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Regulatory CD8\(^+\) T Cells Associated with Erosion of Immune Surveillance in Persistent Virus Infection Suppress In Vitro and Have a Reversible Proliferative Defect

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CD4\(^+\) T cell help is critical for CD8\(^+\) T cell memory and immune surveillance against persistent virus infections. Our recent data have showed the lack of CD4\(^+\) T cells leads to the generation of an IL-10–producing CD8\(^+\) T cell population during persistent murine \(\gamma\)-herpesvirus-68 (MHV-68) infection. IL-10 from these cells is partly responsible for erosion in immune surveillance, leading to spontaneous virus reactivation in lungs. In this study, we further characterized the generation, phenotype, and function of these IL-10–producing CD8\(^+\) T cells by comparing with a newly identified IL-10–producing CD8\(^+\) T cell population present during the acute stage of the infection. The IL-10–producing CD8\(^+\) T cell populations in acute and chronic stages differed in their requirement for CD4\(^+\) T cell help, the dependence on IL-2/CD25 and CD40–CD40L pathways, and the ability to proliferate in vitro in response to anti-CD3 stimulation. IL-10–producing CD8\(^+\) T cells in the chronic stage showed a distinct immunophenotypic profile, sharing partial overlap with the markers of previously reported regulatory CD8\(^+\) T cells, and suppressed the proliferation of naive CD8\(^+\) T cells. Notably, they retained the ability to produce effector cytokines and cytotoxic activity. In addition, the proliferative defect of the cells could be restored by addition of exogenous IL-2 or blockade of IL-10. These data suggest that the IL-10–producing CD8\(^+\) T cells arising in chronic MHV-68 infection in the absence of CD4\(^+\) T cell help belong to a subset of CD8\(^+\) regulatory T cells. The Journal of Immunology, 2013, 191: 000–000.

Two \(\gamma\)-herpesviruses have been identified in humans: EBV, a lymphocryptovirus, and Kapo\'s \textit{murine \(\gamma\)-herpesvirus-68} (MHV-68), a rhadinovirus, which are very prevalent pathogens. Generally, the majority of the population infected with the \(\gamma\)-herpesviruses is asymptomatic into advanced age, but the virus infection can lead to severe lymphoproliferative disease or Kapo\'s sarcoma in AIDS and immunocompromised patients due to immune surveillance failure (1–3). Exploring the mechanisms of how immune surveillance against persistent infection breaks down in such patients will benefit the development of novel approaches for controlling diseases associated with these infections.

Murine \(\gamma\)-herpesvirus-68 (MHV-68) is a rodent pathogen that is genetically closely related to EBV and Kapo\’s \textit{murine \(\gamma\)-herpesvirus}, a rhadinovirus, which are very prevalent pathogens. Generally, the majority of the population infected with the \(\gamma\)-herpesviruses is asymptomatic into advanced age, but the virus infection can lead to severe lymphoproliferative disease or Kapo\’s sarcoma in AIDS and immunocompromised patients due to immune surveillance failure (1–3). Exploring the mechanisms of how immune surveillance against persistent infection breaks down in such patients will benefit the development of novel approaches for controlling diseases associated with these infections.

MHV-68–infected mouse has been used as one of the models for investigating the immune response in chronic viral infections (4, 5). Primary infection by MHV-68 leads to acute replication of the virus, mainly in lungs (4). The acute infection is resolved after 2 wk; however, the virus subsequently establishes a latent infection in B cells (6), macrophages (7), dendritic cells (8), and lung epithelial cells (9). Control of virus replication is mediated by CD8\(^+\) T cells partly through perforin–granzyme B–, IFN-\(\gamma\)–, or Fas-dependent mechanisms (10–12). MHC class II–deficient mice, which contain very few CD4\(^+\) T cells, are able to control the primary acute infection (13) but are unable to prevent viral reactivation in lungs (14), indicating that CD4\(^+\) T cell help is not essential for primary control of MHV-68 by CD8\(^+\) T cells, but is required for long-term immune surveillance. As for other persistent virus infection models, it has become apparent that the clearance or persistence of pathogens and the equilibrium between virus and host are strongly influenced by populations of immune regulatory cells (15).

Regulatory T cells (Tregs) play an important role in the maintenance of immunologic homeostasis by suppressing immune responses in autoimmunity and infection (16, 17). Tregs are a dynamic and diverse T cell population composed of various phenotypically and functionally distinct subsets, and their differentiation and function are controlled by specific signals in the immune environment (18). Most research has focused on CD4\(^+\) Tregs; however, some subsets of regulatory CD8\(^+\) T cells, both natural and induced in humans and mice, have also attracted attention (19, 20). Naturally occurring CD8\(^+\)CD122\(^+\) Tregs mediate suppression through IL-10 (21) and have a programmed death-1 (PD-1)\(^+\) phenotype (22). Hepatitis C virus–specific CD8\(^+\) Tregs, positive for Foxp3, glucocorticoid-induced TNFR (GITR), and CTLA-4, are induced in chronically infected patients and suppress T cell proliferation in a cell contact–dependent manner (23). CD8\(^+\) CD25\(^+\)Foxp3\(^+\)LAG-3\(^+\) Tregs are induced in humans infected with mycobacteria and suppress T cells partly through the secretion of CCL4 (24). HIV Ags can induce TGF-\(\beta\)–producing (25) and IL-10–producing (26) CD8\(^+\) Tregs. However, the HIV–specific IL-10–CD8\(^+\) Tregs mediate suppression through cell–cell contact, but not via IL-10 release (26). EBV–specific CD8\(^+\)Foxp3\(^+\) Tregs induced from PBMCs of immunocompromised transplant patients produce both IL-10 and IFN-\(\gamma\) and display suppressive activity in a cell contact–dependent manner (27). These studies demonstrate that...
CD8+ Tregs can be induced in a range of different systems and exhibit different phenotypes and functions. IL-10 plays a pivotal role in controlling inflammation by suppressing APC function and inflammatory cytokine production (28). IL-10 can be produced by many different myeloid and lymphoid cells, and more than one population of IL-10–producing cells may be induced during a single infection (28). IL-10 operates primarily as a feedback inhibitor of activated T cell responses to limit the magnitude of immune responses to infections (29). Accordingly, IL-10 plays a dual role in infectious disease by preventing immunopathology and impeding pathogen clearance (30, 31). In acute virus infections, such as with influenza virus, effector T cells attenuate lung inflammation by producing IL-10 (32). In persistent virus infections, such as with HIV, IL-10 derived from multiple cell types contributes to the inhibition of virus-specific T cells (33). Blockade of IL-10 signaling has been shown to enhance immune responses in persistent virus infections such as HIV (34), hepatitis C virus (35, 36), and lymphocytic choriomeningitis virus clone 13 (37, 38).

Our previous work has shown that mice lacking CD4+ T cells lost long-term control of MHV-68 infection, and this was accompanied by an elevated level of IL-10 in the serum. The relevant source of IL-10 during the persistent infection was a population of CD8+ T cells (39). Importantly, therapeutic blockade of IL-10 improved control over viral reactivation in lungs, demonstrating IL-10 was responsible for the breakdown in immune surveillance. These data show an association between the lack of CD4+ T cell help and the generation of suppressive IL-10–producing CD8+ T cells that erode immune surveillance to persistent virus infection.

In this study, we investigated the generation, phenotype, and function of the IL-10–producing CD8+ T cells that were induced in MHV-68–infected mice during the acute and chronic stages of infection. A lack of IL-2/CD25 signaling was partially responsible for the increase in the IL-10–producing CD8+ T cell population during the chronic stage. The array of markers expressed by the IL-10–producing CD8+ T cells showed partial overlap with markers expressed by reported CD8+ Tregs. Consistent with other Treg populations, the IL-10–producing CD8+ T cells arising in the chronic phase of the infection suppressed the proliferation of T cells and lacked proliferative ability following CD3 stimulation in vitro. The proliferative defect was reversible and could be restored by addition of exogenous IL-2 or blockade of IL-10. These data suggest that the IL-10–producing CD8+ T cells in chronically MHV-68–infected and CD4+ T cell–depleted mice belong to a subset of CD8+ Tregs.

**Materials and Methods**

**Mice and viral infections**

C57BL/6 (B6) and congenic B6-Ly5.2/Cr mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred or housed in the Dartmouth-Hitchcock Medical Center mouse facility. The Animal Care and Use Program of Dartmouth College approved all animal experiments. MHV-68 (clone G2.4) originally obtained from Dr. A. A. Nash (University of Edinburgh, Edinburgh, U.K.) was propagated and stored by addition of exogenous IL-2 or blockade of IL-10. These T cells and lacked proliferative ability following CD3 stimulation.

**Preparation of splenocytes**

Single-cell suspensions were prepared by passing spleens through cell strainers, and RBCs were lysed using Gey’s solution.

**Abs and flow cytometry**

Surface markers of splenocytes were stained with Abs in PBS with 2% bovine growth serum at 4°C for 20 min. For intranuclear staining of Foxp3 and intracellular staining of CTLA-4, the Foxp3 Staining Buffer Set (eBioscience) was used. Abs used for flow cytometric analysis were as follows: CD8a FITC-eFluor 750 (53-6.7; eBioscience, San Diego, CA), Thy1.1 allophycocyanin (OX-7; BioLegend, San Diego, CA), CD45.1 allophycocyanin (A20; BioLegend), CD45.2 allophycocyanin (104; eBioscience), LAG-3 Alexa Fluor 488 (CB9TW; AbD Serotec, Raleigh, NC), GITR PE (DTA-I; Mileniy Biotech, Cambridge, MA), PD-1 Alexa Fluor 488 (RMP1-30; AbD Serotec), CD122 PE (TM-β1; BioLegend), IL-10R PE (B11.3a; BioLegend), gp130 PE (125623; R&D Systems, Minneapolis, MN), CD25 PE (PC61; BioLegend), IL-10 PE and APC (JESS-163; eBioscience), IFN-γ allophycocyanin (XM1G1.2; BioLegend), granzyme B PE (GB11; Invitrogen, Carlsbad, CA), TNF-α PE (MPE-XT22; BioLegend), IL-2 PE (JS-6E5; H4; BioLegend), CTLA-4 PE (UC10-4B9; BioLegend), and Foxp3 PE (FJK-16-16; eBioscience). Samples were analyzed using FACSFlow (BD Biosciences, San Jose, CA) or Accuri flow cytometers (Accuri, Ann Arbor, MI) in the Dartlab core facility. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) or Accuri software (Accuri).

**Intracellular cytokine/molecule staining**

Splenocytes were restimulated with 50 ng/ml PMA (Sigma-Aldrich, Milwaukee, WI) and 1 μg/ml ionomycin calcium salt from Streptomyces conglubus (Sigma-Aldrich) in complete medium with 10 U/ml rIL-2 and 10 μg/ml brefeldin A (Sigma-Aldrich) at 37°C for 5 h. Cells were stained with Abs against surface markers at 4°C for 20 min, followed by fixation with 2% formaldehyde at room temperature (RT) for 20 min, and permeabilization with 0.5% saponin solution at RT for 10 min. Cells were then stained with Abs against IL-10, IFN-γ, granzyme B, TNF-α, or IL-2 in 0.5% saponin solution at 4°C for 30 min.

**Tetramer staining**

MHC/peptide tetramers for ORF61524–531 (TSINFVKIK) epitope conjugated to allophycocyanin were obtained from the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). Splenocytes were stained for 1 h at RT in dark and analyzed by flow cytometry as previously described (40). To measure the frequency of ORF61524–531+ specific cells within IL-10−CD8+ T cell populations, splenocytes were restimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in complete medium with 10 U/ml rIL-2 and 10 μg/ml brefeldin A at 37°C for 5 h. Splenocytes were then stained with ORF61532–531 tetramer for 1 h at RT in the presence of brefeldin A, further stained with Abs against surface markers, fixed, and permeabilized as described above.

**In vitro cytotoxicity assay**

Splenocytes were prepared from 10BiT mice depleted of CD4+ cells and infected with MHV-68 for 42 d and stained with anti-Thy1.1 and anti-CD8a Abs. Thy1.1+CD8+ T cells were sorted using a FACSaria cell sorter (BD Biosciences). Targets for cytotoxicity assay were prepared from spleens of naive C57BL/6 mice and incubated with or without ORF61524–531 peptide (1 μg/ml) in complete medium at 37°C for 2 h. The ORF61524–531 pulsed and unpulsed splenocytes (106/ml) were labeled with 2 μM and 0.2 μM CFSE (Sigma-Aldrich), respectively, in HBSS at RT for 10 min. The target cells (4 × 105) were cultured with Thy1.1+ or Thy1.1− CD8+ T cells at an E:T ratio of 30:1 at 37°C for 22 h. In the culture, effective E:T ratio was 2:1 because the frequency of ORF61532–531+ specific
CD8+ T cells among Thy1.1+CD8+ T cells was 6.7% (Supplemental Fig. 2). Cells were then stained with 10 μM 7-aminoactinomycin D (BD Biosciences) at 4°C for 10 min. Cells were analyzed by flow cytometry, and specific lysis was calculated using the following formulas: ratio = number of peptide pulsed cells/number of unpulsed cells; and % specific lysis = (1 − [ratio of Thy1.1+ or Thy1.1− CD8 group/ratio of target only group]) × 100%.

Suppression assay

Splenocytes were prepared from 10BiT mice depleted of CD4+ cells and infected with MHV-68 for 42 d, and Thy1.1+CD8+ T cells were sorted. Responder CD8+ T cells were prepared from spleens of naive B6-Ly5.2 mice and purified using EasySep Mouse CD8+ T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada).Responder cells (5 × 10^5) were labeled with 0.5 μM CFSE and cultured with Thy1.1+CD8+ T cells at ratios of 1:2 to 1:6 (responder cell/Thy1.1+CD8+ cell) in 96-well plates coated with 5 μg/ml anti-CD3ε Ab (eBioscience) in complete medium at 37°C for 72 h. As controls, responder cells were cultured alone or with Thy1.1+CD8+ T cells. In some experiments, IL-10R blocking Ab (1B1.3a; BioXCell) or control RatIgG at 20 μg/ml was added to medium in the beginning of culture. For transwell (HTS Transwell-96 system; Corning, Lowell, MA) experiments, Thy1.1+CD8+ T cells (4.5 × 10^5) were cultured in the top well, and CFSE-labeled responder CD8+ T cells (1 × 10^5) were cultured in the bottom well coated with 5 μg/ml anti-CD3ε Ab. Thy1.1+CD8+ T cells or media alone in the top wells were used as controls. Cells were cultured in complete medium at 37°C for 72 h, and CFSE dilution of responder cells was determined by flow cytometry.

CFSE proliferation assay of IL-10–producing CD8+ T cells

Splenocytes were prepared from 10BiT mice depleted of CD4+ cells and infected with MHV-68 for 14, 42, or 105 d. The splenocytes were labeled with 0.5 μM CFSE and then cultured in 96-well plates coated with/without 5 μg/ml anti-CD3ε Ab in complete medium at 37°C for 72 h. After staining with anti-Thy1.1 and anti-CD8α Abs, the proliferation of Thy1.1+CD8+ T cells based on CFSE dilution was analyzed by flow cytometry. For proliferation rescue experiments, 10 U/ml rIL-2, 10 μg/ml agonistic Abs to OX-40 (OX-86; BioXCell) or CD27 (RM27-3E5, provided by Dr. Hideo Yagita, Juntendo University, Tokyo, Japan), 20 μg/ml IL-10R blocking Ab, or 20 μg/ml RatIgG were added to the culture medium at the beginning of the culture.

Statistical analysis

Student t tests were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA). Values of p < 0.05 were considered statistically significant.

Results

Generation of IL-10–producing CD8+ T cells in MHV-68–infected mice

We first examined the kinetics of IL-10–producing CD8+ T cell appearance in MHV-68–infected mice with or without CD4+ T cell help. Intact B6 (wild-type [WT]) mice and CD4+ cell–depleted (anti-CD4) mice were infected with MHV-68. At different time points postinfection (pi), splenocytes were prepared, and IL-10 production was measured by intracellular cytokine staining. During the acute phase of infection (day 14 pi), 1.1% of CD8+ T cells produced IL-10 in the WT mice, whereas the proportion was approximately half of that in the anti-CD4 mice (Fig. 1A). During the chronic phase of infection (day 42 pi), consistent with our previous study (39), the proportion of IL-10+CD8+ T cells in the WT mice decreased slightly from 1.1 to 0.9%, whereas the proportion in the anti-CD4 mice increased from 0.5 to 4.5% (Fig. 1B). The frequency and total number of IL-10+CD8+ T cells in MHV-68–infected mice increased only in the absence of CD4+ cells, reached a peak at day 42 pi, and then declined but remained at significantly higher levels until day 105 pi compared with the WT mice (Fig. 1C).

To assess the proportion of MHV-68–specific cells within the IL-10–producing CD8+ T cells in WT and anti-CD4+ mice, we performed tetramer staining with ORF61524–531, a dominant epitope of MHV-68. The frequencies of ORF61524–531–specific cells within
IL-10+CD8+ T cells were 9.8 and 9.5% at day 14 pi and 7.4 and 6.1% at day 42 pi for WT and anti-CD4 mice, respectively (Fig. 1D).

**Deficiency of IL-2 signaling is partially responsible for the increase in IL-10–producing CD8+ T cells during the chronic phase of infection**

CD4+ T cell–derived IL-2 is an essential mediator of help for CD8+ T cell responses in various systems (41), and CD25 (IL-2 receptor α-chain) expression, which is upregulated by CD4+ T cell help, controls the expansion and differentiation of CD8+ T cells (42). To explore the role of IL-2 signaling in the generation of IL-10–producing CD8+ T cells in our system, we generated mixed BM chimeric mice containing both CD25+/+ (CD45.1+) and CD25−/− (CD45.2+) cells, which could be distinguished by staining for the congenic markers (Fig. 2A). CD25−/− mice could not be used directly because CD25−/− mice develop severe autoimmune disease due to lack of Tregs (43). Intact or CD4-depleted chimeric mice were infected with MHV-68, and the frequency of IL-10+ CD8+ T cells was compared in the presence or absence of IL-2 signaling. At day 14 pi, the frequency of IL-10–producing cells was similar in CD25+/+ and CD25−/− CD8+ T cells (Fig. 2B). In contrast, at day 40 pi, the frequency of IL-10–producing cells was significantly increased in intact mice in the CD25−/− CD8+ T cell compartment compared with the CD25+/+ CD8+ T cell compartment, although not reaching the levels seen in the CD4 T cell–depleted groups. Deficiency in IL-2 signaling was responsible for the increase in IL-10–producing CD8+ T cells, suggesting that IL-2 is partially responsible for transmitting CD4 help during the chronic phases of infection.

**Generation of IL-10–producing CD8+ T cells is mediated by the CD40–CD40L pathway during the acute but not the chronic phase of infection**

Among the multiple mechanisms of CD4 help, interaction of CD40L on CD4+ T cells with CD40 on APCs has also been shown to play a critical role in CD8+ T cell responses (44). Signaling via CD40 can replace CD4 help in priming some CD8+ T cell responses (45–47) and in preventing reactivation of MHV-68 in lungs of CD4+ T cell–deficient mice (48). Accordingly, we investigated the contribution of CD40–CD40L pathway to the generation of IL-10–producing CD8+ T cells. During the acute phase of infection (day 14 pi), the frequency of IL-10+ CD8+ T cells decreased to approximately one half in both CD4+ cell–depleted and CD40L–blocked mice when compared with intact mice (Fig. 3A). In contrast, during the chronic phase (day 42 pi),...
increase of IL-10–producing CD8+ T cells was observed only in CD4+ T cell–depleted mice, but not in intact and CD40L-blocked mice (Fig. 3B). Therefore, CD40-CD40L–mediated CD4 help is required for the generation of IL-10+CD8+ T cells during the acute phase but not the chronic phase of infection. Unlike the absence of CD4+ T cells, the absence of CD40–CD40L signaling is not sufficient to result in the increase of IL-10+CD8+ T cells during the chronic phase.

IL-27 is dispensable for generation of IL-10–producing CD8+ T cells in both acute and chronic phases of infection

IL-27 is a potent inducer of IL-10 in murine CD4+ and CD8+ T cells (49–51) and is composed of the IL-27p28 and Ebi3 subunits (52). Ebi3 is a subunit of both IL-27 and IL-35 (53). By using Ebi3−/− mice, we investigated whether IL-27 was necessary for generation of the IL-10–producing CD8+ T cells in MHV-68 infection. At both days 14 and 55 pi, the absence of the Ebi3 did not affect the tendencies observed in the WT and anti-CD4 mice (Supplemental Fig. 1A, 1B), indicating that IL-27 is not required for the generation of IL-10–producing CD8+ T cells in either acute or chronic phases of infection.

Phenotype of IL-10–producing CD8+ T cells

CD8+ Tregs with various phenotypes have been reported in different systems, but specific markers for identification of these cells are still elusive. To further characterize the IL-10–producing CD8+ T cells in our system, we analyzed seven markers that have been reported to be associated with CD8+ Tregs, including LAG-3, GITR, PD-1, CD122 (IL-2Rβ or IL-15Rβ), Foxp3, CTLA-4, and CD25. We also measured the expression of IL-10R and gp130 (a component of the receptor for IL-27 and other IL-6 family cytokines). Of these markers, CTLA-4 was analyzed by intracellular staining because the majority of CTLA-4 is not expressed on the surface but is sequestered intracellularly (54). We used the 10BiT

FIGURE 4. Phenotypes of IL-10–producing CD8+ T cells. Splenocytes were prepared from CD4+ cell–depleted and infected 10BiT mice at days 14 (A) and day 42 pi (B). Expressions of surface markers, intranuclear Foxp3, and intracellular CTLA-4 were determined by staining with specific Abs. Histograms were gated on Thy1.1+ (bold line) or Thy1.1− (thin line) CD8+ T cell population. Fluorescence minus one (FMO) was used as a control (filled). Mean fluorescence intensity (MFI) value shown on histogram is the mean in a single experiment, calculated by subtracting MFI value of FMO from that of Thy1.1+ or Thy1.1− CD8+ T cells. Percentage shown on histogram is the mean from multiple experiments. Graphs were based on MFI or percent positive within the Thy1.1+ or Thy1.1− CD8+ T cell population. Each point represents a single mouse. Data are representative of three independent experiments with three to four mice per group. *p < 0.05, **p < 0.01, ***p < 0.0001.
mice in which the Thy1.1 gene is under control of the IL-10 promoter. When the IL-10 promoter is active, the cells express Thy1.1 on their surface. We analyzed Thy1.1 expression instead of intracellular IL-10 staining to avoid possible phenotype changes caused by PMA/ionomycin stimulation.

Thy1.1\(^+\)CD8\(^+\) T cells (IL-10 producers) expressed higher levels of inhibitory receptors LAG-3, GITR, and PD-1 than Thy1.1\(^-\) CD8\(^+\) T cells at both day 14 pi (Fig. 4A) and day 42 pi (Fig. 4B). CD122 and IL-10R expression was also higher in Thy1.1\(^+\)CD8\(^+\) T cells. Only a small proportion of Thy1.1\(^+\)CD8\(^+\) T cells expressed Foxp3 (8.6%), CTLA-4 (15%), and CD25 (11.6%) at day 14 pi (Fig. 4A), and this proportion declined further at day 42 pi to 3.6, 3.5, and 5.6%, respectively (Fig. 4B), although the proportion was higher in Thy1.1\(^+\)CD8\(^+\) T cells than Thy1.1\(^-\) CD8\(^+\) T cells. In contrast to the markers described above, a lower frequency of Thy1.1\(^+\)CD8\(^+\) T cells expressed gp130 than the Thy1.1\(^-\) CD8\(^+\) T cells during both the acute (13.7 versus 34.6%) and chronic phase (7.8 versus 50.4%). These data indicated the array of markers expressed by the IL-10–producing CD8\(^+\) T cells in this system shows partial overlap with markers expressed by reported CD8\(^+\) Tregs.

IL-10–producing CD8\(^+\) T cells produce effector cytokines and mediate cytotoxicity in vitro

We have previously reported that a smaller proportion (30%) of Thy1.1\(^+\)CD8\(^+\) T cells produced IFN-\(\gamma\) and TNF-\(\alpha\) after anti-CD3 stimulation compared with the proportion (50%) of Thy1.1\(^+\)CD8\(^+\) T cells (39). In the current study, we stimulated the cells with PMA/ionomycin for 5 h, which is favorable for detection of intracellular IL-10 production. We measured intracellular IL-10 and effector molecule production at the same time. The proportions of the IL-10–producing CD8\(^+\) T cells producing IFN-\(\gamma\), granzyme B, TNF-\(\alpha\), and IL-2 were 95, 74, 57, and 20% at day 14 pi (Fig. 5A) and 94, 52, 58, and 6% at day 42 pi (Fig. 5B), respectively. Comparing the two time points, similar frequencies of IL-10–producing CD8\(^+\) T cells produced IFN-\(\gamma\) and TNF-\(\alpha\), whereas higher frequencies of IL-10–producing CD8\(^+\) T cells from day 14 pi produced granzyme B and IL-2 compared with the cells from day 42 pi (Fig. 5C). These results indicated that most of the IL-10–producing CD8\(^+\) T cells could produce IFN-\(\gamma\), and about half of them could produce granzyme B and TNF-\(\alpha\).

Because granzyme B is one of the effector molecules for cytotoxicity, we tested if IL-10–producing CD8\(^+\) T cells had killing ability. Thy1.1\(^+\) or Thy1.1\(^-\) CD8\(^+\) T cells from day 42 pi were cultured with ORF61\(_{524-531}\)-pulsed target cells at different ratios. At an E:T ratio of 30:1, the specific lysis by the Thy1.1\(^+\) and Thy1.1\(^-\) CD8\(^+\) T cells were 80 and 40%, respectively (Fig. 5D, 5E). This difference of lysis by Thy1.1\(^+\) and Thy1.1\(^-\) cells was likely due to the lower frequency of ORF61\(_{524-531}\)-specific CD8\(^+\) T cells in the Thy1.1\(^+\) CD8\(^+\) T cells (2.8%) compared with the frequency in the Thy1.1\(^+\) CD8\(^+\) T cell population (6.7%) (Supplemental Fig. 2). Therefore, IL-10–producing CD8\(^+\) T cells displayed cytotoxic function comparable to that of non–IL-10–producing CD8\(^+\) T cells in vitro.

IL-10–producing CD8\(^+\) T cells suppress proliferation of naive CD8\(^+\) T cells in vitro in a cell contact–independent manner

To investigate whether the IL-10–producing CD8\(^+\) T cells have suppressive ability, we performed a suppression assay in vitro. Responder cells were prepared from splenocytes of naive mice and labeled with CFSE, an intracellular fluorescent dye for which intensity reduces by half after every cell division. When the responder cells were cultured alone or with Thy1.1\(^+\) or Thy1.1\(^-\) CD8\(^+\) T cells, 83 and 90% of the responder cells proliferated upon anti-CD3 stimulation (Fig. 6A). In contrast, when cultured with Thy1.1\(^+\) CD8\(^+\) T cells, only a small proportion of Thy1.1\(^+\)CD8\(^+\) T cells expressed gp130 than the Thy1.1\(^-\) CD8\(^+\) T cells during both the acute (13.7 versus 34.6%) and chronic phase (7.8 versus 50.4%). These data indicated the array of markers expressed by the IL-10–producing CD8\(^+\) T cells in this system shows partial overlap with markers expressed by reported CD8\(^+\) Tregs.
CD8+ T cells, only 44% of the responder cells proliferated, indicating that the Thy1.1+CD8+ T cells suppressed the proliferation of naive CD8+ T cells in vitro.

To determine if the suppression mediated by IL-10–producing CD8+ T cells requires direct contact with the responder cells, we used a transwell system that can prevent cell–cell contact but allow soluble proteins to pass through. Even without cell contact, the proliferation of responder cells was inhibited to 30%, compared with the responder cells cultured alone (71%) or cultured with Thy1.1+CD8+ T cells (90%) (Fig. 6B). These results indicate that IL-10–producing CD8+ T cells suppress the proliferation of responder cells in a cell contact–independent manner.

**FIGURE 6.** IL-10–producing CD8+ T cells suppress naive CD8+ T cell proliferation in vitro in an IL-10– and cell contact–independent manner. CD4+ cell–depleted 10BiT mice (CD45.2+) were infected for 42 d, and splenic Thy1.1+ or Thy1.1+ CD8+ T cells were purified from naive Ly5.2 mice (CD45.1+), labeled with CFSE, and cultured either alone or with Thy1.1- or Thy1.1+ CD8+ T cells for 72 h in the presence/absence of anti-CD3ε Ab. Cells were then stained with anti-CD8α and anti-CD45.1 Abs. The suppressive ability of Thy1.1+CD8+ T cells was determined by CFSE dilution of responder cells. Histograms were gated on CD45.1+CD8+ T cells. Vertical lines were inserted to compare CFSE dilution between plots, and percentages are frequencies of CFSE low cells within responder cells. (A) Thy1.1- or Thy1.1+ CD8+ T cells were cultured with responder cells in a single well. (B) Cells were cultured in a transwell system. Responder cells were cultured in the bottom wells coated with/without anti-CD3ε Ab, whereas the top wells contained medium alone, Thy1.1-, or Thy1.1+ CD8+ T cells. (C) Cells were cultured in a single well in the presence of either IL-10R blocking Ab or Rat-IgG. Data are representative of five (A) and two (B, C) independent experiments.

**FIGURE 7.** IL-10–producing CD8+ T cells from the acute phase of infection can proliferate in vitro but those from the chronic phase fail to proliferate. CD4+ cell–depleted 10BiT mice were infected for 14, 42, or 105 d. Splenocytes were labeled with CFSE and cultured with/without anti-CD3 stimulation for 72 h. Cells were then stained with anti-Thy1.1 and anti-CD8α Abs and proliferation of Thy1.1+ and Thy1.1- CD8+ T cells were determined by CFSE dilution. (A) Representative FACS plots and histograms. Thy1.1+ (bold) or Thy1.1- (thin) CD8+ T cells were from the same mouse splenocytes stimulated by anti-CD3ε Ab, and the control (filled) was CD8+ T cells from nonstimulated splenocytes. Percentages shown on the histograms are the mean frequency of CFSE-diluted cells within the Thy1.1+ or Thy1.1- CD8+ T cell population. (B) Pooled data from two experiments. Each point represents a single mouse. Data are representative of three independent experiments for day 14 pi, five for day 42, and two for day 105 with three to four mice per group. ***p < 0.0001.
To examine if IL-10 was responsible for the suppressive activity of IL-10–producing CD8+ T cells in vitro, we added IL-10R blocking Ab to the system. Unexpectedly, IL-10R blockade did not antagonize the suppressive activity when the responder cells were incubated with Thy1.1+CD8+ T cells (Fig. 6C), suggesting a possible presence of inhibitory factor(s) besides IL-10 in the system.

We also determined whether TGF-β and IL-35, two suppressive cytokines produced by Tregs (55), were involved in the inhibition of T cell proliferation. The surface expression of latency-associated peptide (LAP), an N-terminal propeptide of TGF-β precursor, has been used to characterize TGF-β-dependent Tregs (56, 57). We tested surface expression of LAP and found that the frequency of LAP cells within IL-10–producing CD8+ T cells was 5.5% at day 59 pi (data not shown). Because ~95% of the IL-10–producing CD8+ T cells were negative for LAP, TGF-β is unlikely to contribute to the suppression in vitro. We further tested IL-35 levels in the supernatants collected from cultures of Thy1.1+CD8+ and Thy1.1+CD8+ T cells in the in vitro suppression assay, and there was no difference between the two supernatants (data not shown). These results indicate that TGF-β or IL-35 are not responsible for the suppression of proliferation in vitro.

**IL-10–producing CD8+ T cells fail to proliferate in vitro but can be rescued by IL-2 or IL-10R blockade**

Failure to proliferate in vitro upon antigenic stimulation is one of the characteristics of Tregs (58, 59). To evaluate whether the IL-10–producing CD8+ T cells in this system also had this property, we measured the proliferative ability of Thy1.1+CD8+ T cells upon anti-CD3 stimulation. At day 14 pi, 80% of Thy1.1+CD8+ T cells and 97% of Thy1.1+CD8+ T cells proliferated (Fig. 7A, 7B). At days 42 and 105 pi, however, only 15 and 23% of Thy1.1+CD8+ T cells proliferated, which was much lower than 78 and 79% of the Thy1.1+CD8+ T cells (Fig. 7A, 7B). These data indicate that most of IL-10–producing CD8+ T cells from the acute phase of infection could proliferate in vitro, whereas most IL-10–producing CD8+ T cells from the chronic phase failed to proliferate.

In the presence of exogenous IL-2, CD4+CD25+ Tregs have been shown to proliferate in vitro following antigenic stimulation (58, 59). Signaling through OX-40, a T cell costimulatory molecule belonging to TNFR family, has also been shown to restore CD4+CD25+ Treg proliferation in vitro (60). Signaling through CD27, another TNFR family, is critical for T cell expansion and survival (61). In this experiment, we tested if rIL-2, anti–OX-40, or anti-CD27 agonistic Abs and IL-10R blockade could rescue the proliferative ability of the IL-10–producing CD8+ T cell in vitro. Exogenous rIL-2 rescued the proliferation of Thy1.1+CD8+ T cells, resulting in increase of CFSE-diluted cells from 18 to 64% (Fig. 8A, 8B). OX-40 and CD27 agonistic Abs had no effect on proliferation of these cells (Fig. 8A, 8B). Interestingly, IL-10R blockade also rescued the proliferation of Thy1.1+CD8+ T cells, with the CFSE-diluted cells increasing from 18 to 82%, which was similar to the levels achieved by Thy1.1+CD8+ T cells (Fig. 8). Therefore, the failure to proliferate in vitro can be reversed by exogenous IL-2 or by blockade of IL-10 signaling.

**Discussion**

Long-term immune surveillance failure is observed in MHV-68–infected mice in the absence of CD4+ T cell help, which is not due to reduced numbers or dysfunction of the virus-specific CD8+ T cells. Actually, the numbers of virus-specific CD8+ T cells increase in the lymphoid tissue, and the antiviral functions such as IFN-γ secretion and cytotoxicity of the CD8+ T cells are mostly normal (39, 62). We have previously identified a population of IL-10–producing CD8+ T cells that arise in CD4+ T cell–depleted MHV-68–infected mice and is associated with breakdown of long-term immune surveillance (39).

In this study, we characterized two populations of IL-10–producing CD8+ T cells during different stages of MHV-68 infection. In the acute phase, we observed more IL-10–producing CD8+ T cells in CD4-sufficient mice, whereas in the chronic phase, IL-10–producing CD8+ T cells increased only in CD4-deficient mice.

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**FIGURE 8.** IL-2 or IL-10R blocking Ab can rescue the proliferation of IL-10–producing CD8+ T cells in vitro. CD4+ cell–depleted 10BiT mice were infected for 42 d. Splenocytes were labeled with CFSE and cultured with anti-CD3 stimulation in the presence of rIL-2, agonistic Abs against OX-40 or CD27, IL-10R blocking Ab, or RatIgG for 72 h. Cells were then stained with anti-Thy1.1 and anti-CD8 Abs, and proliferation of Thy1.1+ and Thy1.1–CD8+ T cells was measured by CFSE dilution. (A) Representative FACS plots and histograms. Percentages shown on the histograms are the mean frequency of CFSE-diluted cells within the Thy1.1+ (bold) or Thy1.1– (thin) CD8+ T cell population. (B) Each point represents a single mouse. Data are representative of three to five independent experiments with three to four mice per group. ***p < 0.0001.
The IL-10–producing populations in acute and chronic phases differed in their requirements for CD4+ T cell help (Fig. 1), the dependence on IL-2/CD25 and CD40–CD40L pathways (Figs. 2, 3) and the ability to proliferate in vitro in response to anti-CD3 stimulation (Fig. 7). IL-10–producing CD8+ T cells increased in the absence of IL-2 signaling during the chronic phase of infection (Fig. 2), suggesting that IL-2 deficiency is partially responsible for the increase of IL-10–producing CD8+ T cells in the absence of CD4+ T cells. The proportion of MHV-68–specific cells within the IL-10–producing CD8+ T cells is still unclear, but we confirmed that 6–10% of the IL-10–producing CD8+ T cells were specific to ORF61524–531, a dominant epitope of MHV-68 (Fig. 1D).

IL-27 has been shown to induce CD4+ and CD8+ T cells to produce IL-10 (49–51). Studies on influenza infection have shown that IL-10 production by CD8+ T cells is dependent in part on IL-27 from innate cells (63). In MHV-68 infection, however, IL-27 was not necessary for the CD8+ T cells to produce IL-10 either during the acute or chronic phases of infection (Supplemental Fig. 1). This was further confirmed by the fact that most of the IL-10–producing CD8+ T cells did not express gp130, one subunit of the IL-27R (Fig. 4).

Various markers have been reported to associate with CD8+ Tregs, such as LAG-3, GITR, PD-1, CD122, Foxp3, CTLA-4, and CD25. In this system, we observed the IL-10–producing CD8+ T cells expressing higher levels of LAG-3, GITR, PD-1, and CD122 compared with non–IL-10–producing CD8+ T cells in both acute and chronic phases of the infection. In contrast, the majority of IL-10–producing CD8+ T cells were negative for Foxp3, CTLA-4, and CD25 (Fig. 4). The IL-10–producing CD8+ T cells show a phenotype that partially overlaps with the reported CD8+ Tregs but have a distinct phenotypic profile.

Tregs suppress immune responses by several mechanisms including the production of anti-inflammatory cytokines, such as IL-10, TGF-β, and IL-35, direct cell–cell contact and the modulation of the functions of APCs (55). In this study, IL-10–producing CD8+ T cells suppressed proliferation of naive CD8+ T cells in vitro in a cell contact–independent manner (Fig. 6A, 6B). Blockade of IL-10R did not antagonize the suppression (Fig. 6C), suggesting the existence of suppressive factors besides IL-10. Although IL-10–mediated suppression of target T cell proliferation was not observed in vitro, we have clear evidence for the suppressive role of IL-10 in vivo, because IL-10R blockade led to increased TNF-α production and better control of MHV-68 reactivation (39). We also confirmed that TGF-β and IL-35 are unlikely to be responsible for the suppression in vitro. However, we cannot rule out the possibility that a combination of IL-10, TGF-β, IL-35, and other suppressive factors may together result in inhibition in this system.

Some regulatory cells can produce effector molecules, such as IFN-γ, perforin, and granzymes A and B (27, 64). In many chronic infections, CD4+ T cells that produce high levels of both IL-10 and IFN-γ have been documented (65). Similarly, the IL-10–producing CD8+ T cells in our system produced effector molecules such as IFN-γ, TNF-α, and granzyme B (Fig. 5A–C) and exhibited cytotoxicity against target cells loaded with an MHV-68 epitope (Fig. 5D, 5E), indicating these IL-10–producing CD8+ T cells are polyfunctional. Foxp3 expression has been reported to prevent deviation of Tregs into effector T cell lineages (66). Loss of Foxp3 or its diminished expression in Tregs leads to acquisition of effector T cell properties including production of cytokines such as IL-2, IL-4, IL-17, and IFN-γ (67, 68). Accordingly, lack of Foxp3 expression in the IL-10–producing CD8+ T cells may have resulted in the production of various effector molecules in our system. However, several studies have also shown Foxp3+ Tregs can produce effector cytokines (27, 64). Further research is needed to clarify this difference. We have previously reported that the IL-10–producing CD8+ T cells are partly responsible for erosion in immune surveillance, leading to spontaneous virus reactivation in lungs of MHV-68–infected mice (39). This indicates the IL-10–mediated suppressive activity is the dominant function of the polyfunctional CD8+ T cell in vivo. The ability of CD8 T cells to elaborate both antiviral effector functions and suppressive factors such as IL-10 highlights the plasticity of T cell differentiation. The lineage relationships between these different CD8 T cell subsets are unclear at present, and it remains an open question whether they represent terminally differentiated cell types or have the ability to covert from an effector to a regulatory phenotype or vice versa.

Tregs have been shown to be nonproliferative in vitro (58, 59). We found that most of the IL-10–producing CD8+ T cells from the acute phase can proliferate, but those from the chronic phase cannot (Fig. 7). Exogenous IL-2 or IL-10R blocking Ab rescued the proliferation of IL-10–producing CD8+ T cells (Fig. 8). IL-2R consists of IL-2Rα (CD25), β (CD122), and γ (CD132) subunits, which form a high-affinity trimeric IL-2R. In the absence of IL-27 expression, IL-2 can still signal if cells express high levels of CD122 and CD132 (69). In our system, although most of the IL-10–producing CD8+ T cells did not express CD25, they expressed high levels of CD122, allowing the cells to be sensitive to IL-2 signaling. Blockade of IL-10R rescued the proliferation of the IL-10–producing CD8+ T cells, suggesting IL-10 may act as an autocrine/paracrine cytokine in vitro. Furthermore, the IL-10–producing CD8+ T cells expressed significantly higher levels of IL-10R than non–IL-10–producing CD8+ T cells (Fig. 4B) and naive CD8+ T cells (data not shown), which could explain why IL-10–producing CD8+ T cells themselves were suppressed by IL-10 (Fig. 8), but the naive CD8+ T cells in the suppression assay were not (Fig. 6C).

We describe an IL-10–producing CD8+ T cell population arising in chronic MHV-68 infection in the absence of CD4+ T cell help and showing an immunophenotypic profile of Treg populations. The increase of IL-10–producing CD8+ T cells during the chronic phase of the infection is associated with immune suppression and may be generated aberrantly as the delicate balance of immune homeostasis is perturbed by the presence of chronic virus replication in the lungs. Enhanced understanding of this balance may contribute to skewing the immune response away from suppression and lead to better control of persistent virus infections.

Disclosures
The authors have no financial conflicts of interest.

References


Regulatory CD8+ T cells in persistent virus infection.


