, MA), which itself was then placed in a Transwell chamber containing 0.6 ml RPMI 1640 supplemented with 0.5% fatty acid–free BSA and 10 mM 5′I (Sigma-Aldrich) or 0.1% methanol as a vehicle control. Cells cultured for 2 h were recovered from the lower chambers and cell numbers were counted.

Microarray analysis

For the isolation of thymic Tregs, CD4+CD8a− CD25th thymocytes were isolated from five 1.5- to 2-wk-old TNF-α−/− RelA−/− or TA-KO male mice by using a FACSaria cell sorter. Sorted cells were suspended in RPMI 1640 supplemented with 0.5% fatty acid–free BSA (Wako, Osaka, Japan). One hundred microrter cells suspension (0.6–1.2 × 10⁶ sorted cells) was placed in a Transwell chamber polycarbonate membrane insert (diameter, 6.5 mm; pore size, 5 μm; Costar, Cambridge, MA), which itself was then placed in a Transwell chamber containing 0.6 ml RPMI 1640 supplemented with 0.5% fatty acid–free BSA and 10 mM 5′I (Sigma-Aldrich) or 0.1% methanol as a vehicle control. Cells cultured for 2 h were recovered from the lower chambers and cell numbers were counted.
populations (enriched thymic stromal cells containing macrophages or dendritic cells) were sorted.

Total RNA was extracted from pooled cells by using TRZol reagent. The quality of the RNA was checked with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and an RNA 6000 Pico kit (Agilent Technologies). Gene expression analysis was performed with a mouse GE 4 x 44K v2 microarray (Agilent Technologies) and a Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies). The microarray data are available on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE64191.

Statistical analysis

For analysis of population frequency, we used the Student t test with Bonferroni correction. For analysis of cell numbers, Student t tests were performed with logarithmically transformed values and Bonferroni correction. For analysis of data obtained from the Transwell migration assay, the paired Student t test was used.

Results

TA-KO mice show abnormal Treg localization

Knockout of RelA in mice, which is a gene in the NF-kB family, results in increased apoptosis in the fetal liver and lethality by embryonic day 14; however, this embryonic lethality can be rescued by also knocking out TNF-α to produce TA-KO mice (16). TA-KO mice show poorer growth than do their TNF-α−/−KO littermates after birth. They also develop autoimmune disorders such as inflammatory cell infiltration in several organs and die within 3 wk after birth (16). This phenotype prompted us to examine the Treg population in TA-KO mice. We found that the frequencies of CD4−Foxp3+ Tregs in the spleen and peripheral blood of TA-KO mice were markedly lower than those in these locations in WT or TNF-α−/−KO mice (Fig. 1A, 1B). To check Treg development, we also analyzed the Treg population in the thymus and found that the frequency of CD4−Foxp3+ Tregs in the TA-KO thymus was similar to that in the thymus of TNF-α−/−KO mice (Fig. 1A, 1B).

TA-KO mice also showed a reduced frequency of CD4 single-positive (SP) PBMCs, a tendency toward reduced (but not significantly so) frequency of CD4SP or CD8SP splenocytes and CD8SP PBMCs (Fig. 1C–E) and a higher frequency of mature (CD24−) CD4SP and CD8SP thymocytes in the thymus (Fig. 1C–G) compared with TNF-α−/−KO mice, suggesting that the tendency toward reduced T cell frequency at the periphery was due to reduced egress from the thymus. Treg frequency at the periphery was drastically reduced compared with that of non-Treg T cells, so we checked the expression of Nrp1, which is a marker of thymus-derived nTregs (20, 21). Most of the Treg population in the TNF-α−/−KO spleen had an Nrp1+ phenotype, whereas in the TA-KO spleen had a trace differential pattern of lower Nrp1 expression (Fig. 1H) and a lower percentage of Nrp1+ Tregs (Fig. 1I). This low frequency of Nrp1+ Tregs in the spleens of TA-KO mice was not due to a lack of Nrp1 expression by RelA-deficient nTregs, because TA-KO thymic Tregs expressed a normal level of Nrp1 (Supplemental Fig. 1B). These results indicated that the splenic Treg population of TA-KO mice contained few thymus-derived Tregs. The calculated fold change between TNF-α−/−KO and TA-KO Nrp1+ Treg frequency in the splenocytes was 21.3 ± 5.0 (Fig. 1I).

TA-KO thymic Tregs do not leave the thymus

We next examined the fate of TA-KO thymic Tregs. We transplanted whole thymi from TA-KO mice or their TNF-α−/−KO littermates under the kidney capsules of Rag1-null mice. We then examined the population of splenic Tregs derived from the donor thymocytes. With TNF-α−/−KO thymus transplantation, we found a splenic Treg population derived from donor TNF-α−/−KO thymocytes (Fig. 2A, 2B). Most of the splenic Treg population had an Nrp1+ phenotype, indicating that they were thymus-derived nTregs. However, with TA-KO thymus transplantation we detected fewer splenic Tregs derived from donor TA-KO thymocytes (Fig. 2A, 2B). This splenic Treg population also had lower Nrp1 expression, indicating that it included fewer nTregs. This reduction of splenic Tregs was not due to transplantation failure, because a definite CD4SP population was observed in the spleen (Fig. 2B). We did not observe the reduction in splenic CD4SP frequency seen in intact TA-KO mice. We also transplanted TNF-α−/−KO or TA-KO thymi to BALB/c nude mice and obtained similar results (Supplemental Fig. 1D).

These results imply either that TA-KO thymic Tregs do not leave the thymus or that they undergo cell death or lose the Treg phenotype (such as Foxp3 expression) once at the periphery. To elucidate whether TA-KO thymic Tregs are held at the thymus by disrupted at the periphery, we transplanted TA-KO thymic Tregs to the periphery of other neonatal TA-KO mice. We hypothesized that if TA-KO thymic Tregs were lost at the periphery, then this transplantation would not rescue the lethality in the recipient TA-KO mice. We harvested enriched CD4+CD8α−CD25+ Tregs from the thymus of TA-KO mice or their TNF-α−/−KO mice (TKO-AHT) littermates, and from the spleens of TKO-AHT mice; the enriched Tregs were then i.v. transplanted into neonatal TA-KO mice. Survivability of the recipient TA-KO mice was then traced (Fig. 2C).

Although intact TA-KO mice died within 3 wk after birth, mice that received TKO-AHT splenic or thymic Treg transplants survived until 3–7.5 wk after birth. Transplantation of TA-KO thymic Tregs had a life-extending effect, demonstrating that TA-KO thymic Tregs were functional at the periphery. We considered that TKO-AHT Tregs were functional, because TKO-AHT mice do not develop autoimmunity. TA-KO thymic Treg transplantation was as effective as TKO-AHT Treg transplantation, indicating that TA-KO Tregs were as functional as TKO-AHT Tregs. These Treg transplantations did not fully rescue the lethality in TA-KO mice, probably because of effects of RelA deficiency other than those on autoimmunity. These results indicated that the absence of TA-KO Tregs at the periphery was due not to loss at the periphery but to arrest in the TA-KO thymus.

TA-KO thymic Tregs are arrested at a late mature stage

To determine at which stage the maturation of TA-KO thymic Tregs was arrested, we analyzed the expression of the T cell maturation markers CD24, CD62L, and CD69 (23) in thymic Tregs. Almost all of the WT or TNF-α−/−KO thymic CD4+CD25−Foxp3+ Treg population expressed a mature (CD62Lhi, CD69lo, or CD24hi) phenotype. We also found that the TA-KO thymic CD4+CD25hi Foxp3+ Treg population had similar, or slightly more mature, CD26Lhi and CD69lo phenotypes (Fig. 3A, Supplemental Fig. 1E).

To further examine the maturation of thymic Tregs, we employed genomic DNA methylation analysis. Stable Foxp3 expression requires the demethylation of CpG motifs in a conserved noncoding sequence element (Treg-specific demethylation region [TSDR]) in the foxp3 locus (22, 24, 25), and this demethylation begins in the thymus (26, 27). We used this methylation state transition to distinguish between mature Tregs (demethylated TSDR) and immature Tregs (methylated TSDR). We isolated CD4+CD25hi Tregs from the thymus of WT, TNF-α−/−KO, or TA-KO male mice and performed bisulfite sequencing of the TSDR. We detected both methylated and demethylated TSDRs in WT and TNF-α−/−KO Treg populations, indicating that WT and TNF-α−/−KO thymic Treg populations contained both mature and immature Tregs. In contrast, the TA-KO Treg population contained mainly demethylated TSDRs (Fig. 3B), revealing that the TA-KO thymic Treg population contained mostly mature Tregs. These results indicated
**FIGURE 1.** TA-KO mice had a trace Treg population at the periphery but a definite Treg population at the thymus. (A) Representative plots of CD4+ Foxp3+ populations in 2.5-wk-old WT, TNF-α-KO, or TA-KO mice. Percentages represent CD4+Foxp3+ cells in whole thymocyte, splenocyte, or erythrocyte-depleted peripheral blood cells (PB). (B) Aggregate data for CD4+Foxp3+ frequencies in thymocytes, splenocytes, and PBMCs from 1.5- to 2.5-wk-old mice (n = 5–16). Bars indicate averages. (C) Representative plots of CD4SP or CD8SP populations in 2-wk-old mice. (D and E) Aggregate data for CD4SP (D) or CD8SP (E) frequencies in thymocytes, splenocytes, and PBMCs from 1.5- to 2.5-wk-old mice (n = 5–13). (F) Representative histograms of CD24 expression levels by CD4SP or CD8SP thymocytes of WT (shaded), TNF-α-KO (broken line), or TA-KO (solid line) mice. (G) Aggregate data for CD24+ mature T cell frequencies in CD4SP- or CD8SP-gated thymocytes from 1.5- to 2-wk-old mice (n = 7–13). (H) Representative histograms of Nrp1 expression levels in CD4+Foxp3+-gated splenocytes (spl.) from TNF-α-KO (shaded) or TA-KO (solid line) mice. (I) Aggregate data for Nrp1+CD4+Foxp3+ frequencies in CD4+Foxp3+-gated or whole splenocytes from 1.5- to 2-wk-old mice (n = 5 or 6). fc, Fold change between TNF-α-KO and TA-KO Nrp1+ Treg frequencies in the splenocyte population. Absolute cell numbers of each population in the thymus and spleen are shown in Supplemental Fig. 1A. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant, spl., splenocytes.
that TA-KO thymic Treg maturation was arrested at a mature state. This was consistent with our results that TA-KO thymic Tregs were functional at the periphery.

T cell egress from the thymus is dependent on both S1P1 (13, 14) and Kruppel-like factor 2 (15), and we found that TA-KO thymic Tregs expressed transcripts of both these factors (Supplemental Fig. 1G). We also found that TA-KO thymic Tregs expressed S1P1 at the same level and frequency as did TNF-α–KO thymic Tregs (Fig. 3C, 3D). Moreover, TA-KO thymic Tregs, similar to TNF-α–KO Tregs, tended to elicit chemotaxis toward S1P (Fig. 3E). These results indicate that TA-KO thymic Tregs retain their competence to respond with appropriate signals for egress from the thymus; this is consistent with our results that TA-KO thymic Tregs are mature and functional. Although the relative expression of slpr1 in TNF-α–KO and TA-KO thymic Tregs was lower than that in WT Tregs (Supplemental Fig. 1G), because TNF-α–KO mice do not show thymic Treg arrest, these results suggested that this level of expression was sufficient to elicit Treg egress.

There are several reports of chemokine receptors being associated with thymocyte egress. The CCR7 axis and its ligand, CCL19, are reported to be a major pathway of neonatal T cell export (26). The CCR5-mediated chemorepellent activity of intrathymic SDF-1/CXCL12 is also reported to be associated with thymocyte egress from the fetal thymus (29, 30). We checked for the expression of CCR7 and CXCRR4 in the thymic Treg populations of TNF-α–KO and TA-KO mice, but we did not find any significant differences in expression frequency between the two populations (Supplemental Fig. 2A, 2B), suggesting that lack of expression of these receptors is not the cause of the impaired thymic Treg egress in TA-KO mice. We also analyzed the expression of chemokine receptors in the thymic Treg population of TNF-α–KO and TA-KO mice by means of microarray analysis; we found upregulation of ccr3 transcripts in the TA-KO Treg population (Supplemental Fig. 2C). It was reported that mature invariant NKT cells are retained in the thymus by CXCR3, and expression of CXCR3 ligands in the medullary TECs promotes the chemotactic retention of mature invariant NKT cells (31). It was also reported that upregulation of CXCR3 was observed in KLF-2-deficient T cells (32). Next, we checked the expression of the corresponding chemokines (cxcl9, cxcl10, and cxcl11) in thymic stromal cells (CD45 EpCAM+ TECs and T cell–depleted CD45 EpCAM– thymic stromal cells) (Supplemental Fig. 2C). The chemokine expression profile of TA-KO CD45 EpCAM+ stromal cells was similar to, or lower than, that of TNF-α–KO. This tendency was also seen in the expression profiles of TECs, although cxcl11 was expressed at a higher level in TA-KO TECs. C57BL/6 mice have a deletion in the coding sequence for CXCL11; this introduces a frame shift that causes truncation of the peptide and almost certainly abolishes biological activity (33). It is therefore unlikely that these chemokines attract CXCR3+ Tregs to the TA-KO thymus and prevent their egress; however, it remains possible that small amounts of these chemokines are enough to prevent CXCR3+ Treg egress.

We also found upregulation of ccr5 transcripts in the TA-KO Treg population (Supplemental Fig. 2C). CCR5 and its ligands (CCL5, CCL4, and CCL5) have been reported to elicit Treg migration (34–39), and we found that ccl4 transcripts were upregulated in the CD45 EpCAM+ TECs of TA-KO mice (Supplemental Fig. 2C). It is therefore possible that ccl4 transcript upregulation in TA-KO TECs attracts CCR5+ Tregs to the TA-KO thymus and prevents their egress. We also found that, in addition to the tendency for ccr10 transcripts to be upregulated in TA-KO Tregs, transcripts of the corresponding chemokine ccl28 also tended to be upregulated in TA-KO CD45 EpCAM+ TECs (Supplemental Fig. 2C). Tumor hypoxia promotes the recruitment of Tregs through induction of the expression of CCL28 (40). Therefore, the CCR10/CCL28 axis may play an important role in Treg arrest in the TA-KO thymus.

**FIGURE 2.** Tregs in TA-KO mice do not leave the thymus. (A) Intact thymus of 1.5-wk-old TNF-α–KO or TA-KO mice were transplanted (Tp) under the kidney capsules of Rag1-null mice; 1 mo after the transplant, splenocytes were harvested from the recipients and analyzed. Left and center: Representative plots of CD4+Foxp3+ populations in spleen of the recipients. Percentages represent CD4+Foxp3+ cells in whole splenocyte. Right: Representative histogram of Nrp1 expression levels by CD4+Foxp3+–gated splenocytes. (B) Aggregate data for population frequencies (n = 3 or 4). Treg, CD4+Foxp3+ population. Absolute cell numbers for each population are shown in Supplemental Fig. 1C. (C) Enriched CD4+CD8α+CD25+ thymocytes (tTregs) from TNF-α–KO/RelA−/− (TKO-AHT) or TA-KO mice, and enriched CD4+CD8α+CD25+ splenocytes (sTregs) from TKO-AHT mice, were transplanted i.v. into 2-d-old TA-KO mice and mouse survivability was traced. *p < 0.02, **p < 0.003. n.s., not significant.
IL-7Rα is a possible Treg late-stage maturation marker

To elucidate the maturation status of thymic Tregs in TA-KO mice, we again compared the gene expression profiles of thymic Treg populations from TNF-α−KO and TA-KO mice by using microarray analysis. In the TA-KO Treg population, we found upregulation of il-7r, which encodes the α-chain of IL-7R (Fig. 4A). Flow cytometric analysis confirmed that the TA-KO thymic Treg population had a marked IL-7Rα+ subpopulation (Fig. 4B, 4C, Supplemental Fig. 3A).

Our finding that the TA-KO thymic Treg population included mainly mature Tregs implied that IL-7Rα+ Tregs are mature. To test this, we isolated CD4+CD25−CD24− (mature conventional CD4SP T cells), CD4+CD25int, CD4+CD25hiIL-7Rα−, and CD4+CD25hiIL-7Rα+ thymocytes from 2- to 3-wk-old WT male mice. Thymocytes were sorted into CD4+CD8α−CD25−, CD4+CD8α−CD25int, CD4+CD8α−CD25hiIL-7Rα−, or CD4+CD8α−CD25hiIL-7Rα+ populations, and bisulfite sequencing was performed (○, demethylated CpG sites; ●, methylated CpG sites). The gating criteria of CD25int and CD25hi were described in Supplemental Fig. 3B. Experiments were performed three times, as shown in Supplemental Fig. 3C. *p, 0.001. n.s., not significant.
TSDR demethylation was correlated with CD25 upregulation to CD25\textsuperscript{hi} and CD25\textsuperscript{hi} (Fig. 4D). Whereas the CD4\textsuperscript{+}CD25\textsuperscript{hi}IL-7R\alpha cell population contained some methylated TSDRs, the CD4\textsuperscript{+}CD25\textsuperscript{hi} IL-7R\alpha population contained mostly demethylated TSDRs (Fig. 4D), suggesting that IL-7R\alpha upregulation follows TSDR demethylation in the Treg maturation process and, therefore, that IL-7R\alpha is a possible late-stage Treg maturation marker. IL-7R\alpha conditional knockout mice generated from CD4-Cre and IL-7R\alpha–floxed mice had reduced numbers of thymic Tregs, suggesting that IL-7R\alpha is required for Treg proliferation but is not essential for the functional maturation of thymic Tregs (41). This is consistent with our results that IL-7R\alpha upregulation comes after Treg maturation and TSDR demethylation.

**Expression of the Treg absence phenotype in the TA-KO mouse spleen involves a non–cell-autonomous mechanism**

To elucidate whether the arrest of TA-KO thymic Tregs in the thymus was caused by the TA-KO Tregs themselves or by the TA-KO thymic environment, we generated chimeric mice in which the hematopoietic system was reconstituted with TA-KO cells. TA-KO or its littermate fetal liver cells were transplanted into lethally irradiated CD45.1\textsuperscript{+}C57BL/6 (ly5.1\textsuperscript{+}) mice (FL–TA-KO or FL–TNF-\alpha–KO, respectively), and 2–3 mo after transplantation we examined the chimeric mice for donor-derived splenic Treg populations. We found that FL–TA-KO mice had definite TA-KO splenic Treg populations (Fig. 5A). Furthermore, the FL–TA-KO Treg population had an Nrp1 expression pattern that was similar to that found in the FL–TNF-\alpha–KO Treg population, the intact TNF-\alpha–KO splenic Treg population (Fig. 1H), and the TNF-\alpha–KO thymus-transplanted Rag1-null-mouse Treg population (Fig. 2A) but different from that found in the Treg population of intact TA-KO mice or TA-KO thymus-transplanted Rag1-null mice. Although the frequencies of the splenic Treg population (CD4\textsuperscript{+}Foxp3\textsuperscript{+}) and Nrp1\textsuperscript{+} Treg subpopulation in FL–TA-KO mice were significantly lower than those in FL–TNF-\alpha–KO mice, the fold change in the Nrp1\textsuperscript{+} Treg population frequency between FL–TNF-\alpha–KO and FL–TA-KO mice recovered to 5.0 \pm 0.8 (Fig. 5B). That most of the Tregs in the FL–TA-KO spleen expressed Nrp1 suggested that most of the splenic TA-KO Tregs in FL–TA-KO chimeric mice were thymus-derived Tregs that had egressed from the thymus. These results strongly indicated that a non–cell-autonomous mechanism contributes to the expression of the Treg absence phenotype in the spleens of (intact) TA-KO mice.

The TA-KO fetal liver chimeric mice that had TA-KO thymocytes but their own WT thymic environment had a TA-KO splenic Nrp1\textsuperscript{+} Treg population; however, in mice that received whole thymus transplants under the kidney capsule (41), the CD4\textsuperscript{+}Foxp3\textsuperscript{+} and Nrp1\textsuperscript{+} Treg subpopulation was not easily distinguished by analysis of CD24, CD62L, or CD69 expression. Use of this new marker will enable the definition of the late-stage mature Treg population, which cannot be easily distinguished by analysis of CD24, CD62L, or CD69 expression. Use of this new marker will enable the definition of the late-stage mature Treg population, which cannot be easily distinguished by analysis of CD24, CD62L, or CD69 expression. Use of this new marker will enable the definition of the late-stage mature Treg population, which cannot be easily distinguished by analysis of CD24, CD62L, or CD69 expression.

**Discussion**

TA-KO mice, which ultimately develop autoimmune disorders and die prematurely, had a definite population of Tregs in the thymus, but few were seen at the periphery. Using TA-KO Treg transplantation followed by a survival assay, TSDR methylation analysis, and expression analysis of maturation markers (including the newly identified possible Treg maturation marker IL-7R\alpha), we showed that TA-KO thymic Tregs were functional, did not leave the thymus, and were arrested at a late mature stage. When we performed fetal liver transplantations, we found that FL–TA-KO mice had a definite TA-KO splenic Treg population, although it was smaller than that of FL–TNF-\alpha–KO mice. We also observed that RelA deficiency reduced thymic Treg development. Most of the Tregs in the FL–TA-KO spleen were Nrp1\textsuperscript{+} Tregs, indicating that they had egressed from the thymus. These data suggested that RelA deficiency affects generation of thymic Tregs in a cell-autonomous manner, but the generated RelA-deficient thymic Tregs egress from the thymus of FL–TA-KO mice. Our results also suggested that Treg arrest in the TA-KO thymus was due to RelA deficiency in the thymic environment. Thus, we suggest that egress of mature Tregs from the thymus is dependent on RelA in the thymic environment.

We identified a new possible late-stage mature Treg marker, IL-7R\alpha, which is expressed by late-stage mature Tregs in which TSDR has been demethylated. Surface expression of IL-7R\alpha distinguishes the late-stage mature Treg population, which cannot be easily distinguished by analysis of CD24, CD62L, or CD69 expression. Use of this new marker will enable the definition of a new subset of immature Tregs that are soon to leave the thymus. Our observations suggest that there is a checkpoint that controls mature Treg egress from the thymus, and that the thymic environment plays a pivotal role in this control mechanism via RelA.
The thymic environment has many types of cells, such as cortex and medullary TECs, dendritic cells, macrophages, fibroblasts, and vascular endothelial cells. Our dGuo-treated fetal thymus transplantation experiments indicated that TECs are not RelA-dependent in the process of Treg egress from the thymus; therefore, further work is required to identify which types of cell are involved in this process. However, candidates can be narrowed down to cells that have radiosensitivity, such as nonproliferating or terminally differentiated cells, because irradiated mice that received TA-KO fetal liver cells had a population of donor-derived splenic Nrp1+ Tregs (Fig. 5A, 5B).

In a gene expression microarray analysis, we detected the up-regulation of cxcr3 and ccr5 transcripts in TA-KO Tregs, as well as the upregulation of cxcl11 and ccl4 transcripts in TA-KO TECs. Because these chemokine receptors are reported to be involved in the attraction of Tregs (and other cells) (31, 32, 34–39), it is possible that the upregulation of cxcr3 and ccr5 transcripts in Tregs is part of the mechanism of Treg arrest in TA-KO thymus. If so, because TECs are not RelA-dependent in the process of Treg egress, and the involvement of other types of cells is suggested, as discussed above, it is likely that the upregulation of ccl4 and cxcl11 transcripts in TECs occurs in a cell-extrinsic manner. Although cxcl11 coding sequence of C57BL/6 mice does not produce functional CXCL11 peptides (33), examination of the dysregulation of the transcription of these genes may provide important information on the types of cells that regulate the expression of these chemokines in TECs, and therefore on the types of cells that are involved in the process of Treg arrest in the TA-KO thymus.

We noted that the size of the thymic Treg population in TA-KO mice was approximately normal compared with those in TNF-α–KO mice, although the mature TA-KO thymic Tregs accumulated in the thymus. We also noted that the thymic Treg population of TA-KO mice was comprised mostly of mature Tregs. Taken together, these results imply that de novo production of (immature) Tregs in the TA-KO thymus is inhibited. We hypothesize that the thymus has a limit to the number of Tregs of which it can control the maturation at one time. TCRs have instructive roles in the development of thymic Tregs, and the selection of individual Treg clones in the thymus depends on small selecting “niches” (43, 44). It is yet to be elucidated whether these selecting niches also maintain Treg maturation, or whether there are distinct selecting and maturation niches within the thymus.

FIGURE 5. A definite Nrp1+ Treg population in the spleen was observed in both TA-KO fetal liver–transplanted and dGuo-treated TA-KO fetal thymus transplanted mice. (A) Fetal liver cells from TNF-α–KO or TA-KO embryonic day 13.5 embryos were transplanted into lethally irradiated CD45.1+ C57BL/6 adult mice (FL–TNF-α–KO or FL–TA-KO, respectively), and the donor-derived splenic Treg population was analyzed 2 or 3 mo after transplantation. Plots in the left panel are gated on donor-derived CD45.1+ (Ly5.2+) splenocytes. Numbers in the figures represent the percentages of CD4+Foxp3+ cells in the Ly5.2+-gated population. Histogram shows Nrp1 expression in donor-derived CD4+Foxp3+ splenocytes. (B) Aggregate data for population frequencies (n = 6 or 7). fc, Fold change between FL–TNF-α–KO and FL–TA-KO Nrp1+ Treg frequencies in donor-derived splenocytes; spl., splenocytes; Treg, CD4+Foxp3+ population. Absolute cell numbers are shown in Supplemental Fig. 3D. (C) Representative plots of Ly5.2+-gated thymocytes in FL–TNF-α–KO or FL–TA-KO mice. (D) Aggregate data for CD4+Foxp3+ frequencies in donor-derived thymocytes (thy.; n = 5 or 7). Absolute cell numbers are shown in Supplemental Fig. 3E. (E) Fetal thymus from TNF-α–KO or TA-KO embryonic day 14.5 to embryonic day 15.5 embryos were treated with dGuo and then transplanted under the kidney capsules of BALB/c nude mice (dG–TNF-α–KO or dG–TA-KO, respectively); the splenic Treg population was analyzed 2 mo after transplantation. Plots in left panel are gated on host-derived H-2Kb+ splenocytes. Histogram shows Nrp1 expression in host-derived splenic CD4+Foxp3+ splenocytes. (F) Aggregate data for population frequencies in host-derived splenocytes or splenic Tregs (n = 3–8). Absolute cell numbers are shown in Supplemental Fig. 3F. *p < 0.01, **p < 0.001. n.s., not significant.
In summary, in this study, we proposed the possible role of RelA in the process of thymic Treg egress. Our findings provide new perspectives on the mechanisms of Treg egress from the thymus.

Disclosures

The authors have no financial conflicts of interest.

References


