FoxP3+ T Cells Undergo Conventional First Switch to Lymphoid Tissue Homing Receptors in Thymus but Accelerated Second Switch to Nonlymphoid Tissue Homing Receptors in Secondary Lymphoid Tissues

Jee H. Lee, Seung G. Kang and Chang H. Kim

J Immunol 2007; 178:301-311; doi: 10.4049/jimmunol.178.1.301
http://www.jimmunol.org/content/178/1/301

Why The JI?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References

This article cites 58 articles, 37 of which you can access for free at:
http://www.jimmunol.org/content/178/1/301.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
FoxP3+ T Cells Undergo Conventional First Switch to Lymphoid Tissue Homing Receptors in Thymus but Accelerated Second Switch to Nonlymphoid Tissue Homing Receptors in Secondary Lymphoid Tissues

Jee H. Lee, Seung G. Kang, and Chang H. Kim

Forkhead box P3 (FoxP3)-positive T cells are a specialized T cell subset for immune regulation and tolerance. We investigated the trafficking receptor switches of FoxP3+ T cells in thymus and secondary lymphoid tissues and the functional consequences of these switches in migration. We found that FoxP3+ T cells undergo two discrete developmental switches in trafficking receptors to migrate from primary to secondary and then to nonlymphoid tissues in a manner similar to conventional CD4+ T cells as well as unique to the FoxP3+ cell lineage. In the thymus, precursors of FoxP3+ cells undergo the first trafficking receptor switch (CCR8/CCR9→CXCR4→CCR7), generating mostly homogeneous CD62L+CCR7+CXCR4lowFoxP3+ T cells. CXCR4 expression is regained in FoxP3+ thymic emigrants in the periphery. Consistent with this switch, recent FoxP3+ thymic emigrants migrate exclusively to secondary lymphoid tissues but poorly to nonlymphoid tissues. The FoxP3+ thymic emigrants undergo the second switch in trafficking receptors for migration to nonlymphoid tissues upon Ag priming. This second switch involves down-regulation of CCR7 and CXCR4 but up-regulation of a number of memory/effector type homing receptors, resulting in generation of heterogeneous FoxP3+ T cell subsets expressing various combinations of trafficking receptors including CCR2, CCR4, CCR6, CCR8, and CCR9. A notable difference between the FoxP3+ and FoxP3− T cell populations is that FoxP3+ T cells undergo the second homing receptor switch at a highly accelerated rate compared with FoxP3− T cells, generating FoxP3+ T cells with unconventionally efficient migratory capacity to major nonlymphoid tissues. The Journal of Immunology, 2007, 178: 301–311.

Received for publication July 6, 2006. Accepted for publication October 16, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

This study was supported in part by Grant AI063064 from National Institutes of Health-National Institute of Allergy and Infectious Diseases, by the American Heart Association, and by the Sidney Kimmel Foundation (to CHK).

Address correspondence and reprint requests to Dr. Chang Kim, Department of Comparative Pathobiology, Purdue University, 725 Harrison Street, West Lafayette, IN 47907. E-mail address: chkim@purdue.edu

Abbreviations used in this paper: FoxP3, forkhead box P3; Treg, regulatory T cell; PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer’s patch; mOVA, membrane-bound OVA; DP, double positive; SP, single positive; DN, double negative.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$2.00

www.jimmunol.org

The Journal of Immunology

www.jimmunol.org

by guest on November 13, 2017 http://www.jimmunol.org/ Downloaded from
DO11.10 TCR rag2−/− transgenic mice were purchased from Taconic Farms. Mice were housed at Purdue University (West Lafayette, IN) under a specific pathogen-free condition and used according to approved protocols and institutional guidelines. PCR genotyping was performed to identify mOVA/DO11.10 Treg mice that contain both mOVA and DO11.10 transgenic genes. Generally, mice used were between age 6 and 8 wk at the start of each experiment.

**Cells and cell culture**

Single-cell suspensions from lymph nodes (mesenteric, inguinal, auxiliary, and brachial), Peyer’s patches (PP), spleen, and liver were prepared by grinding tissues through an iron mesh. Peritoneal lavage was prepared in 10 ml of PBS. Bone marrow cells were flushed from femurs and tibias with 10 ml of PBS per tibia or femur. Lamina propria lymphocytes of the intestinal tract were isolated by flushing out the distal ileum with 10 ml of PBS. Peritoneal cavity (P. cavity) and the lamina propria of small intestine (S. intestine) are also examined. A representative set of data (A) and combined data (B–D) obtained from seven different experiments using 6- to 8-wk-old BALB/c mice are shown.

**FIGURE 1.** Tissue distribution of heterogeneous FoxP3+ T cell subsets in primary lymphoid, secondary lymphoid (2-LT), and nonlymphoid tissues (Non-LT). A, Definitions of FoxP3+ and FoxP3− CD4 T cell subsets. B, Absolute numbers of FoxP3+ T cell subsets in the 10 selected organs. C, Percentage shows relative distribution of FoxP3+ T cells among the 10 selected organs: thymus, blood, spleen, MLN, six PLN (two inguinal, two auxiliary, and two brachial), PP, small intestine, bone marrow (marrow), and liver. D, Percentages of the relative frequencies of CD62L+ and CD62L− FoxP3+ T cell subsets in each organ. Peritoneal cavity (P. cavity) and the lamina propria of small intestine (S. intestine) are also examined. A representative set of data (A) and combined data (B–D) obtained from seven different experiments using 6- to 8-wk-old BALB/c mice are shown.

**FIGURE 2.** Thymus generates CCR7+CXCR4− FoxP3+ T cells. A, DN, DP, and SP FoxP3+ or FoxP3− thymocyte subsets. B, Expression of four major thymocyte chemokine receptors by DN (FoxP3−), DP (FoxP3+ and FoxP3−), and SP (FoxP3− and FoxP3+) subsets and recent FoxP3+ thymic emigrants in PLN and MLN was examined. FoxP3+ thymic emigrants in PLN and MLN were identified as described for Fig. 3. Combined data from four independent experiments are shown. *, p ≤ 0.05 significant difference between FoxP3+ and FoxP3− CD4+ cells.
small intestine were isolated by removing the epithelial cells with 5 mM EDTA (five times) and digesting the remaining tissues for 45 min with 300 U/ml collagenase (type 3; Worthington Biochemical) and 100 μg/ml DNase I (Worthington Biochemical) at 37°C. For removal of RBC from spleen, liver, and bone marrow cells, cells were treated with hypotonic RBC lysis buffer. For one experiment (see Fig. 6, B and C), T cells were isolated from mesenteric lymph nodes (MLN) and labeled with CFSE as previously described (32). CFSE-labeled MLN cells were cultured in the presence of 300 U/ml IL-2 and Ag peptide OVA323–339 (final 1 μg/ml) for 5 days before examination of cell division and trafficking of receptor expression by FACS.

Expression of trafficking receptors

Fresh or Ag-primed mouse T cells, harvested from BALB/c, mOVA × DO11.10, or mOVA × DO11.10 (rag2−/−) mice were stained with Abs to CCR3 (clone 83103; R&D Systems), CCR5 (clone C34-3448; BD Pharmingen), CCR6 (clone 140706; R&D Systems), CCR7 (clone 4B12; BioLegend), CCR9 (clone 242503; R&D Systems), CXCR2 (clone 242216; R&D Systems), CXCR3 (clone 220803; R&D Systems), CXCR4 (clone 2B11; BD Pharmingen), CXCR5 (clone 2G8; BD Pharmingen), CXCR6 (clone 221002; R&D Systems), or rat control IgG2b (clone A95-1; BD Pharmingen) for 30 min on ice. Fc fusion proteins of CCL1, CCL17, and CCL27 were used for staining of CCR5, CCR4, and CCR10, respectively. To generate Fc chemokine receptors, pcDNA3.1 expression vectors containing Fc proteins of CCL17 (33), CCL1 and CCL17 under the CMV early promoter were used to produce Fc chemokine constructs in HEK-293 cells. The specificity of the staining with the constructs was verified by using chemokine receptor transfectants. Mouse cells were stained with biotinylated anti-rat IgG2a (clone RT17;30; BD Pharmingen), anti-IgG2b (clone RT17/11; Caltag Laboratories), anti-rat IgG2c (clone MRG2b; BioLegend), or anti-human IgG Ab (Vector Laboratories) for 30 min, followed by staining with allophycocyanin-streptavidin (BD Pharmingen) and Abs to CD62L, DO11.10, and CD4. The cells were further stained with FoxP3 (eBioscience) according to the manufacturer’s protocol.

Labeling of thymic FoxP3+ T cells and emigration study

BALB/c mice were anesthetized by i.p. injection of 90–120 mg/kg ketamine and 10 mg/kg xylazine. A ventral midline incision was made one-third down the sternum to expose the thymus. A Hamilton syringe was used to inject 5 μl of a 1 mg/ml solution of FITC into each lobe. The skin incision was closed with surgical clips, and mice were allowed to recover for 1–3 days. As the first step, we examined the tissue distribution of FoxP3+ T cells (or CD4+FoxP3+) and their tissue tropisms in primary, secondary, and nonlymphoid tissues (Fig. 3). The majority (on average 65%) of FoxP3+ T cells that migrated from the thymus to each organ were calculated, and the percentage of distribution among the selected secondary and nonlymphoid organs was calculated. Combined data from four independent experiments are shown.

For assessment of FoxP3+ T cell numbers in bone marrow, we used the number of total bone marrow mononuclear cells (2.8 × 10⁹) determined by others (34, 35).

Statistical analyses

Averages with SEM are shown in most of the experiments. Student’s paired and unpaired t tests were used to determine the significance of the differences between two groups of data. Values of p ≤ 0.05 were considered significant.

Results

FoxP3+ T cell subsets and their tissue tropisms in primary, secondary, and nonlymphoid tissues

As the first step, we examined the tissue distribution of FoxP3+ T cells in young (6- to 8-wk-old) BALB/c mice using FoxP3 as a cell lineage marker (Fig. 1, A and B). The majority (on average ~65%) of the FoxP3+ T cells were found in MLN, PLN, and spleen among the 10 organs examined. Other FoxP3+ T cells were present in nonlymphoid tissues, thymus, and bone marrow. When the frequency of FoxP3+ T cells among CD4+ T cells was considered (Fig. 1C), the most FoxP3+ T cell-enriched organ was marrow (~40% of CD4+ T cells) followed by peritoneal cavity (~16%). On average, ~8% of CD4+ T cells in the small intestine were FoxP3+ T cells.

Tregs in the spleen and lymph nodes are divided into CD62L+ and CD62L− cells, which have different phenotype and function (19, 36–38). The majority of FoxP3+CD4+CD8− T cells in the thymus were CD62L+CD44high cells, whereas FoxP3+CD4+CD8− T cells were CD62L−CD44low cells (data not shown). In MLN, PLN, and spleen, many FoxP3+ T cells were CD62L−CD44high, but some were CD62L+CD44high cells. In the peripheral blood, the proportion of CD62L+FoxP3+ cells was increased.

FIGURE 3. Tissue destinations for recent FoxP3+ thymic emigrants in the periphery. A, FITC was injected into the thymus to label FoxP3+ T cells. Three days later, FITC+ FoxP3+ T cells that had migrated to various organs were counted by flow cytometry analysis. B, FoxP3+ T cell frequencies within the FoxP3+ T cell population in each organ, shown as the percentage after normalization for spleen, are shown. Averages with SEM are shown in most of the experiments. Student’s paired and unpaired t tests were used to determine the significance of the differences between two groups of data. Values of p ≤ 0.05 were considered significant.

Adoptive transfer, immunization, and homing experiment

For short-term (20-h) homing experiments, splenocytes containing ~5 × 10⁵ KJ1-26FoxP3+ T cells from mOVA × DO11.10 Treg mice were prepared and injected into BALB/c mice via a tail vein. Host mice were immunized with 250 μg of OVA with 50 μl of complete or IFA per site (CFA or IFA; Sigma-Aldrich). The mice were immunized s.c. at the flank to examine only the T cells in peripheral lymph nodes (PLN), or s.c. and i.p. at the same time to examine T cells in PLN, MLN, and spleen. Comparable results were obtained for T cells in PLN using the two immunization methods. For controls, mice were immunized with CFA or IFA only without OVA. Three or 8 days later, mice were sacrificed to determine the percentages of KJ1-26FoxP3+ T cells in primary, secondary, and nonlymphoid tissues. For short-term homing experiments, Ag-primed KJ1-26FoxP3+ T cells were prepared by transferring KJ1-26FoxP3+ T cells from mOVA × DO11.10 mice into BALB/c mice followed by immunization with OVA as described.

Eight days later, ~5 × 10⁷ splenocytes (containing ~5 × 10⁶ KJ1-26FoxP3+ T cells) isolated from the immunized BALB/c mice were injected into BALB/c mice. At 20 h later, mice were sacrificed to determine homing of KJ1-26FoxP3+ and FoxP3+ T cell subsets to selected organs. KJ1-26FoxP3+ T cells in various organs were counted by flow cytometry. Because the absolute number of migrated T cells varies from experiment to experiment due to differences in the number of transferred cells, we normalized the absolute number of KJ1-26FoxP3+ T cells present in each organ to the number of spleen (spleen = 1) T cells. This system generates a parameter termed “homing index,” which indicates the relative distribution of injected FoxP3+ T cells among the organs examined.
FIGURE 4. Ag priming redistributes FoxP3+ T cells from secondary lymphoid to nonlymphoid tissues. A, Characterization of spleen FoxP3+ T cells (isolated from mOVA × DO11.10 mice and used as input cells) in expression of FoxP3, CD62L, and/or CD25. CD4+ cells from BALB/c and DO11.10 rag2−/− mice are shown for comparison. B, Dynamics of FoxP3+ T cell subset distribution among the six organs after Ag priming. BALB/c mice were injected i.v. with splenocytes containing mOVA × DO11.10 FoxP3+ CD4+ T cells (day 0) followed by immunization s.c. at the flank and i.p. with OVA. Relative distribution of FoxP3+ T cells in multiple tissues was determined based on absolute numbers of KJ1-26 FoxP3+ cells in each organ, which was normalized by that of KJ1-26 FoxP3+ T cells in spleen (spleen = 1). The average absolute number of KJ1-26 FoxP3+ and FoxP3+ T cells in immunized spleens at day 3 was 123 × 10^3 (CD62L+ FoxP3+), 42.9 × 10^3 (CD62L− FoxP3+), 155 × 10^3 (CD62L+ FoxP3−), and 106 × 10^3 (CD62L− FoxP3−). The average absolute number in immunized spleens at day 8 was 46.8 × 10^3 (CD62L− FoxP3+), 3.7 × 10^3 (CD62L− FoxP3−), 37.8 × 10^3 (CD62L+ FoxP3−), and 11.8 × 10^3 (CD62L+ FoxP3+). Combined data from four independent experiments are shown. *, p ≤ 0.05. C, Conversion of OVA-specific FoxP3− CD4+ T cells to FoxP3+ T cells is minimal in the given immunization condition. BALB/c mice were injected i.v. with splenocytes containing DO11.10 rag2−/− FoxP3− CD4+ or mOVA × DO11.10 FoxP3+ CD4+ T cells followed by immunization with OVA.
to ~50% within the FoxP3+ T cell population (Fig. 1D). In bone marrow and nonlymphoid tissue sites, such as liver, peritoneal cavity, and lamina propria of the small intestine, the proportion was increased even more, up to ~95%. In PP (the secondary lymphoid tissues embedded within the intestine), ~70% of FoxP3+ cells were CD62L+ cells. There is a clear trend that the proportion of CD62L+ FoxP3+ cells is increased in nonlymphoid tissues compared with secondary lymphoid tissue and thymus, suggesting that the FoxP3+ cell subsets have different tissue tropisms from each other.

FoxP3+ T cells undergo a trafficking receptor switch in the thymus to generate CD62L+CCR7+CXCR4lowFoxP3+ T cells, and these FoxP3+ thymic emigrants mainly migrate to secondary lymphoid tissues.

Next, we examined the trafficking receptor switch in thymus, the primary lymphoid organ for T cells. FoxP3+ cells appear at double-positive (DP) and single-positive (SP) stages in the thymus (11). Consistently, the majority of thymic FoxP3+ cells were CD4+SP, and some were CD4 and CD8 DP (Fig. 2A). We determined the trafficking receptors expressed by FoxP3+ cells at different development stages. Thymic chemokines, such as CXCL12, CCL1, CCL25, and CCL19, are important for T cell localization, development, and emigration. CXCR4 (the CXCL12 receptor) was up-regulated at the transition from the double-negative (DN) FoxP3− cells to DP FoxP3+ cells but was down-regulated as DP FoxP3+ cells become CD4 SP FoxP3+ cells (Fig. 2B). After emigration to lymph nodes, CXCR4 was again up-regulated. Conversely, CCR9 (the CCL25 receptor) was high on DN FoxP3− cells and DP FoxP3+ cells but was sharply down-regulated afterward. CCR8 (the CCL1 receptor) was high on DN FoxP3− cells but was down-regulated afterward. In contrast, CCR7 (the CCL19 receptor, a secondary lymphoid tissue homing receptor) was gradually up-regulated at the transition from DP FoxP3+ cells to SP FoxP3+ cells. Notable differences between FoxP3+ and FoxP3− cells include expression of CXCR4 and CCR9: CXCR4 was expressed on many more FoxP3+ cells, whereas CCR9 was expressed on many more FoxP3− T cells at the CD4 SP stage. Overall, thymus generates mostly homogeneous CD62L+CCR7+CXCR4low FoxP3+ T cells as the consequence of the switch.

Apparently, the CD62L+CCR7+CXCR4lowFoxP3+ T cells, generated in the thymus, have the homing receptors associated with secondary lymphoid tissues. To determine their homing behavior to secondary lymphoid tissues as well as their emigration behavior to other tissue sites, we used a FITC thymic injection method to label thymic FoxP3+ cells (Fig. 3A). Three days after the injection with FITC, tissue distribution of FITC+ FoxP3+ cells in the periphery was determined. Most FITC+ FoxP3+ cells were found in secondary lymphoid tissues such as spleen and lymph nodes among the eight organs examined (Fig. 3B), suggesting that the thymus-emigrating CD62L+CCR7+CXCR4low FoxP3+ T cells preferentially migrate to secondary lymphoid tissues over nonlymphoid tissues.
Ag priming in secondary lymphoid tissue generates CD62L+FoxP3+ T cells with a tissue tropism for nonlymphoid tissue

Ag priming is a key event that expands the number of T cells and changes their function and tissue tropism. Next, we investigated the changes in FoxP3+ T cell tissue distribution following Ag priming in secondary lymphoid tissues. For this experiment, we used OVA-specific (KJ1-26+) FoxP3+ T cells obtained from mOVA × DO11.10 double transgenic mice. Although the surface and functional phenotypes of these Tregs are indistinguishable from those of wild-type mice, the lymphoid tissues of these mice are highly enriched with OVA-specific FoxP3+ cells as the result of transgenic coexpression of OVA-specific TCR and OVA (39). A 35–50% of KJ1-26+ CD4+ T cells from secondary lymphoid tissues (spleen) of these mice were FoxP3+ T cells, and ~85% of these FoxP3+ T cells were CD25+ (Fig. 4A). Most (>95%) of the FoxP3+ T cells in spleen and lymph nodes were CD62L+. When splenocytes of mOVA × DO11.10 mice were injected i.v. into BALB/c mice, most KJ1-26+ FoxP3+ T cells migrated back to secondary lymphoid tissues such as spleen, PLN, and MLN, but not to nonlymphoid tissues among the organs examined (Fig. 4B) in a manner similar to the recent FoxP3+ thymic emigrants. After immunization of the mice with OVA, CD62L+KJ1-26+FoxP3+ T cells were generated and expanded in secondary lymphoid tissues (data not shown). At day 3 after immunization, KJ1-26+ FoxP3+ (or FoxP3−) T cells were still found mainly in secondary lymphoid tissues (Fig. 4B). By day 8, however, many KJ1-26+ FoxP3+ T cells were found in nonlymphoid tissues such as bone marrow and lamina propria of the small intestine (Fig. 4B). It was notable that it was CD62L+FoxP3+ T cells, but not CD62L−FoxP3− T cells, that were increased in the nonlymphoid tissues (Fig. 4B). The appearance rate for KJ1-26+ CD62L+FoxP3+ T cells in nonlymphoid tissue was significantly higher than the rate for their FoxP3− counterparts. In contrast, the FoxP3+ T cells in control mice, injected with adjuvant only without the Ag, were gradually decreased in numbers in secondary lymphoid tissue at days 3 and 8 and did not appear in the nonlymphoid tissue at significant numbers, suggesting that the redistribution (potentially the result of migration and/or expansion) of FoxP3+ T cells to nonlymphoid tissue is an Ag-specific response.

It has been reported that FoxP3− T cells can convert to FoxP3+ T cells following Ag priming. Therefore, it is possible that the

FIGURE 6. Ag priming in secondary lymphoid tissue induces the second chemokine receptor switch in FoxP3+ T cells. A, Splenocytes containing OVA-specific KJ1-26+FoxP3+ T cells, obtained from mOVA × DO11.10 mice, were injected i.v. into BALB/c mice. Chemokine receptor expression by KJ1-26+FoxP3+ T cells in PLN before and after immunization s.c. at the flank (or s.c. and i.p. at the same time) with OVA plus CFA (or CFA alone) was compared. Multiple control Abs (CTR Ab) with different isotypes were used to determine background staining levels. Combined data from four independent experiments are shown. *, p ≤ 0.05 for significant difference between FoxP3+ and FoxP3− cells. B and C, The second trafficking receptor switch in vitro. MLN KJ1-26+ T cells of mOVA × DO11.10 mice were labeled with CFSE and cultured with OVA323-339 in the presence of irradiated splenocytes as APCs. On day 5, FoxP3+ or FoxP3− KJ1-26+CD4+ T cells at different numbers of cell division were examined for their expression of chemokine receptors. Combined data from three experiments are shown.

Downloaded from http://www.jimmunol.org/ by guest on November 13, 2017
FoxP3⁺ cells in the periphery of the immunized mice in Fig. 4B are from FoxP3⁻ T cells. To examine this possibility, we transferred DO11.10 rag2⁻/⁻ T cells, which are free of KJ1-26⁺FoxP3⁻ T cells as shown in Fig. 4A, into BALB/c mice and immunized the host mice with OVA (Fig. 4C). The average conversion rate of KJ1-26⁺FoxP3⁻ T cells to FoxP3⁺CD4⁺ T cells was ~0.5% at day 3 and ~1.0% at day 8 in the spleen and lymph nodes of BALB/c mice after immunization. These conversion rates are almost negligible, compared with the high FoxP3⁺ T cell frequency (40–60% of KJ1-26⁺CD4⁺ T cells) in the mice injected with mOVA × DO11.10 CD4⁺ cells. These data suggest that most Ag-specific FoxP3⁺ T cells, present in host mice after immunization, were probably from injected FoxP3⁺ but not FoxP3⁻ T cells.

Differential chemokine receptor expression by FoxP3⁺ T cell subsets in secondary lymphoid tissues

Because trafficking receptors are important for organ to organ migration of T cells, we next examined the trafficking receptor phenotype of the CD62L⁺ and CD62L⁻FoxP3⁺ T cell subsets in MLN and PLN of unimmunized BALB/c mice (Fig. 5). CD62L⁺FoxP3⁺ T cells in MLN mainly expressed the two lymphoid tissue-associated chemokine receptors CCR7 and CXCR4, along with CCR6, CCR8, and CCR9 at low frequencies (10–20%) (Fig. 5A). This pattern was a very different from that of the chemokine receptors expressed by CD62L⁻FoxP3⁺ T cells, many of which expressed CCR2, CCR4, CCR5, CCR6, CCR8, CXCR3, and CXCR6 in addition to CCR7 and CXCR4.

In a manner similar to the FoxP3⁺ T cell subsets in MLN, the two FoxP3⁻ T cell subsets in PLNs differentially expressed chemokine receptors (Fig. 5B). CD62L⁻FoxP3⁺ T cells in PLN mainly expressed CCR7 and CXCR4, whereas CD62L⁺FoxP3⁺ T cells had the two receptors plus a number of memory/effector/inflammation-associated chemokine receptors. Reduced expression of CCR7 by some CD62L⁺FoxP3⁺ T cells and up-regulation of memory/effector/inflammation-associated chemokine receptors by FoxP3⁺ T cells compared with FoxP3⁻ cells were again evident.

Ag priming in secondary lymphoid tissue induces the second switch in trafficking receptors in FoxP3⁺ T cells in an accelerated manner compared with FoxP3⁻ T cells

The differences in trafficking receptor expression between CD62L⁺ and CD62L⁻FoxP3⁺ T cells, shown in Fig. 5, and the generation of CD62L⁻FoxP3⁺ T cells from CD62L⁺ cells upon Ag priming, shown in Fig. 4, suggest the presence of another switch in trafficking receptors. To demonstrate the switch, we adoptively transferred splenocytes containing OVA-specific FoxP3⁺ cells, obtained from mOVA × DO11.10 double transgenic mice, into BALB/c mice and examined their up-regulation of chemokine receptors upon antigen challenge. Before cell transfer, the injected KJ1-26⁺FoxP3⁻ T cells largely expressed only two receptors (CCR7 and CXCR4) among the 14 chemokine receptors examined (only seven receptors are shown in Fig. 6A). On day 3 after immunization with OVA, however, many FoxP3⁺ T cells found in PLN acquired CCR4, CCR6, CCR8, and CXCR3 at high frequencies (Fig. 6A). Importantly, FoxP3⁺ T cells gained the expression of the memory/effector/inflammation-associated chemokine receptors at much higher frequencies than FoxP3⁻ T cells. During the 3-day period, 50–60% of FoxP3⁻ and FoxP3⁺ T cells lost the expression of CCR7 and CXCR4. The decrease in CCR7 and increase in memory/effector/inflammation-associated receptor expression suggest the presence of a chemokine receptor switch in FoxP3⁺ T cells following antigenic stimulation. Compared with

![Image](http://www.jimmunol.org/)

**FIGURE 7.** The FoxP3⁺ T cells that populate nonlymphoid tissues following immunization show the trafficking receptor phenotype after the second switch. Splenocytes containing OVA-specific KJ1-26⁺FoxP3⁻ T cells, obtained from mOVA × DO11.10 mice, were adoptively transferred into BALB/c mice. The trafficking receptor phenotype of KJ1-26⁺FoxP3⁻ T cells in secondary lymphoid tissue (2° LT) and nonlymphoid tissue (Non-LT) at day 8 postimmunization with OVA plus CFA is shown. PLN, MLN, peritoneal cavity (PC), and small intestine (SI) are represented. Representative data from three independent experiments are shown.

**FIGURE 8.** The accelerated second switch in FoxP3⁺ T cells, compared with FoxP3⁻ T cells, is not due to relative suppression of FoxP3⁺ T cells by cojected FoxP3⁻ T cells. Splenocytes containing KJ1-26⁺ T cells were transferred from DO11.10 (rag2⁻/⁻) mice or mOVA × DO11.10 (rag2⁻/⁻) mice into BALB/c mice. Twenty hours later, the host mice were immunized with OVA. Three days later, FoxP3⁺ and FoxP3⁻ KJ1-26⁺CD4⁺ T cells were examined for their expression of chemokine receptors. Combined data from four independent experiments are shown. *p ≤ 0.05 is significant difference compared with FoxP3⁻ T cells.
day 3, the numbers of FoxP3+ T cells expressing the nonlymphoid tissues homing chemokine receptors were decreased at day 8 in PLN, MLN, and spleen, whereas the proportions of CXCR4+ and CCR7+ FoxP3+ T cells were increased (data not shown). Control mice were injected with the adjuvant without OVA, and FoxP3+ T cells in these mice did not undergo the chemokine receptor switch that occurred in mice immunized with OVA, suggesting that the trafficking receptor switch of FoxP3+ T cells in secondary lymphoid tissues is an Ag–specific response. Similar switches in trafficking receptors of the FoxP3+ T cells were also observed in spleen, PP, and MLN (data not shown). It has been reported that TLR ligands (e.g., mycobacterial products in CFA) may affect the biology of FoxP3+ cells (40, 41). To rule out the potential modulation effects of CFA on expression of FoxP3 or chemokine receptors, we also used IFA for immunization. The results with IFA (data not shown) were almost identical with those found in mice immunized with CFA, and thus, the differences in trafficking receptor switch between FoxP3+ and FoxP3− T cells are not attributable to the type of adjuvant used.

We, next, examined whether mouse FoxP3+ T cells undergo the same accelerated switch in vitro. For this study, CFSE-labeled MLN cells containing FoxP3+ and FoxP3− T cells obtained from mOVA × DO11.10 double transgenic mice were cultured for 5 days with the Ag peptide OVA323–339. Consistent with the in vivo data (Fig. 6A), the hyperexpression of nonlymphoid tissue homing receptors by primed FoxP3+ T cells compared with FoxP3− T cells was also detected in vitro (Fig. 6B and C). The difference between FoxP3+ and FoxP3− T cells in expression of major receptors was evident for the cells after various numbers of cell divisions. CCR7 expression was similarly down-regulated on both FoxP3+ and FoxP3− cells as they divided. In contrast, CCR4 and α4β7 were gradually up-regulated with cell division. Other chemokine receptors such as CCR8, CCR9, and CXCR6 were gradually down-regulated.

Ag-primed and nonlymphoid tissue-residing FoxP3+ T cells have the postsecond switch trafficking receptor phenotype

To determine the trafficking receptor switch status of the FoxP3+ T cells that appear in nonlymphoid vs secondary lymphoid tissues after Ag priming, KJ1-26+ (OVA-specific), FoxP3+ T cells were isolated from selected nonlymphoid tissues (small intestine and peritoneal cavity) of BALB/c mice, previously injected i.v. with mOVA × DO11.10 cells and immunized with OVA, and examined for their trafficking receptor profiles (Fig. 7). The KJ1-26+FoxP3+ T cells in the nonlymphoid tissues of mice had very different trafficking receptor phenotypes than that of secondary lymphoid tissue at day 8 postimunization. Although the majority of KJ1-26+FoxP3+ T cells in secondary lymphoid tissue expressed CCR7, the majority of KJ1-26+FoxP3− T cells in nonlymphoid tissues did not express it. Instead, many of KJ1-26+FoxP3+ T cells in nonlymphoid tissues expressed tissue-specific homing receptor such as CCR9 (small intestine) and memory/effector type homing receptors such as CCR4, CCR6, and CXCR3 (peritoneal cavity).

The hyperexpression of nonlymphoid tissue homing receptors by Ag-primed FoxP3+ T cells compared with FoxP3− T cells after the second switch is not due to relative suppression of FoxP3− T cells by FoxP3+ T cells during Ag priming

It is possible that the apparent hyperexpression of nonlymphoid tissue homing receptors by secondary lymphoid tissue FoxP3+ T cells (and the relative hypexpression by FoxP3− T cells) would be due to suppression of the up-regulation of the trafficking receptors on FoxP3+ T cells by the FoxP3+ T cells cotransferred together. To rule out this possibility, BALB/c mice were adoptively injected i.v. with splenocytes of DO11.10 (rag2−/−) mice, which do not contain KJ1-26+FoxP3+ cells) (Fig. 4A) and immunized with OVA. In parallel, a separate group of BALB/c mice were
injected i.v. with mOVA × DO11.10 (rag2−/−) splenocytes containing both KJ1-26 FoxP3+ and FoxP3− subsets and immunized with OVA for comparison. Chemokine receptor switch on the KJ1-26 FoxP3+ T cells, Ag-primed in the presence or absence of KJ1-26 FoxP3+ T cells, was compared (Fig. 8). In the situation in which splenocytes containing KJ1-26 FoxP3− CD4+ T cells without KJ1-26 FoxP3+ cells were transferred, the KJ1-26 FoxP3+ cells up-regulated memory-effector-associated receptors (e.g., CCR2, CCR4, CCR6, CCR8, CXCXR3, and CXCR5) at levels similar to (or even lower for some receptors than) the KJ1-26 FoxP3− T cells, primed in the presence of KJ1-26 FoxP3+ T cells. Therefore, the accelerated chemokine receptor switch by FoxP3+ T cells appears not to be due to potential suppression of chemokine receptor expression in FoxP3− T cells by FoxP3+ T cells with the same Ag specificity. When compared with KJ1-26 FoxP3− CD4+ cells, FoxP3+ T cells, primed in both MLN and PLN, more efficiently up-regulated CCR2, CCR4, CCR6, and CCR8, again providing evidence for the presence of the accelerated second switch in chemokine receptors. It was notable that only the FoxP3+ T cells, primed in MLN but not PLN, expressed gut homing-related αβ and CCR9 at significant levels. Skin homing-related CCR8 was expressed on many more FoxP3+ T cells primed in PLN than in MLN. Again, these results suggest that FoxP3+ T cells are programmed to efficiently express the memory/effector-type trafficking receptors upon Ag priming, and, importantly, this occurs at highly accelerated rates for FoxP3+ T cells compared with FoxP3− CD4+ T cells.

Ag-primed FoxP3+ T cells, after the accelerated second switch, can migrate more efficiently to nonlymphoid tissues than FoxP3− T cells

To directly determine the functional consequence of the accelerated chemokine receptor switch, we performed a short-term (20 h) in vivo homing study of Ag-primed FoxP3+ T cells (Fig. 9). The 20-h time period for homing was chosen because it was long enough for the primed cells to migrate to various tissue sites but not enough for their expansion or induction at these sites. Ag-primed FoxP3+ T cells were prepared in vivo by transfer of OVA-specific FoxP3+ T cells into BALB/c mice followed by immunization. Eight days later, splenocytes containing Ag-primed FoxP3+ and FoxP3− T cells were harvested from the immunized mice and then reinfected into BALB/c mice to determine their migration capacity to various tissues within 20 h. Absolute numbers of the OVA-specific FoxP3+ and FoxP3− T cells that migrated to various organs were determined. As shown in Fig. 9A, KJ1-26 CD62L+FoxP3+ and FoxP3− T cells preferentially migrated to secondary lymphoid tissues (spleen, MLN, and PLN) at levels similar to each other. Some CD62L−FoxP3+ T cells migrated to nonlymphoid tissues at low levels (Fig. 9A). KJ1-26 CD62L−FoxP3+ also migrated to secondary lymphoid tissue at somewhat reduced rates but more efficiently migrated to nonlymphoid tissues (i.e., bone marrow and lamina propria of small intestine) than their FoxP3− counterparts. A notable difference between FoxP3+ and FoxP3− cells was that CD62L−FoxP3+ T cells significantly better migrated than their FoxP3− counterparts into the nonlymphoid tissues (Fig. 9A). Only FoxP3+, but not FoxP3−, memory T cells migrated into bone marrow at significant levels. Although the absolute numbers of the CD62L−FoxP3+ cells that migrated to the peritoneal cavity were small due to the small organ size (Fig. 9A), cells were better enriched in the peritoneal cavity than in the secondary lymphoid tissue (Fig. 9, B and C).

Discussion

We investigated the development of FoxP3+ T cell homing program in primary and secondary lymphoid tissues, a process that is important for migration of FoxP3+ T cells to target tissue sites, but has been incompletely understood so far. Our results show the orderly switches of FoxP3+ T cell trafficking receptors and migration behavior during their development in the thymus and secondary lymphoid tissues. FoxP3+ T cells, generated in the thymus, undergo the first trafficking receptor switch for emigration to secondary lymphoid tissues. FoxP3+ thymic emigrants migrate to secondary lymphoid tissues and undergo the second round of trafficking receptor switch to migrate to nonlymphoid tissues. Interestingly, the second switch of FoxP3+ T cells is highly robust, compared with that of conventional CD4+ T cells. Only the FoxP3+ T cells that underwent the second switch in trafficking receptor expression were able to migrate efficiently to nonlymphoid tissues.

Prothymocytes sequentially undergo CD4−CD8− (DN), CD4+CD8+ (DP), and CD4−CD8− or CD4+CD8+ (SP) stages of development. These thymocyte subsets are differentially located in the thymus: DN cells are found in the subcapsular region, whereas DP cells are located in the cortex. CD4−CD8− or CD4+CD8+ SP T cells are located in the medulla of thymus. Previously, others and we found that thymocytes undergo developmental changes in chemokine receptor expression (42–45), and chemokine receptors play important roles in T cell maturation and/or localization within the thymus (46–49). Our data show that FoxP3+ thymocytes undergo a developmental switch in trafficking receptor expression from CXCR4 mediumCCR9 highCCR5 mediumCCR7 low (DN FoxP3− cells) to CXCR4 highCCR9 highCCR5 mediumCCR7 low (DP FoxP3− cells), and finally to CXCR4 mediumCCR9 lowCCR5 lowCCR7 high (mature SP FoxP3− cells). The CCR9 ligand CCL25 is expressed by thymic epithelial cells and dendritic cells (50, 51), and provides the signal important for their localization in the subcapsular region of the thymus (44, 52). The major CCR8 ligand CCL1 is expressed by thymic macrophages and fibrous septa epithelial cells (53). A CCR7 ligand CCL19 is predominantly expressed in the medulla including endothelial venules (54). Considering their expression sites, these chemokine receptors may regulate localization of DP and SP FoxP3− T cells in different microenvironments between subcapsular area and medulla. Consistent with the trafficking receptor phenotype (CXCR4 mediumCCR9 lowCCR5 lowCCR7 high cells), our data show that recent FoxP3+ thymic emigrants almost exclusively migrate to secondary lymphoid tissues. Once within the secondary lymphoid tissues, they regain CXCR4 to become CXCR4 high CCR7 high cells, which are mainly localized in the T cell zones.

As shown in Fig. 1, certain FoxP3+ T cells are highly enriched in selected nonlymphoid tissues, data that support and expand previous reports by others on unconventional enrichment of Tregs in certain tissue sites (22, 24, 27). The fact that FoxP3+ T cells, freshly made in the thymus, almost exclusively migrate to secondary lymphoid tissue points out the need for additional trafficking receptor switches to migrate from secondary to nonlymphoid tissues. Alternatively, it is possible that the enrichment may occur because FoxP3+ T cells can be converted from FoxP3− naïve T cells and/or expanded from existing FoxP3+ cells in nonlymphoid tissues. In the experimental models used in this study, the conversion rate of FoxP3+ T cells to FoxP3− cells within the given time period was negligible (Fig. 4C). Furthermore, the results of our 20-h short-term homing study revealed that migration of Ag-primed FoxP3+ cells does occur in a highly efficient manner. Therefore, the results support that FoxP3+ T cells found in the
nonlymphoid tissues were probably the FoxP3+ T cells that migrated from secondary lymphoid tissues after Ag priming and/or originated from them if the FoxP3+ T cells underwent expansion in the nonlymphoid tissue.

We hypothesize that FoxP3+ T cells acquire the ability to migrate to nonlymphoid tissues through the trafficking receptor switch in secondary lymphoid tissues. Our data indeed show that FoxP3+ T cells in secondary lymphoid tissues undergo an Ag-driven switch in trafficking receptors: down-regulation of lymphoid tissue homing receptors (L-selectin, CCR7, and CXCR4) but up-regulation of a number of memory/effector chemokine receptors (CCR2, CCR4, CCR5, CXCR6, CCR8, CCR9, CXCR3, CXCR5, and CXCR6). These mouse FoxP3+ T cells are particularly efficient at up-regulation of memory/inflammation/nonlymphoid tissue-related trafficking receptors, a universal feature shared with human FoxP3+ T cells (30). We found in this study that FoxP3+ T cells and FoxP3− T cells are intrinsically different in up-regulation of many trafficking receptors in secondary lymphoid tissue. Furthermore, we were able to demonstrate the differences in vitro. It remains to be determined whether the transcription factor FoxP3, together with the TCR signal, would directly or indirectly control expression of the chemokine receptors at the molecular level.

Recently, a report raised a possibility that regulatory T cells may suppress activation of non-FoxP3+ T cells and up-regulation of a chemokine receptor CXCR3 (55). It is possible that activation of FoxP3− T cells may be suppressed by the FoxP3+ T cells sharing the same Ag specificity to make FoxP3+ T cells less efficient in the chemokine receptor switch. However, our data that FoxP3− and FoxP3+ T cells lost CCR7 and CXCR4 at similar rates following antigenic stimulation (Fig. 6A) suggest that both FoxP3+ and FoxP3− T cells were comparably activated. To more rigorously demonstrate the differences in chemokine receptor switch, we performed experiments also with FoxP3+ T cells from DO11.10 rag2−/− mice (Fig. 8). These mice have OVA-specific FoxP3+ CD4+ T cells but not FoxP3− cells, while mOVA × DO11.10 rag2−/− mice are highly enriched with FoxP3+ cells. We did not use wild-type FoxP3− T cells because it is not feasible to observe synchronized chemokine receptor switches using polyclonal wild-type FoxP3− T cells in an Ag-dependent manner in vivo. These experiments demonstrated that up-regulation of memory/effector type chemokine receptors in KJ1-26 FoxP3+ T cells was not suppressed by coinjected KJ1-26 FoxP3− T cells. Therefore, the available evidence, in vivo and in vitro, presented in this report supports the hypothesis that FoxP3+ T cells are intrinsically more efficient in up-regulation of nonlymphoid tissue trafficking receptors than FoxP3− CD4+ T cells. Additionally, we found that FoxP3− T cells, transferred from two different mouse strains (DO11.10 rag2−/− mice and mOVA × DO11.10 rag2−/− mice) behave similarly in expression of many chemokine receptors. However, up-regulation of a few receptors such as CCR4, CCR7, and αββγ was weaker on FoxP3− T cells transferred from DO11.10 rag2−/− mice than those from mOVA × DO11.10 rag2−/− mice. This result is unexpected and warrants further investigation in the future.

It has been undetermined where and when Foxp3+ T cells acquire tissue-specific trafficking receptors such as gut homing-related CCR9 and αββγ, and skin homing-related CCR4 and CXCR8. We found that FoxP3+ T cell expression of CCR9 and αββγ is induced in MLN, but not in PLN (Figs. 7 and 8). In contrast, CCR8 is expressed by many more FoxP3+ T cells primed in PLN than MLN. CCR4 is highly expressed by the FoxP3+ cells in both organs, and thus, cannot distinguish FoxP3+ T cells primed in skin vs gut. These results suggest that it is the second switch after which the naive-like FoxP3+ T cells with homogeneous trafficking potential to secondary nonlymphoid tissue diverge into FoxP3− T cells with heterogeneous trafficking potentials to different tissue sites.

The FoxP3+ cells that efficiently migrate to nonlymphoid tissue sites are CD62L+, but not CD62L−, cells. In contrast, the CD62L+ T cell subset mainly migrates to secondary lymphoid tissues, which is consistent with another report by tracking CD25+ or CD103+ T cells (20). This difference in trafficking behavior is in line with the different yet complementary expression patterns of chemokine receptors by the two FoxP3+ T cell subsets. CD62L+FoxP3+ cells mainly express CCR7 and CXCR4. The ligands of these chemokine receptors, such as CXCL12 (CXCR4), CCL19, and CCL21 (CCR7), are expressed in secondary lymphoid tissues and play important roles in lymphocyte recruitment to secondary lymphoid tissue (56–58). In contrast, CD62L+FoxP3+ cells express reduced levels of CCR7 and CXCR4 but high levels of a panel of memory/effector type chemokine receptors such as CCR2, CCR4, CCR5, CXCR6, CXCR8, CXCR3, CXCR5, and CXCR6. These chemokine receptors are implicated in memory/effector T cell migration to nonlymphoid tissues or sites of inflammation. Importantly, many more CD62L+ FoxP3+ cells than CD62L− FoxP3− cells express such chemokine receptors, consistent with the relative abundance of FoxP3+ T cells in the selected nonlymphoid tissues.

Taken together, our results show that Foxp3+ T cells undergo programmed switches in migratory behavior and trafficking receptors in the thymus and secondary lymphoid tissues. The first switch occurring in the thymus is consistent with the specific emigration of FoxP3+ T cells into secondary lymphoid tissues. The second switch in secondary lymphoid tissues upon Ag priming is also consistent with their migration to nonlymphoid tissues or sites of inflammation. Moreover, our study revealed the high expression of nonlymphoid tissue homing receptors by Foxp3+ T cells compared with Foxp3− T cells after the second switch, which is consistent with their efficient migration and enrichment in major nonlymphoid tissues. Also at the second switch, Foxp3+ T cells can diverge into heterogeneous groups in terms of trafficking receptors. Although the first switch is a programmed one leading to generation of Foxp3+ T cells with a largely homogeneous trafficking potential to secondary lymphoid tissues, the robust second switch upon Ag priming in secondary lymphoid tissues appears to generate Foxp3+ cell subsets with heterogeneous homing potentials to different secondary lymphoid tissues.

Acknowledgments

We thank C. W. Wang and H. W. Lim for helpful input (Kim laboratory). We also thank Dr. A. Abbas (University of California, San Francisco, CA) and Dr. W. Heath (Walter and Eliza Hall Institute) for RIP-mOVA mice and Dr. K. Hieshima (Kinki University) for the CCL27-Fc fusion vector.

Disclosures

The authors have no financial conflict of interest.

References


33. Hieshima, K., H. Ohtani, M. Shibano, D. Izawa, T. Nakayama, Y. Kawasaki,
12. Kawahata, K., Y. Misaki, M. Yamauchi, S. Tsunekawa, K. Setoguchi,
57. Förster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, and
31. The Journal of Immunology
efficacious chemotaxant for human thymocytes, uses GPR-6/CCR9 as a spec-
138. Benz, C. K., Heinzell, and C. C. Bleu. 2004. Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute require-
140. Ruiz, C. A., S. B. Burkett, A. Panoskaltsis-Mortari, S. L. Kirby, A. D. Luster,
141. Crellin, N. K., R. V. Garcia, O. Hadisfar, S. E. Allan, T. S. Steiner, and
132. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
136. Kwon, J., and N. Killeen. 2004. CCR7 directs the migration of thymocytes into sup-
137. Ueno, T., K. Hara, M. S. Willis, M. A. Malin, U. E. Hopken, D. H. Gray,
CCR4+ regulatory T cells in breast cancer express CD25+ and inhibit cytokine 
tracts double- and single-positive thymocytes expressing the TECK receptor
144. Ekstein, B., A. Miles, S. M. Curbishley, C. Tselips, A. J. Grant, L. S. Walker, and D. H. Adams. 2006. Epithelial inflammation is associated with CCL28 produc-
145. Ishida, T., T. Ishii, A. Inagaki, H. Yano, H. Komatsu, S. Iida, H. Inagaki, and
147. Benz, C. K., Heinzell, and C. C. Bleu. 2004. Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute require-
149. Ruiz, C. A., S. B. Burkett, A. Panoskaltsis-Mortari, S. L. Kirby, A. D. Luster,
150. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
151. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
152. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
153. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
154. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
155. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
156. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
157. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
158. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
159. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
160. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
161. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
162. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
163. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
164. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
165. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
166. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
167. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
168. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
169. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
170. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
171. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
172. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,
173. Wurbel, M. A., J. M. Philippe, C. Nguyen, G. Victorero, T. Freeman,