

On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection

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Abstract

Chronic viral infections are often characterized by CD8 T-cell responses with poor cytokine secretion potential and limited expansion of the CD8 T-cell pool, collectively referred to as CD8 T-cell exhaustion. Exhaustion of lymphocytic choriomeningitis virus (LCMV)-specific CD8 T cells was shown to be partially regulated by the inhibitory receptor programmed death 1 (PD-1). Here, we demonstrate that exhausted LCMV-specific CD8 T cells also express the negative regulatory receptor lymphocyte activation gene 3 (LAG-3) which is mainly expressed on cells co-expressing the negative regulatory receptors PD-1 and Tim-3. Expression levels of LAG-3 on anti-viral CD8 T cells remain stable over short-term *in vitro* stimulations in presence of antigenic peptide. Nevertheless, *in vitro* and *in vivo* blockade of LAG-3 did not rescue cytokine production by virus-specific CD8 T cells and did not alter the virus titer in various organs. Likewise, chronic LCMV infection of LAG-3^{-/-} mice led to a comparable degree of T-cell exhaustion as observed in C57BL/6 controls and to similar virus titers. Further, LAG-3 did not influence T-cell activation or cell division during chronic LCMV infection. These data suggest that even though LAG-3 is continuously up-regulated on LCMV-specific exhausted CD8 T cells, it alone does not significantly contribute to T-cell exhaustion.

Keywords: CD8 T cell, chronic viral infection, dysfunction, inhibitory receptor

Introduction

Unlike after acute resolved infections, the development of long-lived antigen-independent memory CD8 T cells that can rapidly exert effector functions is precluded during chronic viral infections (1, 2). During chronic viral infections (i.e. human infection with HIV, hepatitis B or hepatitis C virus), virus-specific CD8 T cells acquire effector functions early after infection but gradually lose them as the antigen persists over extended time periods (3–8). This T-cell dysfunction, also termed CD8 T-cell exhaustion, is well studied after chronic infection of mice with lymphocytic choriomeningitis virus (LCMV) Clone 13 or LCMV Docile (9). In this setting, the LCMV-specific CD8 T-cell pool expands early after infection and acquires effector functions, which, however, are not sufficient to control viral replication. As a result of the ensuing continuous presence of viral antigen, LCMV-specific CD8 T cells are continuously stimulated which leads to gradual decline of their effector functions: first the ability to produce IL-2 and to proliferate, then tumor necrosis factor α (TNF α) secretion and last IFN- γ production is decreased (10–13). Although degranulation of CD8 T cells and killing of target cells was also reported to be impaired (12), this is not a consistent finding (10). The above scenario is observed for

CD8 T cells with specificities for the LCMV glycoprotein-derived peptides gp33 and gp276, whereas CD8 T cells with specificity for the LCMV epitope np396 are physically deleted during protracted LCMV infection (9, 11).

Recent work has highlighted the role of inhibitory receptors in inducing/maintaining exhausted CD8 T cells (14–18). Programmed death 1 (PD-1), an inhibitory receptor of the CD28 superfamily (19), was shown to be expressed at elevated levels on exhausted CD8 T cells compared with functional memory T cells in the LCMV system (16). Blockade of PD-1/programmed death ligand 1 (PD-L1) interactions after chronic LCMV infection not only increased the numbers of LCMV-specific CD8 T cells but also enhanced their functionality resulting in decreased virus titers (16). However, functional restoration was not complete, suggesting the involvement of other negative regulatory pathways in CD8 T-cell exhaustion. CTLA-4 (cytotoxic T lymphocyte antigen 4), another CD28 family member, is also up-regulated on CD8 T cells after chronic LCMV infection [(18) and K. Richter, P. Agnellini and A. Oxenius, unpublished results]. However, *in vivo* CTLA-4 blockade in chronically LCMV-infected mice did not increase T-cell functionality (16).

Gene expression profiling revealed a strong up-regulation of the inhibitory receptor lymphocyte activation gene 3 (LAG-3) on LCMV-specific CD8 T cells in chronically LCMV-infected mice compared with CD8 T cells from acutely infected mice [K. Richter, P. Agnellini and A. Oxenius, unpublished results (18)]. LAG-3 (CD223) is an activation-induced cell surface molecule that is structurally similar to the CD4 molecule and binds to MHC II molecules with high affinity (20, 21). LAG-3 blockade was shown to induce CD69 expression on CD4 and CD8 T cells and to induce T-cell expansion and production of T_H1 cytokines in resting human T cells (22). As murine LAG-3^{-/-} OTII T-cells secrete more IL-2 and IFN- γ following stimulation than their wild-type counterparts (23) and as LAG-3^{-/-} T cells showed increased T-cell expansion *in vivo* after Sendai virus infection (24), LAG-3 was chosen as candidate inhibitory molecule that might have a role in dampening the CD8 T-cell response in chronic LCMV infection. In this study, we show that LAG-3 is up-regulated on LCMV-specific CD8 T cells after chronic LCMV infection. Nevertheless, neither antibody-mediated blockade *in vivo* nor use of LAG-3 knockout mice led to increased T-cell effector functions or reduction of viral titers compared with control treated, respective wild-type mice, indicating that LAG-3 alone does not majorly contribute to CD8 T-cell exhaustion.

Methods

Virus and viral peptides

LCMV Docile was propagated on MDCK cells, whereas LCMV Clone 13 was propagated on BHK21 cells as described (25). Viral peptides gp33–41 (gp33; KAVYNFATM), np396–404 (np396; FQPQNGQFI) and gp61–80 (p13; LNGPDIYKGVYQFKSVEFD) were purchased from NeoMPS (Strasbourg, France).

Antibodies and peptide MHC I tetramers

Allophycocyanin or PE-conjugated peptide/MHC I tetrameric complexes were generated as described (26). Rat anti-LAG-3 IgG2a antibodies used for *in vivo* blocking experiments were received from Klaus Karjalainen (27), Singapore. Blocking antibodies directed against LAG-3, CTLA-4 and PD-L1 that were used in *in vitro* experiments were purchased from eBioscience (THP, Vienna, Austria). Antibodies used for flow cytometry were purchased from BD Pharmingen (Allschwil, Switzerland) and Biolegend (LucernaChem, Luzern, Switzerland).

Mice

Mice were kept under specific pathogen-free conditions and were infected intravenously (i.v.) with 200 pfu or 10^6 pfu of LCMV strains Docile or Clone 13. When indicated, mice were treated *in vivo* with 200 μ g anti-LAG-3 by intraperitoneal administration either on days -3, -1, 3, 6, 9, 12 and 15 or on days 39, 42, 45, 48 and 51 post-infection. Animal experiments were performed according to the regulations of the cantonal veterinary office.

Stimulation of lymphocytes and flow cytometry

Lymphocytes were harvested from the spleens of perfused mice and stimulated with 1 μ g ml⁻¹ gp33, np396 or p13

peptide in the presence of Brefeldin A (10 μ g ml⁻¹; Sigma-Aldrich, St. Louis, MO, USA) and Monensin A (2 μ M; Sigma-Aldrich) for 5 h at 37°C. For assessment of degranulation, 1 μ l anti-mouse CD107a was added during the stimulation. Where indicated, the cells were pre-treated for 1 h or 2 days with 10 μ g ml⁻¹ of blocking antibodies directed against LAG-3, CTLA-4 and/or PD-L1 [functional grade purified; eBioscience (THP)] before stimulation was continued in the presence of antibodies. During 2 day pre-treatment, the cells were incubated in RPMI containing 100 U ml⁻¹ IL-2 and 100 ng ml⁻¹ IL-15 (Biosource, Nivelles, Belgium).

Surface staining was performed for 30 min at 4°C before the cells were fixed and permeabilized in 500 μ l 2 \times FacsLyse (BD Biosciences, Allschwil, Switzerland) containing 0.05% Tween 20 (Sigma-Aldrich) for 10 min at room temperature. After a wash step, cytokines were stained for 30 min at room temperature. Cells were washed and re-suspended in PBS containing 1% PFA (Sigma-Aldrich). Multiparameter flow cytometric analysis was performed using an FACS LSRII flow cytometer (BD Biosciences, Allschwil, Switzerland) with FACSDiva software. Analysis was performed using FlowJo software (Tree Star, San Carlos, CA, USA).

Results

LAG-3 is up-regulated during chronic LCMV infection

Comparative gene profiling analyses of LCMV-specific CD8 T cells during acute or chronic LCMV infection revealed a marked up-regulation of LAG-3 expression in the setting of chronic LCMV infection [for chronic LCMV Docile infection (K. Richter, P. Agnellini and A. Oxenius, unpublished results) and for LCMV Clone 13 infection (18)]. To assess if LAG-3 was not only up-regulated on mRNA level but also on protein level during chronic LCMV infection, C57BL/6 mice were acutely or chronically infected with LCMV strains Docile or Clone 13 and analyzed for LAG-3 expression on endogenous CD8 T cells specific for the LCMV-derived peptide epitope gp33. On day 13 post-infection, gp33-specific CD8 T cells from acutely infected mice showed only slightly elevated expression compared with LAG-3^{-/-} CD8 T cells, while LAG-3 expression on gp33-specific CD8 T cells from chronically infected mice was markedly up-regulated (Fig. 1A and B). Note that LAG-3 expression was slightly stronger in Docile compared with Clone 13-infected mice. Next, we analyzed if LAG-3 is co-expressed with the inhibitory receptor PD-1 which is involved in CD8 T-cell exhaustion after chronic LCMV infection (16) on endogenous anti-viral CD8 T cells. We observed that most cells expressing LAG-3 also expressed PD-1 after Clone 13 infection or after Docile infection (Fig. 1B).

As endogenous CD8 T-cell populations specific for a certain epitope comprise polyclonal T cells with presumably different TCR affinities for the epitope, we went on to assess LAG-3 expression on transferred Ly5.1⁺ TCR transgenic CD8 T cells specific for the gp33 peptide after acute and chronic infection with LCMV Docile. This monoclonal population of T cells showed a similar expression pattern compared with the endogenous polyclonal population with respect to up-regulation of LAG-3, PD-1 and additionally of the inhibitory receptor Tim-3 that was shown to be up-regulated on exhausted CD8 T cells during HIV infection (28). Again,

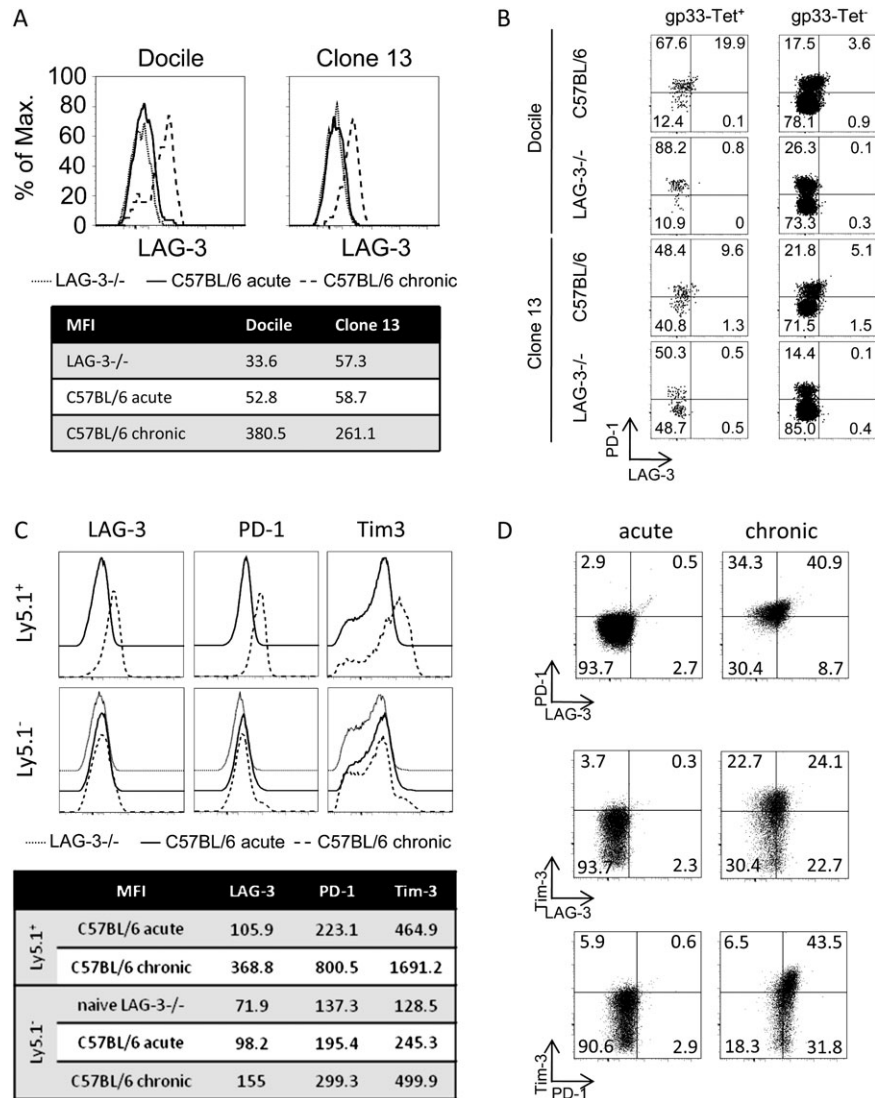


Fig. 1. LAG-3 is up-regulated after chronic LCMV infection and is mainly co-expressed with the inhibitory receptor PD-1. (A) C57BL/6 mice were i.v. infected with 10^6 (dashed) or 200 pfu (black) of LCMV strains Docile or Clone 13. LAG-3^{-/-} mice infected with 200 pfu of the respective virus served as negative control (dotted). Spleen single-cell suspensions were analyzed for the expression of LAG-3 on H-2D^p-gp33-tetramer⁺ CD8 T cells by flow cytometry on day 13 post-infection. One representative staining of three to four mice per group is shown. Data in the table represent the means of the mean fluorescence intensities. (B) Co-expression of LAG-3 and PD-1 was examined on H-2D^p-gp33-tetramer⁺ CD8 splenocytes after i.v. infection with 10^6 pfu of LCMV Docile or Clone 13. (C) Expression of the inhibitory receptors PD-1, LAG-3 and Tim-3 was analyzed on Ly5.1⁺ TCR transgenic CD8 T cells specific for gp33 that were transferred 1 day before acute or chronic infection with LCMV Docile. Splenocytes were analyzed on day 18 post-infection. One representative staining of three mice per group is shown. (D) Analysis of co-expression of inhibitory receptors; cells are gated on CD8 Ly5.1⁺ TCR transgenic T cells.

LAG-3 was largely co-expressed with other inhibitory receptors, in particular with PD-1 (Fig. 1D).

T-cell priming is similar in LAG-3^{-/-} and wild-type mice

To analyze if LAG-3 influences the priming of CD4 and CD8 T cells in the context of LCMV infection, C57BL/6 and LAG-3^{-/-} mice were acutely infected with LCMV Clone 13 or Docile and assessed for the frequency of LCMV-specific CD8 T cells with the ability to degranulate upon antigen-specific re-stimulation. The frequency of degranulating CD8 T cells was similar in LAG-3^{-/-} mice and C57BL/6 controls after Clone 13 infection (as assessed by CD107a staining,

Fig. 2A) and after Docile infection (Fig. 2B). Likewise, similar frequencies of CD8 T cells secreting IFN- γ (Fig. 2C), TNF α (Fig. 2D) and IL-2 (Fig. 2E) were measured in LAG-3^{-/-} and C57BL/6 mice after stimulation with the antigenic peptides gp33 and np396 on day 13 post-infection with LCMV Docile. Comparable results were obtained after infection with Clone 13 (data not shown). Furthermore, the frequency of LCMV-specific CD4 T cells secreting IFN- γ (Fig. 1F) and TNF α (Fig. 1G) after stimulation with p13 peptide were comparable in LAG-3^{-/-} and C57BL/6 mice. Thus, LAG-3 does not influence the quality or quantity of T-cell priming upon acute LCMV infection.

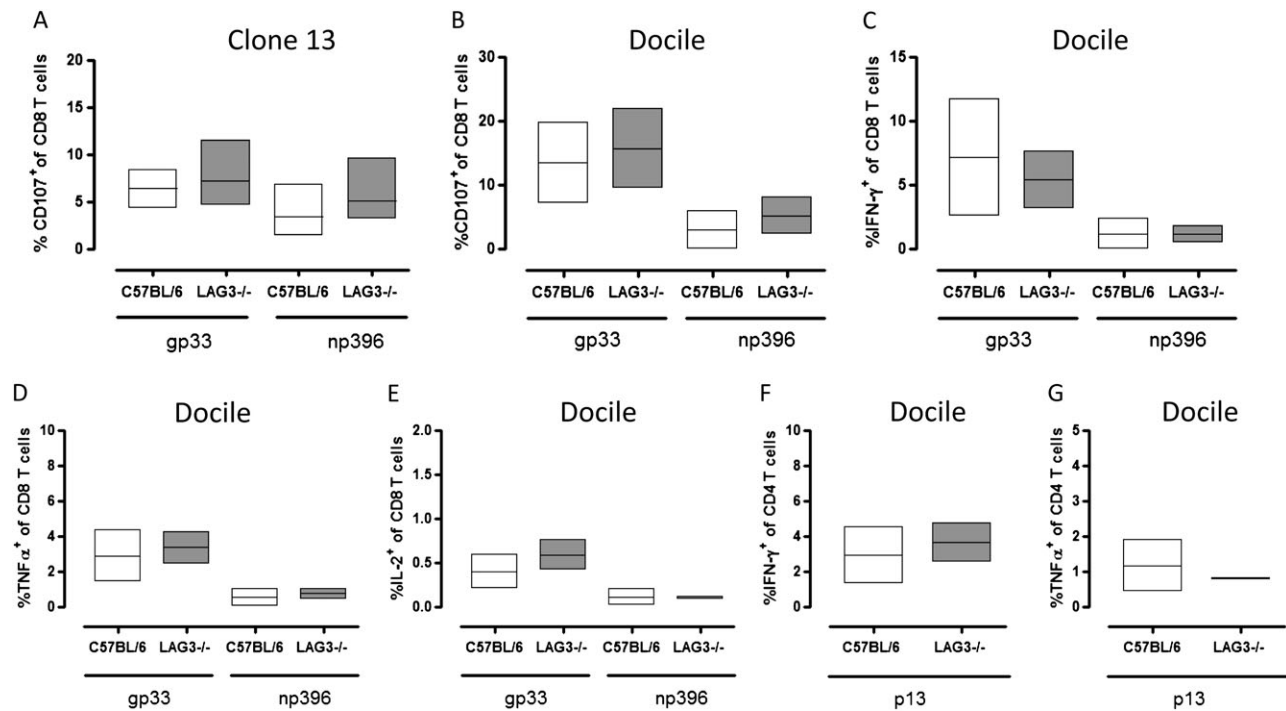


Fig. 2. T-cell priming is similar in LAG-3^{-/-} and C57BL/6 mice. C57BL/6 and LAG-3^{-/-} mice were i.v. infected with 200 pfu of LCMV Clone 13 (A) or LCMV Docile (B–G). Splenocytes were analyzed on day 13 post-infection for the ability of CD8 T cells to degranulate (as assessed by CD107 staining; A and B) and to produce IFN- γ (C), TNF α (D) and IL-2 (E) after 5 h of stimulation in the presence of gp33 respective np396 peptide. CD4 T cells were analyzed for production of IFN- γ (F) and TNF α (G) after stimulation with p13 peptide. Background staining was subtracted from the measured value of each individual mouse. Horizontal lines indicate the arithmetic means of each group of mice while boxes show minimum and maximum of the measured values.

Expression of LAG-3 on LCMV-specific CD8 T cells does not decrease during short-term stimulations in the presence of antigenic peptide

As it was reported that LAG-3 can be cleaved at the cell surface by ADAM 10 and ADAM 17 metalloproteases allowing cytokine production and proliferation (29), we measured LAG-3 expression during short-term stimulations in the presence of antigenic peptide. A total of 10^4 TCR transgenic CD8 T cells specific for the LCMV-derived gp33 peptide were transferred i.v. into C57BL/6 recipients 1 day before chronic infection. On day 18 post-infection, the transferred TCR transgenic CD8 T cells were re-stimulated *in vitro* for 5 h in presence or absence of gp33 peptide and LAG-3 surface expression was analyzed. LAG-3 expression did not significantly decrease upon stimulation (Fig. 3), indicating that signaling events via LAG-3 can occur *in vivo* in temporal proximity to TCR stimulation.

Stimulation of CD8 T cells in the presence of a blocking antibody directed against LAG-3 does not rescue exhausted phenotype

To analyze the effect of LAG-3 blockade on exhausted T cells, we investigated short-term changes in T-cell effector functions in the presence of blocking antibodies *in vitro*. One hour pre-incubation with various blocking antibodies, including anti-LAG-3, did not restore any cytokine defects within CD8 T cells from chronically LCMV-infected mice. However, a 2-day resting period prior to peptide stimulation

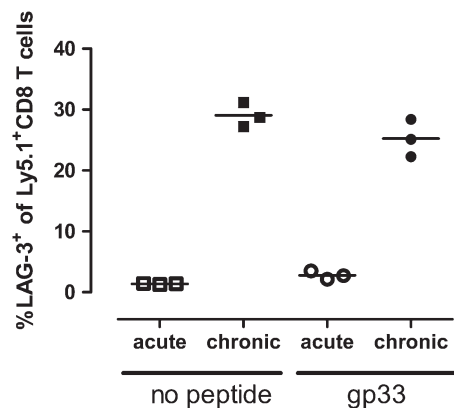


Fig. 3. LAG-3 expression is maintained after stimulation with gp33. A total of 10^4 Ly5.1⁺ TCR transgenic CD8 T cells were transferred into C57BL/6 and LAG-3^{-/-} mice 1 day before i.v. infection with 200 or 10^6 pfu LCMV Docile. On day 18 post-infection, splenocytes were stimulated in the presence of Brefeldin A and in presence or absence of gp33 for 5 h. LAG-3 expression was analyzed on Ly5.1⁺ TCR transgenic CD8 T cells. Each symbol represents one individual mouse. Horizontal bars indicate mean values of each group. Data are representative of two independent experiments.

markedly increased the cytokine production potential within CD8 T cells originating from chronically infected mice (Fig. 4). However, this partial rescue of cytokine production in this short-time period occurred irrespective of whether blocking antibodies (specific for LAG-3, PD-L1 or CTLA-4 or

combinations thereof) were present during the 2-day recovery period or not.

In vivo treatment of chronically LCMV-infected mice with an anti-LAG-3 blocking antibody does not rescue T-cell exhaustion and does not lead to decreased viral titers

Next, we decided to block LAG-3 *in vivo*. If LAG-3 plays a crucial role in inhibiting T-cell effector functions during chronic LCMV infection, we would expect that LAG-3 blockade should elevate cytokine production by CD8 T cells and/or increase the frequency of LCMV-specific CD8 T cells, which could eventually also lead to a decrease in virus titers. In a first experiment, C57BL/6 mice were chronically infected

and treated with anti-LAG-3 or PBS on days 39 until day 51 every 3 days (Fig. 5A). Acutely infected C57BL/6 mice served as controls. On day 54 post-infection, the frequency of gp33-specific CD8 T cells (Fig. 5B) and the capacity to degranulate (Fig. 5C) or secrete IFN- γ (Fig. 5D) after 5 h stimulation with gp33 was not different in anti-LAG-3-treated chronically infected mice compared with PBS-treated chronically infected mice. Viral titers between anti-LAG-3-treated and PBS-treated chronically infected C57BL/6 mice were monitored in the blood over time and did not change in the anti-LAG-3-treated group compared with the control group (Fig. 5E). Note that the viral titers were already slightly lower in the anti-LAG-3-treated group before the treatment was started on day 39 post-infection.

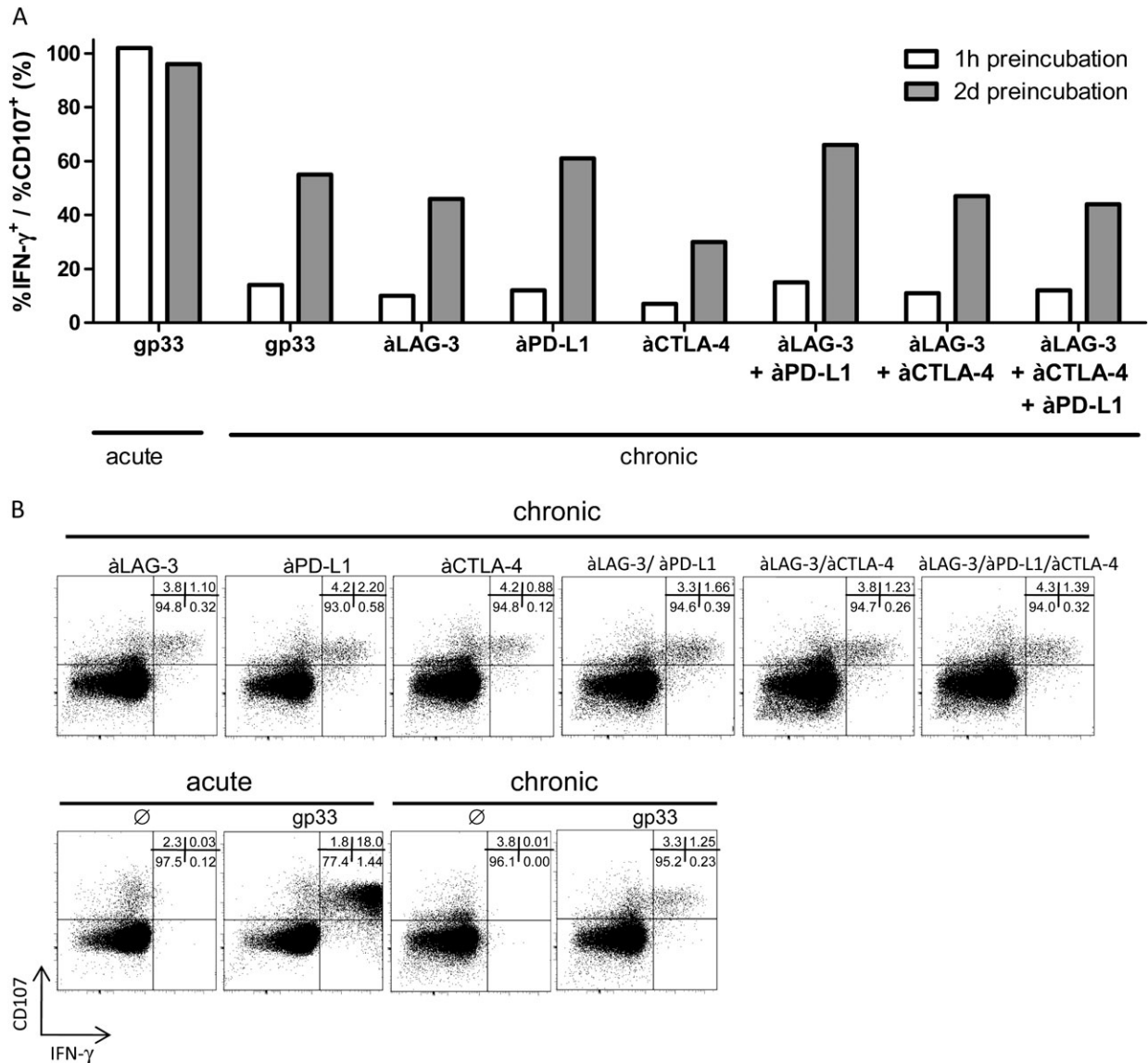


Fig. 4. Short-term stimulation of CD8 T cells in the presence of a blocking antibody directed against LAG-3 does not rescue exhausted phenotype. Groups of three C57BL/6 mice were either infected with 200 (acute) or 10^6 (chronic) pfu LCMV Docile. On day 16 post-infection, splenocytes were seeded at 4×10^5 per well with the indicated blocking antibodies directed against LAG-3, CTLA-4 and PD-L1. After 1 h or 2 days, gp33 was added and the cells were incubated another 5 h before being stained against CD8, CD107 and IFN- γ . Negative controls (\emptyset) were incubated without gp33. (A) The ratio of CD8 T cells producing IFN- γ per CD107 $^+$ cells is shown. (B) Representative FACS plots from day 2 gated on CD8 T cells are depicted.

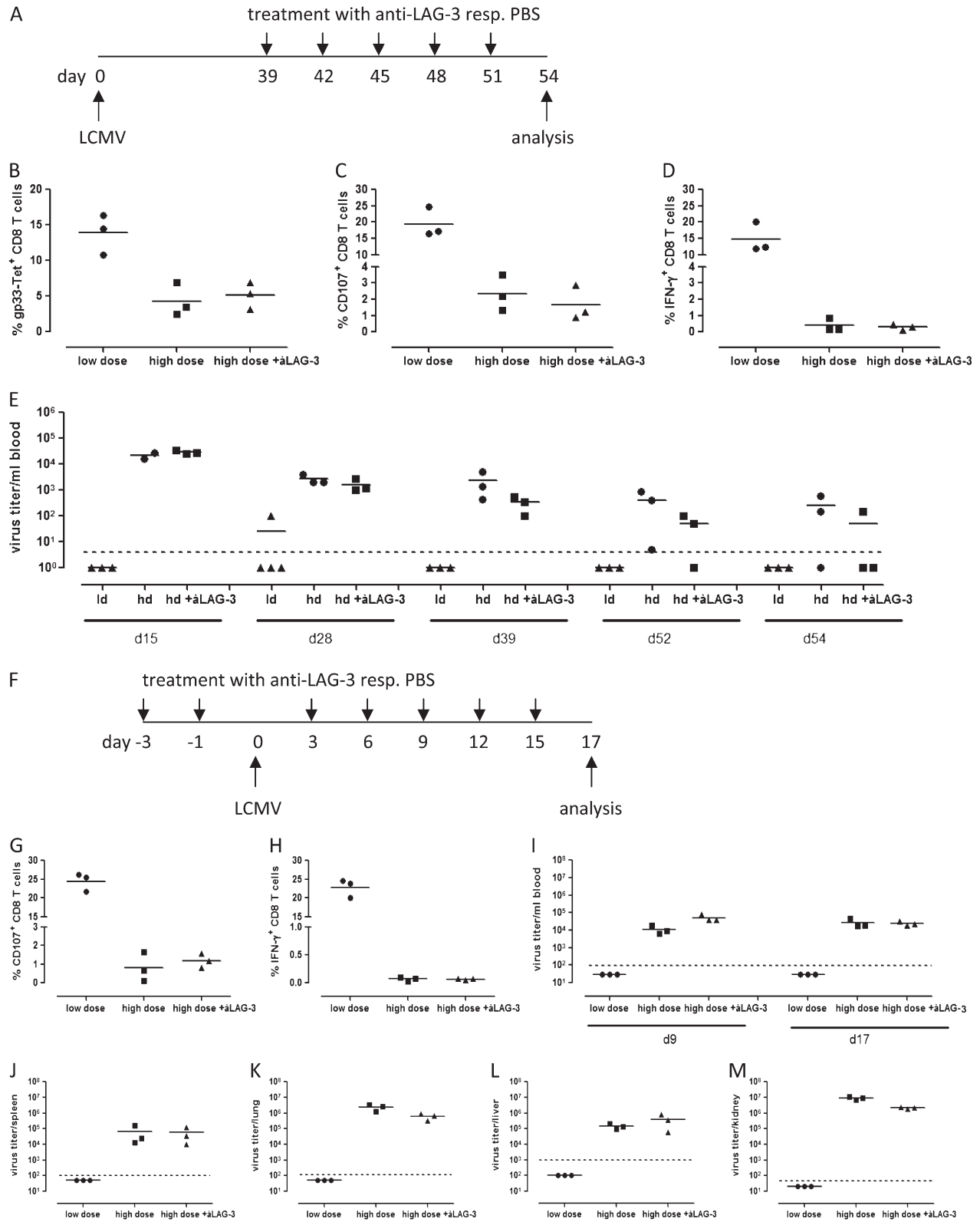


Fig. 5. *In vivo* treatment of chronically LCMV-infected mice with an anti-LAG-3 blocking antibody does not rescue T-cell exhaustion and does not lead to decreased viral titers. (A–E) Groups of three C57BL/6 mice were infected with 200 pfu (low dose, ld) or 10⁶ pfu Docile (high dose, hd) as indicated and treated with PBS or 200 μ g of a blocking antibody directed against LAG-3 on days 39, 42, 45, 48 and 51 (A). On day 54 post-infection, the splenocytes were stained for CD8 and H-2D^p-gp33-tetramer (B) or re-stimulated in the presence of gp33 and analyzed for the capacity of CD8 T cells to degranulate (C) and produce IFN- γ (D). Background staining was subtracted. (E) Virus titers in the blood were

As therapeutic blockade of LAG-3 did not increase CD8 effector functions, we investigated whether anti-LAG-3 treatment initiated already during acute infection would influence CD8 T-cell exhaustion and viral control. Thus, chronically infected C57BL/6 mice were treated during the whole course of infection from day 3 until day 15 post-infection (Fig. 5F). Also in this experimental setting, degranulation and IFN- γ secretion of gp33-stimulated CD8 T cells were similar in anti-LAG-3-treated and control-treated mice (Fig. 5G and H). Viral titers in the blood were not significantly different between anti-LAG-3-treated and PBS-treated mice on day 9 and day 17 post-infection (Fig. 5I). Also, LAG-3 blockade did not impact on virus titers in spleen (Fig. 5J), lung (Fig. 5K), liver (Fig. 5L) and kidney (Fig. 5M). Thus, *in vivo* blockade of LAG-3-MHC II interactions neither induced improved CD8 T-cell effector functions in a therapeutic setting nor when mice were treated during the whole course of the experiment. Therefore, a potential inhibitory function of LAG-3 does not seem to be very crucial for T-cell exhaustion.

Chronic infection of LAG-3^{-/-} and wild-type mice with LCMV Docile induces similar degree of T-cell exhaustion and similar viral titers.

As antibody-mediated blockade of LAG-3-MHC II interactions did not prove to rescue T-cell exhaustion, it is conceivable that this could be due to insufficient *in vivo* blockade of the LAG-3 pathway. We therefore used LAG-3 knockout mice for chronic LCMV infections. Neither the frequency of gp33-tetramer⁺ CD8 T cells nor of np396-tetramer⁺ CD8 T cells in the blood was different between LAG-3^{-/-} and C57BL/6 controls chronically infected with LCMV Docile (Fig. 6A and B). Similar results were obtained after Clone 13 infection (Supplementary Fig. 1 is available at *International Immunology Online*). Also, short-time stimulation of splenocytes with gp33 respective np396 peptide did not yield increased IFN- γ production (Fig. 6C and E) and TNF α (Fig. 6D and F) secretion by CD8 T cells from chronically infected mice at any time point analyzed. Likewise, after chronic infection of LAG-3^{-/-} and C57BL/6 mice with LCMV strain Clone 13, no difference in the frequency of degranulating (Supplementary Fig. 1E and H is available at *International Immunology Online*), IFN- γ (Supplementary Fig. 1F and I is available at *International Immunology Online*) and TNF α (Supplementary Fig. 1G and J is available at *International Immunology Online*) producing CD8 T cells was observed after a 5-h stimulation with gp33 and np396 peptides.

Similarly, when splenocytes from LCMV Docile-infected LAG-3^{-/-} and C57BL/6 mice were stimulated with the CD4 T-cell epitope p13 (30), no elevation of IFN- γ (Fig. 6G) or TNF α (Fig. 6H) production was observed in absence of LAG-3 on days 13, 39 and 58 post-infection. Further, CD4

T cells were similarly exhausted in chronically Clone 13-infected C57BL/6 and LAG-3^{-/-} mice and did not produce significant amounts of IFN- γ (Supplementary Fig. 1K is available at *International Immunology Online*) and TNF α (Supplementary Fig. 1L is available at *International Immunology Online*).

If altered T-cell effector functions had been present at time points that were not included in this analysis, this could be reflected in viral titer. As viral titers in blood, lymph node, spleen, liver, lung, kidney and brain are similar in C57BL/6 and LAG-3^{-/-} mice on days 13, 35 and 58 post-infection with LCMV Docile, this is rather unlikely (Fig. 6I). Similar titers were also observed in Clone 13-infected LAG-3^{-/-} and C57BL/6 mice (Supplementary Fig. 1M is available at *International Immunology Online*).

Thus, LAG-3^{-/-} mice compared with C57BL/6 mice showed improved CD8 T-cell effector functions and/or reduced virus titers neither after LCMV Docile infection nor after LCMV Clone 13 infection. Therefore, a potential inhibitory function of LAG-3 alone does not seem to be very crucial for T-cell exhaustion.

The degree of T-cell exhaustion was also analyzed by assessment of the expression of the inhibitory receptor PD-1. Interestingly, PD-1 was expressed at similar levels in LAG-3^{-/-} and C57BL/6 mice, but on a smaller frequency of gp33-specific CD8 T cells derived from spleen of Clone 13-infected mice than from Docile-infected mice (Fig. 7A). The difference between the virus strains was not apparent if gp33-specific CD8 T cells from blood were analyzed (Fig. 7B), probably reflecting migration of exhausted splenocytes to the periphery.

As LAG-3 was reported to affect T-cell proliferation (17, 23), we next examined whether *in vivo* T-cell division was altered in LAG-3^{-/-} compared with C57BL/6 mice after chronic infection with LCMV strains Docile respective Clone 13. We did not observe any substantial differences in the frequency of Ki67⁺ gp33-tetramer⁺ CD8 T cells derived from spleen (Fig. 7C) and blood (Fig. 7D). Similar proliferation might also account for the similar frequency of gp33-specific CD8 T cells observed in LAG-3^{-/-} and C57BL/6 mice after chronic LCMV infection (Fig. 6 and Supplementary Fig. 1 is available at *International Immunology Online*).

Interestingly, the frequency of Ki67⁺ gp33-specific CD8 T cells is higher in blood than in spleen, possibly reflecting migration of recently activated/proliferating cells from secondary lymphoid organs to peripheral tissues via the blood circulation.

Discussion

In this report, we investigated the expression and importance of the inhibitory receptor LAG-3 on CD8 T cells during

determined on days 15, 28, 39, 52 and 54. (F–M) Groups of three C57BL/6 mice were infected with 200 (low dose, ld) or 10⁶ pfu Docile (high dose, hd) as indicated and treated with PBS or 200 μ g of a blocking antibody directed against LAG-3 on days -3, -1, 3, 6, 9, 12 and 15 (F). On day 17 post-infection, splenocytes were re-stimulated with gp33 for 5 h and analyzed for the capacity of CD8 T cells to degranulate (G) and to produce IFN- γ (H). Background staining was subtracted. Virus titers in the blood were determined on days 9 and 17 post-infection (I). On day 17 post-infection, virus titers were determined in spleen (J), lung (K), liver (L) and kidney (M). One representative of two experiments is shown. Each symbol represents the measured value of one individual mouse. Horizontal lines indicate the arithmetic means of each group of mice. Dashed lines indicate the detection limits.

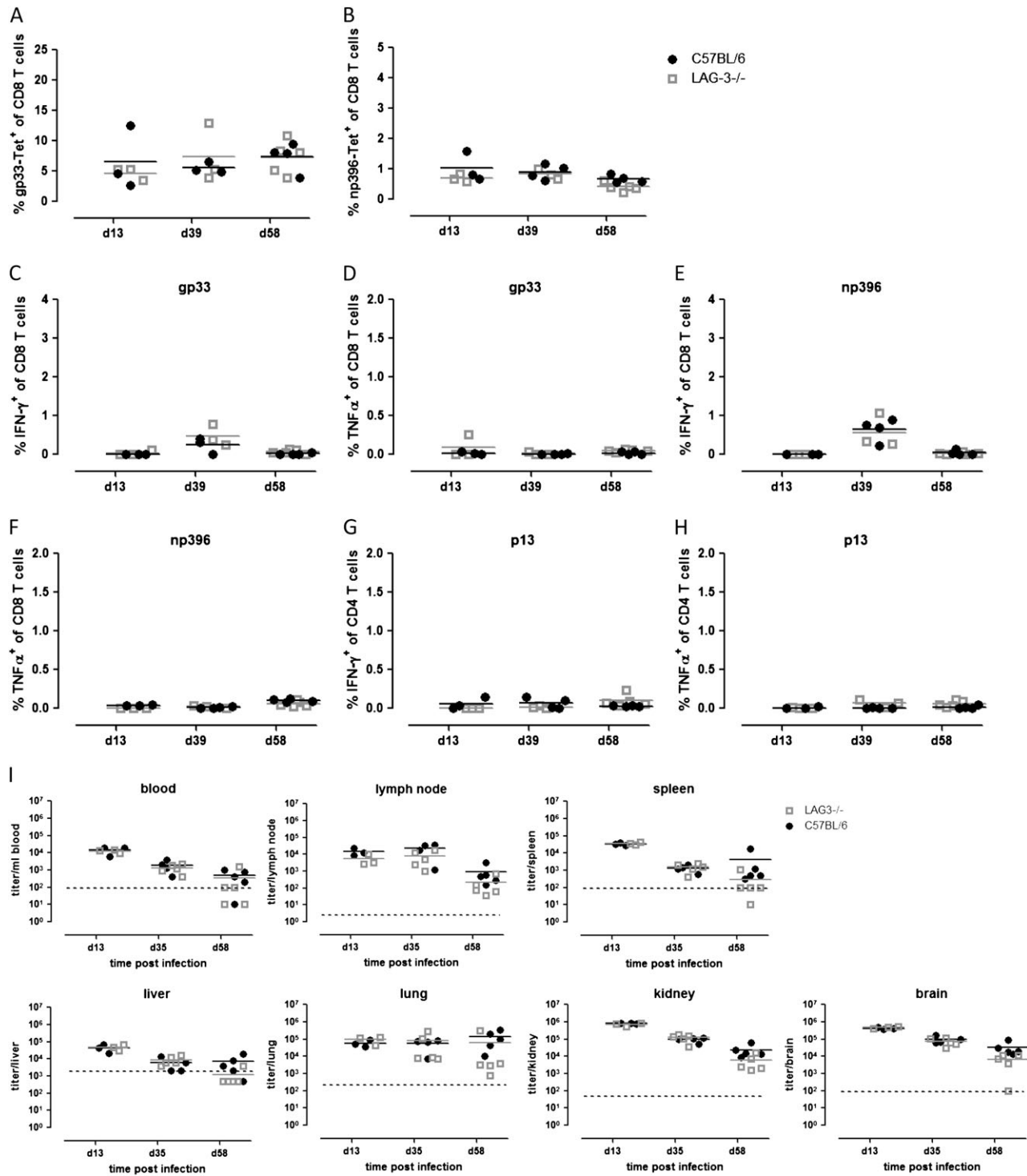


Fig. 6. Chronic infection of LAG-3^{-/-} and wild-type mice with LCMV Docile induces similar degree of T-cell exhaustion and similar viral titers. Groups of three to five LAG-3^{-/-} and C57BL/6 mice were i.v. infected with 10⁶ pfu LCMV Docile and sacrificed on days 13, 39 and 58 post-infection. The frequencies of H-2D^b-gp33- (A) and H-2D^b-np396- (B) tetramer⁺ CD8 T cells were determined in blood. Splenocytes were re-stimulated with gp33 (C and D) and np396 (E and F) and analyzed for the capacity of CD8 T cells to produce IFN- γ (C and E) and TNF α (D and F). CD4 T cells were stimulated with p13 peptide and assessed for their ability to produce IFN- γ (G) and TNF α (H). Virus titers were determined in blood, lymph nodes, spleen, liver, lung, kidney and brain (I). Each symbol represents the measured value of one individual mouse. Horizontal lines indicate the arithmetic means of each group of mice. Dashed lines indicate the detection limits.

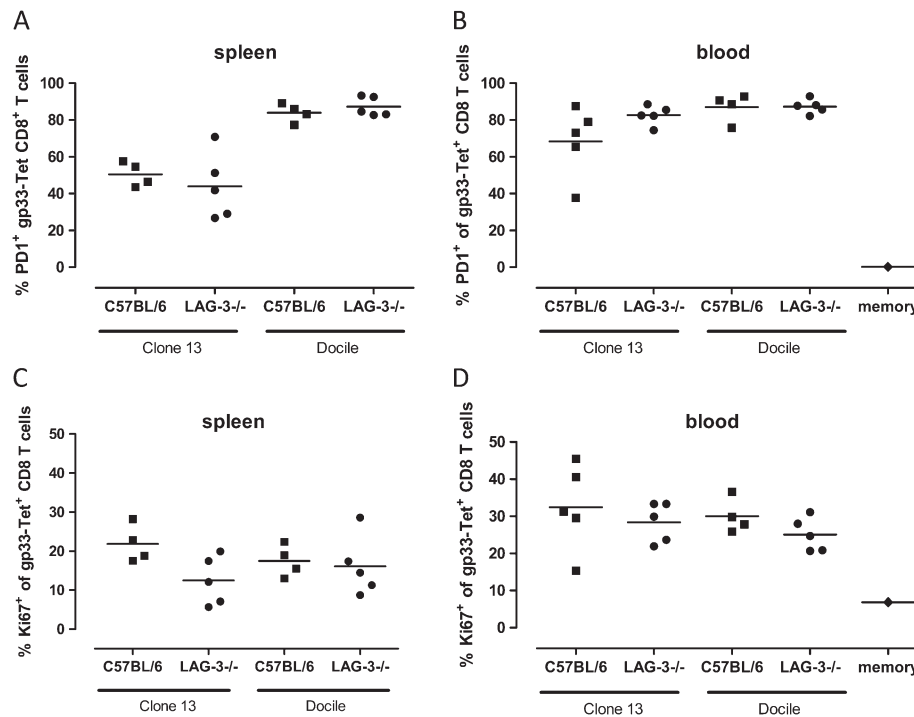


Fig. 7. Expression of PD-1 and proliferation marker Ki67 is unaltered in LCMV-specific CD8 T cells from LAG-3^{-/-} mice compared with C57BL/6 mice after chronic infection with LCMV Docile or Clone 13. LAG-3^{-/-} and C57BL/6 mice were i.v. infected with 10⁶ pfu LCMV Docile or Clone 13 and sacrificed on day 58 post-infection. Expression of PD-1 on H-2D^b-gp33-tetramer⁺ CD8 T cells derived from spleen (A) and blood (B) was assessed. A mouse that was transferred with 10⁴ TCR transgenic CD8 T cells and infected with 200 pfu LCMV-WE several months before analysis served as control (memory). Intracellular expression of Ki67 was analyzed on H-2D^b-gp33-tetramer⁺ CD8 T cells derived from spleen (C) and blood (D). Each symbol represents the measured value of one individual mouse. Horizontal lines indicate the arithmetic means of each group of mice.

acute and chronic LCMV infection. We could demonstrate that exhausted virus-specific CD8 T cells express elevated levels of LAG-3. LAG-3 was mainly expressed on cells co-expressing other inhibitory receptors as PD-1, an inhibitory receptor involved in T-cell exhaustion during LCMV infection (16), and Tim-3, an inhibitory receptor expressed by HIV-specific CD8 T cells (28). These data support and extend data from Blackburn *et al.* describing complex expression patterns of various inhibitory receptors on exhausted CD8 T cells (17). Interestingly, the expression levels of LAG-3 on gp33-specific CD8 T cells were higher after chronic infection with LCMV strain Docile than with strain Clone 13. This might reflect the different severity of T-cell exhaustion in Clone 13 versus Docile infection, with LCMV Docile infection leading to more severe exhaustion (10, 12, 16).

In vitro blockade of LAG-3, also in combination with anti-PD-L1 and/or anti-CTLA-4, did not elevate cytokine production in short-term stimulations of splenocytes from chronically infected mice. Similarly, we (10) and others (17, 31) have previously shown that short-term PD-L1 blockade does not restore T-cell functionality. However, PD-L1 blockade was shown to have a beneficial effect during longer stimulation periods (16). Thus, blockade of inhibitory receptor signaling on exhausted CD8 T cells does not lead to immediate restoration of their functionality but seems to require a 'rejuvenating' proliferation period to gain enhanced effector functions (10, 31).

Next, we showed that the presence of LAG-3 is not crucial for T-cell priming during acute LCMV infection. This is a prerequisite to be able to compare T-cell exhaustion during chronic viral infection with and without LAG-3 signals. If murine LAG-3 can function as negative regulator of activated T cells as it was described for human, the inhibitory role of LAG-3 (20, 32) is controversial as the first analysis of the inhibitory role of LAG-3^{-/-} mice did not reveal a defect in T-cell function with respect to activated and memory subpopulations of CD4 and CD8 T cells and their responses to mitogens and to antigen priming (33). However, in more recent studies, it could be shown that murine LAG-3^{-/-} OTII T cells secrete more IL-2 and IFN- γ following peptide stimulation than their wild-type counterparts (23) and that LAG-3^{-/-} T cells expanded more vigorously *in vivo* after Sendai virus infection than wild-type T cells (24). *In vivo* blockade or genetic ablation of the *Lag-3* gene also led to increased accumulation and effector function of antigen-specific CD8 T cells within tumors and organs that express cognate antigen (34), but in this system, PD-1 and LAG-3 are expressed on different subsets of CD8 T cells (35). Nevertheless, in our experiments, no crucial contribution of LAG-3 during T-cell priming in acute LCMV infection could be observed and goes along with the low and transient expression of LAG-3 on LCMV-specific CD8 T cells after acute LCMV infection.

Despite the fact that initial T-cell priming during LCMV infection was not altered in LAG-3^{-/-} mice, it was still possible

that sustained LAG-3 expression on LCMV-specific CD8 T cells during chronic infection might critically contribute to their dysfunction. However, *in vivo* blockade of LAG-3 during an established chronic infection or during the whole course of an infection did not rescue cytokine production by virus-specific CD8 T cells, or change the frequency of LCMV-specific CD8 T cells or alter the virus titer in various organs. The finding that therapeutic treatment of mice with anti-LAG-3 alone did not rescue exhausted T cells was recently reported by another group (17). Nevertheless, even though no differences were observed in mice treated with anti-LAG-3 and untreated mice with respect to cytokine production, slightly decreased virus titers were observed in the study by Blackburn *et al.* (17) as opposed to our study using *in vivo* LAG-3 blockade or LAG-3^{-/-} mice. The reasons for this discrepancy are currently unclear.

We observed that expression levels of LAG-3 on anti-viral CD8 T cells remained stable over short-term *in vitro* stimulations in the presence of antigenic peptide. It was previously shown that cleavage of LAG-3 by the metalloproteases ADAM 10 and ADAM 17 regulated T-cell proliferation and effector function (29). The unaltered expression of LAG-3 upon antigenic stimulation indicates that repetitive *in vivo* stimulation of LCMV-specific CD8 T cells, as it is likely the case during chronic LCMV infection, does not lead to LAG-3 cleavage, thus maintaining the potential inhibitory function of LAG-3-mediated signals. However, as both *in vivo* blockade and the use of LAG-3^{-/-} mice did not show any changes in T-cell function or control of viral replication upon chronic LCMV infection, we conclude that inhibitory signals mediated via LAG-3 alone are not critically involved in CD8 T-cell exhaustion.

Further, the presence of LAG-3 did not influence T-cell division of gp33-specific CD8 T cells after chronic LCMV infection as assessed by expression of Ki67. This is in contrast to gp276-specific CD8 T cells after chronic LCMV infection which showed increased proliferation after *in vivo* LAG-3 blockade (17). Whether this difference is due to the different T-cell specificity is currently unclear.

These data in combination with the data from Blackburn *et al.* (17) suggest that even though LAG-3 is up-regulated on LCMV-specific exhausted CD8 T cells, it does not significantly contribute to T-cell exhaustion alone. To effectively interfere with T-cell exhaustion, it is very likely that several inhibitory receptors will have to be targeted simultaneously (17).

Supplementary data

Supplementary Fig. 1 is available at *International Immunology Online*.

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