PD-1 Upregulated on Regulatory T Cells during Chronic Virus Infection Enhances the Suppression of CD8+ T Cell Immune Response via the Interaction with PD-L1 Expressed on CD8+ T Cells

Hyo Jin Park, Joon Seok Park, Yun Hee Jeong, Jimin Son, Young Ho Ban, Byoung-Hee Lee, Lieping Chen, Jun Chang, Doo Hyun Chung, Inhak Choi and Sang-Jun Ha

*J Immunol* 2015; 194:5801-5811; Prepublished online 1 May 2015;
doi: 10.4049/jimmunol.1401936
http://www.jimmunol.org/content/194/12/5801

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/05/01/jimmunol.1401936.DCSupplemental

**References**
This article cites 58 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/194/12/5801.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

**Errata**
An erratum has been published regarding this article. Please see next page or:
/content/195/12/5841.full.pdf
PD-1 Upregulated on Regulatory T Cells during Chronic Virus Infection Enhances the Suppression of CD8+ T Cell Immune Response via the Interaction with PD-L1 Expressed on CD8+ T Cells

Hyo Jin Park,*1 Joon Seok Park,*1,2 Yun Hee Jeong,* Jimin Son,* Young Ho Ban,* Byoung-Hee Lee,† Lieping Chen,‡ Jun Chang,§ Doo Hyun Chung,¶ Inhak Choi,** and Sang-Jun Ha*†

Regulatory T (Treg) cells act as terminators of T cell immunity during acute phase of viral infection; however, their role and suppressive mechanism in chronic viral infection are not completely understood. In this study, we compared the phenotype and function of Treg cells during acute or chronic infection with lymphocytic choriomeningitis virus. Chronic infection, unlike acute infection, led to a large expansion of Treg cells and their upregulation of programmed death-1 (PD-1). Treg cells from chronically infected mice (chronic Treg cells) displayed greater suppressive capacity for inhibiting both CD8+ and CD4+ T cell proliferation and subsequent cytokine production than those from naive or acutely infected mice. A contact between Treg and CD8+ T cells was necessary for the potent suppression of CD8+ T cell immune response. More importantly, the suppression required cell-specific expression and interaction of PD-1 on chronic Treg cells and PD-L1 on CD8+ T cells. Our study defines PD-1 upregulated on Treg cells and its interaction with PD-L1 ligand on effector T cells as one cause for the potent T cell suppression and proposes the role of PD-1 on Treg cells, in addition to that on exhausted T cells, during chronic viral infection. The Journal of Immunology, 2015, 194: S801–S811.

Interactions between hosts and chronic pathogens led to the evolution of several immune suppressive strategies to ensure reduced immunopathology against the host and extended pathogen survival; such strategies disable the host’s ability to effectively mediate pathogen clearance (1–3). T cell exhaustion is considered the primary reason for failed immunological control of chronic pathogens, but other suppression mechanisms might exist, because the rejuvenation of exhausted T cells alone is not sufficient to terminate pathogen persistence (1–3). A number of reports have suggested that regulatory T (Treg) cells play a significant role in weakening T cell response against a variety of chronic pathogens, such as viral (4), helminthic (5, 6), and bacterial infections (7–9). Moreover, Treg cells suppress antiviral T cell response against chronic viruses, as previously reviewed (10). It was recently reported that depletion of Treg cells in the mice chronically infected with lymphocytic meningitis virus (LCMV) resulted in a significant expansion of functional LCMV-specific CD8+ T cells (11), demonstrating the strong suppressive activity of Treg cells during chronic virus infection. However, the molecular mechanisms of Treg cell–mediated immune suppression during chronic pathogen infection are poorly understood.

In addition to CD25 and Foxp3, Treg cells express various surface molecules, such as CTLA-4, lymphocyte activation gene-3 (LAG-3), and programmed death-1 (PD-1), on the cell surface or in the intracellular region (2, 12, 13). These molecules expressed by Treg cells might have profound effects on the suppressive function of Treg cells (14–18). The roles of the molecules expressed by Treg cells, such as CTLA-4, LAG-3, and PD-1, are highly upregulated on exhausted T cells, and they mediate the inhibition of pathogen-specific T cell function (2, 3). Among the various inhibitory receptors, PD-1 is a hallmark of exhausted T cells in chronic pathogen infection (2, 3, 14, 19). Interaction between PD-1 and its ligand, PD-ligand 1 (PD-L1) (B7-H1), inhibits TCR signaling, causing the deterioration of T cell immune response against chronic viruses. Treg cells also upregulate PD-1 expression on their surfaces during chronic viral infection (20). PD-1:PD-L1 interactions between hosts and chronic pathogens led to the evolution of several immune suppressive strategies to ensure reduced immunopathology against the host and extended...
interaction has a critical role in promoting the conversion of conventional T cells into Treg cells and maintaining them (21–23). However, whether PD-1 expression by Treg cells during chronic infection is associated with the suppression of pathogen-specific T cell immune response remains unknown (24–26).

To demonstrate whether and how PD-1 contributes to the suppression of T cell response during chronic viral infection, we investigated the function of PD-1 expressed on Treg cells using a mouse model infected with LCMV clone 13 (CL13), a well-characterized chronic viral infection model. We observed that the number of Treg cells increased during chronic LCMV infection and demonstrated that Treg cells isolated from chronically infected mice exhibited greater suppressive activity than Treg cells from uninfected or acutely infected mice. This suppressive activity was mediated by direct contact between Treg cells and CD8+ T cells. We also proved that high expression of PD-1 on T reg cells during chronic LCMV infection was essential for suppressing CD8+ T cell response by PD-1 interaction with PD-L1 on CD8+ T cells. Our findings implicate Treg cell expression of PD-1 as a therapeutic target for chronic viral infections, such as hepatitis B virus, hepatitis C virus (HCV), and HIV.

Materials and Methods

Ethics statement

All animal experiments were carried out in accordance with the Korean Food and Drug Administration guidelines. The protocol was approved by the International Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center at Yonsei University (Permit 2013-0115).

Mice and infections

Five- to six-week-old female C57BL/6 mice and B6.129S7 Rag1−/− mice were purchased from Charles River Laboratories and The Jackson Laboratory. Dr. Rafi Ahmed (Emory University School of Medicine), Dr. Doo Hyun Chung, and Dr. Inhak Choi provided the P14 Thy1.1+ PD-1−/− mice, and PD-L1−/− mice, respectively. PD-1−/− and PD-L1−/− mice were originally generated by Dr. Tasuku Honjo (Kyoto University) and Dr. Lieping Chen (28), respectively. All mice were maintained in a specific pathogen-free facility of the Yonsei Laboratory Animal Research Center of Yonsei University. For viral infections, mice received 2×105 PFU LCMV Arm and 2×105 PFU LCMV CL13 by i.p. and i.v. injection, respectively. LCMV was separated by centrifugation and quantified for cytokine production using the Mouse IFN-γ Platinum ELISA (eBioscience) and BD Cytometric Bead Array Mouse Th1/Th2/Th17 CBA Kit (BD Biosciences).

Cell isolation

Lymphocytes were isolated from spleens, as previously described (14). The lungs were perfused with ice-cold PBS before removal for lymphocyte isolation. For the CFSE or CellTrace Violet dilution assay, CD8+ T cells were isolated from naive mice using a CD8+ T Cell Isolation Kit (Milenyi Biotec), and CD4+CD25− T cells or CD4+CD25+ Treg cells were isolated from naive, LCMV Arm-infected, or LCMV CL13-infected mice using a CD4+CD25+ Regulatory T Cell Isolation Kit (Milenyi Biotec).

Gene expression profiling

Total RNA was extracted, using the RNeasyPlus Micro Kit (Qiagen), from the isolated Treg cells. RNA was hybridized with a Mouse Genome 430 2.0 array (Affymetrix). Gene expression was subsequently profiled using microarray analysis tools. The microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63876) under accession number GSE63876.

In vitro suppression assay

Purified CD8+ or CD4+CD25+ T cells were labeled with 5 μM CFSE or CellTrace Violet. T cells (105 [96-well flat or U-bottom plate] or 104 [24-well plate]), labeled with CD8+ or CD4+CD25−, were stimulated with 2.5 and 25 μl Dynabeads mouse T Activator CD3/CD28 (Invitrogen), respectively, in the presence or absence of an appropriate number of CD4+CD25+ Treg cells for 66–72 h. To block cell migration, Transwell membranes (0.4 μm pore; BD Biosciences) were inserted into 24-well plates. CFSE-labeled CD8+ T cells and CD4+CD25+ Treg cells were placed into lower and upper wells, respectively, and Dynabeads mouse T Activator CD3/CD28 was added into both wells for stimulation. In the same manner as described above, CD8+ T cells isolated from PD-1−/− and PD-L1−/− mice were labeled with CellTrace Violet and cultured with CD4+CD25+ Treg cells isolated from either PD-L1−/− or PD-1−/− mice. In certain experiments, CellTrace Violet–labeled PD-1−/− or CD8+ T cells were cocultured with naive Treg or chronic Treg cells. For stimulation of P14 Thy1.1+ CD8+ T cells, the naive splenocytes were cocultured with P14 Thy1.1+ CD8+ T cells in 1:5 ratios as feeder cells in the presence of 0.2 μg/ml LCMV gp33–41 peptides. For PD-1−/− or PD-L1−/− blockade in chronic Treg cells, chronic Treg cells isolated at 16 d postinfection (p.i.) were incubated in vitro for 1 h with 10 μg/ml PD-1 Ab (RMP1-14; BioXcell), PD-L1 Ab (10F.9G2; BioXcell), rat IgG2a isotype control (2A3; BioXcell), or rat IgG2b isotype control (LTF-2; BioXcell) at 4°C and then washed twice. These chronic Treg cells were cocultured in vitro with CD8+ T cells for 68 h in the presence of Dynabeads mouse T Activator CD3/CD28.

Adoptive cell transfer for in vivo suppression assay

Thy1.1+ CD8+ T cells (2×106) were injected i.v. into recipient Rag1−/− mice alone or with Thy1.2+ naive Treg, acute Treg, or chronic Treg cells (5×106 at 16 d.p.i. At 7 d after cell transfer, splenocytes isolated from Rag1−/− mice were analyzed for homeostatic expansion of the Thy1.1+ CD8+ T cell population using FACs.

In vivo Treg cell depletion

Mice were treated with 0.5 mg CD25 Ab (PC 61.5.3; BioXcell) or rat IgG1 isotype control (HRPN; BioXcell) or rat IgG1 isotype control (HRPN; BioXcell) by i.p. injection at 16 d.p.i. Seven days later, FACs analysis was performed to check Treg cell depletion and measure virus-specific CD8+ T cells and their activity to produce effector cytokines, IFN-γ and TNF-α. Viral titers of Ab-treated mice were measured from the serum at the same day.

Statistical analysis

Statistical analysis was performed with two-tailed, unpaired Student t tests using the Prism program (GraphPad Software).

Results

Altered Treg cell numbers and phenotype during chronic viral infection

To investigate the mechanism of Treg cell contribution to chronic viral infection, we compared the frequency of Foxp3+ Treg cells isolated from mice infected with the LCMV Armstrong (Arm) or CL13 strain, which are known to mediate acute and chronic
infections, respectively (Fig. 1A). Consistent with a previous report (20), at 16 d.p.i., the number of Foxp3+ T<sub>reg</sub> cells among CD4<sup>+</sup> T cells was 2- to 3-fold higher in CL13-infected mice than in Arm-infected mice. This result was observed when serum virus levels were completely different after each infection (Fig. 1B). The number of T<sub>reg</sub> cells dramatically increased above basal levels in CL13-infected mice compared with Arm-infected mice at 16 d.p.i. The number of T<sub>reg</sub> cells in chronically infected mice progressively decreased, according to the reduction in serum virus titer (Fig. 1B).

We next examined whether T<sub>reg</sub> cells from chronically infected mice (chronic T<sub>reg</sub> cells) simply increased in number or also upregulated their function compared with T<sub>reg</sub> cells from acutely infected mice (acute T<sub>reg</sub> cells). We profiled the gene expression patterns of chronic T<sub>reg</sub> and acute T<sub>reg</sub> cells isolated from the spleens of CL13- and Arm-infected mice, respectively, at 16 d.p.i., when the numbers of T<sub>reg</sub> cells and their phenotypes were the most different between the group cells (Fig. 1C). Overall, the relative expression levels for various surface molecules known to be expressed by T<sub>reg</sub> cells were not different between naive T<sub>reg</sub> cells and acute T<sub>reg</sub> cells (data not shown), but consistently higher in chronic T<sub>reg</sub> cells at 16 d.p.i. than in either naive T<sub>reg</sub> cells or acute T<sub>reg</sub> cells. Compared with acute T<sub>reg</sub> cells, chronic T<sub>reg</sub> cells displayed high CD103 expression, which is considered a hallmark of activated T<sub>reg</sub> cells in vivo with stronger suppressive activity than steady-state T<sub>reg</sub> cells (31, 32). The most striking result from the chronic T<sub>reg</sub> cell gene expression profile was the upregulation of the genes encoding T cell inhibitory receptors such as PD-1, TIM-3, CTLA-4, and LAG-3. The gene encoding GITR, a molecule expressed by T<sub>reg</sub> cells, was also upregulated in chronic T<sub>reg</sub> cells than in acute T<sub>reg</sub> cells. Protein levels for inhibitory receptors, including PD-1, TIM-3, CTLA-4, and GITR protein level, were higher in chronic T<sub>reg</sub> cells than in acute T<sub>reg</sub> cells at 16 d.p.i. (Fig. 1D, Supplemental Fig. 1). The upregulation of PD-1 in chronic T<sub>reg</sub> cells was the most significant (∼3-fold) among the inhibitory receptors. Despite the microarray results, the protein level for LAG-3 was not different between chronic T<sub>reg</sub> and acute T<sub>reg</sub> cells (data not shown).

We also examined the expression of T<sub>reg</sub> cell effector function-related genes on chronic T<sub>reg</sub> cells (Supplemental Fig. 2A, 2B). Coincident with the upregulation of T<sub>reg</sub> cell surface marker genes (Fig. 1C), the genes associated with T<sub>reg</sub> cell effector function, such as IL-10 and EBV-induced protein 3, which is a subunit of IL-35 (33), were upregulated in chronic T<sub>reg</sub> cells compared with naive T<sub>reg</sub> cells. We also observed higher expression of the genes encoding B lymphocyte-induced maturation protein-1 (34) and E4 promoter-binding protein 4 (35), which are transcription factors involved in IL-10 expression, in chronic T<sub>reg</sub> cells than in naive T<sub>reg</sub> cells. In addition, the upregulation of genes encoding cytolytic proteins responding to T<sub>reg</sub> cell–killing activity (36, 37) was also observed in chronic T<sub>reg</sub> cells. Especially, IL-10 and granzyme B mRNA expressions were >10-fold higher in chronic T<sub>reg</sub> cells compared with acute T<sub>reg</sub> cells (data not shown).

FIGURE 1. Increase of T<sub>reg</sub> cells and the upregulation of inhibitory receptors and other surface molecules during chronic viral infection. (A) The kinetics of Foxp3 expression on CD4<sup>+</sup> T cells in the spleen at the indicated time points after acute or chronic viral infection with LCMV Arm or CL13, respectively. The percentage of Foxp3<sup>+</sup> T<sub>reg</sub> cells among CD4<sup>+</sup> T cells is shown in the plots. (B) The absolute numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in the spleen and viral titers in the serum obtained from the mice in (A) at the indicated time points postinfection. Data points in the line graphs are shown as mean ± SEM. The dashed black line represents the virus detection limit. (C) Microarray analysis for expression levels of the indicated genes in T<sub>reg</sub> cells isolated from the spleens of mice at 16 d.p.i. with LCMV Arm or CL13. The heat map represents the normalized intensity of each gene from three independent experiments. (D) Protein expression levels of the indicated proteins expressed in splenic CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells at the indicated time points after acute or chronic viral infection. Right top number in histogram showing CD103 expression represents the percentage of CD103<sup>+</sup> cell population. The number in other histograms represents mean fluorescence intensity of the expressed protein. Naive T<sub>reg</sub> acute T<sub>reg</sub> and chronic T<sub>reg</sub> cells indicate T<sub>reg</sub> cells isolated from the spleens of uninfected naive, acutely infected, and chronically infected mice, respectively. Data shown in (A), (B), and (D) are representative of three independent experiments. n = 3–5 mice per group in each experiment.
cells than in naive T<sub>reg</sub> cells. Compared with naive T<sub>reg</sub> cells, strong expression of granzyme B in chronic T<sub>reg</sub> cells was confirmed at the protein level (Supplemental Fig. 2C).

**Preferential expression of inhibitory receptors and GITR in CD103<sup>+</sup> chronic T<sub>reg</sub> cells**

To more closely investigate the phenotypic changes in chronic T<sub>reg</sub> cells, splenocytes obtained at 16 d p.i. were gated by CD4 and Foxp3 expression, and the CD4<sup>+</sup>Foxp3<sup>+</sup> cells were then gated individually depending on CD103 expression (Fig. 2A). Foxp3 and CD25, hallmarks of T<sub>reg</sub> cells, were more highly expressed in CD103<sup>+</sup> chronic T<sub>reg</sub> cells than in CD103<sup>−</sup> chronic T<sub>reg</sub> cells. In addition, higher levels of PD-1, TIM-3, CTLA-4, and GITR were found in CD103<sup>+</sup> chronic T<sub>reg</sub> cells than in CD103<sup>−</sup> chronic T<sub>reg</sub> cells (Fig. 2B). When analyzing the combination of these surface molecules, the coexpression of PD-1 and TIM-3 or CTLA-4 and GITR was observed much more frequently in CD103<sup>+</sup> chronic T<sub>reg</sub> cells than in CD103<sup>−</sup> chronic T<sub>reg</sub> cells (~45 and ~15%, respectively) (Fig. 2A). Interestingly, CD103<sup>+</sup> T<sub>reg</sub> cells generated after chronic virus infection had significantly higher expression levels of inhibitory receptors and GITR than CD103<sup>−</sup> T<sub>reg</sub> cells present in naive mice or CD103<sup>+</sup> T<sub>reg</sub> cells generated after acute virus infection (Fig. 2C, 2D), suggesting that a similar subset of T<sub>reg</sub> cells is further differentiated upon chronic virus infection.

**Enhanced suppressive activity of chronic T<sub>reg</sub> cells**

Because the chronic T<sub>reg</sub> phenotype differed significantly from naive T<sub>reg</sub> and acute T<sub>reg</sub> cells at 16 d p.i., we questioned whether the ability of chronic T<sub>reg</sub> cells to suppress CD8<sup>+</sup> T cell proliferation was also changed. We found that chronic T<sub>reg</sub> cells isolated 16 d p.i. could inhibit much more strongly CD8<sup>+</sup> T cell proliferation than acute T<sub>reg</sub> cells isolated at the same time point or naive T<sub>reg</sub> cells (Fig. 3A, 3B). Potent chronic T<sub>reg</sub> cell–mediated suppression seemed to be strongly dependent on the timing of infection, because chronic T<sub>reg</sub> cells isolated 30 d p.i. did not show strong suppressive activity, similarly to naive T<sub>reg</sub> or acute T<sub>reg</sub> cells (Fig. 3A, 3B). The enhanced suppressive capacity of chronic T<sub>reg</sub> cells at 16 d p.i. was confirmed by the reduction in IFN-γ production (Fig. 3C). In addition, coculture with chronic T<sub>reg</sub> cells showed a dramatically low frequency of IFN-γ–producing CD8<sup>+</sup> T cells than that with naive or acute T<sub>reg</sub> cells, confirming the strong suppression of CD8<sup>+</sup> T cell response by chronic T<sub>reg</sub> cells (data not shown). Moreover, strong suppressive activity displayed by chronic T<sub>reg</sub> cells was dependent on T<sub>reg</sub> cell numbers that were cocultured (Fig. 3D, 3E). Similar to the result of chronic T<sub>reg</sub> cell–mediated potent suppression of CD8<sup>+</sup> T cell response, chronic T<sub>reg</sub> cells also inhibited more strongly CD4<sup>+</sup> T cell proliferation and IFN-γ production than naive T<sub>reg</sub> or acute T<sub>reg</sub> cells (Supplemental Fig. 3A–C). This chronic T<sub>reg</sub> cell–mediated potent suppression of CD8<sup>+</sup> T cell response is not only due to the suppressive activity of T<sub>reg</sub> cells, but also due to the reduced IFN-γ production by CD8<sup>+</sup> T cells. These data suggest that chronic T<sub>reg</sub> cells are more potent than acute or naive T<sub>reg</sub> cells in suppressing the CD8<sup>+</sup> T cell response.

**FIGURE 2.** Upregulation of inhibitory receptors and GITR in CD103<sup>+</sup> chronic T<sub>reg</sub> cells. (A) PD-1, TIM-3, CTLA-4, and GITR expression in CD103<sup>+</sup> or CD103<sup>−</sup> subsets among Foxp3<sup>+</sup>CD4<sup>+</sup> T cells. Splenocytes from chronically infected mice (16 d p.i.) were stained with CD4, CD25, Foxp3, and CD103. CD4<sup>+</sup>Foxp3<sup>+</sup> chronic T<sub>reg</sub> cells were subsequently divided into CD103<sup>−</sup> and CD103<sup>+</sup> subsets (upper panel). The coexpression of either PD-1 and TIM-3 or CTLA-4 and GITR was plotted in two different chronic T<sub>reg</sub> cell subsets. The number in the plots indicates the percentage of the corresponding population. (B) Mean fluorescence intensity (MFI) of protein expressed in CD103<sup>−</sup> or CD103<sup>+</sup> T<sub>reg</sub> cells. The graphs are depicted as individual MFIs with the mean value (red line). (C) PD-1, TIM-3, CTLA-4, and GITR expression in CD103<sup>+</sup> or CD103<sup>−</sup> subsets among Foxp3<sup>+</sup>CD4<sup>+</sup> T cells. The number in the plots indicates MFI of the expressed protein. (D) Comparison of the MFI for inhibitory receptor expression between CD103<sup>+</sup> and CD103<sup>−</sup> T<sub>reg</sub> cells. The bar graphs represent the mean ± SEM for individual MFIs. Data are representative of three independent experiments. n = 4 mice per group in each experiment. *p < 0.05, **p < 0.01, ***p < 0.001.
CD4+ T cell response was also observed in Treg cell number-dependent manner (Supplemental Fig. 3D, 3E). The number of live Treg cells was higher in the naive Treg or acute Treg cell culture than in the chronic Treg cell culture in the presence of a CD3/CD28-coated beads, excluding the possibility that the suppressive capability was caused by increased numbers of chronic Treg cells (data not shown). To confirm the enhanced suppressive capability of chronic Treg cells in an in vivo system, Thy1.1+ CD8+ T cells were transferred into Rag1−/− mice together with naive Treg, acute Treg, or chronic Treg cells in a 1:0.25 ratio. Chronic Treg

**FIGURE 3.** Enhanced suppression of CD8+ T cell immune response by chronic Treg cells. (A) Suppression of CD8+ T cell proliferation by Treg cells isolated at various time points after acute or chronic viral infection. CFSE-labeled CD8+ T cells were stimulated in vitro with αCD3/CD28-coated beads for 72 h in the absence or presence of naive Treg, acute Treg, or chronic Treg cells (16 and 30 d p.i.). CFSE dilution in proliferated CD8+ T cells is depicted in each histogram (top) and summarized by the bar graph (bottom). The left top number in the histogram indicates the percentage of proliferated CD8+ T cells.

(B) Proliferation profile of CD8+ T cells in (A). The percentage of population in each division stage was calculated by cell proliferation analysis. (C) Concentration of IFN-γ in the coculture media of (A). (D) Suppressive activity of each Treg cell population. Treg cells from naive, acutely, or chronically infected mice (16 d p.i.) and CFSE-labeled CD8+ T cells from naive mice were cultured together in vitro for 72 h in the presence of αCD3/CD28-coated beads. The percentage of inhibition was determined according to the following formula: % Inhibition = (% of proliferated CD8+ T cells in the absence of Treg cells − % of proliferated CD8+ T cells in the presence of Treg cells)/(% of proliferated CD8+ T cells in the absence of Treg cells) × 100. (E) Fold reduction in IFN-γ production by CD8+ T cells cocultured with each Treg cell population. The concentration of IFN-γ in the coculture media of (D) was measured, and the fold reduction in IFN-γ production was determined by the ratio of its concentration in the absence of Treg cells to that in the presence of Treg cells. (F) Homeostatic expansion frequency of donor Thy1.1+ CD8+ T cells in the spleen isolated from Rag1−/− mice at 7 d after adoptive cell transfer. (G) Absolute number of donor Thy1.1+ CD8+ T cells in the spleen of Rag1−/− mice. (H) Treg cell–mediated suppression of LCMV-specific CD8+ T cell proliferation (left) and the percentage of recently proliferated (5–6 divisions) CFSE-labeled P14 Thy1.1+ CD8+ T cells after coculture with Treg cells (right). CFSE-labeled P14 Thy1.1+ CD8+ T cells containing Dβ-restricted TCR specific for LCMV gp33−41 were cocultured with gp33−41 peptide-loaded feeder cells for 66 h in the absence or presence of isolated naive Treg cells, acute Treg cells, and chronic Treg cells at 16 d p.i. (I) Concentration of IFN-γ in the coculture media of (H). The data points in the line graphs and bar graphs represent the mean ± SEM and mean + SEM, respectively. Data are representative of three to four independent experiments. n = 3 mice per group in each experiment. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
cells showed greater capability to inhibit proliferation of donor Thy1.1+ CD8+ T cells than did naive Treg and acute Treg cells (Fig. 3F, 3G). These data demonstrate that chronic Treg cells isolated at 16 d p.i. exhibit strong suppressive capability per cell.

Next, we investigated whether chronic Treg cells can inhibit Ag-specific CD8+ T cells. Chronic Treg cells inhibited more efficiently a recent proliferation of LCMV-specific P14 CD8+ T cells and IFN-γ production than naive or acute Treg cells under the optimal condition to stimulate P14 CD8+ T cells (Fig. 3H, 3I). Taken together, this result indicates that the potent suppressive activity of chronic Treg cells is independent of the specificity of TCR expressed by proliferating CD8+ T cells. However, because most of the CD8+ T cells proliferating during chronic viral infection are specific to the particular virus, the Treg cells expanded during the infection may primarily inhibit the proliferation of LCMV-specific CD8+ T cells.

**PD-1 expressed by chronic Treg cells is necessary for their potent suppressive function**

We addressed whether cell contact is a requirement for suppression. Suppression of T cell proliferation and reduction of IFN-γ production by chronic Treg cells were not observed in the presence of the Transwell membrane (Fig. 4A–C), indicating that suppression of CD8+ T cell proliferation by chronic Treg cells requires physical contact between each cell population. Next, we questioned whether the inhibitory receptors upregulated by chronic Treg cells can contribute to the suppressive activity of chronic Treg cells. As the upregulation of PD-1 in chronic Treg cells was more significant than the other inhibitory receptors (Fig. 1D), we investigated the effect of PD-1 on chronic Treg cells. Selective blockade of PD-1 on chronic Treg cells, but not on CD8+ T cells, was performed by the incubation of the PD-1 Ab with chronic Treg cells in vitro, followed by removal of unbound Ab before coculture of the chronic Treg cells with CD8+ T cells. PD-1–blocked chronic Treg cells showed a significantly decreased suppressive activity in the proliferation of CD8+ T cells and their IFN-γ production compared with isotype Ab-treated chronic Treg cells (Fig. 4D–F). However, selective blockade of PD-L1 on chronic Treg cells did not show any effect on their suppressive function. These results indicate that the expression of PD-1, but not of PD-L1, in chronic Treg cells is critical for their potent suppressive capability.

**Chronic Treg cell–mediated suppression requires PD-L1 expressed by CD8+ T cells**

Next, we performed a suppression assay using wild-type (WT) Treg cells from CL13-infected mice and CD8+ T cells from PD-L1−/−.
mice to address whether PD-1 upregulated by chronic Treg cells requires the interaction with PD-L1 expressed by effector CD8+ T cells for their enhanced suppressive activity. In the absence of Treg cells, there was no difference in the proliferation and IFN-γ production between WT CD8+ T cells and PD-L1−/− CD8+ T cells when stimulated in vitro with αCD3/CD28-coated beads, indicating that PD-L1 expressed by CD8+ T cells does not affect CD8+ T cell immune response in the absence of Treg cells (Fig. 5). Of interest, when chronic Treg cells were cocultured, the proliferation and IFN-γ production of PD-L1−/− CD8+ T cells were significantly less inhibited than that of WT CD8+ T cells. Even though suppressive activity of naive Treg cells was not as strong as chronic Treg cells, the pattern that suppression of CD8+ T cell response was weaker in PD-L1−/− CD8+ T cells than in WT CD8+ T cells was similar to the case of coculture with chronic Treg cells. More interestingly, the inhibition of PD-L1−/− CD8+ T cell response by PD-1high chronic Treg cells was similar to that of WT CD8+ T cell response by PD-1low naive Treg cells. These findings clearly demonstrate that upregulation of PD-1 on chronic Treg cells is not enough to exert their strong suppressive function, which can be achieved when PD-L1 is present on CD8+ T cells.

Interaction of PD-1 and PD-L1 between Treg and CD8+ T cells is responsible for the suppression of CD8+ T cell response

To further investigate which combinatorial expression of PD-1 and PD-L1 on chronic Treg cells and CD8+ T cells is required for chronic Treg cell–mediated suppression of CD8+ T cell immune response, first we attempted to obtain chronic Treg cells from PD-1−/− or PD-L1−/− mice. However, we could not obtain Treg cells from CL13-infected PD-1−/− or PD-L1−/− mice at 16 d p.i., because these mice died of severe immunopathology within 1 wk p.i., as previously reported (14). Instead, we isolated Treg cells from uninfected PD-1−/− or PD-1−/− mice and applied them to the suppression assay with CD8+ T cells isolated from PD-1−/− or PD-L1−/− mice (Fig. 6). Coculture of PD-L1−/− Treg cells and PD-1−/− CD8+ T cells inhibits more efficiently both CD8+ T cell proliferation and IFN-γ production in a Treg cell dose-dependent manner than coculture of PD-1−/− Treg cells and PD-L1−/− CD8+ T cells (Fig. 6). Coculture of PD-1−/− Treg cells and PD-L1−/− CD8+ T cells slightly inhibited CD8+ T cell proliferation and limited IFN-γ production in the presence of high numbers of PD-1−/− Treg cells, which might have been caused by triggering PD-1 signaling in CD8+ T cells to inhibit TCR signaling, as previously reported (38). This result indicates that the interaction between PD-1 on Treg cells and PD-L1 on CD8+ T cells is more important for the suppression of CD8+ T cell immune response by Treg cells than that between PD-L1 on Treg cells and PD-1 on CD8+ T cells.

Enhanced restoration of exhausted CD8+ T cell function by depletion of chronic Treg cells

Considering that PD-1 signaling conferred suppressive function in chronic Treg cells, the depletion of chronic Treg cells during chronic virus infection would enhance antiviral T cell immune response and decrease viremia. To test this, CL13-infected mice were treated with CD25 Ab at 16 d p.i., in which CD25-expressing T cells were observed only in Foxp3+ Treg cells. CD4+Foxp3+ CD25+ cells were efficiently depleted, but CD4+Foxp3−CD25− Treg cells were still observed (data not shown). Nonetheless, partial depletion of chronic Treg cells substantially increased the numbers of LCMV gp33- or gp276-specific CD8+ T cells (Supplemental Fig. 4A) and also enhanced the ability of CD8+ and CD4+ T cells to secrete effector cytokines, IFN-γ and TNF-α (Supplemental Fig. 4B). Accordingly, viral titer in the serum slightly decreased in the CD25 Ab-treated mice compared with isotype Ab-treated mice (Supplemental Fig. 4C). This result indicates that in vivo depletion of chronic Treg cells could lead to a partial recovery of antiviral T cell immune response and a slight reduction in viremia.

Discussion

In the current study, we identified the primary phenotypic and functional changes in Treg cells during infections in mice and defined the role of PD-1 expressed by those cells. Although Treg cell–mediated suppression of antiviral T cells during chronic virus infection has been reported (4, 11, 39, 40), the underlying mechanism has not been intensively investigated. We found that either PD-1 blockade on chronic Treg cells or PD-L1 deficiency on CD8+ T cells dramatically diminished the suppression of T cell

FIGURE 5. Effect of PD-L1 expression on CD8+ T cells during chronic Treg cell–mediated suppression. CellTrace Violet-labeled WT and PD-L1−/− CD8+ T cells were stimulated in vitro with αCD3/CD28-coated beads for 72 h in the absence or presence of isolated naive or chronic Treg cells at 16 d p.i. (A) The proliferation of WT and PD-L1−/− CD8+ T cells. CellTrace Violet dilution in proliferated CD8+ T cells is depicted in each histogram. (B) Comparison of proliferation percentage of WT and PD-L1−/− CD8+ T cells cocultured with each Treg cell population. (C) The change of IFN-γ production in WT and PD-L1−/− CD8+ T cells cocultured with Treg cell population in (A). IFN-γ production was measured by intracellular cytokine staining. The bar graphs represent mean ± SEM. Data are representative of three independent experiments. n = 3 mice per group in each experiment. *p < 0.05, **p < 0.01. ns, not significant.
immune response, demonstrating the requirement of PD-1 on T_{reg} cells and PD-L1 on CD8^+ T cells. This was supported by the observation that coculture of PD-L1−/− T_{reg} cells and PD-1−/− CD8^+ T cells, but not that of PD-1−/− T_{reg} cells and PD-L1−/− CD8^+ T cells, suppressed CD8^+ T cell immune response. Our report details a critical role of PD-1:PD-L1 interaction between T_{reg} cells and effector T cells as the mechanism by which T_{reg} cells generated during chronic virus infection display a superior suppressive activity. In summary, our study defines PD-1 upregulation on T_{reg} cells as a prerequisite for T_{reg} cell–mediated potent suppression of T cell immune response.

Our observation that the immunosuppressive potency of chronic T_{reg} cells dynamically changed with regard to frequency, phenotype, and function, depending on the status of chronic LCMV infection (Fig. 3A–C), raises a question regarding T_{reg} cell plasticity. According to a previous report, chronic T_{reg} cells are generated in LCMV CL13-infected mice from a pre-existing pool of T_{reg} cells specific to a retroviral superantigen, the genes of which are encoded in the mouse genome. Therefore, it can be speculated that most of the T_{reg} cells responding to viral infection are enriched to CD103^+ T_{reg} cells expressing CD103 during chronic viral infection. Interestingly, CD103^+ T_{reg} cells showed significantly higher expression levels of PD-1, TIM-3, CTLA-4, and GITR than CD103^− T_{reg} cells, suggesting the difference of their function as well as phenotype. Although we did not examine the relationship between activation phenotype of T_{reg} cells and Ag specificity in the current study, Punsksoby et al. (20) have already demonstrated very nicely that a specific subpopulation of T_{reg} cells that expresses TCR VB5 most prominently expands and upregulates activation markers such as CD103, CD101, CD69, ICOS, PD-1, OX-40, and 4-1BB in C57BL/6 mice after LCMV CL13 infection. A marked expansion of T_{reg} cells was demonstrated to be dependent on retroviral superantigen genes encoded in the mouse genome. Therefore, it can be speculated that most of the T_{reg} cells responding to viral infection are enriched to CD103^+ population, which also expresses high levels of PD-1, TIM-3, CTLA-4, and GITR.

In addition to phenotypical change, chronic T_{reg} cells displayed the upregulation of functional molecules, granzyme B and IL-10, which are involved in the effector function of T_{reg} cells (Supplemental Fig. 2). First, chronic T_{reg} cells might reduce the population of proliferating CD8^+ T cells by inducing granzyme B–mediated apoptosis. Consistent with previous reports that T_{reg} cells express substantial levels of granzyme B and are capable of killing effector T cells through a granzyme B–mediated process.
Second, chronic T reg cells can indirectly inhibit CD8+ T cells through IL-10 production. Chronic T reg cells expressed higher levels of IL10 mRNA than naive T reg cells. Some reports have shown immunosuppressive factors such as IL-10 or TGF-β have a significant effect on T reg cell–mediated suppression (41, 42), whereas it has also been reported that the absence of these cytokines does not affect the prevention of T reg cell–mediated suppression (43, 44). We observed that the suppression of CD8+ T cells by chronic T reg cells did not decline when IL-10-neutralizing or IL-10R–blocking Ab was used in vitro (data not shown). However, the following phenomenon could be assumed because other immune cells exist during chronic viral infection, unlike the in vitro system in which there are only T reg cells and CD8+ T cells. In vivo, IL-10 appeared to be involved in the suppression of CD8+ T cells by inhibiting dendritic cell (DC) maturation and CD4+ T cell function. PD-1 upregulation on chronic T reg cells may confer sustained contact between chronic T reg cells and DCs that upregulate PD-L1 to eliminate the interaction of DCs and CD8+ T cells. Such potential mechanisms must be investigated further, because the T reg cell–DC interaction is one of the critical factors for the induction of T cell inactivation or tolerance.

In this study, we clearly demonstrated the direct interaction between chronic T reg cells and effector T cells is critical for chronic T reg cell–mediated strong suppression of effector T cell immune response (Fig. 4A–C). Therefore, these data made us suggest a direct receptor–ligand interaction between T reg cells and effector T cells. In this study, we focused on the interaction between PD-1 and PD-L1 because chronic T reg cells have a profoundly higher level of PD-1 than other known inhibitory molecules. Although some studies have shown that PD-1 blockade abrogated T reg cell–mediated suppression of T cell immune response (45–48), the exact ligand–receptor interaction was not specifically investigated. Our data showed that either PD-1 blockade on chronic T reg cells (Fig. 4D–F) or PD-L1 deficiency on CD8+ T cells (Fig. 5) abrogated the potent suppression. We also found that coculture of PD-L1+/− T reg cells and PD-1+/− CD8+ T cells, but not that of PD-1+/− T reg cells and PD-L1+/− CD8+ T cells, showed T cell suppression (Fig. 6), confirming the critical role of PD-1:PD-L1 interaction between T reg and CD8+ T cells in the suppression of T cell immune response.

Although it is evident in our study that chronic T reg cell–mediated potent T cell suppression requires the PD-1:PD-L1 interaction, the underlying mechanism should be further investigated. Requirement of PD-1:PD-L1 interaction on chronic T reg cells and CD8+ T cells made us propose two possible molecular mechanisms by which PD-1high chronic T reg cells display such a strong suppressive activity to inhibit CD8+ T cell immune response. One possible mechanism is that a ligation of PD-L1 present in CD8+ T cells onto PD-1 upregulated by chronic T reg cells triggers PD-1 signaling in chronic T reg cells. Although chronic T reg cells isolated at 16 d p.i. expressed higher levels of T reg cell effector molecules such as IL-10 and granzyme B than acute and naive T reg cells, it is not known whether this effect is caused by turn-on of PD-1 signal transduction in T reg cells via the ligation of PD-L1 presented by CD8+ T cells. Alternatively, it could be predicted that a reverse inhibitory signaling via PD-L1 in CD8+ T cells after a ligation of PD-1 provided by chronic T reg cells contributes to the effects of chronic T reg cell–mediated potent suppressive activity. Indeed, as shown in Fig. 5, we observed that the absence of PD-L1 in CD8+ T cells lessened PD-1high chronic T reg cell–mediated potent suppression of CD8+ T cell immune response. Although a reverse signaling from PD-1 to PD-L1 has not been reported yet, Butte et al. (49) observed that PD-L1 triggering via B7-1 ligation dramatically decreased T cell proliferation. Taken together, a more detailed study about bidirectional PD-1:PD-L1 interaction between T reg cells and effector T cells needs to be carried out as a separate experiment.

The role of PD-1 expression by T reg cells is somewhat controversial (21, 24). Franceschini et al. (24) showed that PD-1:PD-L1 signaling limited the proliferation of T reg cells isolated from the livers of patients chronically infected with HCV. However, Francisco et al. (21) reported that PD-L1 induced the development of induced T reg cells and enhanced their suppressive function in vitro. This discrepancy most likely originated from the status of T reg cells. We observed that chronic T reg cells are more sensitive to apoptosis, upregulating Casp3 but downregulating Bcl-2 (data not shown). Similar to the chronic T reg cells in our study, T reg cells isolated from the livers of HCV patients may be undergoing apoptosis, and this process is accelerated by PD-L1 ligation, resulting in less expanded populations. Taken together, these data indicate that PD-1:PD-L1 signaling is differentially regulated, depending on the status of T reg cells or the period of infection. This result suggests that PD-1 ligation mediates T reg cell activation at the initial stage of infection, but ligation accelerates the apoptotic T reg cell susceptibility after continuous TCR stimulus.

There were common phenotypic changes between chronic T reg cells and exhausted T cells generated during chronic viral infection. In addition to the upregulation of inhibitory receptors, both cell types overexpressed intracellular transcription factors, such as Blimp1 (Supplemental Fig. 2A, 2B) and Batf (data not shown), which are associated with T cell exhaustion (50, 51). Among these inhibitory receptors, PD-1 is also expressed on follicular Th (Tfh) cells (52, 53). Because a large population of CD4+ T cells differentiates into Tfh cells during chronic viral infection (54), PD-1 is a hallmark of various T cell subsets, including exhausted T, T reg, and Tfh cells. Although the function of PD-1 expressed on Tfh cells is still controversial (53, 55), PD-1 expressions on chronic T reg cells and exhausted T cells, at least, are associated with the suppression of T cell immune response. Because PD-1 signaling is differentially regulated by cell type–specific downstream intrinsic factors, a direct comparison of gene profiles between exhausted T cells and chronic T reg cells during chronic infections may help identify common and cell-specific regulatory factors and elucidate different signaling pathways in chronic T reg cells versus exhausted T cells.

Chronic environment-induced T reg cell expansion is another obstacle to successful immunotherapy for chronic pathogen infections. We observed that removal of chronic T reg cells by CD25 Ab treatment during chronic virus infection increased the frequency of functional Ag-specific CD8+ T cells (Supplemental Fig. 4). However, CD25 Ab treatment leads to concurrent elimination of non-T reg cells and rapid replenishment of T reg cells (56–58). Therefore, additional research on the side effect caused over a long period after T reg cell blockade and the effect of T reg cell blockade on cellular or humoral immune response is required. The functional inactivation of T reg cells and rejuvenation of exhausted T cells by targeting PD-1 overexpression in combination with the temporal depletion of T reg cells may be a powerful strategy to achieve better control of chronic infection and cancer. Therefore, developing methods for the specific depletion of T reg cells that express inhibitory receptors such as PD-1 could provide fine-tuned clinical strategies against chronic infectious diseases and cancers.

Acknowledgments

We thank Dr. Rafi Ahmed (Emory University School of Medicine) and all the members of the Hyehwa Forum for helpful comments on the manuscript.
Disclosures

S.-J.H. has a patent and receives patent royalties related to the PD-1 pathway. The remaining authors have no financial conflicts of interest.

References


Corrections


In Fig. 3F, the representative data indicating in vivo expansion frequency of donor Thy1.1^{+}CD8^{+} T cells in Rag1^{−/−} mice were incorrect as published. The corrected Fig. 3 is shown. There is no change in the Results section of the article. The figure legend was correct as published and is shown below the figure for reference.

We apologize for the inconvenience caused by this inadvertent error.
FIGURE 3. Enhanced suppression of CD8+ T cell immune response by chronic Treg cells. (A) Suppression of CD8+ T cell proliferation by Treg cells isolated at various time points after acute or chronic viral infection. CFSE-labeled CD8+ T cells were stimulated in vitro with αCD3/CD28-coated beads for 72 h in the absence or presence of naive Treg, acute Treg, or chronic Treg cells (16 and 30 d p.i.). CFSE dilution in proliferated CD8+ T cells is depicted in each histogram (top) and summarized by the bar graph (bottom). The left top number in the histogram indicates the percentage of proliferated CD8+ T cells.

(B) Proliferation profile of CD8+ T cells in (A). The percentage of population in each division stage was calculated by cell proliferation analysis. (C) Concentration of IFN-γ in the coculture media of (A). (D) Suppressive activity of each Treg cell population. Treg cells from naive, acutely, or chronically infected mice (16 d p.i.) and CFSE-labeled CD8+ T cells from naive mice were cultured together in vitro for 72 h in the presence of αCD3/CD28-coated beads. The percentage of inhibition was determined according to the following formula: % Inhibition = ([% of proliferated CD8+ T cells in the absence of Treg cells − % of proliferated CD8+ T cells in the presence of Treg cells]/[% of proliferated CD8+ T cells in the absence of Treg cells]) × 100. (E) Fold reduction in IFN-γ production by CD8+ T cells cocultured with each Treg cell population. The concentration of IFN-γ in the coculture media of (D) was measured, and the fold reduction in IFN-γ production was determined by the ratio of its concentration in the absence of Treg cells to that in the presence of Treg cells. (F) Homeostatic expansion frequency of donor Thy1.1+ CD8+ T cells in the spleen isolated from Rag1−− mice at 7 d after adoptive cell transfer. (G) Absolute number of donor Thy1.1+ CD8+ T cells in the spleen of Rag1−− mice. (H) Treg cell–mediated suppression of LCMV-specific CD8+ T cell proliferation (left) and the percentage of recently proliferated (5–6 divisions) CFSE-labeled P14 Thy1.1+ CD8+ T cells after coculture with Treg cells (right). CFSE-labeled P14 Thy1.1+ CD8+ T cells containing Dα-restricted TCR specific for LCMV gp33–41 were cocultured with gp33-41 peptide-loaded feeder cells for 66 h in the absence or presence of isolated naive Treg cells, acute Treg cells, and chronic Treg cells at 16 d p.i. (I) Concentration of IFN-γ in the coculture media of (H). The data points in the line graphs and bar graphs represent the mean ± SEM and mean ± SEM, respectively. Data are representative of three to four independent experiments. n = 3 mice per group in each experiment. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
Supplemental Figure 1. Expression kinetics of CD103, inhibitory receptors, and GITR in T_{reg} cells during acute or chronic viral infection. Mice were infected with LCMV Arm or CL13 and sacrificed at the indicated times after infection. The splenocytes were stained, gated on CD4^{+}Foxp3^{+} T cells, and analyzed for the expression of CD103 and various surface molecules. The MFI values were plotted for the corresponding protein expression in CD4^{+}Foxp3^{+} subset during acute or chronic viral infection. For CD103, the percentage of the CD103^{+} cell population was plotted. The data points in the line graphs are shown as the mean ± SEM. Data are representative of three independent experiments. n = 3–5 mice per group in each experiment. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Supplemental Figure 2. Increased expression of T<sub>reg</sub> cell-mediated effector molecules, IL-10 and granzyme B during chronic viral infection. (A) Microarray analysis for the expression profile of the indicated genes in naïve T<sub>reg</sub> and chronic T<sub>reg</sub> cells (16 d p.i.). The heat map represents the normalized intensity of each gene from three independent experiments. (B) Relative gene expression levels between naïve T<sub>reg</sub> and chronic T<sub>reg</sub> cells. The bar graphs indicate relative gene expression levels in chronic T<sub>reg</sub> cells to naïve T<sub>reg</sub> cells, and bars represent the mean ± SEM from three independent experiments. (C) Comparison of granzyme B expression levels in naïve T<sub>reg</sub> and chronic T<sub>reg</sub> cells. Splenocytes were isolated from naïve and CL13-infected mice at 16 d p.i. Foxp3 and granzyme B expression in CD4<sup>+</sup> T cells were plotted together. Data are representative of four independent experiments (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Supplemental Figure 3. Enhanced suppression of CD4+ T cell response by chronic Treg cells.

(A) Suppression of CD4+CD25+ T cell proliferation by Treg cells isolated after acute or chronic viral infection (16 d p.i.). CellTrace Violet-labeled CD4+CD25+ T cells were stimulated in vitro with αCD3/CD28-coated beads for 72 h in the absence or presence of naive Treg, acute Treg, or chronic Treg cells (16 d p.i.). CellTrace Violet dilution in proliferated CD4+CD25+ T cells is depicted in each histogram (top) and summarized by the bar graph (bottom).

(B) Proliferation profile of CD4+CD25+ T cells in (A). The percentage of population in each division stage was calculated by cell proliferation analysis.

(C) IFN-γ production of CD4+CD25+ T cells co-cultured with each Treg cell population.

(D) Suppressive activity of each Treg cell population. The percentage of inhibition was determined according to the following formula:

% Inhibition = [(% of proliferated CD4+CD25+ T cells in the absence of Treg cells - % of proliferated CD4+CD25+ T cells in the presence of Treg cells)/(% of proliferated CD4+CD25+ T cells in the absence of Treg cells)] X 100.

(E) Fold-reduction in IFN-γ production by CD4+CD25+ T cells co-cultured with each Treg cell population.

The fold-reduction in IFN-γ production was determined by the ratio of its concentration in the absence of Treg cells to that in the presence of Treg cells. The data points in the line graphs and bar graphs represent the mean ± SEM and mean + SEM, respectively. Data are representative of three independent experiments. n = 3 mice per group in each experiment. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Supplemental Figure 4. Effect of *in vivo* T<sub>reg</sub> cell blockade with αCD25 on antiviral T cell immune response and viral control during chronic viral infection. CL13-infected mice were treated with 500 μg of CD25 antibody at 16 d p.i. and were sacrificed 7 d later. (A) Absolute cell number of DbGP33-41 and DbGP276-286 tetramer-positive CD8<sup>+</sup> T cells in the spleen. (B) Absolute number of IFN-γ- or TNF-α-producing CD8<sup>+</sup> or CD4<sup>+</sup> T cells in the spleen after *in vitro* stimulation. Splenocytes were stimulated with GP<sub>33-41</sub> peptide, GP<sub>276-286</sub> peptide, peptide pool, or GP<sub>66-80</sub> peptide derived from LCMV proteins. The percentage of IFN-γ- or TNF-α-producing cells among the CD8<sup>+</sup> T cells or CD4<sup>+</sup> T cells is shown in the plots. (C) Decrease of serum viral titer after CD25 antibody treatment. Viral titer was measured in the serum at 23 d p.i. Data are representative of two independent experiments. n = 3 mice per group in each experiment. ns, not significant; *, P < 0.05; **, P < 0.01.