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Negative Regulation of T Cell Homeostasis by Lymphocyte Activation Gene-3 (CD223)[1]

Creg J. Workman and Dario A. A. Vignali[2]

Lymphocyte homeostasis is a central biological process that is tightly regulated. However, its molecular and cellular control is poorly understood. We show that aged mice deficient in lymphocyte activation gene 3 (LAG-3), an MHC class II binding CD4 homologue, have twice as many T cells as wild-type controls. $CD4^+$ and $CD8^+$ LAG-3-deficient T cells showed enhanced homeostatic expansion in lymphopenic hosts, which was abrogated by ectopic expression of wild-type LAG-3, but not by a signaling-defective mutant. In addition, in vivo treatment with anti-LAG-3 mAb resulted in enhanced T cell expansion to a level comparable to that in LAG-3-deficient cells. This deregulation of T cell homeostasis also resulted in the expansion of multiple cell types, including B cells, macrophages, granulocytes, and dendritic cells. Lastly, regulatory T cells were dependent on LAG-3 for their optimal control of T cell homeostasis. Our data suggest that LAG-3 negatively regulates T cell homeostasis by regulatory T cell-dependent and independent mechanisms. *The Journal of Immunology,* **2005, 174: 688–695.**

Lymphocyte homeostasis is a process that keeps cell numbers remarkably constant (1–5). T cells undergo homeostatic expansion/proliferation in hosts rendered lymphopenic by genetic manipulation or sublethal irradiation (6, 7) and in unmanipulated neonatal mice (8). There are currently two signaling pathways that are known to positively regulate lymphocyte homeostasis. First, signaling through the TCR after recognition of self-MHC molecules is important in maintaining naive T cell homeostasis and memory T cell function (9, 10). Recent studies have directly demonstrated that TCR expression and signaling are required for the continued survival of naive and memory T cells (11–13). Second, cytokines that signal via the common γ-chain are critical for naive T cell survival and homeostasis, particularly IL-7 (14, 15). It has also recently been shown that IL-7 is important for memory CD4 T cell homeostasis (13).

To date, no molecules have been conclusively shown to negatively regulate T cell homeostasis. CTLA-4 (CD152) and TGF-β have been implicated in this process, but this has yet to be confirmed by T cell transfer into lymphopenic hosts or analysis during neonatal expansion (16–19). In contrast, there is strong evidence that $CD4^+CD25^+$ regulatory T (T_{reg})[3] cells significantly reduce homeostatic T cell expansion (20–22). However, the molecules they use have yet to be defined.

Previous studies have suggested that human lymphocyte activation gene 3 (LAG-3; CD223) may function as a negative regulator of activated T cells (23, 24). LAG-3 is particularly interesting due to its close relationship with CD4, a key coreceptor for T cells. LAG-3 has a similar genomic organization as CD4 and resides at the same chromosomal location (25). LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ $\alpha\beta$ T lymphocytes and a subset of $\gamma\delta$ T cells and NK cells (26–29). The ligand for both CD4 and LAG-3 is MHC class II molecules; however, LAG-3 binds with a much higher affinity (28–30). We have previously shown that the negative regulatory function of LAG-3 in murine T cell hybridomas is dependent on binding to MHC class II molecules and signaling through a conserved KIEELE motif in the cytoplasmic domain of LAG-3 (31). Although the initial analysis of LAG-$3^{-/-}$ mice did not reveal a defect in T cell function (32), we have recently shown that LAG-3 regulates the expansion of activated T cells in vivo (33). These observations prompted us to evaluate whether LAG-3 played a role in the negative regulation of T cell homeostasis.

Materials and Methods

Mice

The following mice were used: LAG-$3^{-/-}$ (obtained from Y.-H. Chen, Stanford University, Palo Alto, CA, with permission from C. Benoist and D. Mathis, Joslin Diabetes Center, Boston, MA) (32), C57BL/6J (The Jackson Laboratory), B6.PL-*Thy1*^{a}/Cy (Thy1.1 congenic; The Jackson Laboratory), RAG-$1^{-/-}$ (The Jackson Laboratory) (34), OT-II TCR transgenic mice (provided by S. Schoenberger, La Jolla Institute for Allergy and Immunology, La Jolla, CA, with permission from W. Heath, Walter and Eliza Hall Institute, Parkville, Australia) (35), B6.129-*Abb*^{tml} N5 mice (MHC-$II^{-/-}$; provided by P. Doherty, St. Jude Children's Research Hospital, Memphis, TN), and OT-I (OVA) TCR transgenic mice (The Jackson Laboratory) (36). Genome-wide microsatellite analysis demonstrated that 97% of the 88 genetic markers tested for LAG-$3^{-/-}$ mice were derived from C57BL/6 mice (Charles River Laboratories). LAG-$3^{-/-}$, OT-I.LAG-$3^{-/-}$, and OT-II.LAG-$3^{-/-}$ colonies were maintained at St. Jude Animal Resource Center. All animal experiments were performed in an Association for Assessment of Laboratory Animal Care-accredited, specific pathogen-free facility, following national, state, and institutional guidelines. Animal protocols were approved by the St. Jude institutional animal care and use committee.

LAG-3 constructs and retroviral transduction

LAG-3 constructs were produced using recombinant PCR as previously described (37). The LAG-3.wild-type (WT) and LAG-3.ΔK^M (LAG-3 with a deletion of the conserved KIEELE motif in the cytoplasmic tail) have been described previously (31). LAG-3 constructs were cloned into a murine stem cell virus-based retroviral vector, which contained an internal

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[3] Abbreviations used in this paper: T_{reg}, regulatory T cell; DC, dendritic cell; LAG-3, lymphocyte activation gene 3; WT, wild type.

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ribosomal entry site and GFP, and retrovirus was produced as previously described (38, 39). Retroviral producer cell lines were generated by repeatedly transducing GPE+86 cells 7–10 times until a viral titer of $>10^5$/ml after 24 h was obtained (40).

Flow cytometry

Single cell suspensions were made from spleens and RBC lysed with Gey's solution. For dendritic cell (DC) staining/purification, spleens were first treated with collagenase (Worthington Biochemical) and DNase I (Sigma-Aldrich) for 1 h, and single cell suspensions were made. Splenocytes were first stained with Fc Block, anti-CD16/CD32 (2.4G2; BD Pharmingen) for 10 min on ice. The cells were then stained for the following cell surface markers using various conjugated Abs from BD Pharmingen: TCR$\alpha\beta$ (H57-597), Vα2 (B20.1), $\gamma\delta$ TCR (GL3), CD4 (RM4-4), CD8a (53-6.7), CD45R/B220 (RA3-6B2), CD11b/Mac1 (M1/70), Gr-1 (RB6-8C5; granulocyte marker), CD44 (IM7), CD25/IL2R (7D4), CD69 (H1.2F3), CD11c (HL3; DC marker), and CD244.2 (2B4; pan NK cell marker). LAG-3 expression was assessed with a biotinylated rat anti-LAG-3 mAb (C9B7W, IgG1 κ) (29) or the same Ab obtained as a PE conjugate (BD Pharmingen). The cells were then analyzed by flow cytometry (BD Biosciences).

BrdU incorporation

At 5, 16, 28, and 52 wk of age, LAG-$3^{+/+}$, LAG-$3^{-/-}$, OTII.LAG-$3^{+/+}$, and OTII.LAG-$3^{-/-}$ mice were given BrdU (Sigma-Aldrich) in their drinking water for 8 days (0.8 mg/ml). The mice were then killed by CO_2 inhalation, and the spleens were removed. Staining for BrdU incorporation was performed as previously described (41). Briefly, the LAG-$3^{-/-}$ and LAG-$3^{+/+}$ splenocytes were stained for TCR$\alpha\beta$, CD4, CD8, and B220 expression. The OTII.LAG-$3^{+/+}$ and OTII.LAG-$3^{-/-}$ splenocytes were stained for Vα2 and CD4 expression (BD Pharmingen). The cells were then fixed with 1.2 ml of ice-cold 95% ethanol for 30 min on ice. The cells were washed and permeabilized with PBS, 1% paraformaldehyde, and 0.01% Tween 20 for 1 h at room temperature. The cells were then washed and incubated with 50 Kunitz units of DNase (Sigma-Aldrich) in 0.15 M NaCl and 4.2 mM $MgCl_2$, pH 5.0, for 10 min at 37°C. BrdU was detected by the addition of anti-BrdU-FITC (BD Biosciences) for 30 min at room temperature and then analyzed by flow cytometry.

Adoptive transfer experiments

T and/or B cells from the spleens of age-matched (within 4 wk) mice were positively sorted by FACS, negatively sorted by MACS, or both. For FACS purifications, splenocytes were stained for TCR$\alpha\beta$, CD4, and CD8 expression and sorted by positive selection on a MoFlo (DakoCytomation). For negative MACS purification, splenocytes were stained with PE-coupled or biotinylated anti-B220, anti-Gr1, anti-Mac1, anti-TER119 (erythrocytes), anti-CD49b (NK cells), and anti-CD8 (for negative purification of $CD4^+$ T cells only). The cells were then incubated with magnetic beads coupled with anti-PE Ab or streptavidin and negatively sorted on an autoMACS (Miltenyi Biotec) to 90–95% purity. For purification of $CD4^+CD25^+$ cells, splenocytes were first negatively sorted on an autoMACS, then the $CD4^+$ T cells were stained for CD25 and separated by FACS using the MoFlo. Unless otherwise stated, 5×10^6 cells were transferred into RAG-$1^{-/-}$ mice via the tail vein. In some experiments, adoptive transfer recipients were treated with either anti-LAG-3 (C9B7W, IgG1 κ; 100 or 50 μg on day 0; 50 or 25 μg on day 3) (29) or an isotype control (R3-34; BD Pharmingen; 200 μg on day 0; 100 μg on day 3).

Retroviral transduction of normal T cells

Spleens from OTII.LAG-$3^{+/+}$ and OTII.LAG3$^{-/-}$ mice were removed, and single cell suspensions were made at 2.5×10^6 cells/ml. The splenocytes were activated with $OVA_{326-339}$ peptide (10 μM) in culture for 2 days. The activated splenocytes were then incubated on a monolayer of GFP alone, LAG-3.WT/GFP, or LAG-3.ΔK^M/GFP retroviral producer cells for 2 days in the presence of polybrene (6 μg/ml). The cells were allowed to rest for 10 days, then sorted for V$\alpha2^+$/$CD4^+$/GFP^+ expression by FACS. The cells were allowed to rest for 2 additional days, then 5×10^6 cells were injected into RAG-$1^{-/-}$ mice via the tail vein. Fifteen days posttransfer, the mice were killed by CO_2 inhalation, and spleens were removed. The splenocytes were stained and analyzed by flow cytometry.

Results

LAG-$3^{-/-}$ mice have more T cells

WT C57BL/6 mice have a constant number of $\alpha\beta^+$ T cells from 5–52 wk of age (Fig. 1*A*). As previously reported, young (5-wk-

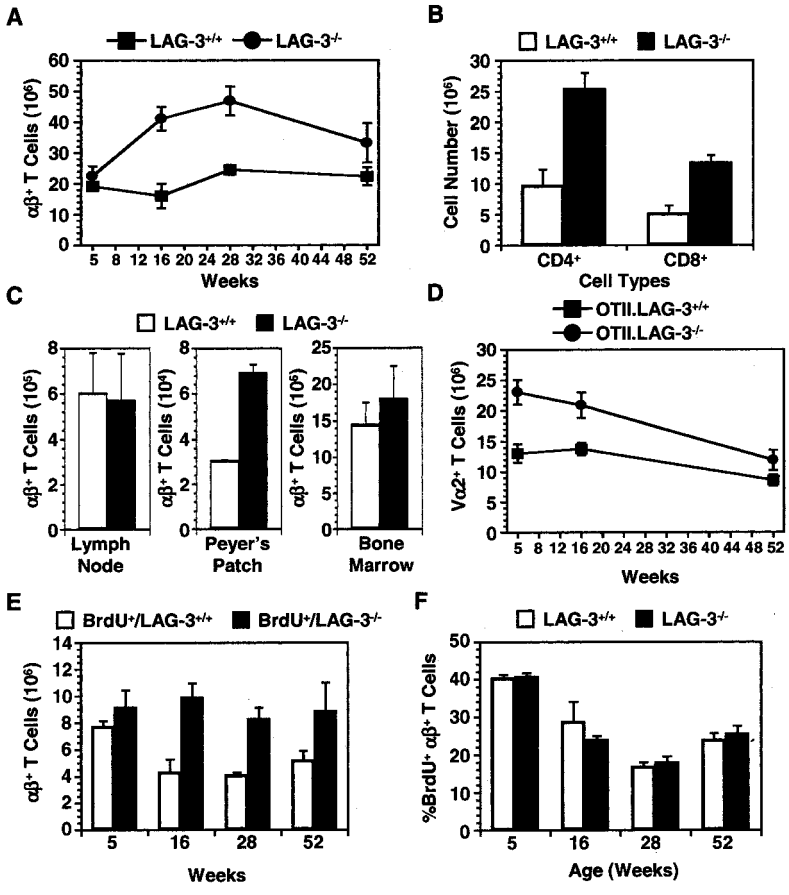

FIGURE 1. Defective T cell homeostasis in LAG-$3^{-/-}$ mice. *A*, LAG-$3^{+/+}$ and LAG-$3^{-/-}$ mice were killed at the times shown, and the number of splenic $\alpha\beta^+$ T cells is indicated. *B*, The number of splenic $CD4^+$ and $CD8^+$ T cells in 16-wk-old mice is shown. *C*, The average number of $\alpha\beta^+$ T cells was determined in axillary, inguinal, and popliteal lymph nodes (average of individual lymph nodes); Peyer's patch (average of individual patches), and bone marrow (taken from the fibula and tibia) from 28-wk-old mice. *D*, LAG-$3^{+/+}$ and LAG-$3^{-/-}$ OT-II TCR transgenic mice were killed at the times shown, and the number of splenic V$\alpha2^+$ T cells is indicated. *E*, LAG-$3^{+/+}$ and LAG-$3^{-/-}$ mice (5, 16, 28, and 52 wk old) were given water containing BrdU (0.8 mg/ml) for 8 days. Splenocytes were counted and stained with anti-$\alpha\beta$ TCR. Cells were then fixed, permeabilized, and stained with anti-BrdU-FITC. *F*, The percentages of $BrdU^+$ $\alpha\beta^+$ T cells at 5, 16, 28, and 52 wk of age were determined. Data in all panels represent the mean ± SE of two to four independent experiments with five to eight mice per group.

old) LAG-3$^{-/-}$ mice have normal T cell numbers (32). However, the number of $\alpha\beta^+$ T cells increases in LAG-3$^{-/-}$ mice to ~2-fold higher than that in WT mice at ~16 wk of age (Fig. 1*A*). This difference is highly significant ($p < 0.005$) given the tight homeostatic regulation of $\alpha\beta^+$ T cell numbers in WT mice and the very low SE. Both CD4$^+$ and CD8$^+$ cells were increased in LAG-3$^{-/-}$ mice, but the CD4:CD8 ratio was unchanged, except in 1-year-old mice (Fig. 1*B* and data not shown). Interestingly, T cells were not significantly elevated in bone marrow or axillary, inguinal, and popliteal lymph nodes (Fig. 1*C*). However, T cell numbers were clearly elevated in Peyer's patches from LAG-3$^{-/-}$ mice, indicating that these observations were not restricted to the spleen, but did differ between different lymphoid organs. LAG-3$^{-/-}$ mice transgenic for OT-II TCR ($OVA_{326-339}$-specific, H-2A^b-restricted) (35) also had an increased number of CD4$^+$ Vα2$^+$ T cells compared with WT control OT-II transgenic mice, except that this difference was evident at 5 wk of age (Fig. 1*D*). The increased cell number observed in LAG-3$^{-/-}$ mice was consistent with an ~50% increase in the number of dividing BrdU$^+$ cells in vivo, although the percentage was largely unchanged (Fig. 1, *E* and *F*). It is important to note that the differences in cell numbers observed between LAG-3$^{-/-}$ and WT mice were highly consistent and reproducible. The absence of LAG-3 did not appear to have any significant effect on the cell surface phenotype of T cells from LAG-3$^{-/-}$ mice or the ratio of naive, memory, or regulatory T cells (data not shown). Taken together, these data suggest that LAG-3 may help regulate T cell numbers in mice.

It has been reported that cytokines are important in the maintenance of T cell homeostasis, in particular IL-7, as well as other cytokines that signal through the common γ-chain (14, 15). However, analysis of serum from 12-wk-old LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice did not reveal differences in IL-7 levels (data not shown). The presence of 16 additional cytokines was also tested, but no differences were found (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-17, KC, MIP-1α, RANTES, GM-CSF, IFN-γ and TNF-α) (data not shown). Because IL-15 levels were not tested, it is possible that this cytokine may be involved in homeostatic control.

Enhanced homeostatic expansion of LAG-3$^{-/-}$ T cells

To determine whether LAG-3 influences the homeostatic expansion of T cells in a lymphopenic environment, purified T cells were adoptively transferred into RAG-1$^{-/-}$ mice, which lack T and B cells, and the T cell number in the spleen was determined 15 days posttransfer. There was a 2.6-fold increase in the number of LAG-3$^{-/-}$ T cells compared with that in the WT control (Fig. 2*A*). Remarkably, there was virtually no LAG-3 expressed on WT T cells despite the clear effect that the absence of LAG-3 has on T cell expansion (data not shown). Increased expansion of both CD4$^+$ and CD8$^+$ T cells was observed, demonstrating that both cell types were equally affected by the absence of LAG-3. There were no obvious differences in cell surface phenotype or the percentage of apoptotic cells, as determined by annexin V staining, between LAG-3$^{-/-}$ and LAG-3$^{+/+}$ T cells (data not shown). As previously reported, these cells display a memory-like phenotype, with increased expression of CD44 and reduced CD62L (42–44). However, very few cells expressed the activation marker CD69. Importantly, this increased cell number was not due to the selective expansion of memory cells, because a comparable difference was seen after transfer of sorted naive CD44^{low} LAG-3$^{-/-}$ and LAG-3$^{+/+}$ T cells (Fig. 2*B*).

To ensure that the increased expansion of LAG-3$^{-/-}$ T cells observed in RAG-1$^{-/-}$ mice was independent of Ag specificity and to further assess the effect of LAG-3 on CD4$^+$ vs CD8$^+$ T cell expansion, we used purified T cells from OT-I ($OVA_{257-264}$-specific, H-2K^b-restricted) (36) and OT-II transgenic mice. OT-II transgenic T cells were of particular interest because they have previously been shown to undergo poor homeostatic expansion in irradiated mice (45). In RAG-1$^{-/-}$ mice, WT CD4$^+$ Vα2$^+$ OT-II T cells do expand, albeit poorly (Fig. 2*C*). In contrast, this did not

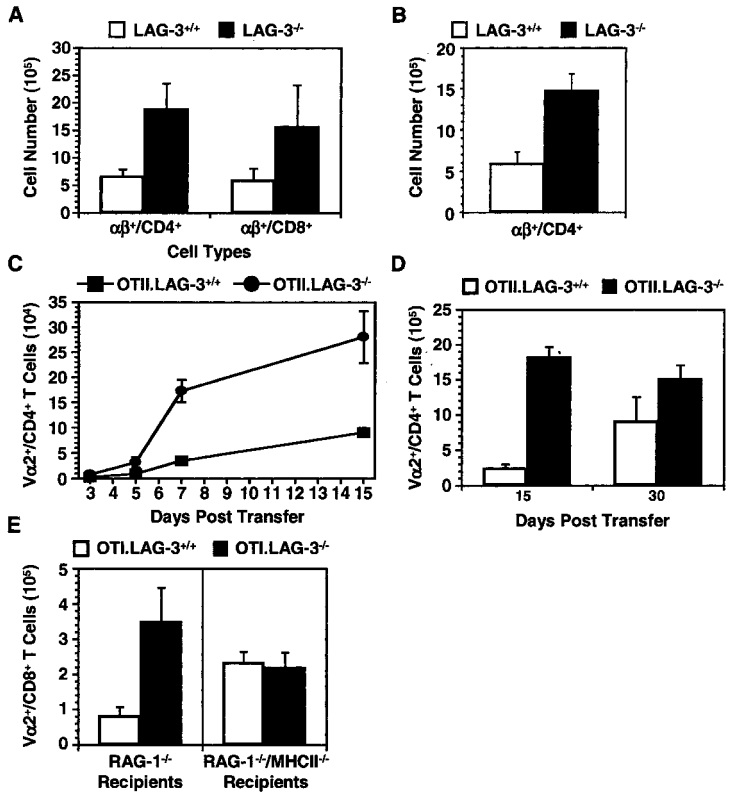

FIGURE 2. T cells lacking LAG-3 undergo greater homeostatic expansion after adoptive transfer into lymphopenic hosts *A*, CD4$^+$ and CD8$^+$ $\alpha\beta^+$ T cells from LAG-3$^{+/+}$ or LAG-3$^{-/-}$ mice were positively sorted from spleens by FACS. CD4$^+$ and CD8$^+$ T cells with the same LAG-3 phenotype were combined at a ratio of 1:1, and 5×10^6 cells (2.5×10^6 CD4$^+$ and 2.5×10^6 CD8$^+$) were injected via the tail vein into RAG-1$^{-/-}$ mice. Fifteen days later, the recipient mice were killed, and the number of T cells in the spleen was determined. *B*, CD4$^+$/CD44^{low} cells from LAG-3$^{+/+}$ or LAG-3$^{-/-}$ mice were positively sorted by FACS and transferred into RAG-1$^{-/-}$ mice. Seven days later, the recipient mice were killed, and the number of CD4$^+$ T cells was determined. *C*, OTII.LAG$^{+/+}$ and OTII.LAG-3$^{-/-}$ transgenic T cells were negatively sorted by MACS, and 5×10^6 transgenic T cells were transferred into RAG-1$^{-/-}$ mice. Recipients were killed at the times indicated, and the number of transgenic T cells in the spleen was determined. *D*, OTII.LAG-3$^{+/+}$ or OTI-I.LAG3$^{-/-}$ T cells were activated with $OVA_{326-339}$ peptide for 4 days and allowed to rest for 10 days. Vα2$^+$/CD4$^+$ cells were sorted and allowed to rest for an additional 7 days. T cells (5×10^6) were transferred into RAG-1$^{-/-}$ mice and analyzed 15 or 30 days posttransfer. *E*, OTI.LAG-3$^{+/+}$ and OTI.LAG-3$^{-/-}$ T cells were purified by FACS using anti-Vα2 and anti-CD8 Abs. The cells were then adoptively transferred into RAG-1$^{-/-}$ and RAG-1$^{-/-}$/MHC class II$^{-/-}$ recipients. The number of transgenic T cells was determined on the days indicated. Data in all panels represent the mean ± SE of two to four independent experiments with three to nine mice per group.

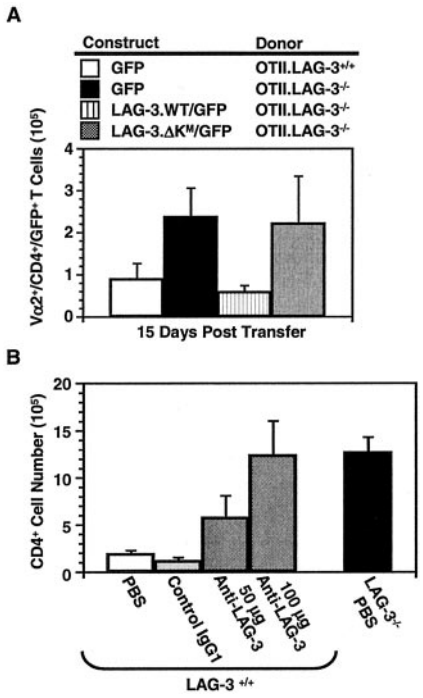

FIGURE 3. LAG-3 is directly responsible for the increased homeostatic expansion seen after transfer of LAG-3$^{-/-}$ T cells into lymphopenic hosts. *A*, Splenocytes from OTII.LAG-3$^{+/+}$ or OTII.LAG3$^{-/-}$ mice were activated as described in Fig. 2*D* for 2 days and transduced with retrovirus carrying either GFP alone, LAG-3.WT and GFP, or LAG-3.ΔK^M and GFP by incubating the T cells with retroviral producer cell lines for 2 days. Cells were rested for 10 days, and Vα2$^+$GFP$^+$ cells were sorted by FACS. After an additional 2-day rest, 5×10^6 cells were transferred into RAG-1$^{-/-}$ mice. Fifteen days posttransfer, the mice were killed, the spleens were removed, and the number of Vα2$^+$CD4$^+$GFP$^+$ transgenic T cells was determined. Data represent the mean $\pm$ SE of two independent experiments with 8–10 mice per group. *B*, In vivo manipulation of T cell homeostasis by anti-LAG-3 mAb treatment. LAG-3$^{+/+}$ and LAG-3$^{-/-}$ T cells were negatively sorted by MACS. T cells (5×10^6) plus anti-LAG-3 (C9B7W; 100 or 50 μg), isotype control (200 μg), or PBS were injected into RAG-1$^{-/-}$ mice via the tail vein. Mice were given a second treatment of the appropriate Ab at half the original dose (50 or 25 μg) or PBS i.v. on day 3. On day 7, the number of CD4$^+$ T cells in the spleen was determined. Data represent the mean $\pm$ SE of two independent experiments with two to eight mice per group.

apply to T cells from LAG-3$^{-/-}$ OT-II transgenic mice, which expanded vigorously in lymphopenic hosts to numbers 3.1-fold higher than those of WT T cells by 15 days posttransfer. This enhanced expansion was not due to a nonspecifically induced hyperactivated state, because no proliferation was observed after transfer of cells into lympho-replete mice (data not shown). The influence of LAG-3 expression on homeostatic expansion in lymphopenic mice is not limited to naive T cells. Transfer of Ag-experienced memory OT-II T cells also resulted in a 7.4-fold expansion of LAG-3$^{-/-}$ T cells compared with WT control cells (Fig. 2*D*). This appeared to reach a plateau at 15 days posttransfer, whereas WT cells continued to expand to day 30.

Similar studies with H-2K^b-restricted OT-I T cells again emphasize that CD4$^+$ and CD8$^+$ T cells are comparably affected. The number of LAG-3$^{-/-}$ CD8$^+$ Vα2$^+$ OT-I transgenic T cells recovered from RAG-1$^{-/-}$ mice was 4.3-fold higher than that of WT control OT-I T cells 7 days posttransfer (Fig. 2*E*). We have previously shown that ligation of LAG-3 by MHC class II is required for LAG-3 function in vitro (31). To address this issue in vivo, we transferred OTI.LAG-3$^{+/+}$ and OTI.LAG-3$^{-/-}$ transgenic T cells into RAG-1$^{-/-}$/MHC-II$^{-/-}$ mice. As predicted, both OTI.LAG-3$^{+/+}$ and OTI.LAG-3$^{-/-}$ T cells expanded equivalently in RAG-1$^{-/-}$/MHC-II$^{-/-}$ mice 7 days posttransfer (Fig. 2*E*). Furthermore, OTI.LAG-3$^{+/+}$ T cells expanded more after transfer into RAG-1$^{-/-}$/MHC-II$^{-/-}$ vs RAG-1$^{-/-}$ mice (2.9-fold). These data suggest that ligation of MHC class II molecules is required for LAG-3 to control homeostatic expansion.

T cell homeostasis is controlled by a KIEELE motif in the cytoplasmic domain of LAG-3

Were the defects observed due to the absence of LAG-3 or were they caused by disruption of a closely linked gene by the original targeting strategy? Furthermore, was LAG-3 directly or indirectly involved in this process? To address these questions, we transduced LAG-3$^{-/-}$ OT-II T cells with murine stem cell virus-based retrovirus that contained either WT LAG-3 or a signaling-defective mutant, LAG-3.ΔK^M, which lacks a conserved KIEELE motif (31). The vector also contained an internal ribosomal entry site and GFP cassette to facilitate analysis of transduced cells (38). LAG-3$^{-/-}$ and LAG-3$^{+/+}$ OT-II T cells were also transduced with an empty vector/GFP-alone control. It is important to note that the transduction efficiencies of WT LAG-3, LAG-3ΔK^M, and empty vector/GFP-alone were equivalent (data not shown). Transduced cells were sorted on the top 30–40% of GFP$^+$ cells and transferred into RAG-1$^{-/-}$ recipients. Fifteen days posttransfer, the number of GFP$^+$/OT-II T cells was determined. As expected, the control LAG-3$^{-/-}$ T cells transduced with GFP alone expanded more than the WT GFP$^+$ T cells (2.6-fold; Fig. 3*A*). Ectopic expression of LAG-3 reduced the number of OT-II T cells to a level comparable to that of the WT control, whereas expression of the LAG-3 signaling-defective mutant had no effect on homeostatic expansion. These data demonstrate that LAG-3 is directly involved in the regulation of T cell homeostasis and that this requires the KIEELE motif in the cytoplasmic domain of LAG-3 (31).

LAG-3-mediated control of T cell homeostasis can be blocked in vivo by anti-LAG-3 mAb treatment

We have previously described a mAb to murine LAG-3 that blocks its function in vitro without interfering with its ability to bind to MHC class II molecules (29). RAG-1$^{-/-}$ recipients of LAG-3$^{-/-}$ and LAG-3$^{+/+}$ CD4$^+$ T cells were treated with purified anti-LAG-3 or an irrelevant rat IgG1 control on days 0 and 3. The data show that anti-LAG-3 treatment blocked LAG-3 function in vivo, resulting in accelerated homeostatic expansion of WT T cells to a value comparable to the number of LAG-3$^{-/-}$ cells in control PBS-treated mice (Fig. 3*B*). Despite the dramatic effect of the Ab on blocking LAG-3 function, the mechanism of Ab blocking is still under investigation. Although our previous in vitro results suggest that the mAb is not blocking LAG-3 interaction with MHC class II, we cannot exclude the possibility that it is doing so in vivo (45). Another possibility is that the Ab is blocking the interaction of LAG-3 with another molecule we have yet to identify. Although the exact mechanism of Ab blocking is unclear, the results are striking with negligible background. Taken together, these data suggest that anti-LAG-3 treatment may allow for the therapeutic manipulation of homeostasis.

Cells that express LAG-3 regulate the expansion of cells that do not

More extensive analysis of 16-wk-old LAG-3$^{-/-}$ mice revealed a generalized increase in many cell types. The numbers of $\gamma\delta^+$ T cells and CD244.2$^+$ NK cells were significantly increased in LAG-3$^{-/-}$ mice (Fig. 4*A*). This might be expected given that ~20% of these cell types constitutively express LAG-3 in WT mice (29).

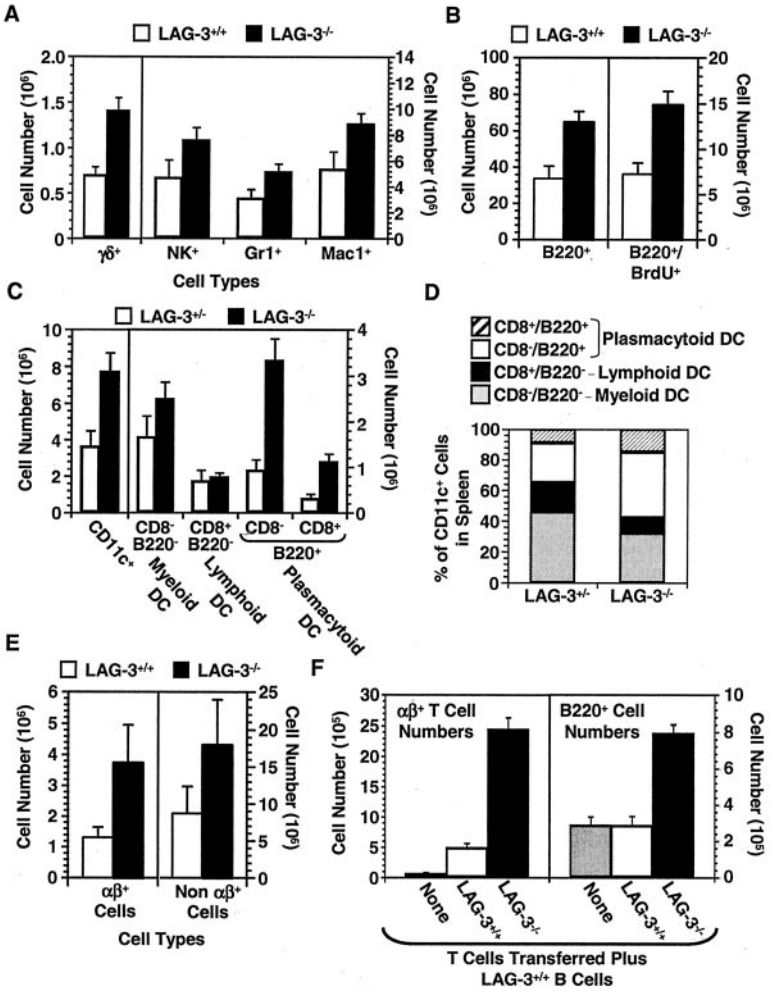

FIGURE 4. Cells that express LAG-3 regulate the expansion of cells that do not. *A*, Splenocytes from 16-wk-old mice were stained with anti-γδTCR, anti-CD49b (pan-NK), anti-Gr1 (granulocyte marker), and anti-Mac-1 (monocytes/macrophages), and cell number was determined. *B*, LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice (16 wk old) were given water containing BrdU (0.8 mg/ml) for 8 days. Splenocytes were counted and stained with anti-B220. Cells were then fixed, permeabilized, and stained with anti-BrdU-FITC. *C*, LAG-3$^{+/-}$ and LAG-3$^{-/-}$ mice (28 wk old) were killed, and their spleens were removed and treated with collagenase/DNase. The splenic cells were stained with anti-CD11c (DC marker), anti-B220, and anti-CD8, and cell numbers were determined. *D*, The percentage of CD11c$^+$ splenic cells that were either B220$^+$ and/or CD8$^+$ was determined. *E*, $\alpha\beta^+$ T cells from LAG-3$^{+/+}$ or LAG-3$^{-/-}$ mice were purified and transferred as described in Fig. 2*A*. Fifteen days later, the recipients were killed, and the numbers of splenic $\alpha\beta^+$ donor-derived T cells and $\alpha\beta^-$ host-derived cells were determined. *F*, Lymphocytes from the spleens of LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were sorted by negative MACS and then separated into B cells (from LAG-3$^{+/+}$ mice only) and Thy 1.2$^+$ T cells by positive MACS. T and B cells were mixed at a 1:1 ratio, and 5×10^6 of each cell type were transferred into RAG-1$^{-/-}$ recipients. Control mice were injected with 5×10^6 B cells only. On day 7 posttransfer, the numbers of splenic T and B cells recovered from the spleens of the adoptively transferred recipients were determined by staining with anti-$\alpha\beta$ TCR and anti-B220, respectively. Data represent the mean ± SE of one or two independent experiments with three to six mice per group.

However, we were surprised to find that several other cell types, such as B220$^+$ B cells, Gr-1$^+$ granulocytes, Mac-1$^+$ macrophages, and CD11c$^+$ DC, none of which expresses LAG-3 (26, 27, 29), were also increased in LAG-3$^{-/-}$ mice (Fig. 4, *A-C*). The increased B cell number observed in LAG-3$^{-/-}$ mice was also reflected in a 2-fold increase in the number of BrdU$^+$ cells (Fig. 4*B*). We were intrigued to find that the proportion of each DC subset was altered. Although the number of myeloid CD8$^-$B220$^-$ DCs and lymphoid CD8$^+$B220$^-$ DCs was largely unaffected, there was a substantial increase in the number and percentage of plasmacytoid CD8$^{+/-}$B220$^+$ DCs in LAG-3$^{-/-}$ mice (Fig. 4, *C* and *D*).

A direct role for T cells in mediating this multilineage increase was implied by a 2-fold increase in the number of $\alpha\beta^-$ host-derived cells observed in RAG-1$^{-/-}$ recipients of LAG-3$^{-/-}$ T cells (Fig. 4*E*). These data imply that an alteration in the homeostatic control of T cells due to the absence of LAG-3 may directly alter the control of other leukocyte cell types. To test this directly, B cells were cotransferred with either LAG-3$^{-/-}$ or WT T cells into RAG-1$^{-/-}$ mice. As seen previously, there was a 5.0-fold increase in the number of LAG-3$^{-/-}$ T cells compared with WT control cells when transferred with B cells (Fig. 4*F*). In the presence of WT T cells, the number of B cells recovered from the spleen 7 days posttransfer was identical with that in mice receiving B cells alone. In contrast, there was a 2.8-fold increase in the number of B cells recovered from LAG-3$^{-/-}$ T cell recipients.

To date, our data suggest that there is not a generalized activated phenotype that causes the multilineage expansion. This idea is further supported by some preliminary studies that suggest that the ability of CD11c$^+$ DCs from LAG-3$^{-/-}$ or LAG-3$^{+/+}$ mice to stimulate T cells is comparable (data not shown), although additional studies would be required to analyze this issue more extensively. Taken together, these data provide a direct demonstration that the generalized multilineage increase is due to the deregulation of T cells lacking LAG-3.

T_{reg} cells control T cell homeostasis via LAG-3

How does LAG-3 regulate T cell homeostasis? First, signaling through LAG-3 may directly control homeostatic T cell expansion or induce the secretion of molecules that cause enhanced T cell expansion. Second, LAG-3 may function indirectly via another cell population. Because CD4$^+$CD25$^+$ T_{reg} cells have been shown to control T cell homeostasis, it is possible that LAG-3 is required for optimal T_{reg} cell function. These possibilities were tested directly in cotransfer experiments. LAG-3$^{-/-}$ Thy1.2$^+$ T cells and LAG-3$^{+/+}$ Thy1.1$^+$ T cells were transferred alone or together into RAG-1$^{-/-}$ recipients. The number of $\alpha\beta^+$ CD4$^+$ or CD8$^+$ T cells in the spleen was determined 7 days posttransfer, and LAG-3 genotype was distinguished on the basis of Thy1 isotype expression. Previous studies have shown that the number of cells transferred can affect the extent of homeostatic expansion (20). To ensure that this factor did not complicate data interpretation, we chose a cell dose that could be doubled without significantly increasing the number of cells recovered (Fig. 5*A*). These experiments showed that transfer of either 5×10^6 or 1×10^7 cells resulted in the recovery of comparable numbers of cells even though there was still a 2-fold

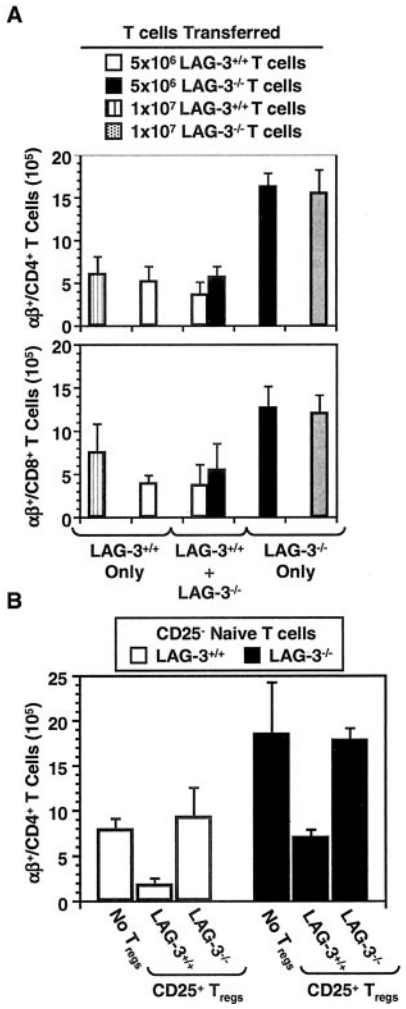

FIGURE 5. T_{reg} cells require LAG-3 to control homeostatic proliferation. *A*, Splenocytes from LAG-3$^{+/+}$ (B6.PL; Thy1.1^{+}) and LAG-3$^{-/-}$ (Thy1.2^{+}) mice were negatively sorted by MACS, and 5×10^6 or 1×10^7 T cells were transferred separately or mixed (1:1 ratio of 5×10^6 cells/T cell type) into RAG-1$^{-/-}$ recipients. Splenocytes were analyzed on day 15 posttransfer. The genotype of transferred cells was determined by Thy1 expression. Data represent the mean ± SE of two independent experiments with 10–13 mice per group *B*, Splenocytes from LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were negatively sorted by MACS for CD4^{+} T cells and then positively sorted by MACS again for CD4^{+}CD25^{+} (50–60% purity) or CD4^{+}CD25^{-} T cells (92–95% purity). CD4^{+}CD25^{-} T cells (4×10^6 cells) were transferred separately or mixed with CD4^{+}CD25^{+} T_{reg} cells (1.5×10^6 cells) for final T_{reg} percentage of 15% and transferred into RAG-1$^{-/-}$ recipients. Splenocytes were analyzed 7 days posttransfer. Data represent the mean ± SE of seven experiments with two to four mice per group.

increase in the number of LAG-3$^{-/-}$ vs LAG-3$^{+/+}$ T cells recovered (Fig. 5*A*). Cotransfer of both populations clearly demonstrated that LAG-3$^{+/+}$ T cells prevented LAG-3$^{-/-}$ T cells from expanding. These data clarify an important point. The ability of LAG-3$^{+/+}$ T cells to control the expansion of LAG-3$^{-/-}$ T cells suggests an indirect effect, perhaps involving T_{reg} cells.

This possibility was tested directly by cotransfer experiments with naive CD4^{+}CD25^{-} T cells in the presence or the absence of CD4^{+}CD25^{+} T_{reg} cells. LAG-3$^{-/-}$ and LAG-3$^{+/+}$ T cells were first negatively sorted by MACS to isolate CD4^{+} cells, then positively sorted by FACS to separate CD4^{+}CD25^{+} and CD4^{+}CD25^{-} T cell populations. CD4^{+}CD25^{-} T cells were transferred alone or with CD4^{+}CD25^{+} T_{reg} cells of the same or opposing genotype (15% of the total cell number transferred). Two important observations were made. First, the enhanced expansion of LAG-3$^{-/-}$ T cells occurs independently of T_{reg} cells (no T_{reg}, 2.3-fold difference; LAG-3$^{+/+}$ T_{reg}, 3.8-fold difference; LAG-3$^{-/-}$ T_{reg}, 1.9-fold difference). This suggests that LAG-3 can affect homeostatic T cell expansion directly. Second, T_{reg} cells from LAG-3$^{+/+}$ mice clearly reduced homeostatic proliferation of both LAG-3$^{+/+}$ and LAG-3$^{-/-}$ T cells (Fig. 5*B*). However, T_{reg} cells sorted from LAG-3$^{-/-}$ mice did not reduce LAG-3$^{+/+}$ or LAG-3$^{-/-}$ CD4^{+}/CD25^{-} T cell expansion. This suggests that LAG-3 is required for optimal T_{reg} cell control of homeostatic expansion.

Discussion

Our data clearly show that LAG-3 negatively regulates the homeostatic expansion of T cells. Previous studies have suggested that CTLA-4 and TGF-β may also be negative regulators of T cell homeostasis (18, 19, 46). However, T cells from mice that lack CTLA-4 or carry a dominant negative TGF-β receptor have an activated phenotype; therefore, it is unclear whether the increased T cell number in these mice is due to a true breakdown of homeostasis or simply the continuous proliferation of activated T cells (16, 17, 19). In contrast, T cells from LAG-3$^{-/-}$ mice are indistinguishable, in terms of cell surface markers, from their WT counterparts. Thus, LAG-3 may be a true regulator of T cell homeostasis. Although it is clear that LAG-3 does not completely prevent T cell expansion, it may serve as a molecular brake to control homeostatic proliferation.

As previously reported (32) and shown in this study, no difference in T cell numbers are seen in younger mice. However, a significant increase in T cells in LAG-3$^{-/-}$ mice is seen in older mice. Three possibilities are worth consideration. First, one possible explanation is that the slightly increased turnover of LAG-3$^{-/-}$ T cells is not evident until the mice are ~3 mo old and is too subtle to be seen in BrdU experiments. Second, T_{reg} cells are not required for homeostatic control until after 5 wk of age. Third, LAG-3$^{-/-}$ T_{reg} cells may be able to control T cell homeostasis up to 5 wk of age due to their residual regulatory activity, which becomes insufficient after this time. This latter possibility may be supported by our observations with OT-II.LAG-3$^{-/-}$ mice, which display a significantly increased T cell number at 5 wk, because these mice have a much lower number of T_{reg} cells and thus manifest deregulated homeostasis at a younger age. It is also noteworthy that the differences observed between LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice are almost lost by 52 wk of age. The reason for this is unknown, but it is possible that the expanded T cells die off due to exhaustion, leaving a more homeostatically balanced population.

LAG-3 appears to both directly and indirectly regulate T cell homeostasis. Our data support a direct role for LAG-3 in regulating homeostatic T cell expansion independently of CD4^{+}CD25^{+} T_{reg} cells (see Figs. 2*E*, 3*A*, and 5*B*). Importantly, the enhanced expansion of purified LAG-3$^{-/-}$ CD8^{+} T cells, which were devoid of CD4^{+}CD25^{+} T_{reg} cells, in lymphopenic mice also supports this premise (see Fig. 2*E*). However, as yet we cannot rule out the possibility that a small percentage of CD25^{-} T cells have regulatory activity or that cells within the expanding population acquire regulatory activity and indirectly affect T cell expansion. These data suggest that LAG-3 may impart regulatory potential on any cell in which it is expressed. This idea is supported by our demonstration that ectopic expression of LAG-3 confers regulatory potential on activated T cells (unpublished observations) (47), a property that to date has only been demonstrated with the forkhead/winged helix transcription factor (48, 49).

Our data also support an indirect role for LAG-3 in regulating T cell homeostasis. In the absence of LAG-3, CD4^{+}CD25^{+} T_{reg} cells are completely unable to control T cell expansion, suggesting

that LAG-3 is required for T_{reg} control of T cell homeostasis. Recently, a more general role for LAG-3 in T_{reg} function has been suggested (47). A comparative analysis of gene expression arrays from Ag-specific $CD4^+$ T cells differentiating to either an effector/memory or a regulatory phenotype was performed to identify molecules that were selectively expressed on T_{reg} cells. LAG-3 was found to be the most differentially expressed gene of those whose identity is currently known. This analysis revealed that LAG-3 is differentially expressed on induced T_{reg} cells, and this correlated with their in vitro regulatory activity. Importantly, the regulatory activity in this system could also be blocked with anti-LAG-3 mAb in vitro and in vivo. Thus, using two very distinct, but complimentary, experimental systems, we provide strong data suggesting that LAG-3 is required for maximal T_{reg} function.

Despite the clear effect that LAG-3 has on T cell homeostasis, only low levels of LAG-3 could be detected on WT T cells. Interestingly, we have recently shown that there is a 10-fold increase in LAG-3 mRNA in $CD4^+CD25^+$ compared with $CD4^+CD25^-$ T cells (47). Thus, there is an apparent discrepancy between the minimal LAG-3 cell surface expression observed on resting $CD4^+CD25^+$ T cells and cells undergoing homeostatic expansion and the mRNA data. An explanation for this may come from our recent observation that LAG-3 is readily cleaved from the cell surface (50). Thus transient, low level expression of LAG-3 may be sufficient to mediate its effect, perhaps emphasizing the importance of its high affinity for MHC class II molecules (29, 51). Likewise, given that LAG-3 appears to be required for T_{reg} control of homeostasis, LAG-3 may only need to be transiently expressed on the small population of regulatory T cells to have a significant effect on homeostatic T cell expansion. Collectively, these data suggest that cell surface expression of LAG-3 is carefully regulated and thus is difficult to detect on the surface of resting T_{reg} cells. Despite this low level expression, it is very evident from our data that signaling through the cytoplasmic domain of LAG-3 is essential for it to mediate its negative regulatory function (Fig. 3*A*) (31). Indeed, we have recently shown that ectopic expression of LAG-3 is sufficient to confer both cell intrinsic and extrinsic regulatory activity (49). Thus, transient expression on either the expanding $CD25^-$ population or the $CD25^+$ regulatory cells could be sufficient to mediate homeostatic control. Identification of molecules that bind to the KIEELE motif in LAG-3 is clearly required to gain further insight into LAG-3 function.

Our data clearly show that LAG-$3^{-/-}$ T cells mediate the enhanced homeostatic expansion of multiple cell types. This is evident from the increased numbers of all cell types analyzed in LAG-$3^{-/-}$ mice and data from the adoptive transfer experiments. Given that T_{reg} cells are unable to control T cell expansion in these studies, it is possible that they may also directly or indirectly regulate other cell types. Alternatively, the increased number of total T cells and/or DCs may also influence multiple cell types. Although we did not see differences in cytokine levels in sera from LAG-$3^{+/+}$ and LAG-$3^{-/-}$ mice, we cannot exclude the possibility that such differences do exist, but that these are consumed by the increased number of T cells in LAG-$3^{-/-}$ mice. It is evident, however, that these increased cell numbers are not due to cells having an activated state, implying a true alteration of the homeostatic balance in these mice.

One intriguing finding was the differential expansion of different DC subsets in the LAG-$3^{-/-}$ mice. Although the number of myeloid $CD8^-B220^-$ DCs and lymphoid $CD8^+B220^-$ DCs was largely unaffected, there was a substantial increase in the number of plasmacytoid $CD8^{+/-}B220^+$ DCs in LAG-$3^{-/-}$ mice. The reason for this is unclear, but it is worth noting that plasmacytoid DCs have been referred to as tolerogenic under certain circumstances and produce large amounts of IFN-α, which inhibits cell proliferation (52). Thus, the increased number of plasmacytoid DCs may help to limit the consequence of the absence of LAG-3.

Although there was clearly an increased number of T cells in spleen and Peyer's patch, this difference was not seen in lymph nodes or bone marrow. The significance of this intriguing finding is unclear. It is conceivable that bone marrow and lymph nodes only contain T cells that are in transit and thus are less affected by the absence of LAG-3. In contrast, spleen and Peyer's patch have more resident T cells that are not migrating and thus can accumulate as a consequence of homeostatic deregulation. Our data may indicate that the contributions of different organs to homeostatic regulation are distinct, and as such, LAG-$3^{-/-}$ mice may represent a very useful model to dissect this issue further.

It is intriguing that a molecule that binds to MHC class II regulates both $CD4^+$ and $CD8^+$ T cell homeostasis. Our data imply a requirement for LAG-3:MHC class II ligation in vivo to regulate homeostatic expansion. Is the restricted expression of MHC class II important for the consequences of LAG-3 function? It is likely that in lymphoid organs or other sites where a high number of MHC class II^+ B cells or DCs are present, T cell expansion would be controlled by LAG-3. However, in instances where T cells would normally undergo homeostatic expansion, such as in neonates (8), MHC class II^+ cells would also be largely absent, thus allowing for the discriminatory use of LAG-3.

Hemopoietic stem cell and bone marrow transplantation are becoming standard therapies in the treatment of both malignant and nonmalignant disorders (53). However, for patients that receive myeloablative conditioning, there is a long period of profound immunodeficiency that can result in significant complications and mortality. Full T cell reconstitution can take >1 year for autologous bone marrow transplantation (53, 54). Some peripheral expansion of transplanted donor T cells in the graft occurs in the first few months and provides some level of protection (55, 56). However, de novo generation of thymus-derived T cells is known to be essential for complete immunocompetence (57, 58). Any therapeutic intervention that leads to accelerated T cell reconstitution could have significant clinical benefits. Our data suggest that interfering with LAG-3 function could be a legitimate target. Indeed, our data show that in vivo treatment with anti-LAG-3 mAb resulted in accelerated T cell expansion (Fig. 3*B*). Data from this study also suggest that LAG-3 is required for T_{reg} cell control of homeostasis, suggesting that LAG-3 modulation may be used to enhance or abrogate T_{reg} cell activity. Identification of additional molecules that negatively regulate lymphocyte homeostasis will be important in furthering our understanding of this essential, biological process and in providing additional targets for therapeutic intervention.

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