IL-2 Receptor Signaling Is Essential for the Development of Klrg1+ Terminally Differentiated T Regulatory Cells

Guoyan Cheng, Xiaomei Yuan, Matthew S. Tsai, Eckhard R. Podack, Aixin Yu and Thomas R. Malek

*J Immunol* 2012; 189:1780-1791; Prepublished online 11 July 2012; doi: 10.4049/jimmunol.1103768

http://www.jimmunol.org/content/189/4/1780

References

This article cites 50 articles, 27 of which you can access for free at:

http://www.jimmunol.org/content/189/4/1780.full#ref-list-1

Why *The JI*? Submit online.

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

*average

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
IL-2 Receptor Signaling Is Essential for the Development of Klrg1+ Terminally Differentiated T Regulatory Cells

Guoyan Cheng,*1 Xiaomei Yuan,*1 Matthew S. Tsai,* Eckhard R. Podack,* Aixin Yu,* and Thomas R. Malek*†

Thymic-derived natural T regulatory cells (Tregs) are characterized by functional and phenotypic heterogeneity. Recently, a small fraction of peripheral Tregs has been shown to express Klrg1, but it remains unclear as to what extent Klrg1 defines a unique Treg subset. In this study, we show that Klrg1+ Tregs represent a terminally differentiated Treg subset derived from Klrg1− Tregs. This subset is a recent Ag-responsive and highly activated short-lived Treg population that expresses enhanced levels of Treg suppressive molecules and that preferentially resides within mucosal tissues. The development of Klrg1+ Tregs also requires extensive IL-2R signaling. This activity represents a distinct function for IL-2, independent from its contribution to Treg homeostasis and competitive fitness. These and other properties are analogous to terminally differentiated short-lived CD8+ T effector cells. Our findings suggest that an important pathway driving Ag-activated conventional T lymphocytes also operates for Tregs. The Journal of Immunology, 2012, 189: 1780–1791.

natural CD4+ Foxp3+ T regulatory cells (nTregs) are a dedicated thymic-derived population that critically functions to maintain self-tolerance by actively suppressing autoreactive T cells that escape thymic negative selection (1). However, upon Ag stimulation, conventional peripheral CD4+ T cells sometimes express Foxp3 and adopt an induced Treg (iTreg) fate (2). These iTregs are more numerous in mucosal tissues and are thought to limit immune responses to antigenic stimulation, especially to environmental Ags, such as food and allergens, and commensal microorganisms (3).

nTregs show extensive phenotypic heterogeneity with respect to surface markers of T cell activation (CD62L, CD69), adhesion molecules (CD103), and chemokine receptors (CCR7 and CCR6), analogous to subpopulations of naive, effector, and memory Tregs (4–10). More recently, Klrg1 has been found on a small proportion of Tregs in the periphery with enhanced suppressive function, and these Klrg1+ Tregs exhibit a gene expression profile of “activated” effector Tregs (11–13). Tregs also exhibit functional heterogeneity in that they use the transcription factors (i.e., T-bet, IRF4, and Stat3) for distinctive suppressive programs to inhibit inflammatory Th1, Th2, and Th17 responses, respectively (14–16). However, the extent to which this phenotypic and functional heterogeneity defines unique versus interrelated Treg subpopulations and the cell extrinsic factors favoring particular Treg subsets remain poorly defined.

IL-2 provides essential nonredundant signals at several levels for Tregs (17). First, IL-2 is essential for nTreg thymic development (18–20). Second, IL-2 induces proliferation of recent thymic emigrants in neonatal life to amplify nTreg numbers to establish an immune tolerant state (21). Third, IL-2 is the main cytokine for homeostasis and competitive fitness of peripheral nTregs in adult mice (22–24). Lastly, Ag-activated conventional T cells readily differentiate into iTregs when stimulated with IL-2 and TGF-β (25, 26). More recently, we showed that thymic Treg development and peripheral homeostasis were normal in mice that expressed mutations within signaling domains of IL-2Rβ that greatly diminished, but did not abrogate, Stat5 activation (27). Such mice were overly healthy, but with age exhibited inflammatory infiltrates in several tissues, particularly the salivary gland, lung, liver, and intestine. Consistent with this phenotype, gene expression profiling of nTregs with impaired IL-2R showed that several key IL-2–dependent genes of Tregs (e.g., Foxp3, and Il2ra) were normally expressed. However, many other genes remained IL-2 dependent, including Klrg1, Itgae (CD103), Il10, Gzmb, and Prdm1 (Blimp-1) (27). This finding suggests that IL-2 provides additional important functions in the periphery for Tregs besides homeostasis and raises the possibility that IL-2R is required for the development of Treg subsets marked by expression of Klrg1 and/or CD103.

In this study, we examined the properties associated with Klrg1+ Tregs and their relationship to the other Treg subsets. As extensive IL-2R signaling is necessary for optimal expression of Klrg1 (27), we also examined whether this reflected a distinct role for IL-2 in the persistence and development of a unique subset based on this surface marker. In comparison with other Treg subsets based on expression of CD62L and CD69, we find that Klrg1 defines a terminally differentiated Treg subpopulation with a distinctive molecular profile. Klrg1+ Tregs preferentially reside within mucosal sites and require strong IL-2R signaling for their development.
Materials and Methods

Mice

C57BL6 (B6) mice and TCRε−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CD45.1-.congenic B6 and IL-15−/− mice were obtained from Taconic. The reporter mice, Foxp3/GFP (28) (kindly provided by A. Rudensky, Memorial Sloan-Kettering Cancer Center), Foxp3/RFP (29) (kindly provided by R.A. Flavell), and Blimp-1/GFP (30) (kindly provided by S.N. Watt and Eliza Hall Institute of Medical Research) were previously described. IL-2Rβ transgenic mice, IL-2RγWT (Y0), IL-2RγY341,395Aβ (Y3) mice, on the IL2R−/− genetic background, were previously described (27). Blimp-1/GFP mice and Y3 mice were each crossed to Foxp3/RFP mice to mark their Tregs with the RFP reporter. All mice were maintained in an animal facility under VAF conditions at the University of Miami. Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Cell preparation and purification

Single-cell suspensions from spleen, mesenteric lymph node (MLN), and Peyers’ patch (PP) were prepared by mechanical disruption. Lamiporta (LP) cells were prepared as previously described (31) with minor modifications. In brief, the small intestine was obtained after cutting 0.5 cm below the stomach and 1 cm above the cecum and flushed with HBSS containing 5% FBS and 1.3 mM EDTA. Gut pieces (2–5 mm) and treated (shaking at 200 rpm for 20 min at 37˚C) with Ca2+/Mg2+-free HBSS containing 5% FBS and trypsin inhibitor (0.24 mg/ml). LP cells were collected after filtration using a 70-μm cell strainer and then purified on a 44%/66% Percoll gradient (800 μl) and conventional CD4+ T cells were purified by sorting. In brief, splenic CD4+ T cells were washed with RMPI 1640 and further treated (shaking at 200 rpm for 20 min at 37˚C) with Ca2+/Mg2+-free HBSS containing 5% FBS and 1.3 mM EDTA. Gut pieces were washed with RPMI 1640 and further treated (shaking at 200 rpm for 60 min at 37˚C) with RPMI 1640 containing 5% FBS, collagenase VIII (30 U/ml), and trypsin inhibitor (0.24 mg/ml). LP cells were collected after filtration using a 70-μm cell strainer and then purified on the IL2R−/− genetic background, were previously described (27). Blimp-1/GFP mice and Y3 mice were each crossed to Foxp3/RFP mice to mark their Tregs with the RFP reporter. All mice were maintained in an animal facility under VAF conditions at the University of Miami. Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Flow Inhalation Exposure System (CH Technologies). Mice were sacrificed with an i.p. boost on day 5. On day 12, mice were aerosol challenged with g OVA (crystallized OVA, Sigma-Aldrich, St. Louis, MO) adsorbed to 6.6 μg alum (aluminum potassium sulfate; Sigma-Aldrich) in 200 μl PBS with an i.p. boost on day 5. On day 12, mice were aerosol challenged with 0.5% w/v OVA (Sigma-Aldrich) in PBS for 1 h using a BANG nebulizer (CH Technologies, Westwood, MA) into a Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (CH Technologies). Mice were sacrificed either on day 12 without aerosol or on day 15 (i.e., 3 d after the aerosol challenge). Bronchoalveolar lavages were obtained and lung lobes were perfused and processed for single-cell suspensions made from lung homogenate for flow cytometry analysis as described previously (32). Draining bronchial lymph nodes and spleens were also procured for subsequent flow cytometry analysis.

BrdU incorporation assay

Mice received 0.8 mg/ml BrdU containing drinking water for 5 d. The incorporation of BrdU was assessed by FACs using anti-BrdU by a minor modification of the manufacturer’s instructions (BD Pharmingen). Briefly, splenic cells were stained with surface markers for 15 min on ice. After washing, cells were fixed with Cytofix/Cytoperm Buffer for another 15 min and then incubated with Cytoperm Plus Buffer for 20 min on ice. Cells were washed again and refixed with Cytofix/Cytoperm Buffer for 5 min. After DNase (300 μg/ml) treatment, cells were then stained with fluorescent anti-BrdU and other intracellular Abs for 20 min at room temperature.

Treg suppression and survival assays in vitro

Suppression was assessed by the capacity of purified Treg subsets to inhibit anti-CD3 (0.25 μg/ml) induced proliferation by conventional CD4+ T cells as previously described (18). To assess Treg survival, magnetic bead-enriched splenic CD4+ T cells from B6 mice were cultured in 24-well plates (1 × 10^6 to 2 × 10^6/well) in complete medium (33) for 3 d. At the indicated times, Tregs were enumerated by staining for Foxp3 and viability assessed by using the Live/Dead fixable stain kit (Invitrogen). Briefly, cells were harvested and incubated with the reconstituted fluorescent reactive dye for 30 min at room temperature. After washing by Hank’s buffer, the cells were stained with surface markers and then fixed for intracellular Foxp3 staining. These data in conjunction with the number of recovered Tregs were used to calculate the survival of Tregs.

Microarray analysis

Total RNA was isolated using TRIzol and further purified with RNAeasy Minikit (Qiagen). RNA quantity and quality was assessed by analyzing with an Agilent 2100 BioAnalyzer. Twenty to fifty nanograms of RNA was used in a single round of linear RNA probe amplification and labeling using NuGEN Ovation Pico WT A system, WT-Ovation Exon module, and Encore Biotin Module (NuGEN, San Carlos, CA). Probe preparation and microarray analyses using Affymetrix Mouse Gene ST 1.0 arrays were performed at the Microarray and Gene Expression Core within the John P. Hussman Institute for Human Genomics at the University of Miami. Image analysis was performed using the Affymetrix Command Console Software. Resulting data were normalized with the RNA method using software at GeneSlicer (Seattle, WA). Multigroup comparisons of the transformed data of at least two independent biological replicates were performed using ANOVA applying the Benjamini Hochberg correction for false positives. Genes expressed ≥2.0-fold up or down (p < 0.05) between groups were considered differentially expressed. Gene enrichment analyses for differentially expressed genes for Gene Ontology processes or KEGG pathways were performed using GeneSlicer. The expression data are available through the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE36527.

Adoptive transfer

The indicated purified Treg population was adoptively transferred by i.v. injection through the tail vein into B6, Y3, CD45.1-B6, or TCRε−/− recipients, as indicated in each illustration. In most cases, TCRε−/− recipients were coincubated with Treg-depleted conventional CD4+ T cells using Foxp3/GFP reporter mice to achieve an ∼1:10 ratio of Treg/ conventional T cells. To study developmental progression, Tregs were labeled with CFSE using the Vibrant CFDA SE Cell Tracer Kit (Sigma-Aldrich) prior to transfer. The Tregs (5 × 10^6/ml) were incubated with 5 μM CFSE in RPMI 1640 containing 5% FBS for 15 min at 37˚C according to the manufacturer’s instructions.

IL-2 immune complex treatment

Mouse IL-2 and the JES6-1A12 mAb to mouse IL-2 were purchased from BioLegend (San Diego, CA). B6 mice were inoculated daily with 200 μl IL-2 (100 U) containing 5% FBS through an 18-20 gauge IV catheter placed in the tail vein under isoflurane anesthesia or received only 200 μl PBS by daily i.p. injections for 3 consecutive days. As a control, mice received only 200 μl PBS.

Statistical analysis

Data were analyzed using Prism 5.0 software. All data are represented as the mean ± SD. Multigroup and two-group statistical analyses were performed using a one-way ANOVA with Tukey’s multiple comparison test and unpaired t test, and p < 0.05 was considered significant. Significant differences are designated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. BrdU turnover data were subjected to linear regression analysis and assessed for significantly different slopes.

Results

Klrγ1 Tregs are a recent, Ag-responsive, highly activated subset

Klrγ1 is minimally expressed on conventional CD4+ T cells but is found at a relatively high level on a small population of Tregs in peripheral immune tissues such as the spleen, MLN, and PP on nearly 50% of the Tregs from the LP of the small intestine (Fig. 1A). Klrγ1 Tregs in the spleen (Fig. 1B) expressed a more activated phenotype (CD69+, CD62Llow, CD103+, and CD44high) (Fig. 1B) compared with Klrγ1 Tregs. Blimp-1, which marks IL-10-producing activated Tregs (35), was also highly expressed by Klrγ1
Tregs (Fig. 1B). This comparison also revealed that Klrg1+ Tregs expressed higher levels of Foxp3 and several important Treg functional molecules (CTLA4, CD39, CD73) (Fig. 1B). In the LP, most Tregs expressed an activated phenotype, and expression of CD69, CD103, CD25, Blimp-1, and Foxp3 was slightly, but significantly, greater for Klrg1+ Tregs (Fig. 1C).

The high prevalence of Klrg1+ Tregs within the LP at the steady state suggests that these Tregs preferentially home and/or are activated within tissue sites. To test this notion, Klrg1 expression by Tregs in the lung and the bronchial draining lymph node (dLN) was examined after mice were either sensitized with OVA and alum or after aerosol challenge to induce allergic hypersensitivity (Fig. 2A). After sensitization, the number of total and Klrg1+ Tregs increased in the dLN and lung (Fig. 2B). Noticeably, 3 d after the aerosol challenge with OVA, more total and Klrg1+ Tregs were detected in the lung but not the dLN in comparison with mice that were only sensitized (Fig. 2B). Therefore, most of the responding Klrg1+ Tregs are probably a result of a response in situ rather than migration into this mucosal tissue site. In addition, increased proliferation as assessed by Ki67 expression was noted by Klrg1+ Tregs in the dLN and lung after OVA sensitization and aerosol challenge (Fig. 2C). Collectively, these data indicate that Klrg1+ Tregs are a highly activated Treg subpopulation with enhanced expression of several Treg functional molecules that readily respond to antigenic challenge.

Distinct gene expression by Klrg1+ Tregs

To define further the molecular properties of Klrg1+ Tregs and explore their relationship to other Tregs, gene expression profiling of ex vivo-isolated splenic nTreg subsets was performed based on expression of CD62L, CD69, and Klrg1 (Fig. 3A). These markers discriminate conventional T cells with properties of resting naive to activated effector and memory cells. Tregs from Foxp3/RFP reporter mice were FACS purified to obtain four subsets, which are CD62Lhi CD69lo (Fr1 Klrg1lo), CD62Llo CD69lo (Fr2 Klrg1lo), and CD62Llo CD69hi (Fr3 Klrg1hi) subsets. Klrg1+ Treg (Fr3 Klrg1+) cells were purified to coexpress CD69 because most (60–70%) Klrg1+ Tregs are CD69+ (Fig. 1B) and represent the fourth subset.

When comparing genes differentially expressed by $\geq 2$-fold only between the four Treg subsets, 745 unique Affymetrix targets were identified. To verify this list, we confirmed that several genes exhibited cell surface expression in accordance with the mRNA levels (Fig. 3B, 3D). In addition, our expression pattern for Klrg1+ Tregs resembled that of another study that compared Klrg1+ Tregs to total Tregs from distinct tissues (13). Euclidean clustering of differentially expressed genes between Treg subsets was performed
FIGURE 2. Distribution and proliferative activity of Klrg1+ Tregs in allergic hypersensitive mice. Allergy was induced in B6 mice as described in Materials and Methods. Just before the aerosol challenge, the Treg composition was assessed for some of the sensitized mice, as indicated. (A) The expression of Klrg1 by Tregs from the indicated tissues before and after induction of acute asthma. (B) Enumeration of the number of total and Klrg1+ Tregs. (C) Proliferative activity of the indicated Klrg1+ and Klrg1- Tregs based on Ki67 expression. Data are representative of three to eight mice/group.

to evaluate globally the relationship between these subsets. This analysis revealed two clades, with Fr1 Klrg1- and Fr2 Klrg1- versus Fr3 Klrg1- and Fr3 Klrg1+ Tregs more related to each other (Fig. 3C). Gene enrichment analyses annotated 233 of 781 (29.8%) genes into 13 Gene Ontology functional classification pathways with z scores >2.0. The four largest clusters of genes that varied between and distinguished these subsets are related to immune system processes, lymphocyte activation, proliferation, and cell death. Other notable characteristic that differed between subsets were tissue migration, cell localization and homing, cytokine expression, but from Fr1 Klrg1- to Fr3 Klrg1+ (lowest) to Fr3 Klrg1+ (highest). In contrast, decreased expression for mRNAs that function in lymphoid tissue homing, survival, hemopoiesis, and Wnt signaling were found in these three subsets, again with a gradient of highest to lowest, but detectable, p-Stat5 associated with Y3 (27). Unlike IL-2–dependent tyrosine Stat5 phosphorylation (p-Stat5) with the level of CTLA4 mRNA in Tregs. In contrast, CD39 expression by Klrg1+ Tregs was significantly increased only when compared with activated Fr3 Klrg1- Tregs (Fig. 4A). As CTLA4 was not detected as a differentially expressed gene (Fig. 3D), this increase may represent a posttranscriptional mechanism or reflect an inability to detect a mRNA difference due to the high levels of CTLA4 mRNA in Tregs. In contrast, CD39 expression by Klrg1+ Tregs was significantly increased only when compared with “resting” Fr1 Klrg1- Tregs. Thus, all Treg suppressive molecules are not selectively overrepresented in the Klrg1+ subset. In vitro suppression was also consistently greater for splenic Klrg1+ Tregs compared with Fr1 Klrg1- Tregs (Fig. 4B), which expressed lower amounts of many Treg suppressive molecules (Fig. 3D). Collectively, these data in conjunction with our gene array analysis support the notion that Klrg1+ Tregs are armed to deliver optimally a number of molecules associated with Treg suppressive function.

Varied suppressive function in Treg subsets

The potency of a T effector response is often enumerated based on the frequency and level of expression of molecules (e.g., cytokines), which are characteristic of T cell function. In this regard, the level of CD39 was substantially increased in splenic Klrg1+ Tregs even when compared with activated Fr3 Klrg1- Tregs (Fig. 4A). As CTLA4 was not detected as a differentially expressed gene (Fig. 3D), this increase may represent a posttranscriptional mechanism or reflect an inability to detect a mRNA difference due to the high levels of CTLA4 mRNA in Tregs. In contrast, CD39 expression by Klrg1+ Tregs was significantly increased only when compared with “resting” Fr1 Klrg1- Tregs. Thus, all Treg suppressive molecules are not selectively overrepresented in the Klrg1+ subset. In vitro suppression was also consistently greater for splenic Klrg1+ Tregs compared with Fr1 Klrg1- Tregs (Fig. 4B), which expressed lower amounts of many Treg suppressive molecules (Fig. 3D). Collectively, these data in conjunction with our gene array analysis support the notion that Klrg1+ Tregs are armed to deliver optimally a number of molecules associated with Treg suppressive function.

IL-2R signaling is essential for Klrg1+ Tregs

The only studies were performed to address the role of IL-2R in Tregs that were performed to address the role of IL-2R in Tregs. The only studies were performed to address the role of IL-2R in Tregs. We previously showed that mutations of Y341 (Y1), Y395 and Y498 (Y2), or Y341, Y395, and Y498 (Y3) to phenylalanine in the cytoplasmic tail of IL-2Rβ resulted in a dose-dependent reduction of IL-2–dependent tyrosine Stat5 phosphorylation (p-Stat5) with the lowest, but detectable, p-Stat5 associated with Y3 (27). Unlike IL-2Rβ–deficient mice, which develop rapid lethal systemic autoimmunity due to failed maturation of Tregs, mice expressing each mutant IL-2Rβ were overtly healthy and contained a normal ratio and number of Tregs and normal levels of Foxp3, but Y2 and Y3 mice eventually showed symptoms of autoimmune disease. Thus,
nTreg development and homeostasis are readily supported by a low level of IL-2R signaling. Gene expression profiling, however, from Tregs with impaired IL-2R signaling revealed a substantial number of genes that were IL-2 dependent. Two such genes are Klrg1 and Itgae (CD103).

When Tregs were examined from mice bearing mutant IL-2Rβ, fewer Klrg1+ Tregs were found in the spleen (Fig. 5A). A similar decrease was detected in peripheral lymph node, MLN, and PP (data not shown), and these effects were generally proportional to increased Y→F IL-2Rβ mutations. This decrease was especially striking in the LP where Klrg1 is normally expressed by 40–50% of the Tregs. The decrease in Klrg1+ Tregs occurred even though the number of total Tregs was not reduced in the LP of Y1 and Y3 mice (data not shown), and these effects were generally proportional to the lower level of IL-2R signaling.

With respect to other T cell activation Ags expressed by Tregs from Y3 mice, a decrease in CD103+ Tregs was noted in the spleen and peripheral lymph node (data not shown), but this effect was absent in the LP (Fig. 5B). Thus, the requirement for IL-2Rβ signaling differs for CD103+ and Klrg1+ Tregs. Noticeably, there was no reduction, but a relatively higher proportion, of CD69+ and CD62L+ activated Tregs (Fig. 5B) Thus, impaired IL-2R signaling, as supported by Y3, does not generally dampen Treg activation but somewhat alters Treg subset composition.

Klrg1 marks a unique Treg subset

Fewer Tregs that express Klrg1 in Y3 mice might simply reflect decreased expression of these molecules by common related Tregs or might indicate the absence of a specific IL-2–dependent Treg subset. To address this issue, we examined whether WT Klrg1+ Tregs selectively repopulate Y3 mice after adoptive transfer of WT Tregs from Foxp3/GFP reporter mice. Because IL-2 contributes to peripheral Treg homeostasis, donor Tregs with WT IL-2R, which delivers normal strong IL-2R signaling, are expected to have a competitive advantage over the more numerous recipient-derived Y3 Tregs, which are maintained by suboptimal IL-2R signaling (27). Therefore, we reasoned that WT Klrg1+ Tregs should only be favored if this population was selectively diminished in Y3 mice.

At 4 wk posttransfer, the percentage (Fig. 6A) and number (Fig. 6B) of total splenic Tregs were similar in WT and Y3 recipient mice. Donor GFP+ Tregs, however, composed a substantially lower fraction (27%) of the total Tregs in WT mice (Fig. 6B, 6C), demonstrating the competitive advantage of WT Tregs within the Y3 recipients. Importantly, the donor WT GFP+ Tregs in Y3 recipient mice were highly enriched in Klrg1+ Tregs such that the composition of the total Treg pool reflected that in normal WT mice. In contrast, the proportion of activated splenic Tregs expressing CD69, which is related to Klrg1+ Tregs (see Fig. 3), was largely unaltered (Fig. 6D). This relationship strikingly held for the LP in Y3 recipients where 27% of Tregs were GFP+ donor-derived, >50% of the GFP+ Tregs expressed Klrg1 (Fig. 6E), and nearly all Klrg1+ Tregs were of donor origin. Collectively, these data indicate that the Tregs expressing a WT IL-2R have a competitive advantage over Y3 Tregs and favor the development of Klrg1+ Tregs in Y3 recipients. This finding supports the notion that Klrg1 marks a distinct IL-2–dependent Treg subset and that this subset depends on more extensive IL-2R signaling.

**IL-2 drives preferential development of Klrg1+ Tregs**

The near lack of Klrg1+ Tregs in Y3 mice indicates that these cells depend on more extensive IL-2R signaling than other Tregs. To test further this notion, we evaluated the effect of extensive IL-2R

---

**FIGURE 3.** Gene expression profile by Treg subsets from B6 mice. Klrg1-depleted Fr1, Fr2, Fr3, and Fr3 Klrg1+ Tregs were purified from the spleen by FACS sorting. Total RNA was isolated, and gene expression profiling was performed using Affymetrix Mouse Gene ST 1.0 arrays. Samples (n = 2, except Fr3 Klrg1+neg, n = 4) represent independent biological replicates. (A) Representation of the three main Treg fractions (Fr) used for gene array analysis. (B) Protein expression of selected genes by FACS for Treg subsets. Data are mean ± SD for four mice per group. (C) Euclidean clustering of sample relatedness for genes that varied by 2-fold (over- or underrepresented) between the indicated Treg subsets. (D) Genes differentially expressed (over- or underrepresented) between the indicated Treg subsets. Shown are the expression levels (log2) of selected genes that varied by ≥2-fold between any two subsets except those underlined, which varied by a lower level. Data were analyzed by one-way ANOVA: p < 0.05 (Benjamini and Hochberg correction).

---

**FIGURE 4.** Treg suppressive activity of Klrg1+ Tregs. (A) Distribution of CTLA4 and CD39 by the indicated WT B6 splenic Treg subsets. (B) In vitro suppression by the indicated splenic Treg subsets isolated from Foxp3/RFP reporter mice. The percent inhibition was determined in comparison with [3H]thymidine incorporation by responder cells lacking Tregs. Results are from three experiments.

---

**FIGURE 5.** CD69 expression by Treg subsets from B6 mice. (A) Flow cytometric analysis of CD69 expression on the indicated Treg subsets. Data are mean ± SD for four mice per group. (B) Protein expression of selected genes by FACS for Treg subsets. Data are mean ± SD for four mice per group. (C) Euclidean clustering of sample relatedness for genes that varied by 2-fold between any two subsets except those underlined, which varied by a lower level. Data were analyzed by one-way ANOVA: p < 0.05 (Benjamini and Hochberg correction).

---

**FIGURE 6.** In vivo effect of extensive IL-2R signaling on Treg homeostasis. (A) Flow cytometric analysis of CD69 expression on the indicated Treg subsets. Data are mean ± SD for four mice per group. (B) Protein expression of selected genes by FACS for Treg subsets. Data are mean ± SD for four mice per group. (C) Euclidean clustering of sample relatedness for genes that varied by 2-fold between any two subsets except those underlined, which varied by a lower level. Data were analyzed by one-way ANOVA: p < 0.05 (Benjamini and Hochberg correction).

---

**FIGURE 7.** In vitro effect of extensive IL-2R signaling on Treg homeostasis. (A) Flow cytometric analysis of CD69 expression on the indicated Treg subsets. Data are mean ± SD for four mice per group. (B) Protein expression of selected genes by FACS for Treg subsets. Data are mean ± SD for four mice per group. (C) Euclidean clustering of sample relatedness for genes that varied by 2-fold between any two subsets except those underlined, which varied by a lower level. Data were analyzed by one-way ANOVA: p < 0.05 (Benjamini and Hochberg correction).
signaling on Treg subsets by treating normal mice with agonist anti–IL-2–IL-2 complexes (IL-2–IC) using the JES6-1A12 mAb that preferentially expands Tregs (34, 36). Three days after the last injection of IL-2–IC, a selective expansion of total and Klrg1+ splenic Tregs was noted in their relative percentage (Fig. 7A) and numbers (Fig. 7B). The increase was ~2.5-fold for total Tregs, but almost 4-fold for Klrg1+ Tregs. IL-2–IC also markedly increased CD25 expression (Fig. 7A), which is known to be upregulated by IL-2 in conventional activated T cells (37). At the peak of the response, nearly all Tregs, including Klrg1+ cells, were recently in cell cycle as evident by the high percentage of Ki67+ cells, but Bcl-2 levels minimally varied (Fig. 7C), suggesting that this form of IL-2R signaling favors proliferation rather than enhanced cell survival. Unlike the transient increase in Klrg1+ Tregs (Fig. 7B), the proportion of splenic Tregs that expressed CD103, CD62L, and CD69 minimally varied (Fig. 7D). Thus, even though these latter Treg subsets proliferated in response to IL-2–IC, they are stably maintained in their relative proportions. Thus, extensive IL-2R signaling through IL-2–IC in lympho-replete mice is particularly efficient in driving development of Klrg1+ Tregs.

To test the extent that Klrg1+ Tregs directly expand in response to IL-2, Klrg1+ and Klrg1-2RFP+ Tregs were purified, transferred into TCRα/2 mice with Treg-depleted conventional CD4+ T cells, and the recipients were treated with IL-2–IC. Under these conditions, only the Klrg1+ Treg population expanded, and ~20% developed into Klrg1+ Tregs (Fig. 7E). Notably, for recipients of Klrg1+ Tregs, the few donor cells detected still expressed Klrg1. Thus, the expansion of Klrg1+ Tregs by IL-2–IC likely reflects proliferation by Klrg1-2 precursors that are driven by IL-2 to develop into Klrg1+ Tregs.

**FIGURE 5.** IL-2R signaling is required for the development of Klrg1+ Tregs. The expression of Klrg1 (A) and other activation markers (B) by Tregs from the spleen and LP of the indicated mice. Data are from 3–11 mice per group.

**FIGURE 6.** Klrg1 marks a splenic Treg subset. Purified Foxp3/GFP+ WT Tregs (4 × 10^5) were transferred into the B6 or Y3 adult recipient mice. (A–C) Four weeks posttransfer, (A) the percent of total Tregs in CD4+ T cells, (B) the number of host (GFP−) versus donor (GFP+) Tregs, and (C) the percentage of donor GFP+ Tregs in total Tregs were determined. (D and E) Total CD4+ Foxp3+ Tregs were gated in spleen (D) and LP (E), and the expression of Klrg1 and CD69 in the GFP+, GFP−, or total Tregs was determined. Data are representative of three to four mice per group.
Tregs within the spleen and LP of WT and Y3 mice. Proliferation as assessed by Ki67 expression of Klrg1+ and Klrg1− Tregs from the spleen and LP was similar between B6 and Y3 mice (Fig. 8A). This finding suggests that homeostatic proliferation of Tregs is maintained by low IL-2R signaling associated with Y3 IL-2Rβ. In both types of mice, higher Ki67 expression was associated with Klrg1− Tregs in the spleen, but with Klrg1+ Tregs in the LP. Thus, the environments associated within a peripheral immune tissue reflects a requirement for relatively high IL-2R signaling to promote their development.

For the LP, the turnover between Klrg1+ and Klrg1− Tregs was similar when examined in B6 or Y3 mice (Fig. 8B). However, Klrg1− LP Tregs from Y3 mice showed significantly greater turnover (p = 0.03) than Klrg1+ LP Tregs from B6 mice (Fig. 8C). Thus, there is a requirement for stronger IL-2R signaling to properly support the homeostasis of Tregs in the LP. As Treg proliferation between B6 and Y3 subsets was similar (Fig. 8A), this likely reflects a role for IL-2 in contributing to Treg survival in the LP. Thus, the striking lack of Klrg1+ Tregs within the LP (Fig. 5A) may reflect failed IL-2–dependent development that is due in part to poor survival of Klrg1− cells that serve as precursor cells.

Klrg1+ Tregs are terminally differentiated

The preceding experiments demonstrate that Klrg1+ Tregs exhibit phenotypic and molecular properties of a late and perhaps terminally differentiated Treg subset. A hallmark of terminally differentiated Tregs is a lack of plasticity to express traits of other differentiated cells related within its lineage. To determine whether Klrg1+ Tregs lack developmental heterogeneity, Klrg1+ and Klrg1− nTreg subsets and Treg cell-depleted CD4 conventional T cells were purified by sorting using Foxp3/RFP and Foxp3/GFP reporter mice, respectively. These cells were mixed at an ~1:10 ratio (Foxp3/RFP+ to Foxp3/GFP−) and transferred into untreated TCRα−/− recipients (Fig. 9A). The addition of conventional T cells provides a potential source of growth factors (e.g., IL-2) required by underwent substantial recent proliferation and is short-lived with very similar behavior in both B6 and Y3 mice. Thus, the relative lack of this subset within the spleen of Y3 mice most likely reflects a requirement for relatively high IL-2R signaling to promote their development.

FIGURE 7. The effect of IL-2–IC on Treg subsets. (A–D) WT B6 mice received three daily injections of IL-2–IC, and 3, 5, or 7 d after the last injection, splenic Tregs were evaluated. Control represents mice that were injected with PBS and were analyzed only 3 d after the last injection. Representative FACS profiles of the indicated markers 3 d after the last injection of IL-2–IC (A) and time course of Treg numbers (B) of the indicated populations. (C) Expression of Ki67 and Bcl-2 3 d after the last injection of IL-2–IC. (D) Time course for the expression of the indicated markers. Data are from at least three mice per group per time point. (E) Splenic Klrg1+ or Klrg1− Tregs (1 × 10^6) from the Foxp3/RFP reporter mice were injected with splenic conventional CD4+ T cells (1 × 10^5) from the Foxp3/GFP reporter mice at the ratio of 1:10 into TCRα−/− mice. One day later, the recipients received three daily injections of IL-2–IC. Six days after the cell transfer, the indicated cell populations from the spleen were analyzed for the expression of RFP and Klrg1. Data are from four to five mice/group where each point on the graph represents an individual mouse.
FIGURE 8. Proliferative activity and turnover of Klrg1+ and Klrg1 nTregs. (A) Bcl-2 and Ki67 expression by the indicated CD4+ Foxp3+ Tregs. Data are from six to eight mice/group. (B and C) Mice received BrdU for 5 d and then were switched to normal water (day 0). BrdU incorporation was determined for the indicated Treg populations. Linear regression analysis was performed for BrdU loss and compared between Klrg1+ or Klrg1 nTreg subsets from the WT B6 and Y3 mice (B) or compared for an indicated individual subset between B6 and Y3 mice (C). The p values of whether the slopes are significantly different are shown within each graph. Data represent three mice/group. (D) Purified CD4+ T cells were cultured in medium for the indicated time. Cell viability and recovery were determined for the Klrg1+ and Klrg1 CD4+ Foxp3+ Tregs and compared with the number of Tregs at culture initiation. Data are from three experiments.

the donor nTregs and permits assessment of iTreg development through detection of GFP+ cells.

At 4 wk posttransfer, RFP nTregs were readily found in the spleen (Fig. 9B) and LP (Fig. 9C) that received Klrg1, but not Klrg1, nTregs. The few GFP+ nTregs in the LP from recipients that received Klrg1 Tregs indicate that the overall low number of donor Klrg1 cells does not simply reflect preferential homing of this subset to tissue sites. In these TCRα−/− recipients where a few RFP+ Tregs were found, they remained as Klrg1+ Tregs (Fig. 9B, 9C, upper panel). However, a substantial proportion of the Klrg1+ nTregs developed into Klrg1+ cells (Fig. 9B, 9C lower panel). Furthermore, in the absence of conventional T cells, Thy-1.1 RFP+ donor Tregs were transferred into TCRα−/− mice, an environment favoring de-differentiation of Tregs into Foxp3− cells (exTregs) (38). In this setting Klrg1−, but not Klrg1+, donor Tregs readily generated a large population of exTregs (Fig. 9D). Thus, the low number of donor cells detected after the transfer of Klrg1+ nTregs is not because Klrg1+ Tregs express an unstable phenotype and then expand as exTregs. Collectively, these findings indicate that Klrg1+ Tregs are derived from Klrg1− cells and are terminally differentiated. The poor recovery of Klrg1+ Tregs in TCRα−/− recipients is consistent with them being mostly short-lived. However, their lack of expansion suggests that the high Ki67 expression and BrdU incorporation associated with Klrg1+ Tregs (Fig. 7) may actually represent proliferation by precursors to the Klrg1+ Treg subset.

When Foxp3/GFP-depleted conventional CD4+ T cells were transferred with Klrg1+ and Klrg1− nTregs into TCRα−/− mice, a readily measurable (~1%) population of GFP+ iTregs was also detected in the spleen and LP (Fig. 9E). A substantial fraction (30–60%) of these iTregs also expressed Klrg1. This distribution and the high purity of donor conventional CD4+ T cells (>99.7% GFP+) make it unlikely that these GFP+ cells posttransfer are solely accounted for by a few contaminating GFP+ nTregs in the conventional T cells inoculum. These data indicate that iTregs express Klrg1 and are consistent with the possibility that both nTregs and iTregs develop into the Klrg1+ subset.

Developmental progression of Klrg1+ Tregs

Klrg1+ Tregs are not found in the thymus (11, 12), raising the possibility that splenic Klrg1+ nTregs might actually reflect selective development by iTregs. To examine directly the development and stability of Klrg1+ Tregs by nTregs in a lympho-replete setting, Y3 mice were adoptively transferred with spleen- or thymus-derived Tregs. Y3 mice have normal numbers of conventional T cells and Tregs (27), but transferred WT Tregs have a competitive advantage over recipient Y3 Tregs due to normal IL-2R signaling and homeostasis, greatly facilitating their identification (Fig. 6). By transferring Foxp3/RFP Thy-1.1+ Tregs (>98% pure) into Thy-1.2+ Y3 recipients, we followed the fraction of Thy-1.1+ donor Tregs that lost expression of RFP and became exTregs.

When donor thymic or splenic Klrg1− Tregs were transferred into Y3 mice, both populations substantially engrafted (Fig. 10A, right) and readily (>40%) developed into Klrg1+ Tregs (Fig. 10A). The development of Klrg1+ Tregs from thymic donor cells directly shows that nTregs readily yield Klrg1+ cells. Thus, nTregs and iTregs (Fig. 9F) develop into the Klrg1+ subset. Analogous to TCRα−/− recipients (Fig. 9), very few splenic Klrg1+ donor Tregs were found in Y3 recipients, and nearly all of these remained as Klrg1+ Tregs. Furthermore, in all three types of transfers, virtually all (>98%) input Tregs were stable and remained RFP+ cells (Fig. 10A, left). This contrasts with TCRα−/− recipients where exTregs were readily detected and indicates that the lymphopenic environment preferentially supports exTreg production.

If Klrg1+ Tregs represent an end-stage terminally differentiated cell, expression of Klrg1 should be acquired late during Treg activation in vivo. To test this notion, the developmental progression of Klrg1+ Tregs was examined in vivo by following the cell surface phenotype and cell division status of CFSE-labeled
FIGURE 9. Klrg1+ Tregs are terminally differentiated and derived from distinct Klrg1− subsets. (A–C) Klrg1+ or Klrg1− Treg subsets (1 × 10^5) were purified from Thy-1.1+ Foxp3/RFP reporter mice and adoptively transferred with (A–C) conventional CD4+ T cells (1 × 10^5) from Foxp3/GFP reporter mice at 1:10 ratio. The purity of injected cells was verified by FACS analysis (A). Four weeks posttransfer, the spleen (B) and LP (C) of TCRα−/− recipients were examined for percent donor RFP+ nTregs and their expression of Klrg1+. (D) RFP+ Thy-1.1+ Klrg1+ and Klrg1− Treg subsets were transferred alone into Thy-1.2+ TCRα−/− mice, and Treg stability was assessed 2 wk later by enumerating the indicated RFP+ Treg and RFP− exTreg Thy-1.1+ donor cells from the spleen and MLN. (E) Foxp3/GFP-marked conventional CD4+ T cells (depleted of Tregs) were transferred with Klrg1− Foxp3/RFP Tregs into TCRα−/− recipients, and iTreg development was assessed by enumerating CD4+ GFP+ T cells (left) and their development into Klrg1+ iTregs (right) from the spleen and LP 4 wk posttransfer. All the data were derived from three to four mice/group.

Discussion

Our understanding concerning effector T cell responses and the development of memory has been greatly facilitated by defining phenotypic and molecular properties that delineate important cell subsets and by establishing extrinsic and intrinsic factors favoring development of one subset over another. Several lines of recent work indicate that this paradigm also holds for Tregs. First, transcription factors important for Th development also function in Tregs in a manner distinctively to coordinate Treg suppressive programs toward the effector subtype that they inhibit (14–16). Second, the gene profiles of Treg subtypes, particularly as they relate to nTreg versus iTreg, were distinguished from each other by their transcriptional profile (13). Lastly, TCR-transgenic Tregs give rise to memory-like Tregs after response to a model self Ag (39). By examining polyclonal Tregs, our current study extends this notion by establishing that ex vivo-derived, phenotypically distinct nTregs express distinctive functional and gene expression programs toward the effector subtype that they inhibit (14–16). Expression of Klrg1 by CD8+ T cells only occurs after extensive Ag-dependent proliferation (41, 42). The CD8+ Klrg1hi cells are associated with Blimp-1–dependent terminally differentiated CD62Llo tissue-seeking effector cells, whereas the Klrg1low cells are enriched in precursors of memory cells (41, 43). For Klrg1hi Tregs, a large majority are Klrg1hi with ∼30–40% as Ki67hi, >95% Bcl-2hi and CD62Llo, indicative of a highly proliferative short-lived population. After adoptive transfer of “resting” CD62Llo Tregs into Y3 recipients, development of Klrg1+ Tregs requires at least eight cell divisions. Collectively, these observations are consistent with Klrg1 marking a Treg population with a highly replicative history. Key genes associated with Treg function, particularly Il10, Fgl2, and Entpd1 (CD39), as well as chemokine receptors associated with cells within inflammatory sites were
Klrg1+ phenotype was stable. Overall, these properties are similar to lympho-replete Y3 recipients, and for the few cells found, the Klrg1 expression as a function of cell division was determined on the indicated day posttransfer. (CFSE-dilution) was determined 6 d posttransfer. Data are representative of three mice/time point.

FIGURE 10. Developmental progression of Klrg1+ Tregs. (A) The indicated Thy-1.1+ RFP+ Tregs (5 x 10^5) were transferred into Thy-1.2+ Y3 recipients. Two weeks posttransfer, the donor cells were examined from the spleen of each recipient. Data are from three mice/group. (B) CFSE-labeled CD62L hi CD69 hi CD103+ Klrg1+ Tregs were transferred into Y3 mice, and the phenotype of the donor cells was determined on the indicated day posttransfer. (C) The relationship of CD103 and Klrg1 expression as a function of cell division (CFSE-dilution) was determined 6 d posttransfer. Data are representative of three mice/time point.

most highly expressed in the Klrg1+ Treg subset. Furthermore, Blimp-1 expression was highly enriched in Klrg1+ Tregs with essentially all as Blimp-1+. By evaluating naive and Ag-challenged airway hypersensitive mice and the patterns of Ki67 expression and BrdU incorporation, most Klrg1+ Tregs behave as recent Ag-stimulated highly proliferative, but short-lived, cells. In marked contrast to Klrg1+ Tregs, transferred purified Klrg1− Tregs did not undergo homeostatic expansion in lymphopenic TCRα−/− or lympho-replete Y3 recipients, and for the few cells found, the Klrg1− phenotype was stable. Overall, these properties are similar to short-lived terminally differentiated CD8+ CTL. This characteristic and their high prevalence in the LP and the lung of allergic hypersensitive mice are consistent with the notion that most Klrg1+ Tregs are terminally differentiated tissue-residing suppressor cells. However, we cannot exclude that a small fraction of Klrg1+ Tregs are long-lived and might be analogous to memory precursor cells.

IL-2 is well known to provide essential homeostatic signals for nTregs (21, 23). IL-2 also shapes the competitive fitness of Tregs through its ability to maintain Foxp3 levels (24). Our past work showed that these key activities of IL-2 readily occur under conditions that support weak suboptimal IL-2R signaling for Tregs within peripheral immune tissues (27). As shown here, weak IL-2R signaling by Tregs bearing Y3 mutant IL-2Rβ supports relatively normal homeostasis but failed to support Klrg1+ and CD103+ Tregs, the latter to a lesser extent. One explanation for this finding is that high IL-2R signaling is required for developmental activities distinct from IL-2-dependent growth/survival. Alternatively, more extensive proliferation that is linked to high IL-2R signaling is required for production of Klrg1+ Tregs, and this effect is distinct from IL-2-mediated homeostasis. These two possibilities are not mutually exclusive. Furthermore, after transfer into TCRα−/− recipients, IL-2−IC readily stimulated Klrg1+ Tregs to proliferate and develop into Klrg1− cells, whereas IL-2−IC did not stimulate proliferation of purified Klrg1+ Tregs. This finding suggests that

IL-2 may drive the development of the Klrg1− subset from Klrg1+ Tregs and raises the possibility that the selective increase of Klrg1+ Tregs in B6 mice after IL-2−IC may at least in part reflect development by Klrg1− Tregs.

An important new finding from this study is that we have defined another function for IL-2 signaling in the periphery; that is, the development of terminally differentiated Klrg1+ Tregs. This IL-2−dependent activity on Tregs is also highly analogous to the contribution of IL-2 for CD8+ T effector cells (44–46). Thus, Tregs use a similar strategy as effector CD8+ T cells to promote their development into cells with heightened suppressive effector function. Additionally, this study has uncovered a distinctive tissue-specific requirement for IL-2R signaling for Treg homeostasis. Within a peripheral lymphoid tissue, weak IL-2R signaling readily supported Treg homeostasis as Ki67 expression as well as BrdU uptake and loss were very similar for WT B6 and Y3 Tregs. However, the turnover of Tregs, particularly Klrg1− cells, in the LP was accelerated in Y3 mice, indicating that Tregs in the LP require stronger IL-2R signaling for increased life span. Other signals, therefore, must compensate for lower IL-2R activity to maintain these cells. This altered homeostasis coupled with failed development of Klrg1+ Tregs likely contributes to tissue-specific inflammation that occurs in older Y3 mice (27). Some T effector activity is also attenuated in Y3 mice, which might offset IL-2R−dependent defects associated with Y3 Tregs (27).

On the basis of our gene profiling and direct analysis of Klrg1+ and Klrg1− Tregs, we favor a model where distinct expression of CD62L, CD69, and Klrg1 represents distinct activation states. The heterogeneity in expression of these and other cell surface molecules has been appreciated for some time (4–12), but little is known concerning their potential interrelationships. This study establishes one such relationship by showing that high IL-2R signaling supports the proliferation of Klrg1− Tregs and causes them to develop into a short-lived Klrg1+ Treg population with heightened expression of a number of molecules associated with...
suppressive function. These Klrg1+ Tregs are also poorly proliferative, unresponsive to IL-2, and lack plasticity. These properties are highly analogous to the association of Klrg1 expression with CD8+ T cells and NK cells that exhibit replicative senescence (47–50) and behave as terminally differentiated cells (41, 42). As illustrated for Treg suppressive activity (14–16), our findings indicate that Tregs also co-opt strategies used by conventional Ag-activated T cells to drive development of Tregs of distinct activation states. The linkage of high IL-2R signaling for the production of Klrg1+ Tregs suggests that this subset is favored under conditions where a stronger or more persistent autoreactive T cell response occurs leading to greater levels of IL-2. High IL-2 levels by the autoreactive T cells may then be counterbalanced by the development of Klrg1+ Tregs that overexpress Treg suppressive molecules. This may occur in peripheral lymphoid tissues but is likely important in inflamed tissues that contain pathogenic effector cells. The short-lived nature of most Klrg1+ Tregs leads to a diminished contribution by these Tregs if the autoreactive response is restrained. However, chronic immune stimulation, as occurs within the gut mucosa, appears to favor continued development of Klrg1+ Tregs. Thus, lower levels of Klrg1+ Tregs may represent a risk factor for autoimmunity. Indeed, the autoimmune symptoms associated with older Y3 mice may be due in part to the lower level of Klrg1+ Tregs in these mice. In any case, this model provides a framework for future studies to understand more fully the interrelationship and development of Treg subpopulations that encounter self or foreign Ags.

Acknowledgments

We thank I. Castro for assistance with the adoptive transfer experiments and L. Nathanson and E. Echandia for the microarrays.

Disclosures

The authors have no financial conflicts of interest.

References


