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 published online 1 May 2015
http://www.jimmunol.org/content/early/2015/05/01/jimmunol.1401936

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

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

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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: 000–000.

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). Some of the molecules expressed by Treg cells, such as CTLA-4, LAG-3, and PD-1, are also 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
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 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 infection is associated with the suppression of pathogen-specific 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−/− , and PD-L1−/− mice, respectively. PD-1−/− and PD-L1−/− mice were originally generated by Dr. Tasuku Honjo (Kyoto University) (27) 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 × 10⁶ 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 (Miltenyi 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 (Miltenyi 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 (10⁶ [96-well flat or U-bottom plate] or 10⁷ [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 in 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-L1−/−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 postinfecion (p.i.) were incubated in vitro for 1 h with 10 μg/ml PD-1 Ab (RMP1-14; BioXcell), PD-1 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 or chronic Treg cells (5 × 10⁶) at 16 d.p.i. At 7 d after adoptive 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) 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+ Treg cells among CD4+ 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 Treg cells dramatically increased above basal levels in CL13-infected mice compared with Arm-infected mice at 16 d p.i. The number of Treg cells in chronically infected mice progressively decreased, according to the reduction in serum virus titer (Fig. 1B).

We next examined whether Treg cells from chronically infected mice (chronic Treg cells) simply increased in number or also upregulated their function compared with Treg cells from acutely infected mice (acute Treg cells). We profiled the gene expression patterns of chronic Treg and acute Treg cells isolated from the spleens of CL13- and Arm-infected mice, respectively, at 16 d p.i., when the numbers of Treg 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 Treg cells were not different between naive Treg cells and acute Treg cells (data not shown), but consistently higher in chronic Treg cells at 16 d p.i. than in either naive Treg cells or acute Treg cells. Compared with acute Treg cells, chronic Treg cells displayed high CD103 expression, which is considered a hallmark of activated Treg cells in vivo with stronger suppressive activity than steady-state Treg cells (31, 32). The most striking result from the chronic Treg 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 Treg cells, was also upregulated in chronic Treg cells than in acute Treg cells. Protein levels for inhibitory receptors, including PD-1, TIM-3, CTLA-4, and GITR protein level, were higher in chronic Treg cells than in acute Treg cells at 16 d p.i. (Fig. 1D, Supplemental Fig. 1). The upregulation of PD-1 in chronic Treg 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 Treg and acute Treg cells (data not shown).

We also examined the expression of Treg cell effector function-related genes on chronic Treg cells (Supplemental Fig. 2A, 2B). Coincident with the upregulation of Treg cell surface marker genes (Fig. 1C), the genes associated with Treg cell effector function, such as IL-10 and EBV-induced protein 3, which is a subunit of IL-35 (33), were upregulated in chronic Treg cells compared with naive Treg 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 Treg cells than in naive Treg cells. In addition, the upregulation of genes encoding cytolytic proteins responding to Treg cell–killing activity (36, 37) was also observed in chronic Treg cells. Especially, IL-10 and granzyme B mRNA expressions were >10-fold higher in chronic Treg

**FIGURE 1.** Increase of Treg cells and the upregulation of inhibitory receptors and other surface molecules during chronic viral infection. (A) The kinetics of Foxp3 expression on CD4+ 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+ Treg cells among CD4+ T cells is shown in the plots. (B) The absolute numbers of CD4+Foxp3+ Treg 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 Treg 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+ Foxp3+ Treg 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+ cell population. The number in other histograms represents mean fluorescence intensity of the expressed protein. Naive Treg, acute Treg, and chronic Treg cells indicate Treg 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_{reg} cells. Compared with naive T_{reg} cells, strong expression of granzyme B in chronic T_{reg} cells was confirmed at the protein level (Supplemental Fig. 2C).

**Preferential expression of inhibitory receptors and GITR in CD103\(^+\) chronic T_{reg} cells**

To more closely investigate the phenotypic changes in chronic T_{reg} cells, splenocytes obtained at 16 d p.i. were gated by CD4 and Foxp3 expression, and the CD4\(^+\)Foxp3\(^+\) cells were then gated individually depending on CD103 expression (Fig. 2A). Foxp3 and CD25, hallmarks of T_{reg} cells, were more highly expressed in CD103\(^+\) chronic T_{reg} cells than in CD103\(^-\) chronic T_{reg} cells. In addition, higher levels of PD-1, TIM-3, CTLA-4, and GITR were found in CD103\(^+\) chronic T_{reg} cells than in CD103\(^-\) chronic T_{reg} 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\(^+\) chronic T_{reg} cells than in CD103\(^-\) chronic T_{reg} cells (~45 and ~15%, respectively) (Fig. 2A). Interestingly, CD103\(^+\) T_{reg} cells generated after chronic virus infection had significantly higher expression levels of inhibitory receptors and GITR than CD103\(^+\) T_{reg} cells present in naive mice or CD103\(^+\) T_{reg} cells generated after acute virus infection (Fig. 2C, 2D), suggesting that a similar subset of T_{reg} cells is further differentiated upon chronic virus infection.

**Enhanced suppressive activity of chronic T_{reg} cells**

Because the chronic T_{reg} phenotype differed significantly from naive T_{reg} and acute T_{reg} cells at 16 d p.i., we questioned whether the ability of chronic T_{reg} cells to suppress CD8\(^+\) T cell proliferation was also changed. We found that chronic T_{reg} cells isolated 16 d p.i. could inhibit much more strongly CD8\(^+\) T cell proliferation than acute T_{reg} cells isolated at the same time point or naive T_{reg} cells (Fig. 3A, 3B). Potent chronic T_{reg} cell–mediated suppression seemed to be strongly dependent on the timing of infection, because chronic T_{reg} cells isolated 30 d p.i. did not show strong suppressive activity, similarly to naive T_{reg} or acute T_{reg} cells (Fig. 3A, 3B). The enhanced suppressive capacity of chronic T_{reg} cells at 16 d p.i. was confirmed by the reduction in IFN-\(\gamma\) production (Fig. 3C). In addition, coculture with chronic T_{reg} cells showed a dramatically low frequency of IFN-\(\gamma\)--producing CD8\(^+\) T cells than that with naive or acute T_{reg} cells, confirming the strong suppression of CD8\(^+\) T cell response by chronic T_{reg} cells (data not shown). Moreover, strong suppressive activity displayed by chronic T_{reg} cells was dependent on T_{reg} cell numbers that were cocultured (Fig. 3D, 3E). Similar to the result of chronic T_{reg} cell–mediated potent suppression of CD8\(^+\) T cell response, chronic T_{reg} cells also inhibited more strongly CD4\(^+\) T cell proliferation and IFN-\(\gamma\) production than naive T_{reg} or acute T_{reg} cells (Supplemental Fig. 3A–C). This chronic T_{reg} cell–mediated potent suppression of
CD4+ T cell response was also observed in T reg cell number-dependent manner (Supplemental Fig. 3D, 3E). The number of live Treg cells was higher in the naive T reg or acute T reg cell culture than in the chronic T reg 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 T reg 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 T reg cells. (A) Suppression of CD8+ T cell proliferation by T reg cells isolated at various time points after acute or chronic viral infection. CFSE-labeled CD8+ T cells were stimulated in vitro with αCD3/αCD28-coated beads for 72 h in the absence or presence of naive T reg, acute T reg, or chronic T reg 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 T reg cell population. T reg 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 chronic T reg cells. 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 T reg cells to that in the presence of T reg 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) T reg 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 T reg 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−/−

---

**FIGURE 4.** Necessity of cell-to-cell contact and PD-1 expression on chronic Treg cells for CD8+ T cell suppression. (A) Contact-dependent Treg cell–mediated suppression of CD8+ T cell proliferation. CFSE-labeled naive CD8+ T cells were stimulated in vitro with αCD3/CD28-coated beads and cocultured with chronic Treg cells (16 d p.i.) for 66 h in the absence or presence of a Transwell membrane. The left top number in the histogram indicates the percentage of proliferated CD8+ T cells. CFSE dilution in proliferated CD8+ T cells is depicted in each histogram (top) and summarized by the bar graph (bottom). (B) Proliferation profile of CD8+ T cells in (A). (C) Effect of contact-dependent chronic Treg cell–mediated suppression on IFN-γ production. Concentration of IFN-γ in the coculture media of (A) was measured. (D) Restored proliferation of CD8+ T cells by PD-1 blockade on chronic Treg cells. Isolated chronic Treg cells at 16 d p.i. were treated in vitro with isotype, αPD-1, or αPD-L1 Abs for 1 h. CellTrace Violet–labeled naive CD8+ T cells were stimulated in vitro with αCD3/CD28-coated beads for 68 h in the absence or presence of Ab-treated chronic Treg cells. CellTrace Violet 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. (E) Proliferation profile of CD8+ T cells in (D). (F) IFN-γ production of CD8+ T cells cocultured with each Ab-treated Treg cell population. Concentration of IFN-γ in the coculture media of (D) was measured. The data points in the line graphs and bar graphs represent the mean ± SEM and mean ± SEM, respectively. Data are representative of two independent experiments. n = 3 mice per group in each experiment. *p < 0.05, ***p < 0.001, ns, not significant.
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-L1 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 naive 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
immune response, demonstrating the requirement of PD-1 on Treg cells and PD-L1 on CD8+ T cells. This was supported by the observation that coculture of PD-L1−/− Treg cells and PD-1−/− CD8+ T cells, but not that of PD-1−/− Treg 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 Treg cells and effector T cells as the mechanism by which Treg cells generated during chronic virus infection display a superior suppressive activity. In summary, our study defines PD-1 upregulation on Treg cells as a prerequisite for Treg cell–mediated potent suppression of T cell immune response.

Because it has been known that activated Treg cells upregulate CD103, the Treg cells expressing CD103 during chronic viral infection in our study might dominate the population that responds to viral infection. Interestingly, CD103+ Treg cells showed significantly higher expression levels of PD-1, TIM-3, CTLA-4, and GITR than CD103− Treg cells, suggesting the difference of their function as well as phenotype. Although we did not examine the relationship between activation phenotype of Treg cells and Ag specificity in the current study, Punkosdy et al. (20) have already demonstrated very nicely that a specific subpopulation of Treg cells that expresses TCR Vβ5 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 Treg cells was demonstrated to be dependent on retroviral superantigen genes encoded in the mouse genome. Therefore, it can be speculated that most of the Treg 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 Treg cells displayed the upregulation of functional molecules, granzyme B and IL-10, which are involved in the effector function of Treg cells (Supplemental Fig. 2). First, chronic Treg cells might reduce the population of proliferating CD8+ T cells by inducing granzyme B–mediated apoptosis. Consistent with previous reports that Treg 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. Diagonostically potent T cell suppression requires the PD-1:PD-L1 interaction, having a strong suppressive activity to inhibit CD8~+~ T cell immune response (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 or 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 between chronic T~reg~ cells and CD8~+~ T cells made us propose two possible molecular mechanisms by which PD-1~high~ 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-1~high~ 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 Bim (Supplemental Fig. 2A, 2B) and Baf46 (data not shown), which are associated with T cell exhaustion (50, 51). Among these inhibitory receptors, PD-1 is also expressed on follicular Th (T~fh~) cells (52, 53). Because a large population of CD4~+~ T cells differentiates into T~fh~ cells during chronic viral infection (54), PD-1 is a hallmark of various T cell subsets, including exhausted T~reg~ and T~fh~ cells. Although the function of PD-1 expressed on T~fh~ 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 T\(_{reg}\) cells. (A) Suppression of CD8\(^+\) T cell proliferation by T\(_{reg}\) 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 T\(_{reg}\), acute T\(_{reg}\), or chronic T\(_{reg}\) 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 T\(_{reg}\) cell population. T\(_{reg}\) 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 T\(_{reg}\) cells — % of proliferated CD8\(^+\) T cells in the presence of T\(_{reg}\) cells]/[% of proliferated CD8\(^+\) T cells in the absence of T\(_{reg}\) cells]) × 100. (E) Fold reduction in IFN-γ production by CD8\(^+\) T cells cocultured with each T\(_{reg}\) 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 T\(_{reg}\) cells to that in the presence of T\(_{reg}\) 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) T\(_{reg}\) 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 containing D\(\beta\)-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 T\(_{reg}\) cells, acute T\(_{reg}\) cells, and chronic T\(_{reg}\) 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.