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Premature Terminal Exhaustion of Friend Virus-Specific Effector CD8<sup>+</sup> T Cells by Rapid Induction of Multiple Inhibitory Receptors

Shiki Takamura,* Sachiyu Tsuji-Kawahara,* Hideo Yagita,† Hisaya Akiba,† Mayumi Sakamoto,* Tomomi Chikaishi,*++ Maiko Kato,*++ and Masaaki Miyazawa*+

During chronic viral infection, persistent exposure to viral Ags leads to the overexpression of multiple inhibitory cell-surface receptors that cause CD8<sup>+</sup> T cell exhaustion. The severity of exhaustion correlates directly with the level of infection and the number and intensity of inhibitory receptors expressed, and it correlates inversely with the ability to respond to the blockade of inhibitory pathways. Friend virus (FV) is a murine retrovirus complex that induces acute high-level viremia, followed by persistent infection and leukemia development, when inoculated into immunocompetent adult mice. In this article, we provide conclusive evidence that FV infection results in the generation of virus-specific effector CD8<sup>+</sup> T cells that are terminally exhausted. Acute FV-induced disease is characterized by a rapid increase in the number of virus-infected erythroblasts, leading to massive splenomegaly. Most of the expanded erythroblasts strongly express programmed death ligand-1 and MHC class I, thereby creating a highly tolerogenic environment. Consequently, FV-specific effector CD8<sup>+</sup> T cells uniformly express multiple inhibitory receptors, such as programmed cell death 1 (PD-1), T cell Ig domain and mucin domain 3 (Tim-3), lymphocyte activation gene-3, and CTLA-4, rapidly become nonresponsive to restimulation and are no longer reinvigorated by combined in vivo blockade of PD-1 and Tim-3 during the memory phase. However, combined blockade of PD-1 and Tim-3 during the priming/differentiation phase rescued FV-specific CD8<sup>+</sup> T cells from becoming terminally exhausted, resulting in improved CD8<sup>+</sup> T cell functionality and virus control. These results highlight FV’s unique ability to evade virus-specific CD8<sup>+</sup> T cell responses and the importance of an early prophylactic approach for preventing terminal exhaustion of CD8<sup>+</sup> T cells. The Journal of Immunology, 2010, 184: 000–000.

Viruses have acquired, through evolution, a multitude of strategies for evading immune surveillance and persisting in the host. A prominent example of such an evasion mechanism is the exhaustion of virus-specific CD8<sup>+</sup> T cells, which is caused by a continuous triggering of virus-specific TCRs owing to a high Ag load in persistently infected animals (1). Programmed cell death-1 (PD-1) has been identified as a major cell-surface inhibitory receptor that regulates CD8<sup>+</sup> T cell exhaustion (2). The levels of PD-1 expression on CD8<sup>+</sup> T cells critically correlate with Ag load and the severity of exhaustion (3–7). For example, in the case of hepatitis C virus infection, highly PD-1<sup>+</sup> and profoundly dysfunctional CD8<sup>+</sup> T cells were located at the sites where the relevant Ag persisted at high levels, such as the liver (8). Although blockade of PD-1/programmed death ligand (PD-L1) inter-

**Department of Immunology and †Department of Dermatology, Kinki University School of Medicine, Osaka-Sayama; ‡Department of Immunology, Juntendo University School of Medicine, Tokyo; and †Unmet Medical Needs Pharma, Yokohama, Japan

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Address correspondence and reprint requests to Prof. Masaaki Miyazawa and Dr. Shiki Takamura, Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. E-mail addresses: masaaki@med.kindai.ac.jp and stakamura@immunol.med.kindai.ac.jp

Abbreviations used in this paper: B6, C57BL/6; F-MuLV, Friend murine leukemia virus; FV, Friend virus complex; LCMV, lymphocytic choriomeningitis virus; LDV, lactate dehydrogenase-elevating virus; nTreg, naturally occurring Treg; PD-1, programmed cell death 1; PD-L1, programmed death ligand; SFFV, spleen focus-forming virus; Tim-3, T cell Ig domain and mucin domain 3; Treg, regulatory T.

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postinfection (15, 20). This rapid and vigorous expansion of erythroid progenitor cells accelerates the viral replication that leads to the high-level viremia and provides a large pool of additional target cells for repeated integration of F-MuLV and SFFV proviruses. Ultimately, FV-infected mice develop erythroleukemia through promoter insertion or the silencing of a tumor suppressor gene (21) or, if able to recover from acute FV-induced disease, develop a life-long chronic infection (22).

Although it is well known that the control of Moloney murine leukemia virus infection largely depends on CD8+ T cells (23), the actual contribution of virus-specific CD8+ T cell responses to the control of FV infection is still controversial and inconclusive. Previous studies reported that although virus-specific CD8+ T cells acquired effector functions at an early stage of infection, these cells displayed a profound functional impairment during the chronic phase (24–26). This virus-specific memory CD8+ T cell dysfunction in the chronic phase has been attributed to suppression by virus-induced regulatory T (Treg) cells, because FV infection results in more Treg cells during the chronic phase (24).

These results demonstrate that FV successfully evades host CD8+ T cells among purified cells were stained with FITC-conjugated anti–CD8+ Ab, PE-conjugated anti–CD44+ Ab, and anti-CD19+ Ab. The percentage of CD8+ T cells among stimulated splenocytes was calculated as described above.

Materials and Methods

Viruses, mice, cells, and infection

A stock of LDV-free B-tropic FV complex was kindly provided by Kim Hasenkrug, National Institutes of Health, National Institute of Allergy and Infections Diseases, Rocky Mountain Laboratories (Hamilton, MT). FV was expanded, stored, and titrated, as previously described (28). BFL3 is an F-MuLV–induced leukemia of C57BL/6 (B6) origin that expresses FV-related Ags and is known to be highly immunogenic; injection of 105 cells in syngeneic B6 mice results in rejection within 4 wk (29). C57BL/6 CD8+ T cells were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed and bred in the experimental animal facilities at Kinki University School of Medicine under specific pathogen-free conditions. Male and female (B6 S129) mice, 6 to 10 wk old, were infected i.v. with 1000 spleen focus-forming units of FV or 1000 fluorescent focus units of F-MuLV. As a control, mice were injected s.c. with 5 × 106 FBL3. All animal studies were approved by Kinki University.

Tissue harvest and flow cytometry

Mice were sacrificed at the indicated time points, and single-cell suspensions of splenocytes were obtained by straining through nylon mesh, depleted of erythrocytes by lysis with ammonium chloride, and pooled on goat anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for pentamer staining (30). Live-cell counts were determined by trypan blue exclusion. Iso- cinated cells were incubated with anti–CD8+ DCMoAb (eBioscience, San Diego, CA) to detect intracellular granzyme B (without in vitro restimulation) or Foxp3, respectively. The pentamer used was purchased from ProImmune (Oxford, U.K.). All three cytokine residues in the epitope peptide were replaced with amino- butyric acid to prevent interpeptide disulfide bonding (25). This variant peptide was shown to be recognized by CD8+ T cells specific for gag257–265 (25). Abs to the following molecules were purchased from BD Biosciences (BD3, C4D, 8T11, and 11B12); BioLegend (CD43 [1B11], CD25, PD-1, CD69, CD62L, CD122, CD127, Tim-3, LAG-3, CTLA-4, Fasl, TRAIL, PD-L1, PD-L2, CD11c, and Foxp3), or Invitrogen (Carlsbad, CA; granzyme B), purified anti-F-V MuLV gp70 mAb, clone 720 (19), was labeled with biotin by NHS-PeG2-Biotin (Thermo Scientific, Waltham, MA), as described (31). Labeled streptavidin was purchased from BioLegend. Samples were run on a FACScalibur cytometer (BD Biosciences). All data were analyzed with FlowJo software (Tree Star, Ashland, OR).

In vitro restimulation and intracellular cytokine staining

Splenocytes isolated from infected mice, as described above, were incubated in a 96-well plate at a concentration of 1 × 106 cells per well. Cells were incubated in the presence of Alexa488-conjugated anti-CD107a, monensin A (Biolegend), and either gag257–265 peptide (5 μM), FBL3 (1 × 106 cells/well), or anti-CD3 (4 μg/ml) (eBioscience, San Diego, CA) for 2 h at 37°C; brefeldin A (50 μg/ml) was added, and the incubation was continued for an additional 4 h. Surface staining for CD8 was performed as described above, and the cells were fixed and permeabilized with the Cytofix Cyperm kit (BD Biosciences). For the detection of intracellular IFN-γ and granzyme B, the cells were incubated for 30 min in the Perm/Wash buffer followed by incubation in the same buffer with anti–IFN-γ and anti-granzyme B for 30 min; the cells were then washed and analyzed as described above. To correct for background variations between experiments, we subtracted a background using medium-stimulated (uninfected) CD8+ T cells without stimulation from the percentage of IFN-γ+ or CD107a+ cells following peptide stimulation, respectively, for each individual mouse.

Cytotoxicity assay

Fourteen days postinfection, splenocytes were depleted of erythroblasts using a magnetic depletion kit (Anti-mouse Ter119 Particles-DM; BD Biosciences) and further enriched for CD8+ T cells using a negative selection kit (Mouse CD8 T Lymphocyte Enrichment Set-DM, BD Biosciences). Cells were then cocultured for 5 h with [3H]–labeled EL-4 cells that had been loaded with gag257–265 peptide. The ratio of gag257–265+ D3-specific CD8+ T cells to target cells (E:T ratio) was adjusted to 5:1. Frequencies of gag257–265+ D3-specific CD8+ T cells among purified cells were >1.5% in each mouse, and spontaneous release was <1.4% in all experiments.

In vitro suppression assay

In vitro suppression assay was performed as described in a previous report, with minor alterations (32). CD4+CD25+ cells were sorted from the splenocytes of FV-infected or FBL3-injected mice at day 14. The frequency of Foxp3+ cells among sorted cells in each group was ~65% for FV-infected mice and 95% for FBL3-injected mice. As a target, CD4+CD8+ splenocytes from naive mice were sorted and then labeled with 5 μM CFSE (Invitrogen). Cells were then combined such that the numbers of Foxp3+ and CD8+ cells were equal and stimulated with...
anti-CD3 Ab (8 μg/ml). This ratio of Treg cells to CD8+ target cells (1:1) is similar to that of total Treg cells to CD8+ T cells specific for the single dominant FV epitope (gag75–83) at day 14 in FV-infected mice (data not shown). For IFN-γ staining, brefeldin A (10 μg/ml) was added for the last 4 h of culturing. Six hours after stimulation, cells were stained for CD8, fixed, permeabilized, and stained for intracellular IFN-γ, as described above. Separate cultures of stimulated cells in the presence or absence of Treg cells were rested for 48 h to assess the CFSE dilution. Similar suppression between each group was also observed when the suppression/target ratio was adjusted based on the number of CD4+ CD25+ cells instead of FoxP3+ cells (data not shown).

In vivo Ab blockade

Anti–PD-1 (250 μg; RMP1-14) (33), anti–Tim-3 (250 μg; RMT3-23) (34), anti–IL-10 (500 μg; JESS-2AS) (35), or control rat IgG (500 μg; Sigma-Aldrich, St Louis, MO) was administered i.p. every 3 d for 2 wk. The above doses of the blocking Abs used are sufficient to inhibit the relevant receptor–ligand interaction in vivo (36–38). RMP1-14 and RMT3-23 do not deplete PD-1− or Tim-3−expressing cells in vivo, and they have no agonistic activity (33, 34). For dual blockade of PD-1 and Tim-3, 250 μg each Ab was used.

Ligand-binding assay

Single-cell suspensions of splenocytes were obtained from Ab-treated mice, as described above. Blockade of PD-L1 interaction with PD-1 on the surface of CD8+ T cells was measured by the ex vivo binding of the chimeric protein B7-1/Fc protein (R&D Systems, Minneapolis, MN). In brief, after FcR blocking, cells were incubated with the chimeric protein (1 μg/10^6 cells) for 1 h on ice and stained with anti-human IgG (Valent Pharmaceuticals, Costa Mesa, CA), anti-CD8, and anti–PD-1 (RMP1-30), which is noncompetitive with RMP1-14. Because the Tim-3 ligand, galectin-9, can bind CD44, blockade of cell-surface Tim-3 was determined by the binding of fluorescent-conjugated anti–Tim-3 mAb (RMT3-23). Tim-3 expression on RMT3-23–treated cells was detected by using a different clone of anti–Tim-3 mAb (RMT3-8), which is noncompetitive with RMT3-23.

Infectious-center assays

Spleen cell suspensions were serially diluted and plated in duplicate at concentrations between 1 × 10^6 and 1 × 10^3 cells onto monolayers of M. dunni cells. After being washed and fixed with methanol on the second day of coculturing, cells were stained with biotinylated 720 mAb, and F-MuLV–infected foci were visualized using an Elite ABC Kit (Vector Laboratories, Burlingame, CA), as described (31).

Statistical analysis

One- and two-way ANOVA with Bonferroni or Dunnet posttests, as indicated, were used to determine statistically significant differences between groups of repeated measurements. For the comparison of groups or repeated measurements with different sample numbers, the Student or Welch t test was used, depending on whether variances were regarded as equal or not, and Bonferroni correction was used to determine α values based on the number of independent hypotheses. Prism software (GraphPad, San Diego, CA) was used to calculate p values.

Results

FV infection results in the generation of excessively activated CD8+ T cells

Previous studies demonstrated that although FV infection elicits vigorous Ag-specific CD8+ T cell responses, they are insufficient to totally eradicate FV-producing cells, and the virus persists after regression of acute splenomegaly even in the resistant strains, suggesting that FV-specific CD8+ T cell responses are compromised during an early stage of infection (25). It has also become clear that FV stocks used in the previous studies were contaminated with LDV, which compromises CD8+ T cell responses (27). To thoroughly investigate the potential factors that influence the onset of virus-specific CD8+ T cell responses following FV infection in the absence of LDV, we compared the Ag-specific CD8+ T cell responses elicited by FV infection or injection of FBL3 tumor cells, a highly immunogenic murine leukemia cell line induced in a B6 mouse by F-MuLV (39). As shown in Fig. 1A, FV infection elicited a robust virus-specific CD8+ T cell response in the spleen at 14 d postinfection, with >5% of the CD8+ T cell population stained brightly with the gag75–83/D3− pentamer. As a control, an s.c. injection of FBL3 elicited comparable numbers of gag75–83/D3− specific CD8+ T cells at day 14 (Fig. 1A). However, despite the elicitation of comparable levels of acute CD8+ T cell responses, there was a notable difference in the effector phenotypes between Ag-specific CD8+ T cells elicited by FV infection and those elicited by FBL3 tumor injection. Although only less than one quarter of Ag-specific CD8+ T cells generated by FBL3 injection displayed a highly activated effector phenotype, as determined by CD43 expression and intracellularly stored granzyme B, Ag-specific CD8+ T cells generated by FV infection were markedly skewed toward the CD43+/granzyme B+ population; detection of this without in vitro restimulation (Fig. 1B) indicates that this was excessive. Substantial accumulation of granzyme B was also observed in the CD43+ population of gag75–83/D3− negative CD8+ T cells in FV-infected mice, indicating that excessive activation is not a unique feature of gag75–83/D3−specific CD8+ T cells. The frequency of CD8+ T cells that contained granzyme B reached up to 48% of the total CD8+ T cell population at 2 wk postinfection, and it declined after 3 wk postinfection (Fig. 1C). Importantly, this decrease in granzyme B expression was apparently associated with the course of virus elimination from the spleen (Fig. 1D). Because the expression of cytolytic molecules detected intracellularly without in vitro restimulation depends mainly on the recent Ag exposure in vivo (40), the reduction in granzyme B+ cells through the late stage of infection is likely due to the
combined consequences of the contraction of Ag-experienced effector CD8+ T cells, the transition of CD8+ T cells from effector to memory stages, and the resolution of FV infection in vivo. A relatively low number of CD8+ T cells was positive for the virally encoded envelope protein (gp70), even at the peak of FV infection (Fig. 1E), indicating that direct infection of CD8+ T cells with FV does not have much impact on the generation of excessively activated effector CD8+ T cells.

**FV-specific CD8+ T cells are not responsive to restimulation**

Because there is considerable evidence showing that gag75–83/Db-specific CD8+ T cells generated by FBL3 injection are fully functional (39), it is of interest to compare the actual functionalities of gag75–83/Db-specific CD8+ T cells generated by FV infection with those generated by FBL3 injection, especially during the early stages postinfection/infection. To do so, splenocytes from mice infected with FV or injected with FBL3 were cultured in vitro with gag75–83 peptide for 6 h and evaluated for intracellular expression of IFN-γ and cell-surface expression of CD107a in conjunction with granzyme B expression. Upon peptide restimulation, CD8+ T cells from FBL3-injected mice showed a significant increase in the intracellular expression of IFN-γ and the surface expression of CD107a at 2 and 3 wk after inoculation compared with those prior to the tumor injection (Fig. 2A, 2B). In contrast, CD8+ T cells from FV-infected mice were nonresponsive to the restimulation; neither upregulation of cell-surface CD107a nor an increase in intracellular IFN-γ was observed (Fig. 2A). Surprisingly, we could not find any responses to the peptide restimulation during the entire course of FV infection (Fig. 2B), despite the presence of significant numbers of epitope-specific CD8+ T cells (Fig. 1A). This contrasts remarkably with other models of chronic infection, such as infection with LCMV clone 13, in which virus-specific CD8+ T cells still exhibit an ability to produce IFN-γ upon restimulation during the early stage of infection (10, 41). Furthermore, the excessively activated (granzyme B+; CD8+ T cell populations in FV-infected mice displayed severely impaired IFN-γ production even following anti-CD3 stimulation (Fig. 2A), suggesting that a vast majority of Ag-experienced CD8+ T cells in FV-infected mice have a possible defect in TCR signaling. A strong staining for CD107a was observed intracellularly, supporting the notion that there was a defect in CD107a relocalization from intracellular lytic granules to the plasma membrane in response to TCR signaling in FV-infected mice (data not shown). The impaired responsiveness was not due to a dysfunction of APCs in the spleens of FV-infected mice, because similar results were observed even when restimulated with FBL3 (Fig. 2A). Thus, we hypothesized that although Ag-specific CD8+ T cells from FV-infected mice expressed significant amounts of effector molecules, they were incapable of responding to the target, thereby failing to exert cytotoxicity. To directly test this, we performed ex vivo [51Cr]-release assays. CD8+-enriched splenocytes from FV-infected or FBL3-injected mice at day 14 were cocultured with gag75–83 peptide-loaded EL-4 cells at an equivalent E:T ratio, based on the number of gag75–83/Db pentamer+ cells. As shown in Fig. 2C, virus-specific CD8+ T cells from FV-infected mice exerted only marginal levels of cytotoxicity, whereas an equivalent number of virus-specific CD8+ T cells from FBL3-injected mice exhibited significantly greater cytolytic activities. Collectively, these data provide convincing evidence that FV infection results in the generation of virus-specific effector CD8+ T cells that are functionally impaired.

**Minor contribution of Treg cells to the early loss of FV-specific CD8+ T cell function**

Given that infection of mice with FV plus LDV induced CD4+ Treg cells that suppressed virus-specific CD8+ T cells during the chronic phase of infection (24), we first considered the possibility that FV-induced Treg cells might contribute to the dysfunction of Ag-specific effector CD8+ T cells. Because previous studies evaluated the presence of virus-induced CD4+ Treg cells by the expression of CD25, we first compared the frequencies and the actual numbers of CD4+CD25+ T cells in FV-infected and FBL3-injected mice at day 14 and stimulated with gag75–83 peptide-loaded EL-4 cells at an equivalent E:T ratio, based on the number of gag75–83/Db pentamer+ cells. As shown in Fig. 2C, virus-specific CD8+ T cells from FV-infected mice exerted only marginal levels of cytotoxicity, whereas an equivalent number of virus-specific CD8+ T cells from FBL3-injected mice exhibited significantly greater cytolytic activities. Collectively, these data provide convincing evidence that FV infection results in the generation of virus-specific effector CD8+ T cells that are functionally impaired.

**FIGURE 2.** FV-specific CD8+ T cells are not responsive to restimulation. Mice were infected with FV or injected with FBL3, as described for Fig. 1. A, Splenocytes were isolated at day 14 and stimulated with gag75–83 peptide, FBL3, or anti-CD3 mAb for 6 h. The intracellular expression of IFN-γ and granzyme B and the surface expression of CD107a were measured by flow cytometry. Shown are representative staining patterns for granzyme B, IFN-γ, and CD107a of CD8+ T cells. The numbers indicate the percentage of cells gated in each quadrant. As CD8+ T cells were stimulated for 6 h in the presence of anti-CD107a Ab, granzyme B+ and CD107a+ cells among CD8+ T cells calculated by (the percentage of gag75–83/Db pentamer+ cells. As shown in Fig. 2C, virus-specific CD8+ T cells from FV-infected mice exerted only marginal levels of cytotoxicity, whereas an equivalent number of virus-specific CD8+ T cells from FBL3-injected mice exhibited significantly greater cytolytic activities. Collectively, these data provide convincing evidence that FV infection results in the generation of virus-specific effector CD8+ T cells that are functionally impaired.
T cells in the spleen between FV-infected and FBL3-injected mice. No significant groupwise differences were observed in the frequency or actual numbers of CD4+CD25+ cells between FV-infected and FBL3-injected mice, and the frequency and absolute numbers of CD4+CD25+ cells increased significantly from those before infection only at days 14 and 7 after FV infection, respectively (Fig. 3A, 3B). An additional phenotypic analysis showed that ~30% of the CD4+CD25+ cells in FV-infected mice were negative for FoxP3, whereas most of the CD4+CD25+ cells in FBL3-injected mice were FoxP3+ (Fig. 3C, 3D). Moreover, a large majority of the FoxP3+ CD4+CD25+ cells in FV-infected mice displayed highly activated phenotypes, as determined by the expression of PD-1 and CD69 (Fig. 3C, 3D), suggesting that the increased numbers of activated cells, but not an increase in Treg cells, were responsible for the slightly elevated levels of CD4+CD25+ T cells after FV infection. In fact, the actual numbers of FoxP3+ Treg cells in FV-infected mice at day 14 were not different from those in FBL3-injected mice (Fig. 3E). To exclude the possibility that Treg cells from FV-infected mice possess higher suppressive ability than those from FBL3-injected mice, we performed in vitro suppression assays. Spleenocytes from FV-infected or FBL3-injected mice were flow cytometrically sorted on the basis of CD4 and CD25 expression, and CD8+ T cells were stimulated in the presence or absence of Treg cells, such that the ratio of CD8+ cells to FoxP3+ Treg cells was 1:1, >5-fold higher Treg cell density compared with the physiological condition. Treg cells from FV-infected mice showed a significant suppression of CD8+ T cell functions but no increase in their suppressive ability compared with those from FBL3-injected mice (Fig. 3F). Taken together, we conclude that the observed early dysfunction of effector CD8+ T cells in FV-infected mice is not explained by the Treg cell-mediated suppression.

**FV-specific CD8+ T cells show highly exhausted phenotypes.** It is well established that virus-specific CD8+ T cells eventually become dysfunctional during chronic diseases as the result of increased expression of inhibitory receptors, including PD-1. To test whether this could be the case for the observed dysfunction of FV-specific effector CD8+ T cells in FV-infected mice, we analyzed the surface expression of PD-1 on the gag75–83/Db-specific CD8+ T cells. Strikingly, as early as 14 d after FV infection, nearly all gag75–83/Db-specific CD8+ T cells expressed high levels of PD-1 (Fig. 4A). Such a high expression of PD-1 on the gag75–83/Db-specific CD8+ T cells was maintained until ≥10 wk postinfection (Fig. 4B). In contrast to other models of chronic

**Figure 3.** Minor contribution of Treg cells to the early loss of FV-specific CD8+ T cell function. A. Representative staining patterns of CD4 and CD25 in FV-infected or FBL3-injected mice at day 14. B. Frequencies of CD25+ cells among CD4+ T cells and the actual numbers of CD4+CD25+ cells in the spleen at the indicated time points after FV infection or FBL3 injection. Bars indicate mean ± SEM. Because the number of samples at each time point differs, t tests with Bonferroni correction for the number of hypotheses were performed.

**Figure 4.** FV-specific CD8+ T cells show highly exhausted phenotypes. A. Relative levels of PD-1 expression and the pentamer binding on CD8+ T cells and their granzyme B contents in the spleens of FV-infected or FBL3-injected mice at day 14. Profiles shown are of gated CD4+CD25+ cells and their granzyme B contents in the spleens of FV-infected or FBL3-injected mice at day 14 and evaluated for intracellular IFN-γ expression (6 h) and proliferation (48 h). Data are representative of two independent experiments with essentially the same results. **B.** Representative FACS plots showing the coexpression of inhibitory molecules with PD-1 on gag75–83/Db+ CD8+ T cells in FV-infected and FBL3-injected mice at day 14. Profiles shown are of gated gag75–83/Db+ CD8+ T cells. All data are representative of at least three independent experiments with essentially the same results.
infection in which the expression levels of PD-1 on exhausted CD8\(^+\) T cells were heterogeneous, Ag-specific CD8\(^+\) T cells in FV-infected mice were predominantly PD-1\(^{hi}\), regardless of the stage of infection (Fig. 4B), revealing a unique severity of exhaustion caused by FV infection. Highly elevated expressions of PD-1 were also observed in the entire population of CD8\(^+\) T cells that contained granzyme B in FV-infected mice (Fig. 4A), suggesting that a vast majority of Ag-experienced CD8\(^+\) T cells, not just those specific for the gag\(_{75–83}\) epitope, shared a propensity for severe exhaustion. Additional phenotypic profiles of Ag-specific CD8\(^+\) T cells in FV-infected mice included higher expression levels of CD69 and lower expression of all three of the other markers (CD62L, CD122, and CD127) in comparison with those in FBL3-injected mice, revealing typical characteristics of exhaustion (Fig. 4C) (41). To more thoroughly pursue the severity of exhaustion, Ag-specific CD8\(^+\) T cells were examined for the expression of additional inhibitory receptors. Concomitant with the elevated expression of PD-1, gag\(_{75–83}/D^{β}\)-specific effector CD8\(^+\) T cells from FV-infected mice expressed three other cell-surface inhibitory receptors at high levels: Tim-3, LAG-3, and CTLA-4; this expression was obviously distinct from CD8\(^+\) T cells with the same epitope specificity in FBL3-injected mice (Fig. 4D). A lack of the elevated expression of FasL and TRAIL, suggests that these cells are not yet programmed to undergo activation-induced cell death (Fig. 4D). Taken together, these data suggest that the severe exhaustion of Ag-specific CD8\(^+\) T cells occurs even in the early stage of FV infection.

**Elevated expression of PD-L1 and MHC class I on FV-infected erythroblasts**

The pronounced expression of PD-1 on FV-specific effector CD8\(^+\) T cells prompted us to investigate the impact of FV infection on the expression of PD-1 ligands: PD-L1 and PD-L2. In contrast to a modest influence of FV infection on PD-L2 expression, significant upregulation of PD-L1 was observed in subpopulations of CD11b\(^+\) dendritic cells and erythroblasts at 14 d after FV infection (Fig. 5A). The elevated expression of PD-L1 on erythroblasts was of particular interest because of the rapid expansion of these cells in the early phase of FV infection. In fact, the percentage of Ter119\(^+\) erythroblasts among nucleated spleocytes increased to 75%, with a >45-fold increase in their number at its peak, which was accompanied by the expansive increase in FV-infected cells (Fig. 5B). Because an engagement of PD-1 and concomitant TCR stimulation, but not the engagement of PD-1 alone, is necessary for providing the negative signal to T cells (42), we further examined the possible expression of MHC class I molecules on the erythroblasts. In agreement with the previous findings that inflammatory stimuli activate hematopoiesis, resulting in increased numbers of nucleated erythroblasts that express MHC class I molecules (43), elevated expression of H-2D\(^{β}\) was even observed on the surfaces of erythroblasts from FBL3-injected mice (Fig. 5C). However, in the case of FV infection, nearly all expanded erythroblasts were positive for H-2D\(^{β}\) (Fig. 5C). Visualization of the erythrocyte cell expression of PD-L1 and viral protein revealed that ∼50% of virus-infected cells were PD-L1\(^{hi}\) (Fig. 5D). Together, these results suggest that the FV-infected erythroblasts can be a potent provider of PD-1–mediated negative signals to CD8\(^+\) T cells.

**Exhaustion of virus-specific CD8\(^+\) T cells is irreversible in FV-infected mice**

Thus far, the data have demonstrated that virus-specific CD8\(^+\) T cells are exposed to abundant cell-associated viral Ags and immunosuppressive signals; therefore, they are highly exhausted at the early stage of FV infection. To determine whether the functionally exhausted CD8\(^+\) T cells could be revived by a blockade of immunosuppressive signals, we attempted to interrupt two pathways by administering blocking Abs to PD-1 and Tim-3, alone or in combination. In the initial experiments, in vivo blockade was started at day 14 (Fig. 6A), when most Ag-specific CD8\(^+\) T cells had already expressed these inhibitory receptors at high levels (Fig. 4). Despite the effective blockade of these inhibitory receptors on cell surfaces (Fig. 6B), no reversal of exhaustion was observed, even when in vivo blockade was performed by a combination of anti-PD-1 and anti–Tim-3 (Fig. 6C, 6D). Furthermore, the combined blockade of PD-1 and Tim-3 had no impact on the pathogen control, because the numbers of virus-producing spleen cells detected in the Ab-treated mice were similar to those in the control mice (data not shown). The failure of reinvigoration was probably not caused by the presence of abundant Ags during the blockade, because FV-specific memory CD8\(^+\) T cells were still in the non-responsive state, even when the blockade was performed between days 28–42 postinfection, when initial splenomegaly had regressed (data not shown). Given that CD8\(^+\) T cell production of IFN-γ is the last function to be ablated in the stepwise hierarchy of T cell exhaustion (1), it is tempting to hypothesize that high-level viral replication in FV-infected mice results in the generation of terminally exhausted FV-specific effector CD8\(^+\) T cells at a very early stage.

**Acute SFFV-induced pathogenesis is responsible for the terminal exhaustion of effector CD8\(^+\) T cells in FV-infected mice**

We next asked whether SFFV-induced acute pathogenesis causes the terminal exhaustion of effector CD8\(^+\) T cells in FV-infected animals. To test this, we examined the severity of CD8\(^+\) T cell exhaustion in....
mice infected with F-MuLV alone. At the peak of infection, FV infection induced severe splenomegaly due to the massive expansion of virus-infected erythroid progenitor cells, whereas such pathologies were not observed in mice infected with F-MuLV (Fig. 7A). Although ~25% of erythroid cells were infected with F-MuLV, these cells did not show any increase in the expression of PD-L1 (Fig. 7B), indicating that infection with F-MuLV alone results in a less immunosuppressive environment compared with that created by infection with FV complex. In the absence of the SFFV-induced acute pathogenesis, dual blockade of PD-1 and Tim-3 resulted in a significantly higher percentage of IFN-γ-producing CD8+ T cells in response to gag75–83 peptide stimulation, which is comparable to the responses of CD8+ T cells from FBL3-injected mice (Fig. 8B, 8C). These Ag-specific effector CD8+ T cell populations that escaped terminal exhaustion were now able to establish memory CD8+ T cell pools capable of producing IFN-γ upon restimulation in the later stage (Fig. 8D). Despite the effectiveness of dual blockade, nearly all gag75–83/D3-specific effector CD8+ T cells still remained positive for PD-1 (Fig. 8E); however, the intensity of PD-1 on effector CD8+ T cells was significantly decreased in mice simultaneously treated with PD-1 and Tim-3 (Fig. 8E), supporting the previous finding that the expression levels of PD-1 dictate the functional ability of CD8+ T cells (9). In agreement with the enhanced functionality of Ag-specific effector CD8+ T cells, we observed significantly improved viral control and reduced splenomegaly in mice simultaneously treated with PD-1 and Tim-3 (Fig. 8F). The analysis of total gag75–83/D3-specific CD8+ T cell numbers revealed that, despite the quantitatively identical CD8+ T cell responses during the early stage of infection (data not shown), dual blockade of PD-1 and Tim-3 resulted in the maintenance of substantially higher numbers of memory CD8+ T cells at 10 wk postinfection (Fig. 8G), which is likely due to a significantly improved survival ability of Ag-experienced CD8+ T cells, as indicated by the significantly reduced expression of...
Annexin V on their surfaces at day 14 (Fig. 8H). Finally, in contrast to the model of LCMV clone 13 infection (46–48), blockade of IL-10 during the early stage of infection had no impact on the responsiveness of FV-specific effector CD8+ T cells or the viral clearance, indicating that FV-specific effector CD8+ T cell exhaustion occurred in an IL-10–independent fashion (Fig. 8B–F). Overall, these data demonstrate that, in contrast to F-MuLV infection, infection with FV complex induces a massive expansion of virus-infected PD-L1Hi and MHC class I+ erythroblasts during the priming/differentiation phase, and virus-specific effector CD8+ T cells rapidly succumb to terminal exhaustion through accelerated high-level expression of multiple inhibitory receptors.

Discussion

It is well established that chronic Ag exposure leads to the stepwise functional exhaustion of CD8+ T cells (1, 41). The cells lose the ability to produce IL-2, exert cytolyis, and proliferate in early stages of exhaustion, followed by the loss of TNF-α production. IFN-γ production is eventually compromised in the final stage of exhaustion. Although this hierarchical loss of effector functions takes place during the initial phase of chronic infection and causes the failure in viral clearance, terminal exhaustion of Ag-specific CD8+ T cells is observed exclusively in the late stages of infection (41). In this study, we have revealed an exception to the above stepwise exhaustion: following FV infection, gag75–83/Db-specific effector CD8+ T cells rapidly lose their ability to produce IFN-γ, a function most resistant to being lost, even during the early stage of infection. CD8+ T cells specific for an immunodominant epitope are generally more prone to functional exhaustion than are those specific for other less-immunogenic epitopes, resulting in the inverted immunodominance (1). However, FV-driven terminal exhaustion of virus-specific effector CD8+ T cells may occur at the population level, because the failure to produce IFN-γ upon restimulation was observed in the entire population of Ag-experienced CD8+ T cells in FV-infected mice, not just in the cells specific for the...
immunodominant gag epitope. This global nonresponsiveness of Ag-specific CD8+ T cells is consistent with the previous finding that FV-infected mice were incapable of rejecting tumor cells that express FV-related epitopes, although these mice still have an ability to reject other tumor cells that express FV-unrelated epitopes (49).

The most notable feature of these dysfunctional effector CD8+ T cells is the excessive expression of multiple inhibitory receptors that can negatively regulate the CD8+ T cell function, such as PD-1, Tim-3, LAG-3, and CTLA4. PD-1 has been identified as a major cell-surface inhibitory receptor capable of regulating CD8+ T cell exhaustion in mice, as well as in humans and nonhuman primates (50). Tim-3 and CTLA-4 were recently found to be overexpressed on HIV- and hepatitis C virus-specific CD8+ and CD4+ T cells and to act to suppress effector functions of activated T cells (11–13, 51). Upregulation of LAG-3 was also shown to correlate with the impaired effector functions and exhaustion of CD8+ T cells (10, 52, 53). In addition to these inhibitory receptors, it was demonstrated that a variety of other inhibitory receptors were expressed on exhausted CD8+ T cells in chronic virus infections, and the increased levels of expression were critically associated with the severity of infection and the lower functionality of CD8+ T cells (10). Although the precise inhibitory mechanisms of each individual pathway are unclear, some inhibitory receptors, such as Tim-3, LAG-3, and CTLA4, are known to negatively regulate CD8+ T cell function cooperatively with PD-1, because combined blockade of these inhibitory receptors, along with the PD-1/PD-L1 pathway, synergistically improved CD8+ T cell responses (10–13). Based on the present observations, Ag-specific effector CD8+ T cells in FV-infected mice seemed to be undergoing unusually rapid and extremely severe exhaustion. This was further confirmed by the current observation that combined blockade of PD-1 and Tim-3 during the memory phase had no effect in reinvigorating already exhausted CD8+ T cells. The data obtained by using the model of nonpathogenic F-MuLV infection clearly demonstrated that acute SFFV-mediated pathogenesis was responsible for the rapid expression of multiple inhibitory receptors that cause unexpectedly rapid terminal exhaustion of effector CD8+ T cells in FV-infected animals.

There has been considerable evidence that the initial viral load inversely correlates with the functionality of Ag-specific CD8+ T cells (10, 54, 55). Recently, a combined in situ tetramer staining and in situ hybridization approach showed that relative rates of increase in Ag-specific effector CD8+ T cells and virally infected targets during the early stage predict the outcome of viral infection (56). For example, LCMV clone 13 preferentially infects fibroblastic reticular cells,
Rapid Exhaustion of FV-Specific Effector CD8+ T Cells

One of the key findings from the current study is that severe FV-specific effector CD8+ T cell dysfunction seemingly occurred, irrespectively of increased Treg cell numbers or functions. In fact, the suppressive ability of Treg cells did not differ between FV infection and FBL3 injection, despite the fact that the latter did not induce CD8+ T cell exhaustion. Several studies have shown that FV infection results in a slight increase in the number and suppressive ability of Treg cells during the chronic phase (24, 25, 59); however, the precise role of FV-induced Treg cells in inducing the dysfunction of FV-specific CD8+ T cells in vivo has not been directly elucidated. In fact, previous evidence for the involvement of Treg cells in the CD8+ T cell dysfunction in vivo is based entirely on the adoptive transfer approach, in which the abilities to secrete effector cytokines were determined by using TCR-transgenic CD8+ T cells that had been transferred into virus-infected animals at different stages [e.g., acute phase versus memory phase (24)]. However, these studies did not take into consideration the possible impact of different Ag/inflammation levels at the time of transfer on the priming of TCR transgenic CD8+ T cells and the subsequent acquisition of effector function. The contribution of Treg cell-mediated suppression to the dysfunction of FV-specific CD8+ T cells in vivo was also demonstrated by depletion of total Treg cells (24, 60). However, all animals have naturally occurring Treg (nTreg) cells that confer basal suppression of effector cell functions; the depletion of nTreg cells results in the induction of autoimmune and/or inflammatory diseases, which are associated with enhanced autoreactive cellular immune responses (61). Therefore, it is common that total Treg cell depletion in mice results in stronger cellular immune responses than do those in Treg cell-deficient mice, and this could be observed with any kind of infection (62, 63). A strategy to deplete virus-induced Treg cells alone, without affecting the numbers and functions of nTreg cells, would be necessary to precisely determine the role of induced Treg cells in vivo, which has never been demonstrated. Thus, it is unknown whether FV-induced Treg cells have any roles in inducing FV-specific CD8+ T cell dysfunction. In addition, because FV-infected animals are capable of mounting a CD8+ T cell response against FV-unrelated tumor cells (49), the severe CD8+ T cell dysfunction seems to occur in an Ag-independent manner. Because Treg cells seem to suppress a broad spectrum of effector cells, it is unlikely that virus-induced Treg cells provide a selective contribution to the FV-specific CD8+ T cell dysfunction. Moreover, direct cell-to-cell interaction is required for Treg cells from FV-infected mice to exert suppressive activity on CD8+ T cells (59), and Treg cell-mediated suppression of CD8+ T cell function in vivo is known to be reversible (64). These facts strongly support our conclusions on the rather minor role of Treg cells in inducing FV-specific CD8+ T cell dysfunction, because the opportunity for Treg cells to interact with virus-specific CD8+ T cells in FV-infected mice must be lower than that in FBL3-injected mice because of the presence of expanded erythroblasts as potential interceptors. Considering the current observations that Ag-specific CD8+ T cells rapidly succumbed to terminal exhaustion in the early phase of FV infection and could not be reinvigorated, memory CD8+ T cell dysfunction that has been observed in FV-infected animals may be better explained as a consequence of the currently observed premature terminal exhaustion of effector CD8+ T cells rather than by Treg cell-mediated suppression.

Another issue to be considered is the possible requirement of virus-specific effector CD8+ T cells for the control of FV infection. It has been believed that FV-specific CD8+ T cells initially acquire effector functions during the early phase of infection, based mainly on the observation of their significant expression of granzyme B (25). However, our results draw attention to the importance of determining the responsiveness to restimulation as an indicator of CD8+ T cell functionality, which is severely compromised in FV-infected mice. Another piece of evidence for the functionality of FV-specific effector CD8+ T cells also relies on the approach of using supraphysiological numbers of TCR-transgenic CD8+ T cells specific for the gag epitope (24). However, we clarified the nonresponsiveness of effector CD8+ T cells by focusing on the endogenous FV-specific CD8+ T cells induced in FV-infected mice. Because the optimal induction of functionally competent FV-specific CD8+ T cell responses by early blockade of inhibitory receptors significantly improved the virus control and led to diminished FV-induced splenomegaly, it is reasonable to conclude that FV infection results in the generation of virus-specific effector CD8+ T cells that are functionally impaired. One question is whether virus-specific CD8+ T cells contribute to the early control of FV in vivo. Although previous studies demonstrated that the depletion of CD8+ T cells significantly accelerated the acute splenomegaly (65–67), the FV stock used in these studies had been contaminated with LDV, a potent suppressor of CD8+ T cell responses (27). Using the LDV-free FV stock, we confirmed an exacerbated acute splenomegaly in CD8+ T cell-depleted animals upon FV infection (S. Takamura, S. Tsuji-Kawahara, and M. Miyazawa, unpublished observations), which seems to conflict with our current observations regarding the early loss of FV-specific CD8+ T cell function. One potential explanation is that a short lag exists between the functional maturation and the subsequent exhaustion of CD8+ T cells in the early stage of FV infection, during which FV-infected cells are at least partially eliminated by effector CD8+ T cells. Alternatively, because weak, but spontaneous, degranulation was observed on granzyme B+CD8+ T cells in FV-infected, but not FBL3-injected, mice, the expanded FV-infected erythroblasts that dominated in the spleen can be eliminated in an Ag-independent manner. We are currently investigating these possibilities.

Overall, the data presented in this article indicate that FV, but not F-MuLV, silences antiviral CD8+ T cell responses during the early phase of infection by inducing the rapid and high expression of multiple inhibitory receptors, leading to irreversible terminal exhaustion. These findings have to be considered a newly recognized strategy for FV to escape from acute CD8+ T cell responses. Finally, manipulation of CD8+ T cell exhaustion by early blockade of multiple inhibitory receptors would be necessary to control the pathogens, such as HIV, that exhibit widespread terminal exhaustion of CD8+ T cells.

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Disclosures
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