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# Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection

Shawn D Blackburn[1], Haina Shin[1], W Nicholas Haining[2,3], Tao Zou[1], Creg J Workman[6], Antonio Polley[1], Michael R Betts[5], Gordon J Freeman[4], Dario A A Vignali[6] & E John Wherry[1]

**T cell exhaustion often occurs during chronic infection and prevents optimal viral control. The molecular pathways involved in T cell exhaustion remain poorly understood. Here we show that exhausted CD8+ T cells are subject to complex layers of negative regulation resulting from the coexpression of multiple inhibitory receptors. Exhausted CD8+ T cells expressed up to seven inhibitory receptors. Coexpression of multiple distinct inhibitory receptors was associated with greater T cell exhaustion and more severe infection. Regulation of T cell exhaustion by various inhibitory pathways was nonredundant, as blockade of the T cell inhibitory receptors PD-1 and LAG-3 simultaneously and synergistically improved T cell responses and diminished viral load *in vivo*. Thus, CD8+ T cell responses during chronic viral infections are regulated by complex patterns of coexpressed inhibitory receptors.**



After acute viral infection, memory CD8+ T cells rapidly reactivate effector functions, have high proliferative potential and are maintained by antigen-independent homeostatic proliferation[1–3]. These properties allow memory T cells to confer protective immunity. In contrast, during chronic viral infection, antigen-specific CD8+ T cells initially acquire effector functions but gradually become less functional as the infection progresses. This loss of function, known as 'exhaustion', is hierarchical, with properties such as proliferative potential and production of interleukin 2 lost early, production of tumor necrosis factor (TNF) persisting for somewhat longer and production of interferon-γ (IFN-γ) lost only at the most extreme stages of exhaustion[4]. First described with lymphocytic choriomeningitis virus (LCMV)[5], CD8+ T cell exhaustion during persistent infection is a common feature of many experimental models and chronic human infection, including infection with human immunodeficiency virus (HIV) and hepatitis B and C viruses[4]. T cell exhaustion is probably a chief reason for the ineffective viral control in these situations.

An important function for PD-1 and its ligand PD-L1 has been reported for CD8+ T cell exhaustion during chronic viral infection[6]. PD-1, an inhibitory receptor in the CD28 'superfamily'[7], is highly expressed by exhausted CD8+ T cells from chronically infected mice but not by functional LCMV-specific CD8+ T cells from mice that have cleared the infection[6]. Blocking the PD-1–PD-L pathway *in vivo* increases virus-specific CD8+ T cell responses, enhances 'per-cell' function and decreases the viral load[6]. Involvement of the PD-1 pathway has also been shown during infection with HIV and hepatitis B and C viruses[8–15]. Such studies have indicated that exhausted CD8+ T cells could be 'rejuvenated' to enhance antiviral immunity. However, functional restoration by PD-1–PD-L blockade is incomplete, and defects in CD8+ T cells remain after PD-1 pathway blockade[6], which suggests the involvement of other negative regulatory pathways in CD8+ T cell exhaustion. Comparison of global gene expression profiles of exhausted CD8+ T cells with functional virus-specific effector and memory CD8+ T cells has identified upregulation of many inhibitory receptors in addition to PD-1 (ref. 16). HIV-specific CD4+ T cells have been shown to coexpress PD-1 and another inhibitory molecule, CTLA-4 (ref. 17). The effect of upregulation of these additional inhibitory receptors on CD8+ T cell dysfunction during chronic viral infection is not known. It is also unclear whether these inhibitory receptors are expressed by distinct subpopulations of exhausted CD8+ T cells or whether there are exhausted CD8+ T cells that coexpress multiple inhibitory receptors. Finally, there is little information about whether these potential inhibitory pathways serve different functions in regulating T cell exhaustion.

We addressed these issues with a mouse model of chronic LCMV infection. Exhausted CD8+ T cells coexpressed multiple inhibitory receptors, and the pattern of inhibitory receptor coexpression affected the functional quality of these virus-specific CD8+ T cells during chronic infection. The severity of chronic infection was associated with the number and 'intensity' of inhibitory receptors expressed. In addition, *in vivo* blockade of the PD-1 and LAG-3 inhibitory receptor pathways together led to much greater reversal of T cell exhaustion and viral control than did blockade of either pathway alone. These observations indicate that layers of negative regulation exist in

[1]Immunology Program and Wistar Vaccine Center, The Wistar Institute, Philadelphia, Pennsylvania 19104, USA. [2]Department of Hematology/Oncology, Children's Hospital, Boston, Massachusetts, USA. [3]Pediatric Oncology and [4]Medical Oncology, Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA. [5]Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [6]Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. Correspondence should be addressed to E.J.W. (jwherry@wistar.org).

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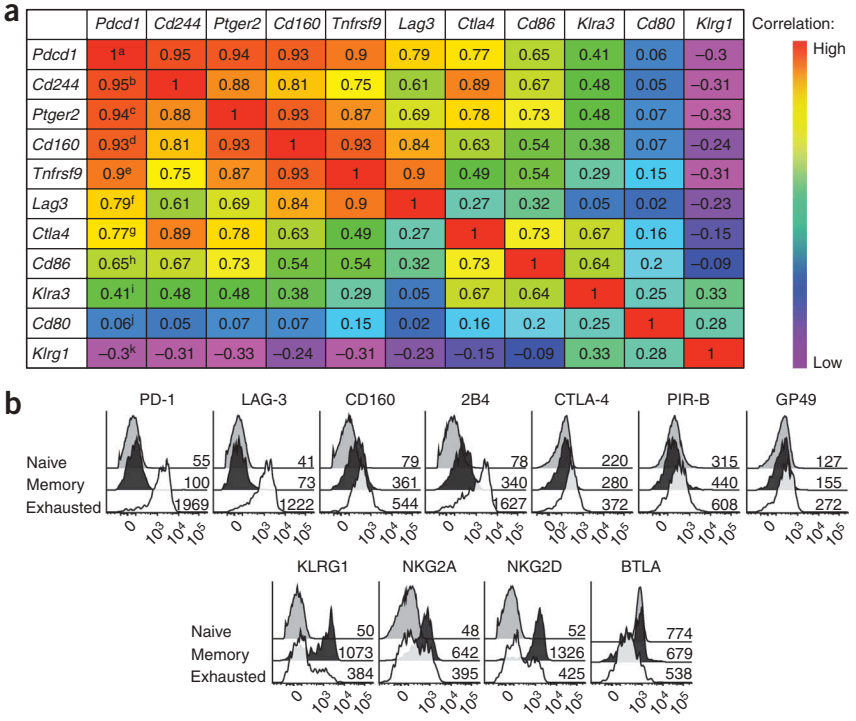


**Figure 1** Memory and exhausted CD8$^+$ T cells express multiple inhibitory receptors. (**a**) Nearest-neighbor analysis with gene expression profiles from ref. 16. Values represent Pearson correlation coefficients, with numbers closer to 1 representing better correlation with the pattern of *Pdcd1* expression. Superscripted letters (a–k) indicate nearest (a) to furthest (k) neighbors for this set of genes. (**b**) Flow cytometry of the expression of 11 receptors on H-2D$^b$ gp33$^+$, CD8$^+$ splenocytes from mice infected with LCMV Arm or clone 13, assessed on day 30 after infection with single-marker staining in four-color panels. Naive CD8$^+$ T cells are total CD44$^{lo}$CD8$^+$ cells from Arm-immune mice. Numbers above bracketed lines indicate mean fluorescence intensity. Data are representative at least three independent experiments with three to five mice each.

exhausted CD8$^+$ T cells as a result of the coexpression of multiple inhibitory receptors and have implications for therapeutic interventions during chronic infection.

## RESULTS

### Inhibitory receptors expressed by exhausted T cells

To identify the genes with expression patterns closest to that of *Pdcd1* (which encodes PD-1) in naive, effector, memory and exhausted CD8$^+$ T cells, we did a nearest-neighbor analysis[18] using published gene expression data[16]. Among the top 100 neighbors of *Pdcd1*, we found several genes encoding surface receptors with inhibitory functions, including *Cd244* (which encodes 2B4)[19], *Cd160* (ref. 20), *Ctla4* and *Lag3* (ref. 21; **Supplementary Table 1** online); the expression of several of these potential inhibitory genes was highly correlated with *Pdcd1* expression (**Fig. 1a**). In contrast, the expression of genes encoding other potential inhibitory receptors, such as *Klra3*, *Cd80* and *Klrg1*, was either neutrally or negatively correlated with *Pdcd1* expression. Thus, at the population level, exhausted cells showed coordinated upregulation of specific genes encoding inhibitory receptors.

We then examined the expression of inhibitory and natural killer cell receptors by flow cytometry of naive and LCMV-specific memory and exhausted CD8$^+$ T cells. We used various strains of LCMV to generate either acute infection and functional memory CD8$^+$ T cells or chronic infection and exhausted CD8$^+$ T cells. Initially, we used the Armstrong (Arm) strain of LCMV, which results in acute infection and functional memory T cells, and the clone 13 strain, which results in chronic infection and T cell exhaustion[22–24] (discussed below). Exhausted CD8$^+$ T cells specific for the H-2D$^b$-restricted gp33-41 epitope of the LCMV glycoprotein (amino acids 33–41; called 'gp33' here) from clone 13–infected mice had higher expression of PD-1, LAG3, 2B4, CD160, CTLA-4, PIR-B and GP49 and lower expression of KLRG1, NKG2A, BTLA and the potentially activating receptor NKG2D than did the functional H-2D$^b$ gp33 tetramer–positive memory CD8$^+$ T cells from Arm-infected mice (**Fig. 1b**). Each of these upregulated receptors has been reported to negatively regulate the function of T cells or other hematopoietic cells[19,20,25–27]. Thus, multiple inhibitory receptors were expressed by exhausted CD8$^+$ T cells but not by memory or naive CD8$^+$ T cells. In addition, the upregulation of inhibitory receptors by exhausted CD8$^+$ T cells was selective, as the inhibitory pathways KLRG1, NKG2A and BTLA were not enhanced.

### Varying severity of LCMV infection in mice

We next used four types of LCMV infection to examine how the severity of infection affects virus-specific CD8$^+$ T cell responses and expression of inhibitory receptors. In addition to the infections with Arm and clone 13 mentioned above, we used infection with LCMV T1B, as well as infection with clone 13 plus transient depletion of CD4$^+$ T cells with antibody to CD4 (anti-CD4). We first defined the kinetics of viral replication with these four infection types. Infection of adult C57BL/6 mice with Arm resulted in high viral replication on day 3 after infection and complete clearance by days 8–10 after infection[28] (**Fig. 2a,b**). For infection with T1B, systemic viral replication lasted about 3–4 weeks, whereas for clone 13, viremia lasted about 2–3 months (**Fig. 2a**). Anti-CD4 plus infection with clone 13 resulted in lifelong viremia[5,23,29] (**Fig. 2a**). Thus, the use of Arm, T1B, clone 13 alone and clone 13 plus anti-CD4 would allow us to compare CD8$^+$ T cell function and the expression of inhibitory receptors during infections of different severity.

All four types of LCMV infection generated robust LCMV-specific CD8$^+$ T cell responses that peaked at about 1–2 weeks after infection, contracted and were then maintained (**Fig. 2c,d**). After infection with Arm, virus-specific CD8$^+$ T cells followed a typical pattern of functional memory CD8$^+$ T cell differentiation[30–32], including upregulation of CD127 and CD62L over time (**Fig. 2e**). During the three chronic viral infections, with the exception of some expression of CD127 on day 100 after infection with T1B, the expression of these markers of normal memory CD8$^+$ T cell differentiation remained low for at least about 100 d (refs. 24,33,34; **Fig. 2e**).

### Infection severity and expression of inhibitory receptors

To assess the regulation of the expression of inhibitory receptors on exhausted CD8$^+$ T cells, we examined the expression of PD-1, LAG-3, 2B4 and CD160 on virus-specific CD8$^+$ T cells with the four infections

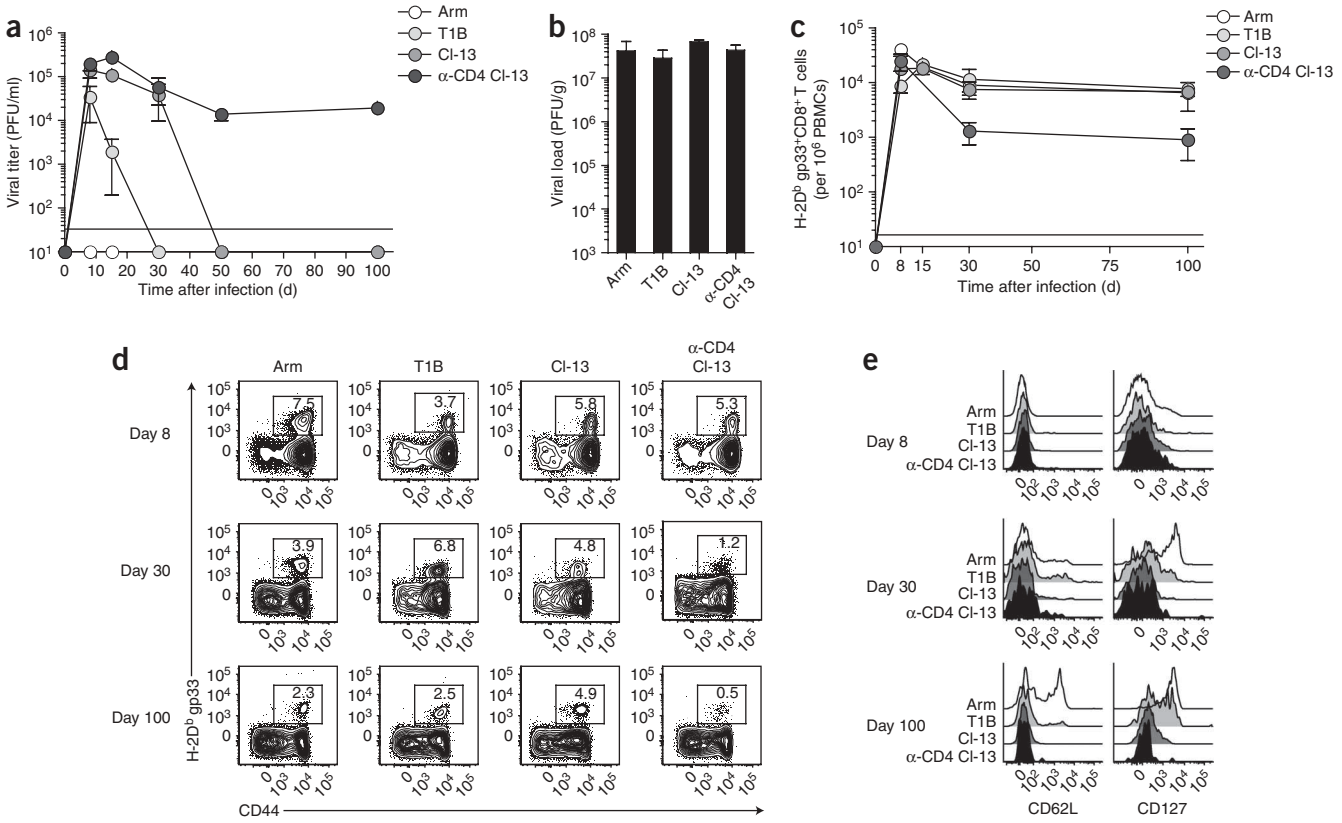


**Figure 2** Four types of LCMV infection. (**a**) Plaque assay of serum viral titers of C57BL/6 mice infected with LCMV Arm, T1B or clone 13 (Cl-13) or depleted of $CD4^+$ T cells 1 d before and 1 d after infection with clone 13 (α-CD4 Cl-13). (**b**) Plaque assay of the viral load in spleens from LCMV-infected mice at 3 d after infection. (**c**,**d**) Flow cytometry of the frequency of H-$2D^b$ gp33$^+$ $CD8^+$ T cells in the peripheral blood. Numbers in outlined areas (**d**) indicate percent $CD8^+$ T cells positive for tetramers. PBMCs, peripheral blood mononuclear cells. Data are representative of two independent experiments with five mice per time point. (**e**) Flow cytometry of the expression of CD62L and CD127 on H-$2D^b$ gp33$^+$, $CD8^+$ T cells. Data are representative of two independent experiments with five (**a**,**c**–**e**) or four (**b**) mice per time point (error bars (**a**–**c**), s.d.).



described above. On day 30 after infection with Arm, H-$2D^b$ gp33$^+$$CD8^+$ T cells had mostly low expression of PD-1, LAG-3 and CD160 and low or intermediate expression of 2B4 (**Fig. 3**). Most virus-specific $CD8^+$ T cells produced both IFN-γ and TNF after peptide stimulation, an indicator of functional competence[23,35]. During infection with T1B, most H-$2D^b$ gp33$^+$$CD8^+$ T cells expressed PD-1 but expressed somewhat less 2B4 than after infection with Arm. Expression of LAG-3 and CD160 on these cells was low. Approximately half of the gp33-specific $CD8^+$ T cells from T1B-infected mice produced both IFN-γ and TNF. During infection with clone 13, H-$2D^b$ gp33$^+$$CD8^+$ T cells had high expression of PD-1 and 2B4 and substantial expression of LAG-3, and some cells expressed CD160. Only about 10% of these gp33-specific $CD8^+$ T cells produced both IFN-γ and TNF. In the most severe condition (anti-CD4 plus clone 13), all four inhibitory receptors were expressed, and these virus-specific $CD8^+$ T cells were very exhausted. As the severity of infection increased, we noted a higher frequency of inhibitory receptor–positive, virus-specific $CD8^+$ T cells, and the H-$2D^b$ gp33$^+$$CD8^+$ T cells had substantially higher expression of each inhibitory receptor per cell and produced less IFN-γ per cell (**Fig. 3c**,**d**). When we examined the PD-$1^{int}$ and PD-$1^{hi}$ subsets of exhausted $CD8^+$ T cells[36], it was apparent that PD-$1^{hi}$, H-$2D^b$ gp33$^+$ exhausted $CD8^+$ T cells expressed more 2B4, LAG-3 and CD160 than did their PD-$1^{int}$ counterparts (**Supplementary Fig. 1** online). Thus, virus-specific $CD8^+$ T cells with high expression of PD-1 also coexpressed other inhibitory receptors.

### Patterns of coexpression of inhibitory receptors

We next used multiparameter flow cytometry to analyze coexpression of PD-1, LAG-3, CD160 and 2B4. On day 8 after infection, a small proportion of H-$2D^b$ gp33$^+$, $CD8^+$ T cells from Arm-infected mice expressed more than one inhibitory receptor (**Fig. 4a**). Conversely, most H-$2D^b$ gp33$^+$, $CD8^+$ T cells from mice infected with T1B or clone 13 alone or clone 13 plus anti-CD4 coexpressed three or four inhibitory receptors. On day 30 after infection, the percentage of H-$2D^b$ gp33$^+$, $CD8^+$ T cells from Arm-immune mice that expressed two or more inhibitory receptors had decreased. The initially high inhibitory receptor coexpression during T1B infection also decreased to some extent by day 30. In contrast, the more severe infections with clone 13, alone or with anti-CD4, resulted in high coexpression of three or four inhibitory receptors on day 8 after infection, which persisted on day 30. Coexpression of inhibitory receptors was similar for gp33-specific $CD8^+$ T cells from P14 mice (**Supplementary Fig. 2** online), which suggested that T cell antigen receptor (TCR) diversity is not the main determinant of the pattern of coexpression of inhibitory receptors. Thus, during chronic LCMV infection, there was substantial coexpression of multiple inhibitory receptors on the same virus-specific $CD8^+$ T cells. Moreover, the extent of inhibitory receptor coexpression was associated with the severity of infection.

Multiparameter staining allowed us to determine which subpopulations of exhausted $CD8^+$ T cells expressing various combinations of

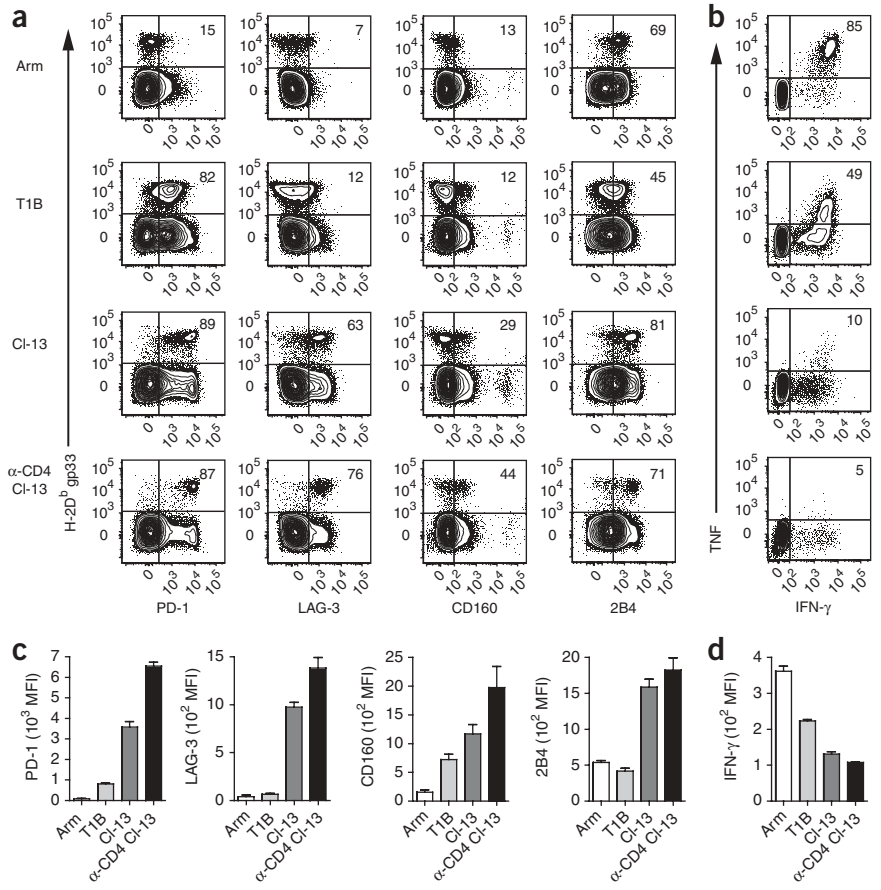


**Figure 3** Influence of the severity of infection on the expression of inhibitory receptors and CD8$^+$ T cell function. (**a**) Multiparameter flow cytometry of the expression of four of the exhaustion-associated inhibitory receptors on H-2D$^b$ gp33$^+$CD8$^+$ splenocytes from mice infected with Arm, T1B, clone 13 or anti-CD4 (α-CD4) plus clone 13, assessed on day 30 after infection. Numbers in top right quadrants indicate percent tetramer-positive CD8$^+$ T cells expressing the receptor (below plot). Similar inhibitory receptor expression profiles were obtained for H-2D$^b$ gp276 tetramer–positive CD8$^+$ T cells (data not shown). (**b**) Flow cytometry of the production of IFN-γ and TNF by splenocytes obtained from mice infected as described in **a** and stimulated with gp33, assessed on day 30 after infection. Numbers in top right quadrants indicate percent IFN-γ$^+$CD8$^+$ T cells also producing TNF. (**c**,**d**) Expression of inhibitory receptors (**c**) and IFN-γ (**d**) by the cells in **b**. MFI, mean fluorescence intensity. Data are representative of three independent experiments with three to five mice per experiment (error bars (**c**,**d**), s.d.).



inhibitory receptors were present during chronic infection. Of the 16 possible combinations, we detected 11, but over 90% of the exhausted H-2D$^b$ gp33$^+$, CD8$^+$ T cells from clone 13–infected mice were in five subpopulations (**Fig. 4b**). Notably, during infection with clone 13 (and infection with clone 13 plus anti-CD4), PD-1 was expressed by most virus-specific CD8$^+$ T cells, followed by LAG-3 and 2B4 (**Fig. 4b** and **Supplementary Fig. 3** online). CD160 was expressed by the lowest percentage of exhausted CD8$^+$ T cells and was associated with coexpression of the other three molecules (**Fig. 4b** and **Supplementary Fig. 3**).

To determine whether the pattern of inhibitory receptor coexpression was associated with T cell function, we did intracellular cytokine staining (ICS) for IFN-γ and TNF after peptide stimulation and stained for the four inhibitory receptors. During infection with clone 13, the degree of CD8$^+$ T cell exhaustion was directly associated the number of inhibitory receptors coexpressed (**Fig. 4c**). Thus, during chronic viral infection, coexpression of more inhibitory receptors was associated lower T cell function.

### *In vitro* blockade of inhibitory pathways

To assess the effect of inhibitory receptors on exhausted CD8$^+$ T cells, we investigated short-term (less than 18 h) changes in T cell properties in the presence of blocking antibodies with three separate *in vitro*

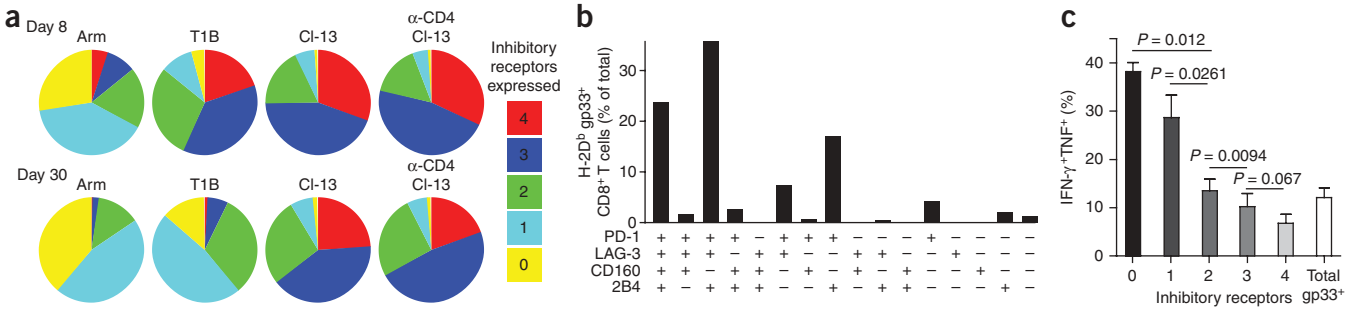


**Figure 4** Concurrent expression of multiple inhibitory receptors increases with viral load and is associated with lower functionality. Boolean gating analysis of the simultaneous expression of multiple inhibitory receptors (PD-1, LAG-3, 2B4 and CD160) on H-2D$^b$ gp33$^+$, CD8$^+$ splenocytes from mice infected with Arm, T1B, clone 13 or anti-CD4 plus clone 13 on days 8 and 30 after infection. (**a**) Individual populations grouped according to total number of inhibitory receptors expressed. (**b**) Relative abundance of each possible individual inhibitory receptor expression profile. (**c**) ICS of IFN-γ and TNF with gp33-41 peptide stimulation of exhausted CD8$^+$ T cells (day 30 after infection with clone 13), in conjunction with staining for four inhibitory receptors; after Boolean gating, the percentage of gp33-responding cells that produced both IFN-γ and TNF was determined and individual populations were grouped according to the total number of inhibitory receptors expressed. Expression of the inhibitory receptors examined here did not change during the 5 h of stimulation for ICS (data not shown). Data are representative of three independent experiments with three to five mice per experiment (error bars (**c**), s.d.).

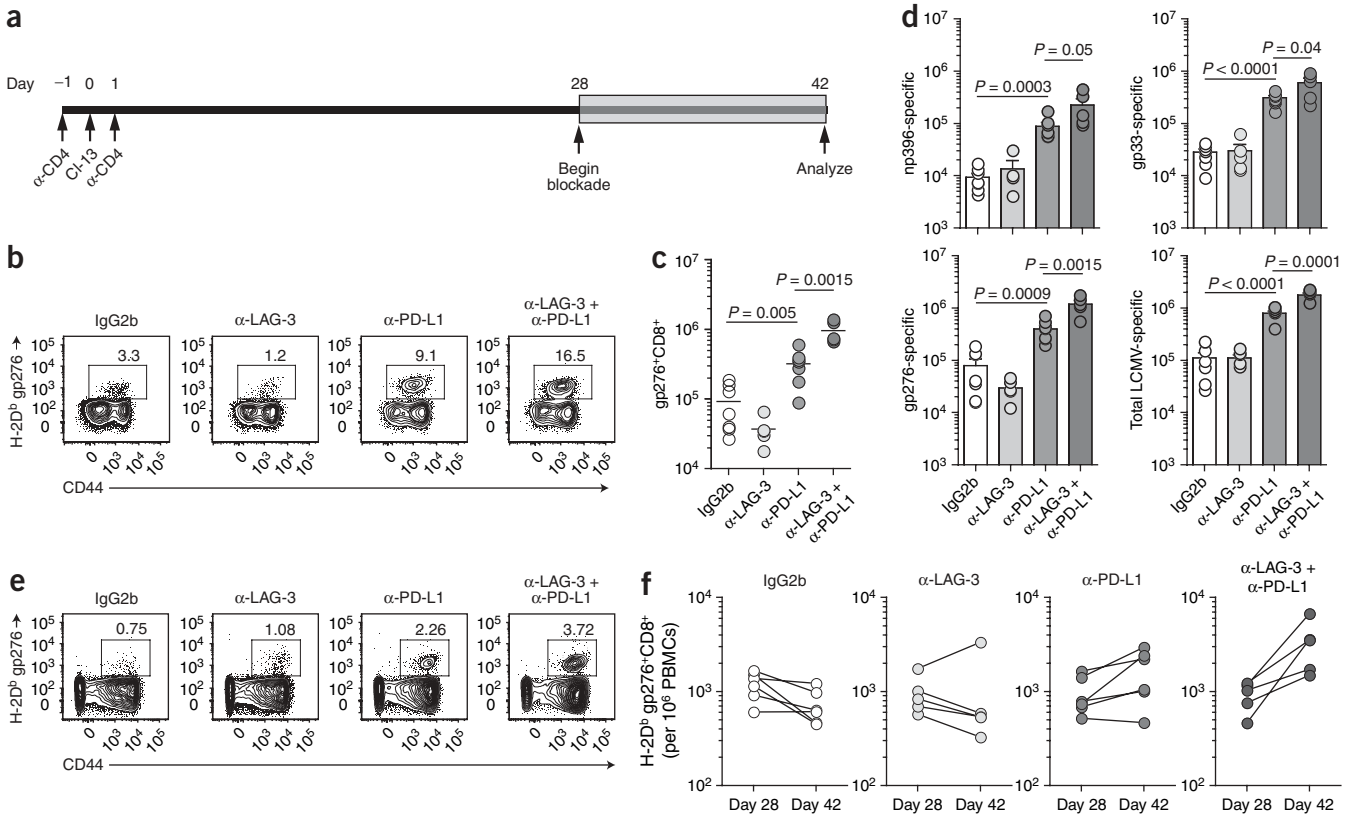


**Figure 5** Blockade of PD-L1 and LAG-3 during chronic LCMV infection enhances antiviral CD8$^+$ T cell responses. (**a**) Protocol for receptor blockade used in **b**–**f**: C57BL/6 mice were depleted of CD4$^+$ T cells (α-CD4) and infected with clone 13, then, starting on day 28 (Begin blockade), were treated every third day for 2 weeks with isotype-matched control antibody, anti-LAG-3, anti-PD-L1, or anti-LAG-3 and anti-PD-L1. (**b**) Staining for H-2D$^b$ gp276 tetramer on CD8$^+$ splenocytes after 2 weeks of treatment. Numbers above outlined areas indicate percent CD8$^+$ T cells that are tetramer positive. (**c**) Total number of H-2D$^b$ gp276$^+$CD8$^+$ T cells in the spleen after treatment. Similar results were obtained for staining for H-2D$^b$ np396 and H-2D$^b$ gp33 tetramers (data not shown). (**d**) Total number of degranulating (CD107a$^+$) and/or IFN-γ producing CD8$^+$ splenocytes in response to peptide stimulation after 2 weeks of treatment. (**e**) Staining for H-2D$^b$ gp276 tetramer on CD8$^+$ peripheral blood mononuclear cells (PBMCs) after 2 weeks of treatment. Numbers above outlined areas indicate percent CD8$^+$ T cells that are tetramer positive. (**f**) Frequency of H-2D$^b$ gp276$^+$CD8$^+$ T cells before and after treatment. Data are representative of three independent experiments with five to seven mice per group in each experiment.



assays: an *in vitro* killing assay[37], an *in vitro* apoptosis assay[36] and an ICS assay for cytokine production (**Supplementary Fig. 4** online). *In vitro* cytotoxicity was enhanced by anti-CD160 but not by anti-PD-L1 or anti-LAG-3 or by blockade of the 2B4 ligand CD48. To examine T cell apoptosis and survival, we separated exhausted CD8$^+$ T cells into PD-1$^{int}$ and PD-1$^{hi}$ subsets as described[36]. Expression of active caspase 3 was lower in the PD-1$^{int}$ subset in the presence of anti-PD-L1 but not in the presence of anti-LAG-3 or anti-CD160. In contrast, anti-CD160, but not anti-PD-L1 or anti-LAG-3, improved the survival of the PD-1$^{hi}$ subset. Moderate 2B4 expression can deliver a costimulatory signal, whereas high 2B4 expression can be inhibitory[38]. Blocking the 2B4 pathway with anti-CD48 decreased cytokine production by 2B4$^{int}$ LCMV-specific CD8$^+$ T cells from chronically infected mice but significantly increased IFN-γ production by exhausted 2B4$^{hi}$ CD8$^+$ T cells ($P = 0.026$). CD48 can also bind CD2 and deliver a costimulatory signal. Blockade of CD2 decreased IFN-γ production from exhausted 2B4$^{int}$ and 2B4$^{hi}$ CD8$^+$ T cells. These observations are consistent with a costimulatory function for CD48 binding to either CD2 or 2B4 on 2B4$^{int}$ virus-specific CD8$^+$ T cells and an inhibitory function of 2B4 through interactions with CD48 on exhausted 2B4$^{hi}$ CD8$^+$ T cells. Although additional *in vivo* studies are warranted, these *in vitro* experiments suggest that the CD160 and 2B4 pathways can contribute to CD8$^+$ T cell exhaustion.

### Simultaneous blockade of PD-L1 and LAG-3 *in vivo*

It is unclear whether different inhibitory receptors have overlapping functions or whether these distinct pathways contribute independently to T cell dysfunction during chronic viral infection. To distinguish between those possibilities, we blocked two pathways simultaneously with blocking antibodies to PD-L1 and LAG-3, either alone or in combination during chronic LCMV infection *in vivo* (**Fig. 5a**). Two weeks of treatment with anti-LAG-3 alone did not improve the frequency or absolute number of tetramer-positive CD8$^+$ T cells specific for the H-2D$^b$-restricted gp276-286 epitope of the LCMV glycoprotein (amino acids 276–286; H-2D$^b$ gp276), whereas blocking the PD-1 pathway augmented the H-2D$^b$ gp276 response (**Fig. 5b**,**c**). Despite the lack of effect of anti-LAG-3 alone on T cell numbers, combining LAG-3 blockade with PD-1–PD-L1 blockade resulted in an 81% higher frequency of H-2D$^b$ gp276$^+$CD8$^+$ splenocytes relative to that of the group treated with anti-PD-L1 and a five-fold improvement relative to the untreated group (**Fig. 5b**), with corresponding increases in absolute numbers (**Fig. 5c**). Dual blockade also resulted in significantly greater CD8$^+$ T cell responses to the np396-404 epitope of the LCMV nucleoprotein (amino acids 396–404; np396), the gp33 and gp276 peptides and a pooled peptide mixture containing 20 known LCMV epitopes than did either single treatment or control treatment, as measured by ICS instead of tetramer staining (**Fig. 5d**).

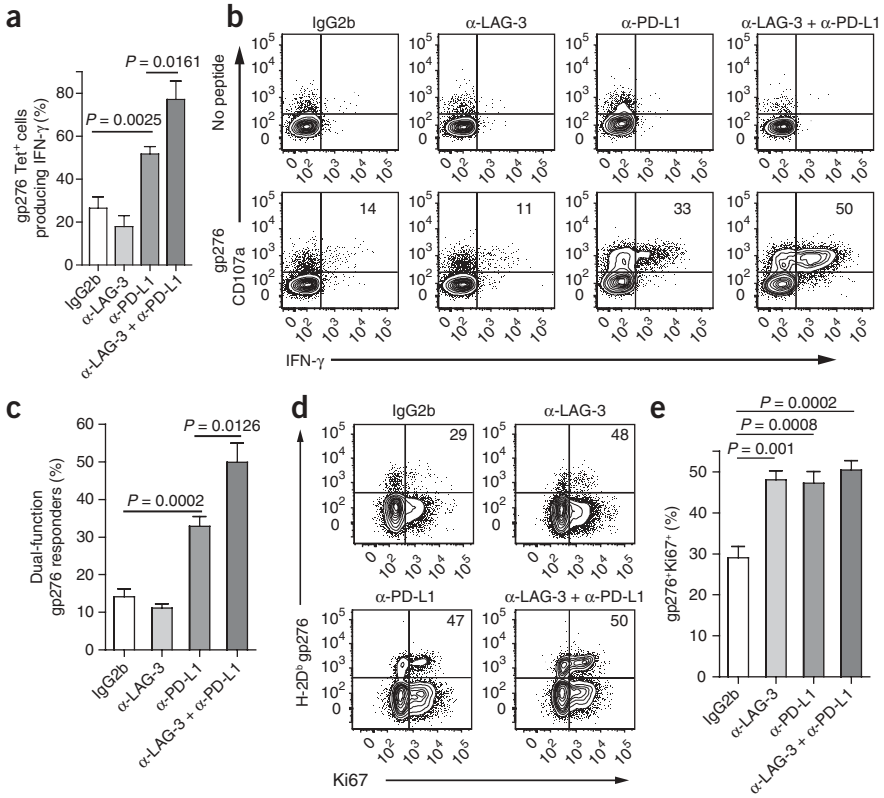


**Figure 6** Improved function of exhausted CD8+ T cells after dual blockade of LAG-3 and PD-L1. (**a**) H-2D$^b$ gp276$^+$, CD8$^+$ splenocytes that produce IFN-γ in response to stimulation with gp276 peptide. Tet, tetramer. (**b**,**c**) CD107a degranulation assay in conjunction with ICS for IFN-γ with and without stimulation with gp276 peptide. Values indicate percent responding cells that both produce IFN-γ and degranulate, for individual mice (**b**) and many mice (**c**). (**d**,**e**) Intracellular staining for Ki67 in H-2D$^b$ gp276$^+$, CD8$^+$ splenocytes. Values indicate percent H-2D$^b$ gp276$^+$, CD8$^+$ splenocytes that express Ki67, for individual mice (**d**) and many mice (**e**). A similar trend was obtained for incorporation of the thymidine analog BrdU (data not shown). IgG2b, isotype-matched control antibody. Data are representative of three independent experiments with five to seven mice per group in each experiment (error bars (**a**,**c**,**e**), s.d.).

We also measured the frequency of LCMV-specific CD8$^+$ T cells in the blood before and after treatment (**Fig. 5e**,**f**). The frequency of circulating CD8$^+$ T cells positive for H-2D$^b$ gp276 tetramers remained essentially constant in control mice and mice treated with anti-LAG-3 only. PD-1–PD-L1 blockade alone resulted in responses that were about 1.8-fold higher ($P = 0.0456$), whereas dual blockade of both LAG-3 and PD-1–PD-L1 led to a circulating pool of H-2D$^b$ gp276$^+$CD8$^+$ T cells that was about 3.7-fold larger ($P = 0.0307$). Thus, blocking the PD-1–PD-L pathway *in vivo* identified substantial involvement of the LAG-3 pathway in coregulating CD8$^+$ T cell exhaustion during chronic viral infection.

## Greater function with LAG-3 and PD-L1 coblockade

To determine whether inhibitory receptor blockade improved the quality of the antiviral CD8$^+$ T cells, we examined function on a 'per-cell' basis. Treatment with anti-PD-L1 alone resulted in a significantly higher percentage of H-2D$^b$ gp276 tetramer–positive CD8$^+$ T cells that produced IFN-γ (**Fig. 6a**). Although treatment with anti-LAG-3 alone had little effect on the severity of exhaustion, combined treatment with anti-LAG-3 and anti-PD-L1 led to substantially greater 'per-cell' function than did anti-PD-L1 alone, anti-LAG-3 alone or control treatment (**Fig. 6a**,**b**). Combined treatment with anti-LAG-3 and anti-PD-L1 also resulted in a significantly greater percentage of dual-function (CD107$^+$IFN-γ$^+$) gp276-specific CD8$^+$ T cells than did treatment with anti-PD-L1 or anti-LAG-3 alone (**Fig. 6b**,**c**). Although the percentage of cells producing both IFN-γ and TNF was only slightly different in the groups (**Supplementary Fig. 5** online), the absolute number of dual-cytokine producers and CD107$^+$IFN-γ$^+$ virus-specific CD8$^+$ T cells was much greater after *in vivo* blockade (data not shown). Thus, combining LAG-3 blockade with PD-1–PD-L1 blockade resulted in substantially better reversal of exhaustion than did PD-1–PD-L1 blockade alone.

Despite the lack of effect of anti-LAG-3 alone on antiviral T cell numbers or effector function, this treatment seemed to have an effect on exhausted CD8$^+$ T cells. Blocking LAG-3 alone, as well as PD-L1

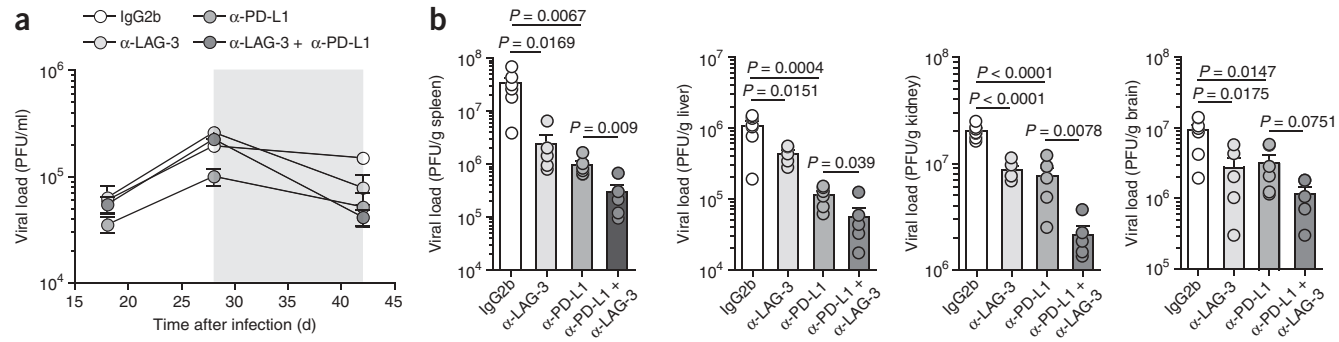


**Figure 7** Greater viral control after dual blockade of PD-L1 and LAG-3. (**a**) Longitudinal analysis of viral load in serum of mice depleted of CD4$^+$ T cells and infected with clone 13 and treated with isotype-matched control antibody (IgG2b), anti-LAG-3, anti-PD-1, or anti-LAG-3 and anti-PD-1. (**b**) Viral load in spleen, liver, kidney and brain of mice infected and treated as described in **a**. Each symbol in bars indicates an individual mouse. Data are representative of three independent experiments with five to seven mice per group in each experiment (error bars, s.d.).

alone or both LAG-3 and PD-L1, resulted in higher expression of PD-1 on virus-specific exhausted CD8$^+$ T cells *in vivo* (**Supplementary Fig. 6** online). As TCR signals can increase PD-1 expression[39], these results suggest that blockade of either LAG-3 or PD-L1 or both can modulate TCR signaling by persistent antigen. Although it is possible that the PD-L1 and LAG-3 blockade operates through other cell types, our results are consistent with the idea that blockade of these inhibitory receptors acts directly on exhausted, virus-specific CD8$^+$ T cells.

We next examined whether the blockade treatments were associated with more T cell division. Although about 30% of the H-2D$^b$ gp276$^+$CD8$^+$ T cells from control mice expressed the proliferation marker Ki67, about 50% of the virus-specific CD8$^+$ T cells in all three treatment groups expressed Ki67 (**Fig. 6d**,**e**). In contrast to the minimal effect of anti-LAG-3 alone on effector function, blockade of LAG-3 and PD-1–PD-L1 alone or together resulted in similarly improved entry of exhausted CD8$^+$ T cells into the cell cycle. However, many more virus-specific CD8$^+$ T cells accumulated after treatment with anti-PD-L1, and especially after treatment with both anti-LAG-3 and anti-PD-L1, than after treatment with anti-LAG-3 alone.

### Improved viral control with blockade of both PD-L1 and LAG-3

To determine whether the blockade of inhibitory receptors affected viral control during treatment with anti-CD4 and infection with clone 13, we measured viral load in the blood before and after treatment. The viral load of control mice treated with immunoglobulin G2b (IgG2b) was only slightly lower (by about 22%) during this time. In contrast, viral load was 70% lower in mice treated with anti-LAG-3 alone, 48% lower with anti-PD-L1 and 81% lower with dual treatment (**Fig. 7a**). Treatment with anti-LAG-3 or anti-PD-L1 alone also resulted in significantly lower viral loads in the spleen, liver and kidney (**Fig. 7b**). However, combined blockade of LAG-3 and PD-L1 resulted in viral loads that were significantly lower in several organs than did blockade of LAG-3 or PD-L1 alone.

## DISCUSSION

Here we have investigated the expression of various inhibitory receptors by exhausted CD8$^+$ T cells during chronic viral infection, with the following results. First, exhausted CD8$^+$ T cells expressed up to seven distinct inhibitory receptors. Second, inhibitory receptors were coexpressed by exhausted CD8$^+$ T cells, and complex patterns of coexpression were apparent. Third, the severity of viral infection affected the diversity and amounts of inhibitory receptors expressed. Fourth, the inhibitory receptors LAG-3 and PD-1 operated as at least partially nonoverlapping pathways that negatively regulated T cell responses during chronic viral infection. Finally, combined blockade of LAG-3 and PD-L1 acted in synergy to enhance T cell responses and viral control during chronic LCMV infection. These observations collectively indicate that multiple negative regulatory pathways contribute to CD8$^+$ T cell exhaustion and suggest that the pattern of inhibitory receptor coexpression is a useful correlate of the amount of T cell exhaustion. Thus, reversal of T cell exhaustion could be improved by therapeutic targeting of multiple inhibitory receptor pathways simultaneously, and approaches to 'fine-tune' T cell responses to persisting pathogens might be developed by targeting of inhibitory receptors that regulate specific T cell properties.

Our results obtained with different LCMV infections suggest that the patterns of coexpression of inhibitory receptors are influenced by the severity and/or type of infection. Our studies indicate the pathogenesis of infection as an important parameter influencing inhibitory receptor expression and suggest the involvement of viral load. Additional studies are needed to investigate the influence of inflammation and CD4$^+$ T cell help, as well as to investigate the patterns of the coexpression of inhibitory receptors during other types of persistent infections. Many studies have emphasized the relationship between the severity of infection and the extent of CD8$^+$ T cell dysfunction[6,8,23,40–42]. The improved CD8$^+$ T cell responses after blockade of inhibitory receptors could benefit from both the blockade of an inhibitory pathway and enhanced control of infection and lower viral load. Blockade of inhibitory receptors could therefore be a pivotal event that initially improves T cell function and viral control. The resulting lower viral load could then have a secondary positive effect on the severity of exhaustion. Given the potential for clinical applications, it will be necessary to understand the contribution of continued inhibitory receptor blockade versus lower viral load to improved T cell responses.

The pathway of PD-1 and PD-L1 or PD-L2 has received considerable attention for its involvement in regulating T cell exhaustion. There has been some debate, however, about precisely what T cell functions are regulated by PD-1 (refs. 7,43). Our data provide a potential explanation for these apparently disparate results, if the coexpression of other inhibitory receptors differed in the T cell populations analyzed in different studies. For example, the greater effect of PD-1 pathway blockade alone on PD-1$^{int}$CD8$^+$ T cells[36] could result from the presence of multiple additional inhibitory receptors, such as CD160, on the PD-1$^{hi}$ subset of exhausted CD8$^+$ T cells. Thus, it may be necessary to consider the full repertoire of inhibitory receptors expressed when investigating the function of any individual inhibitory pathway.

The diversity of inhibitory receptors expressed by exhausted CD8$^+$ T cells is remarkable. Among the set of inhibitory receptors examined here, exhausted CD8$^+$ T cells expressed two inhibitory molecules in the CD28 'superfamily' (PD-1 and CTLA-4) but downregulated a third (BTLA). PD-1–PD-L1 is a chief pathway that regulates T cell exhaustion during many chronic viral infections[7]. During chronic LCMV infection, CTLA-4 does not seem to have a notable effect on exhausted CD8$^+$ T cells[6], but an effect of CTLA-4 on HIV-specific CD4$^+$ T cells has been described[17]. LAG-3 binds to major histocompatibility complex class II molecules, consistent with homology between LAG-3 and CD4 (ref. 44). LAG-3 negatively regulates T cell activation and proliferation in several settings[21,45], but its function during chronic viral infection has remained unknown. Although a function for LAG-3 has been reported on regulatory T cells[46], there is a CD8$^+$ T cell–intrinsic function for LAG-3 in antitumor responses[47]. LAG-3 limits T cell proliferation and memory T cell homeostasis through cell cycle arrest[25,48–50]. The function of LAG-3 as a negative regulator of cell cycle progression of CD8$^+$ T cells[25,48–50] fits well with the higher Ki67 expression we noted after LAG-3 blockade during chronic LCMV infection. The *in vivo* studies presented here are consistent with a CD8$^+$ T cell–intrinsic function for LAG-3 but do not exclude a function for LAG-3 on other cell types.

In addition to PD-1 and LAG-3, which have been shown by *in vivo* blocking studies to be involved in regulating dysfunction, exhausted CD8$^+$ T cells coexpressed a variety of other inhibitory receptors that might be future therapeutic targets. Exhausted CD8$^+$ T cells expressed PIR-B and GP49, both of which have been linked to negative regulation of mast cells, neutrophils, eosinophils, macrophages and natural killer cells[26,27]. Notably, GP49A is not inhibitory, whereas GP49B is a negative regulator of Fc receptor signaling[27,51]. Because antibodies do not distinguish between GP49A and GP49B, future studies are needed to further delineate this pathway. CD160 was also upregulated on exhausted CD8$^+$ T cells; this molecule inhibits human CD4$^+$ T cell responses[20]. Our preliminary *in vitro* studies are

consistent with an inhibitory function for CD160 on exhausted CD8$^+$ T cells, but the exact function of this molecule in natural killer cells and T cells and during viral infection remains poorly understood. Finally, 2B4 (also called CD244) is a SLAM family member that can be either activating or inhibitory and has been best characterized on T cells in the intestinal mucosa and natural killer cells[19,52,53]. The amount of 2B4 per cell and the expression of the 'downstream' adaptor molecule SAP are key determinants of whether 2B4 delivers an inhibitory or an activating signal[53]. Crosslinking of 2B4 on 2B4$^{lo}$ or 2B4$^{int}$ cells delivers a positive signal[38]. However, when 2B4 expression is high and SAP expression is low, 2B4 is inhibitory[38]. SAP amounts are probably limiting in exhausted CD8$^+$ T cells but not effector CD8$^+$ T cells on the basis of gene expression profiles[16]. The high expression of 2B4 by exhausted CD8$^+$ T cells and the effects of *in vitro* CD48 blockade are consistent with an inhibitory function for 2B4 on 2B4$^{hi}$ exhausted CD8$^+$ T cells. The diverse potential of these inhibitory receptors suggests that these pathways contribute in qualitatively different ways to T cell exhaustion. Indeed, the results from the dual blockade of PD-L1 and LAG-3 indicate that these two pathways impart distinct regulatory effects on exhausted CD8$^+$ T cells.

A question that emerges from these and other studies is whether inhibitory receptors cause T cells to become exhausted in the first place. Functional effector CD8$^+$ T cells upregulate PD-1 and LAG-3 during the first week of an acute viral infection[6,16], however, and some of these cells form functional memory T cells once the infection is cleared and expression of inhibitory receptors decreases. Although prolonged expression of inhibitory receptors could be involved, the pathways involved in the induction of T cell exhaustion and the lineage fate 'decisions' of T cell exhaustion and T cell memory remain poorly understood. Published studies have identified about 500 genes that are differently expressed in exhausted CD8$^+$ T cells and naive, effector and memory T cells[16], and it is likely that many pathways in addition to those activated by inhibitory receptors are involved in the early events controlling T cell exhaustion.

In summary, here we have identified coexpression of inhibitory receptors as a key feature of exhausted CD8$^+$ T cells responding to chronic viral infection. Blockade of the PD-1–PD-L pathway identified a previously unknown function for coregulation of exhausted CD8$^+$ T cells by a second inhibitory receptor, LAG-3, and the complex pattern of coexpression of these and other inhibitory receptors suggests a 'tunable' array of inhibitory pathways acting on exhausted CD8$^+$ T cells. Combination therapies for chronic viral infection have considerable potential, and studies combining blockade of PD-1–PD-L or the interleukin 10 receptor with therapeutic vaccination have shown promising results[54,55]. Our studies have shown synergistic effects of dual blockade of two inhibitory receptors during chronic viral infection. Future studies should help define the intracellular targets of these inhibitory pathways and test the potential of therapeutic interventions that target combinations of negative regulatory pathways during chronic viral infection.

## METHODS

**Mice, viruses and infections.** Four-week-old C57BL/6 mice were from Jackson Laboratories. Ly5.1$^+$ P14 mice bearing H-2D$^b$ gp33–specific TCR were backcrossed ten generations or more to C57BL/6 mice and were maintained in the Wistar Institute animal colony. LCMV strains were propagated, titered and used as described[23]. C57BL/6 mice were infected intraperitoneally with $2 \times 10^5$ plaque-forming units (PFU) of LCMV Armstrong, intravenously with $2 \times 10^6$ PFU LCMV T1B or intravenously with $2 \times 10^6$ PFU LCMV clone 13. In experiments in which mice depleted of CD4$^+$ cells were used, C57BL/6 mice were injected intraperitoneally with 200 μg anti-CD4 (GK1.5; BioXcell) on days –1 and +1 relative to infection with LCMV clone 13 on day 0. Chimeric P14 mice were generated by adoptive transfer of $5 \times 10^2$ naive TCR-transgenic T cells into naive C57BL/6 mice, followed by infection with LCMV Armstrong, T1B or clone 13. All mice were used in accordance with guidelines of the Institutional Animal Care and Use Committee of the Wistar Institute.

**Flow cytometry and intracellular cytokine staining.** Lymphocytes were isolated from the spleen and peripheral blood as described[23]. Tetramers of major histocompatibility class I peptide were made and used as described[23]. Antibodies were from eBioscience, BioLegend, Invitrogen, Abcam, Accurate Chemical, R&D Systems and BD Biosciences (**Supplementary Table 2** online). Lymphocytes were stained and analyzed as described[23]. ICS and the CD107a assay were done as described[6,23]. Cells were stimulated with individual LCMV peptides or a pool of 20 LCMV epitopes[56] (**Supplementary Table 3** online). Cells were then analyzed on a LSR II flow cytometer (BD Immunocytometry Systems). Approximately $1 \times 10^6$ events were collected per sample. Data were analyzed with FlowJo v.8.2 (TreeStar). Doublets were removed by gating on a plot of forward-scatter area versus forward-scatter height. Dead cells were removed by gating on Live/Dead Aqua (Invitrogen) versus forward-scatter area. Cells were subjected to a lymphocyte gate on a plot of forward-scatter area versus side scatter, and then were sequentially gated on CD4$^-$, CD8$^+$ and H-2D$^b$ gp33$^+$ events. Positive and negative gates for each inhibitory receptor on H-2D$^b$ gp33$^+$, CD8$^+$ T cells were drawn on the basis of 'fluorescence-minus-one' controls. The Boolean gating function of FlowJo was used to assess each possible inhibitory receptor expression pattern. Coexpression patterns were analyzed with the SPICE program from the Exon distribution server.

***In vivo* antibody blockade.** Rat anti-PD-L1 (200 μg; 10F.9G2; prepared 'in house'), rat IgG2b isotype-matched control antibody (200 μg; BD Biosciences) or rat blocking anti–mouse LAG-3 (200 μg; C9B7W; prepared 'in house') was administered intraperitoneally every third day for 2 weeks. Anti-PD-L1 and anti-LAG-3 do not produce depletion *in vivo*[6,46]. For dual blockade of PD-L1 and LAG-3, 200 μg antibody to each was used. Control groups were left untreated or were injected with 200 μg of isotype-matched control antibody; no differences were noted between these control mice (data not shown).

**Nearest-neighbor analysis.** Nearest-neighbor analysis was done essentially as described[18] with the GeneNeighbor marker analysis algorithm to calculate the nearest neighbors to PD-1 in gene expression profiles. Gene expression profiles from the naive, effector, memory and exhausted CD8$^+$ T cells used here have been published[16].

**Statistical analyses.** *P* values were calculated by pairwise *t*-tests and analysis of variance (where more than two groups were compared). Pearson correlation coefficients were calculated with the GeneNeighbor marker analysis algorithm.

*Note: Supplementary information is available on the Nature Immunology website.*

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AUTHOR CONTRIBUTIONS

S.D.B. and E.J.W. designed the experiments; S.D.B. did the experiments with assistance from H.S., T.Z. and A.P.; S.D.B. and E.J.W. analyzed results with input from W.N.H. and M.R.B. and wrote the manuscript; and C.J.W., G.J.F. and D.A.A.V. provided crucial reagents and intellectual input.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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1. Williams, M.A. & Bevan, M.J. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* **25**, 171–192 (2007).

2. Kaech, S.M. & Wherry, E.J. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27**, 393–405 (2007).
3. Wherry, E.J. & Ahmed, R. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* **78**, 5535–5545 (2004).
4. Shin, H. & Wherry, E.J. CD8 T cell dysfunction during chronic viral infection. *Curr. Opin. Immunol.* **19**, 408–415 (2007).
5. Zajac, A.J. *et al.* Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med.* **188**, 2205–2213 (1998).
6. Barber, D.L. *et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682–687 (2006).
7. Sharpe, A.H., Wherry, E.J., Ahmed, R. & Freeman, G.J. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* **8**, 239–245 (2007).
8. Day, C.L. *et al.* PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **443**, 350–354 (2006).
9. Petrovas, C. *et al.* PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J. Exp. Med.* **203**, 2281–2292 (2006).
10. Trautmann, L. *et al.* Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat. Med.* **12**, 1198–1202 (2006).
11. Zhang, J.Y. *et al.* PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. *Blood* **109**, 4671–4678 (2007).
12. Urbani, S. *et al.* PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J. Virol.* **80**, 11398–11403 (2006).
13. Radziewicz, H. *et al.* Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J. Virol.* **81**, 2545–2553 (2006).
14. Boni, C. *et al.* Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J. Virol.* **81**, 4215–4225 (2007).
15. Boettler, T. *et al.* Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. *J. Virol.* **80**, 3532–3540 (2006).
16. Wherry, E.J. *et al.* Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* **27**, 670–684 (2007).
17. Kaufmann, D.E. *et al.* Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat. Immunol.* **8**, 1246–1254 (2007).
18. Golub, T.R. *et al.* Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286**, 531–537 (1999).
19. McNerney, M.E., Lee, K.M. & Kumar, V. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Mol. Immunol.* **42**, 489–494 (2005).
20. Cai, G. *et al.* CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat. Immunol.* **9**, 176–185 (2008).
21. Triebel, F. LAG-3: a regulator of T-cell and DC responses and its use in therapeutic vaccination. *Trends Immunol.* **24**, 619–622 (2003).
22. Ahmed, R. *et al.* Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med.* **160**, 521–540 (1984).
23. Wherry, E.J. *et al.* Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J. Virol.* **77**, 4911–4927 (2003).
24. Wherry, E.J. *et al.* Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. USA* **101**, 16004–16009 (2004).
25. Workman, C.J. & Vignali, D.A. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur. J. Immunol.* **33**, 970–979 (2003).
26. Takai, T. Paired immunoglobulin-like receptors and their MHC class I recognition. *Immunology* **115**, 433–440 (2005).
27. Rojo, S., Burshtyn, D.N., Long, E.O. & Wagtmann, N. Type I transmembrane receptor with inhibitory function in mouse mast cells and NK cells. *J. Immunol.* **158**, 9–12 (1997).
28. Lau, L.L., Jamieson, B.D., Somasundaram, T. & Ahmed, R. Cytotoxic T-cell memory without antigen. *Nature* **369**, 648–652 (1994).
29. Matloubian, M., Concepcion, R.J. & Ahmed, R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056–8063 (1994).
30. Kaech, S.M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* **111**, 837–851 (2002).
31. Wherry, E.J. *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* **4**, 225–234 (2003).
32. Badovinac, V.P., Haring, J.S. & Harty, J.T. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8+ T cell response to infection. *Immunity* **26**, 827–841 (2007).
33. Shin, H., Blackburn, S.D., Blattman, J.N. & Wherry, E.J. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J. Exp. Med.* **204**, 941–949 (2007).
34. Fuller, M.J. *et al.* Cutting edge: emergence of CD127$^{high}$ functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. *J. Immunol.* **174**, 5926–5930 (2005).
35. Betts, M.R. *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781–4789 (2006).
36. Blackburn, S.D., Shin, H., Freeman, G.J. & Wherry, E.J. Selective expansion of a subset of exhausted CD8 T cells by αPD-L1 blockade. *Proc. Natl. Acad. Sci. USA* **105**, 15016–15021 (2008).
37. Hermans, I.F. *et al.* The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *J. Immunol. Methods* **285**, 25–40 (2004).
38. Chlewicki, L.K. *et al.* Molecular basis of the dual functions of 2B4 (CD244). *J. Immunol.* **180**, 8159–8167 (2008).
39. Butte, M.J. *et al.* Programmed death-1 ligand 1 interacts specifically with the B7–1 costimulatory molecule to inhibit T cell responses. *Immunity* **27**, 111–122 (2007).
40. Kostense, S. *et al.* High viral burden in the presence of major HIV-specific CD8+ T cell expansions: evidence for impaired CTL effector function. *Eur. J. Immunol.* **31**, 677–686 (2001).
41. Wherry, E.J., Blattman, J.N. & Ahmed, R. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *J. Virol.* **79**, 8960–8968 (2005).
42. Streeck, H. *et al.* Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. *PLoS Med.* **5**, e100 (2008).
43. Freeman, G.J., Wherry, E.J., Ahmed, R. & Sharpe, A.H. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J. Exp. Med.* **203**, 2223–2227 (2006).
44. Baixeras, E. *et al.* Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J. Exp. Med.* **176**, 327–337 (1992).
45. Hannier, S., Tournier, M., Bismuth, G. & Triebel, F. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J. Immunol.* **161**, 4058–4065 (1998).
46. Huang, C.T. *et al.* Role of LAG-3 in regulatory T cells. *Immunity* **21**, 503–513 (2004).
47. Grosso, J.F. *et al.* LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J. Clin. Invest.* **117**, 3383–3392 (2007).
48. Workman, C.J. & Vignali, D.A. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J. Immunol.* **174**, 688–695 (2005).
49. Workman, C.J. *et al.* Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J. Immunol.* **172**, 5450–5455 (2004).
50. Byun, H.J. *et al.* Proliferation of activated CD1d-restricted NKT cells is down-modulated by lymphocyte activation gene-3 signaling via cell cycle arrest in S phase. *Cell Biol. Int.* **31**, 257–262 (2007).
51. Katz, H.R. *et al.* Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE. *Proc. Natl. Acad. Sci. USA* **93**, 10809–10814 (1996).
52. Laouar, A. *et al.* Cutting edge: distinct NK receptor profiles are imprinted on CD8 T cells in the mucosa and periphery during the same antigen challenge: role of tissue-specific factors. *J. Immunol.* **178**, 652–656 (2007).
53. Kumar, V. & McNerney, M.E. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nat. Rev. Immunol.* **5**, 363–374 (2005).
54. Ha, S.J. *et al.* Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J. Exp. Med.* **205**, 543–555 (2008).
55. Brooks, D.G. *et al.* IL-10 blockade facilitates DNA vaccine-induced T cell responses and enhances clearance of persistent virus infection. *J. Exp. Med.* **205**, 533–541 (2008).
56. Kotturi, M.F. *et al.* The CD8+ T-cell response to lymphocytic choriomeningitis virus involves the L antigen: uncovering new tricks for an old virus. *J. Virol.* **81**, 4928–4940 (2007).