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Lymphocyte Activation Gene-3 (CD223) Regulates the Size of the Expanding T Cell Population Following Antigen Activation In Vivo[1]

Creg J. Workman,* Linda S. Cauley,[2†] In-Jeong Kim,[†] Marcia A. Blackman,[†] David L. Woodland,[†] and Dario A. A. Vignali[3*]

Lymphocyte activation gene-3 (LAG-3) is a CD4-related, activation-induced cell surface molecule that binds to MHC class II with high affinity. In this study, we used four experimental systems to reevaluate previous suggestions that LAG-3$^{-/-}$ mice had no T cell defect. First, LAG-3$^{-/-}$ T cells exhibited a delay in cell cycle arrest following in vivo stimulation with the superantigen staphylococcal enterotoxin B resulting in increased T cell expansion and splenomegaly. Second, increased T cell expansion was also observed in adoptive recipients of LAG-3$^{-/-}$ OT-II TCR transgenic T cells following in vivo Ag stimulation. Third, infection of LAG-3$^{-/-}$ mice with Sendai virus resulted in increased numbers of memory CD4$^+$ and CD8$^+$ T cells. Fourth, CD4$^+$ T cells exhibited a delayed expansion in LAG-3$^{-/-}$ mice infected with murine gammaherpesvirus. In summary, these data suggest that LAG-3 negatively regulates T cell expansion and controls the size of the memory T cell pool. ***The Journal of Immunology*****, 2004, 172: 5450–5455.**

Relatively few cell surface molecules have been identified that negatively regulate T cell activation. Notable examples are CTLA-4 and programmed death receptor 1, which have been shown to control T cell proliferation and expansion following activation (1). In the case of CTLA-4, absence of this molecule results in a striking lymphoproliferative disorder, mediated by the costimulation dependent activation of CD4$^+$ T cells (2–4). However, this does not occur in CTLA-4/recombinant-activating gene (RAG)$^{-/-}$ TCR transgenic mice (5, 6) or CTLA-4/B7-1/B7-2$^{-/-}$ (CD80/CD86) mice (7), suggesting that CTLA-4 may not play a major role in the regulation of primary activated T cells (5, 6, 8).

Lymphocyte activation gene-3 (LAG-3)[4] is expressed on activated CD4$^+$ and CD8$^+$ T cells and a subset of $\gamma\delta$ T cells and NK cells (9–12). Although it is similar to CD4 in structure and genomic organization, the two molecules share <20% amino acid sequence homology (13, 14). Like CD4, LAG-3 binds to MHC class II molecules but with a much higher affinity, prompting suggestions from studies with human T cells that LAG-3 might act as a negative competitor of CD4 (11, 12, 15). The initial analysis of LAG-3$^{-/-}$ mice did not reveal a defect in T cell function (16, 17). Positive and negative selection appeared normal. The number and distribution of CD4$^+$ and CD8$^+$ T cells, their activated and memory subpopulations were also normal. Furthermore, the T cell proliferative response to mitogens and Ag priming, and the generation and function of CTLs to several viruses seemed unperturbed (16). It was also shown that LAG-3 was not responsible for selecting Th cells in CD4$^{-/-}$ mice (17). In contrast, it was proposed that LAG-3 may define different modes of NK killing (16), although this was not supported by studies with human NK cells (18). Despite the suggestion that there was no T cell role for LAG-3, it was conceivable that LAG-3 performed a function that was not revealed in the original study. Indeed, we have recently shown that murine LAG-3 can act as a negative regulator of T cell function in vitro (19). Furthermore, we have shown that LAG-3 function is dependent on binding to MHC class II molecules and signaling through a conserved KIEELE motif in its cytoplasmic domain (19, 20).

In this study, we set out to reevaluate previous suggestions that LAG-3$^{-/-}$ mice had no T cell defect (16, 17), and to determine whether there was a role for LAG-3 in T cell activation and expansion in vivo or in the generation of a memory T cell response. This was accomplished by using four systems: 1) LAG-3$^{-/-}$ mice treated with the superantigen staphylococcal enterotoxin B (SEB), 2) OTII.LAG-3$^{-/-}$ and OTII.LAG-3$^{+/+}$ transgenic T cells stimulated with peptide in vivo following adoptive transfer into Thy-1.1$^+$ B6.PL mice, 3) LAG-3$^{-/-}$ mice infected with Sendai virus, and 4) LAG-3$^{-/-}$ mice infected with murine gammaherpesvirus 68 (MHV-68).

Materials and Methods

Mice

LAG-3$^{-/-}$ mice were provided by Y.-H. Chien (Stanford University, Palo Alto, CA) with permission from C. Benoist and D. Mathis (Joslin Diabetes Center, Boston, MA) (16). Genome-wide microsatellite analysis demonstrated that 85 (97%) of 88 genetic markers tested were derived from C57BL/6 mice (Charles River Breeding Laboratories, Troy, NY). For some

*Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105; and †Trudeau Institute, Saranac Lake, NY 12983

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[2] Current address: Department of Medicine, MC1319, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-1319.

[3] Address correspondence and reprint requests to Dr. Dario A. A. Vignali, Department of Immunology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105-2794. E-mail address: dario.vignali@stjude.org

[4] Abbreviations used in this paper: LAG-3, lymphocyte activation gene-3; SEB, staphylococcal enterotoxin B; MHV-68, murine gammaherpesvirus-68; NP, nucleoprotein; HN, hemagglutinin-neuraminidase; ORF, open reading frame; MLN, mediastinal lymph node.

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experiments, these mice were crossed with OT-II TCR transgenic mice (kindly 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, Victoria, Australia) (21). B6.PL-*Thy1a*/Cy mice (Thy1.1 congenic) were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific pathogen-free facilities following national, state, and institutional guidelines. Animal protocols were approved by the St. Jude and the Trudeau Institute Institutional Animal Care and Use Committees.

Activation with SEB in vivo

LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice (age and sex matched) were injected i.p. with 100 or 10 μg of SEB (Toxin Technology, Sarasota, FL) in PBS. Mice were sacrificed by CO_2 inhalation and the number of live, splenic Vβ7/8$^+$ T cells determined by trypan blue exclusion and flow cytometric analysis following staining with anti-Vβ7.PE (TR310) and anti-Vβ8.PE (F23.1) (BD PharMingen, San Diego, CA). The percentage increase in Vβ7/8$^+$ T cells was determined by comparing the numbers of Vβ7/8$^+$ T cells from SEB-treated mice to untreated mice. For cell cycle analysis, Vβ7/8.FITC-stained splenocytes were resuspended in 1 ml of PBS and 1 ml of 70% ethanol added dropwise while vortexing. Following incubation at room temperature for 30 min, cells were washed with PBS and treated with RNase A (20 μg/ml) in PBS for 30 min at room temperature. Cells were incubated with propidium iodide (5 μg/ml) for 20 min on ice and then analyzed by flow cytometry using MODFit (Verity Software House, Topsham, ME).

Adoptive T cell transfer and OT-II T cell activation in vivo

OTII.LAG-3$^{+/+}$ and OTII.LAG-3$^{-/-}$ splenocytes were stained with anti-Vα2.PE and anti-CD4.allophycocyanin, and sorted on a MoFlow (Cytomation, Fort Collins, CO). Cells were labeled with 5 μM CFSE for 10 min at 37°C in PBS plus 0.1% BSA at 1×10^7 cells/ml and washed twice. B6.PL mice were injected with 5×10^6 cells i.v. and 24 h later with 100 μm of $OVA_{326-339}$ peptide in 500 μl of PBS i.p. Splenocytes were counted by trypan blue exclusion, stained with anti-Thy1.2.PE and anti-CD4.allophycocyanin and analyzed for CFSE levels by flow cytometry.

Viruses and infections

LAG3$^{+/+}$ and LAG3$^{-/-}$ mice were anesthetized with 2,2,2-tribromoethanol and intranasally infected with 250 EID_{50} (50% egg infectious doses) of Enders strain of Sendai virus or 400 PFU MHV-68 (WUMS strain), as previously described (22, 23).

MHC reagents, staining and analysis

MHC staining reagents were generated and used as previously described (28, 29)

ELISPOT assay

MHV-68-specific T cells were enumerated by IFN-γ ELISPOT in response to splenic APCs pulsed with virus (for CD4$^+$ T cell responses) or with virus-specific peptides, open reading frame (ORF)$6_{487-495}$ (AGPHNCMEI) or $ORF61_{524-531}$ (TSINFVKI) (for CD8$^+$ T cell responses) (23, 24). Sendai virus-specific T cells were enumerated using splenic APCs pulsed with hemagglutinin-neuraminidase $(HN)_{419-433}$ or nucleoprotein $(NP)_{324-332}$.

Results

Splenomegaly and increased Vβ7/8$^+$ T cell number in SEB-treated LAG-3$^{-/-}$ mice

The role of murine LAG-3 in vivo was first investigated by analyzing the T cell response to SEB in LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice. As expected, there was a substantial increase in the number of SEB-reactive Vβ7/8$^+$ T cells in the spleens of LAG-3$^{+/+}$ mice 2 days after i.p. injection of 100 μg SEB (Fig. 1*A*). By day 7 and day 14 posttreatment, the Vβ7/8$^+$ T cell numbers had declined to nearly one-half that of naive mice. In contrast, Vβ7/8$^+$ T cell expansion in LAG-3$^{-/-}$ mice was significantly higher (~2-fold) and slightly delayed (peaking at day 3 rather than day 2). In addition, the subsequent decline in T cell numbers in LAG-3$^{-/-}$ mice was delayed and remained higher than wild-type mice at all time points examined. These differences were also observed in mice treated with 10 μg SEB, a dose that normally induces only weak T cell expansion in vivo (Fig. 1*A*). Interestingly, there was noticeable splenomegaly in LAG-3$^{-/-}$ mice on day 2 post–SEB injection, which was not evident in wild-type mice (Fig. 1*B*). This splenomegaly could not be accounted for by the increase in Vβ7/8$^+$ T cells alone, and appeared to result from a generalized increase in non-T cells as well. These data suggest that deregulation of SEB-reactive T cells resulted in bystander expansion.

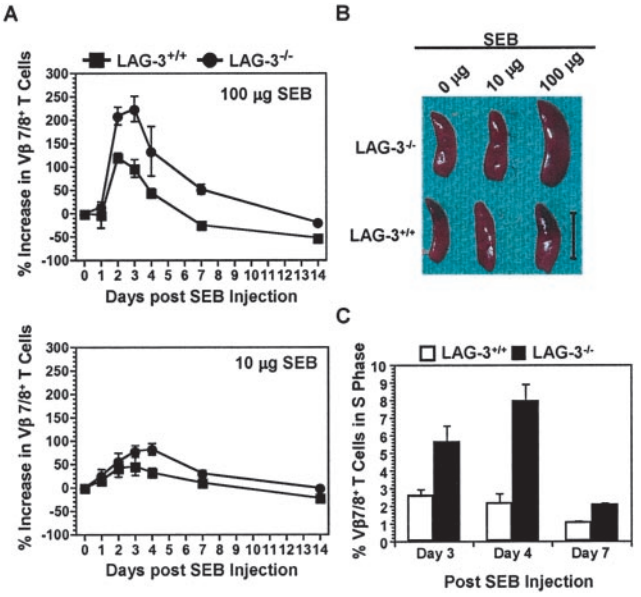

FIGURE 1. Increased numbers of Vβ7/8$^+$ T cells in LAG-3$^{-/-}$ mice treated with SEB in vivo. *A*, LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice were injected i.p. with 100 μg (*top panel*) or 10 μg (*bottom panel*) SEB. The numbers of splenic Vβ7/8$^+$ T cells were determined on the days indicated and the data presented as the percentage change from unmanipulated mice (mean ± SE of four mice analyzed individually from two independent experiments, except day 14 which was two mice). *B*, Picture shows spleens from LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice removed on day 2 posttreatment. Scale bar = 1 cm. *C*, Cell cycle analysis was performed on splenocytes from LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice following SEB treatment. Data were gated on Vβ7/8$^+$ cells and the percentage of cells in S phase determined using MODfit. Data are the mean ± SD of four to six mice analyzed individually.

To further investigate the increase in Vβ7/8$^+$ T cell numbers in vivo, we investigated whether there was a defect in cell cycle arrest. Cell cycle analysis clearly showed that a significantly higher percentage of LAG-3$^{-/-}$ Vβ7/8$^+$ T cells were in S phase compared with the wild-type control 3 and 4 days post SEB treatment (Fig. 1*C*). Taking into account the increased T cell expansion in LAG-3$^{-/-}$ mice (Fig. 1*A*), the difference in the numbers of cells in S phase is even greater: total number of Vβ7/8$^+$ T cells in S phase on day 3, LAG-3$^{+/+}$ (6.2×10^5), LAG-3$^{-/-}$ (20.7×10^5), 3.3-fold increase; day 4, LAG-3$^{+/+}$ (3.8×10^5), LAG-3$^{-/-}$ (21.0×10^5), 5.5-fold increase; day 7, LAG-3$^{+/+}$ (1.0×10^5), LAG-3$^{-/-}$ (3.6×10^5), 3.6-fold increase. It is noteworthy that the number of T cells in S phase decreased by almost 40% from day 3 to day 4 in wild-type mice, whereas this number remained high and unchanged over the same period in LAG-3$^{-/-}$ mice. These data imply that the regulation of T cell number by cell cycle arrest and cell death following antigenic stimulation in vivo is significantly delayed in the absence of LAG-3. Taken together, these data suggest that LAG-3 helps to control the expansion of activated T cells in vivo following stimulation by delaying the initiation of cell cycle arrest.

Increased expansion of OTII.LAG-3$^{-/-}$ T cells following in vivo stimulation

To further examine the role of LAG-3 following T cell activation in vivo, we crossed the LAG-3$^{-/-}$ mice with OT-II mice which express a transgenic TCR specific for the $OVA_{326-339}/A^b$ epitope. OTII.LAG-3$^{-/-}$ and OTII.LAG-3$^{+/+}$ Thy1.2$^+$ CD4$^+$ Vα2$^+$ splenic T cells were purified by FACS, labeled with CFSE and adoptively transferred into Thy1.1$^+$ B6.PL mice. On the following day, $OVA_{326-339}$ peptide was injected i.p. to stimulate the adoptively transferred OT-II transgenic T cells. Two days after peptide administration, both OTII.LAG-3$^{-/-}$ and OTII.LAG-3$^{+/+}$ T cells had begun to divide as evidenced by reduced CFSE levels. By day 4, there was a significant difference in the percentage of OTI-I.LAG-3$^{-/-}$ vs OTII.LAG-3$^{+/+}$ T cells that had proliferated extensively and were now CFSE-negative (Fig. 2, *A* and *B*). This difference was reflected in both the percentage and number of OTI-I.LAG-3$^{-/-}$ CFSE$^-$ T cells on day 4, which was ~2- and ~4-times higher than wild-type T cells, respectively (Fig. 2*C*). As seen with the SEB experiments, these differences were transient as the numbers and percentages of LAG-3$^{-/-}$ and LAG-3$^{+/+}$ T cells were comparable by day 7. Further analysis of the CFSE data on day 4 using a Proliferation Wizard basic model program in MODfit revealed that there was a general increase in the number of cells at generations 2–5 and a significant (3.8-fold) difference in the number of cells that had undergone 6+ cellular divisions (Fig. 2*B*). It is interesting to note that the number of cells that did not proliferate (generation 0) was slightly higher with OTII.LAG-3$^{+/+}$ T cells than with OTII.LAG-3$^{-/-}$ T cells (Fig. 2*B*). This may be due to an increase sensitivity of the LAG-3$^{-/-}$ T cells to Ag stimulation although this was not observed in in vitro proliferation assays (19). Another possibility is that the percentage of transferred OTI-I.LAG-3$^{+/+}$ that did not undergo proliferation (1.68% ± 0.94) was less for the OTII.LAG-3$^{-/-}$ cells (0.78% ± 0.46), suggesting that more OTII.LAG-3$^{-/-}$ T cells responded to Ag stimulation than the OTII.LAG-3$^{+/+}$ T cells. The significance of this finding may only become apparent after further analysis.

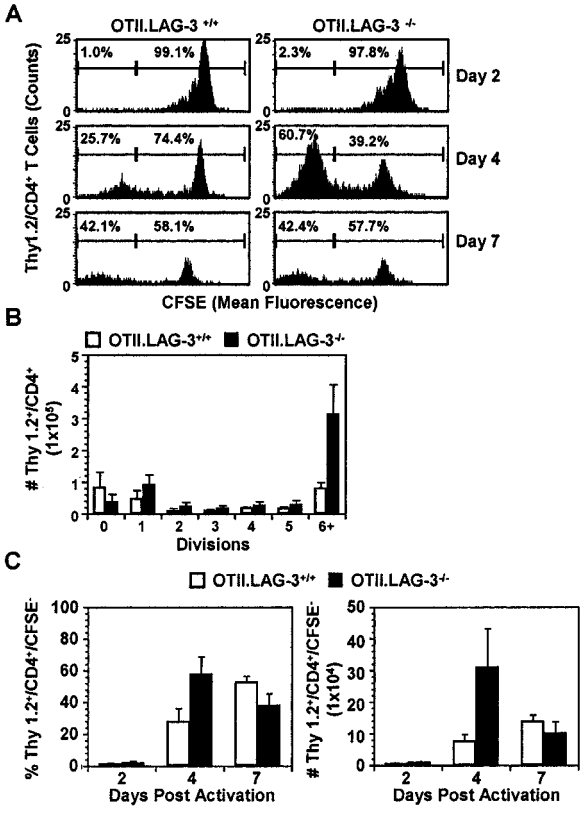

FIGURE 2. OTII.LAG-3$^{-/-}$ T cells expand more following Ag stimulation in vivo. *A*, Transgenic T cells from OTII.LAG-3$^{+/+}$ and OTII.LAG-3$^{-/-}$ mice were FACS purified, labeled with CFSE, adoptively transferred into Thy-1.1$^+$ B6.PL mice and stimulated in vivo 24 h later with $OVA_{326-339}$. Spleens were removed on the days shown and the percentages of CFSE$^+$/Thy1.2$^+$/CD4$^+$ and CFSE$^-$/Thy1.2$^+$/CD4$^+$ T cells determined. Data are representative of three independent experiments. *B*, The number of Thy1.2$^+$/CD4$^+$ T cells in each cellular division was calculated from the spleens on day 4 above using the Proliferation Wizard basic model program in MODfit. *C*, The percentage (*left panel*) and the number (*right panel*) of Thy1.2$^+$/CD4$^+$/CFSE$^-$ cells postactivation in vivo is shown. Data are the mean ± SE of three independent experiments with a total of five to six mice per group per time point.

LAG-3$^{-/-}$ mice generate increased numbers of memory T cells following Sendai virus infection

We next evaluated the influence of LAG-3 on the effector T cell response and establishment of T cell memory following distinct viral infections. Sendai virus (a murine parainfluenza virus) infection in mice is a well-defined system for the study of T cell immunity to respiratory virus infections (25–28). Following resolution of an acute infection with Sendai virus, large populations of memory CD8$^+$ and CD4$^+$ T cells persist in both the secondary lymphoid organs and peripheral tissues. We took advantage of this system to assess the effect of LAG-3 on the expansion of virus-specific CD4$^+$ and CD8$^+$ effector T cells and the development of T cell memory. LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were intranasally infected with Sendai virus and the lungs, mediastinal lymph nodes (MLN), and spleen removed on days 7 and 9 postinfection for analysis. The numbers of Sendai $HN_{419-433}/A^b$-specific CD4$^+$ T cells were determined using an MHC class II:Ig multimer [$sHN_{419-433}$:H-2A^b.γ2aFc] (29, 30). Likewise, the numbers of $NP_{324-332}/K^b$-specific CD8$^+$ T cells were determined using an $NP_{324-332}/K^b$ tetramer (31). The numbers of $HN_{419-433}/A^b$-specific CD4$^+$ T cells were essentially equivalent in the LAG-3$^{-/-}$ vs the wild-type mice in the lung and MLN, but slightly higher in the spleen (Fig. 3*A*). However, some significant differences were noted for the CD8$^+$ T cell response. At day 7 postinfection, there was a significant reduction in the numbers of $NP_{324-332}/K^b$-specific CD8$^+$ T cells in the MLN, and to some extent the lungs, of LAG-3$^{-/-}$ mice that was not evident in the spleen. Although the basis for this reduction remains to be clarified, it could be due to differences in either homing or local expansion of LAG-3$^{-/-}$ T cells. Nonetheless, this difference appeared to be transient as the number of $NP_{324-332}/K^b$-specific CD8$^+$ T cells in LAG-3$^{-/-}$ and LAG-3$^{+/+}$ mice was comparable by day 9. Concomitantly, there was a 2-fold increase in the number of $NP_{324-332}/K^b$-specific CD8$^+$ T cells in the spleens of LAG-3$^{-/-}$ mice. This latter observation is consistent with our findings following SEB and OVA peptide treatment previously described.

To examine the role of LAG-3 in the generation of memory, the numbers of memory T cells were determined in LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice that had recovered from a Sendai virus infection (day 30 postinfection). CD4$^+$ and CD8$^+$ T cells were isolated from the lungs and MLN and the numbers of IFN-γ producing, $HN_{419-433}/A^b$-, and $NP_{324-332}/K^b$-specific cells determined by ELISPOT analysis (Fig. 3*B*). There was a significant increase (2- to 3-fold) in the total number CD4$^+$ and CD8$^+$ T cells found in the lungs and MLN of the LAG-3$^{-/-}$ mice compared with LAG-3$^{+/+}$ mice. These differences were even more substantial for peptide-specific, IFN-γ^+ T cells. There was a 10- to 30-fold difference between the LAG-3$^{-/-}$ and LAG-3$^{+/+}$ T cells specific for both peptides in the lungs and a 3- to 4-fold difference in the MLN.

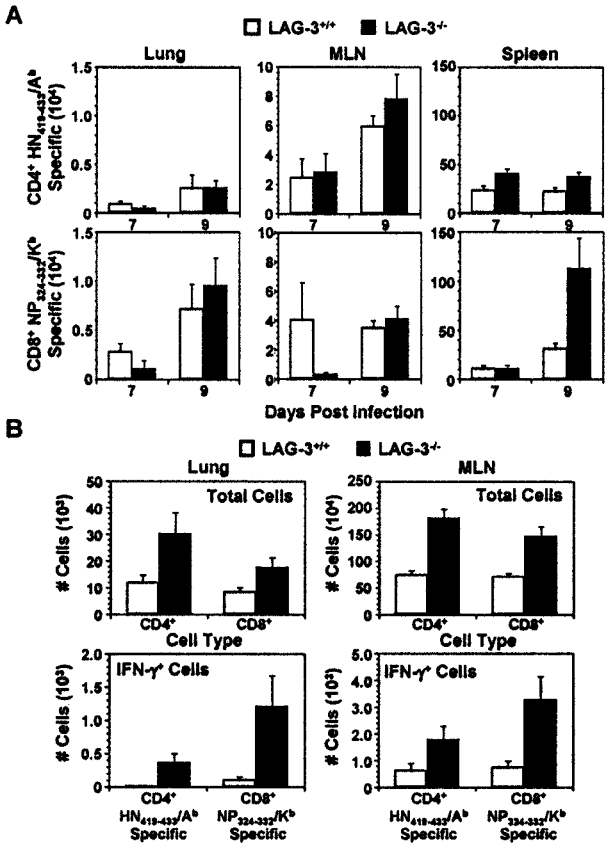

FIGURE 3. LAG-3$^{-/-}$ mice have a larger memory T cell pool following a viral infection. *A*, LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were infected with Sendai virus and the number of CD4$^+$ Sendai $HN_{419-433}/A^b$-specific and CD8$^+$ $NP_{324-332}/K^b$-specific T cells 7 and 9 days postinfection was determined by staining with the appropriate tetramer/multimer. *B*, The total numbers of CD4$^+$ and CD8$^+$ T cells were determined in the lung and MLN 30 days postchallenge. The number of IFN-γ-producing, CD4$^+$ Sendai $HN_{419-433}/A^b$-specific and CD8$^+$ $NP_{324-332}/K^b$-specific T cells in the lung and MLN were determined by ELISPOT. Data are the mean ± SE of five mice per group per time point.

These data suggest that LAG-3 may play a role in determining the size of the memory T cell pool.

Differences in the CD4$^+$ but not CD8$^+$ T cell response to MHV-68 infection in LAG-3$^{-/-}$ mice

If LAG-3 affects the generation of memory T cells to an acute viral infection, would it also influence the T cell response to a chronic viral infection? To evaluate this, we analyzed mice infected with MHV-68 (24). Upon intranasal inoculation, MHV-68 establishes an acute lytic infection in the respiratory tract, which is cleared largely by CD8$^+$ T cells, followed by the establishment of a lifelong latent infection, which can be readily detected in the spleen 14 days after infection. Immune control of latency and the prevention of recrudescence of lytic virus are mediated by poorly understood, probably redundant, immune mechanisms involving Ab as well as CD4$^+$ and CD8$^+$ T cells (24). LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were infected with MHV-68, and the numbers of virus-specific CD4$^+$ T cells, and CD8$^+$ T cells were determined by IFN-γ ELISPOT analysis. Virus-specific CD8$^+$ T cells were examined with peptides specific for two well-characterized viral epitopes, $ORF6_{487-495}/D^b$ and $ORF61_{524-531}/K^b$. Due to the low frequency of CD4$^+$ T cells specific for the few MHC class II epitopes currently defined (32), virus-specific CD4$^+$ T cells were assessed by stimulation with cells pulsed with virus. Interestingly, the numbers of virus-specific LAG-3$^{+/+}$ CD4$^+$ T cells reached a peak on day 21 postinfection, consistent with previous results (32), whereas the virus-specific LAG-3$^{+/+}$ CD4$^+$ T cells did not reach their maximal numbers until day 36 and remained twice as high as the corresponding cells in wild-type mice by day 62 (Fig. 4).

In contrast, no significant differences were observed in the CD8$^+$ T cell response to the two MHV-68 epitopes examined (Fig. 4). These results suggest that LAG-3 had no effect on the CD8$^+$ T cell response to a chronic MHV-68 infection, in contrast to our observations following Sendai virus infection. However, we cannot rule out the possibility that differences may be observed for responses to other epitopes and clearly additional analysis will be required to dissect these findings further.

Discussion

The goal of this study was to reevaluate previous suggestions that LAG-3$^{-/-}$ mice had no T cell defect (16, 17). Using four distinct in vivo systems, we have demonstrated that LAG-3 performs an important role in regulating both the expansion of activated primary T cells and the development of the memory T cell pool.

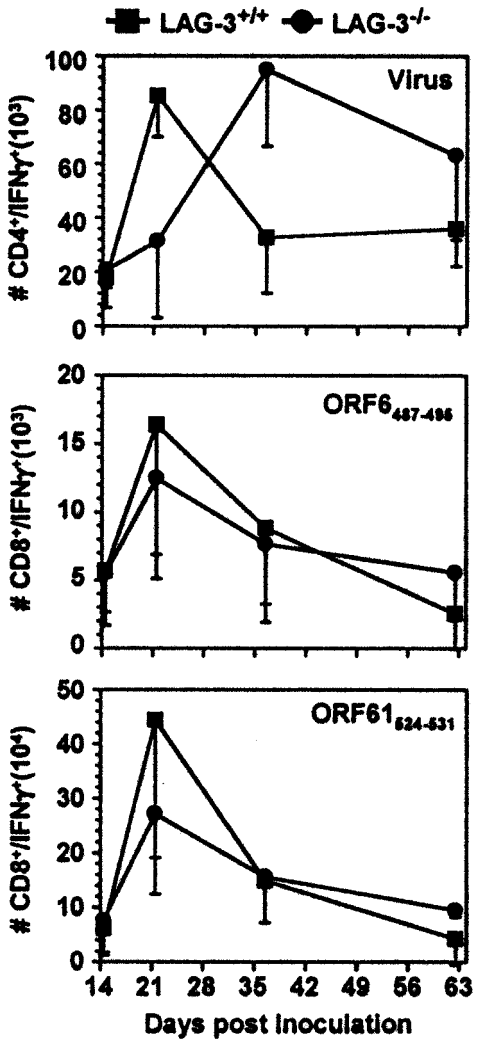

FIGURE 4. LAG-3 plays a limited role in the T cell response to persistent MHV-68 infection. LAG-3$^{+/+}$ and LAG-3$^{-/-}$ mice were inoculated with MHV-68 on day 0 and the number of IFN-γ$^+$ T cells specific for whole virus (CD4$^+$, *top panel*), $ORF6_{487-495}$ (CD8$^+$, *middle panel*), and $ORF61_{524-531}$ (CD8$^+$, *bottom panel*) determined on the days indicated. Data represent the mean ± SE of three mice per time point.

Although additional experiments will need to be performed to further dissect the role of LAG-3 in each of these systems, these studies establish the important principle that there is a role for LAG-3 in T cell function in vivo, contrary to the initial analysis of the LAG-$3^{-/-}$ mice (16, 17). Our findings also support previous suggestions that LAG-3 may be an important negative regulator (11, 12, 15, 19, 20).

Our previous in vitro studies have shown that, although T cells from LAG-$3^{-/-}$ mice proliferate normally in response to a superantigen, there was an increase in cell death following in vitro T cell activation/expansion with SEB (19). As noted previously, this increased cell death in vitro was probably due to over-expansion of LAG-3-deficient cells resulting in cytokine starvation and/or Ag-induced cell death (19). Interestingly, the in vivo cell cycle analysis presented in this study revealed that LAG-$3^{-/-}$ mice stimulated with SEB resulted in a delay in cell cycle arrest causing an increase in both the percentage and number of LAG-$3^{-/-}$ Vβ7/8^+ T cells in S phase. This led to mass expansion of SEB-reactive T cells resulting in splenomegaly. At first glance, the in vitro and in vivo data appear to be contradictory. However, this may not be the case. Following SEB treatment there is clearly an enhanced expansion, and thus total number, of LAG-$3^{-/-}$ Vβ7/8^+ T cells in vivo. However, by day 14 the number LAG-$3^{-/-}$ and LAG-$3^{+/+}$ Vβ7/8^+ T cells had contracted to near comparable numbers. Thus, from day 3 to day 14 there is a considerably greater reduction in LAG-$3^{-/-}$ Vβ7/8^+ T cell number suggesting more cell death. The enhanced T cell expansion in vivo may be due to increased local cytokine production. Indeed, we have observed increased IL-2 and IFN-γ production by LAG-$3^{-/-}$ T cells following stimulation in vitro (19). As the environment in vivo may be more optimal for survival, less initial cell death may have been observed that we found in our in vitro studies. Importantly, these observations are not restricted to superantigen-stimulated T cells, as the absence of LAG-3 had a similar effect on the response of OT-II TCR transgenic T cells to peptide in vivo.

By using the Sendai viral system, we were able to examine the role of LAG-3 in both an acute infection as well as in the development of a memory T cell pool following viral infection. Although the data suggest no clear differences in the $CD4^+$ specific acute response to $NP_{419-433}/A^b$ 7 and 9 days post infections, there were some differences in the $CD8^+$-specific $NP_{324-332}/K^b$ acute response. On day 7 in the MLN, there was an increase in the number $CD8^+$ LAG-$3^{+/+}$ $NP_{324-332}/K^b$-specific T cells compared with the LAG-$3^{-/-}$ T cells. However, this appeared to be transient as this difference was not apparent by day 9. As was seen in the SEB model and OT-II transgenic model, there was also an increase in the number of $CD8^+$ Ag-specific T cells in the spleen by day 9 in the Sendai-infected LAG-$3^{-/-}$ mice. Surprisingly, these differences were not seen in the lung and MLN. The reason for this is not clear but may be due to homing issues or differences in the local cytokine/chemokine environment. Additional experiments would need to be performed to address these issues.

Overall, the differences between the acute viral response and results seen in the SEB model and OT-II model may be due to the fact that the initial viral-specific response is not as robust as is seen following superantigen stimulation or peptide-specific transgenic T cell stimulation. However, the delay in cell cycle arrest observed in LAG-$3^{-/-}$ T cells may cause an accumulation of viral-specific T cells over time with differences not readily apparent at days 7 and 9. This could explain why we see a significant difference in the number of viral specific T cells in the lungs and MLN of LAG-$3^{-/-}$ mice 30 days later following recovery from Sendai viral infection. Taken together our studies clearly show a difference in the development of a memory T cell pool following Sendai viral infection in LAG-$3^{-/-}$ mice compared with LAG-$3^{+/+}$ mice.

The analysis of virus-specific T cell responses to a persistent virus, MHV-68, showed that there were differences in the long-term $CD4^+$ T cell response, but not the $CD8^+$ T cell response. This delayed enhancement of the $CD4^+$ T cell response is somewhat analogous to our observations following SEB treatment (Fig. 1*A*). $CD4^+$ T cells participate in both the acute and long-term response to this chronic viral infection (32, 33). Unfortunately, latent MHC class II epitopes have not yet been identified, so it is not currently possible to distinguish whether the absence of LAG-3 is having an impact on the $CD4^+$ T cell response to reactivating lytic virus or latent virus.

It is intriguing that a molecule, MHC class II, which is classically considered a ligand for $CD4^+$ Th cells should also influence the expansion of $CD8^+$ T cells via LAG-3. What is the physiological relevance of this finding? It is a possibility that the cellular distribution of MHC class II is of more significance for this issue than the fact that it is a ligand for CD4. The more sparse and restricted expression of MHC class II may modulate the LAG-3-mediated control of T cell expansion, which would be influenced by its location and proximity to MHC class II^+ APCs. For instance, T cells may expand to a greater extent in peripheral tissues than in secondary lymphoid organs in which the number of APCs would be higher. This may be an important facet of LAG-3-mediated control of T cell expansion and memory T cell development that is worthy of further investigation.

In conclusion, by using four distinct in vivo systems we have demonstrated that LAG-3 performs an important role in regulating both the expansion of activated primary T cells and the development of the memory T cell pool, contrary to the initial analysis of the LAG-$3^{-/-}$ mice (16). Our findings also support previous in vitro work with both human and murine LAG-3 (11, 12, 15, 19, 20). Molecules involved in the negative regulation of T cell activation and expansion are critical for tight regulation of T cell homeostasis. A defect in immune regulation could cause lymphopenia, which could lead to an inadequate or reduced T cell response to a foreign pathogen, whereas a hyper-expanded T cell pool could cause a lymphoproliferative disorder or autoimmunity. Our data raise the possibility that blockade of LAG-3 function, for instance with an anti-LAG-3 mAb (12 and C. J. Workman and D. A.A. Vignali, unpublished observation), could be used to enlarge the memory T cell pool following vaccination, thereby enhancing vaccine efficacy.

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