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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 Defect¹

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Abstract

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 γ -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 acute stage of the infection. The IL-10 producing CD8⁺ 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 naïve CD8⁺ T cells. Notably, they retained the ability to produce effector cytokines and the 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⁺ T regulatory cells.

Introduction

Two γ -herpesviruses have been identified in humans: EBV, a lymphocryptovirus, and Kaposi's sarcoma-associated herpesvirus (KSHV), a rhadinovirus, which are very prevalent pathogens. Generally, the majority of the population infected with the γ -herpesviruses are asymptomatic into advanced age, but the virus infection can lead to severe lymphoproliferative disease or Kaposi's sarcoma in AIDS and immunocompromised patients due to immune surveillance failure (1–3). Exploring the mechanisms 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 γ -herpesvirus-68 (MHV-68) is a rodent pathogen that is genetically closely related to EBV and KSHV. 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

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resolved after 2 weeks, 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- γ - 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).

T regulatory 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 PD-1⁺ (programmed death 1) phenotype (22). Hepatitis C virus (HCV)-specific CD8⁺ Tregs, positive for Foxp3 (transcription factor forkhead box p3), GITR (glucocorticoid-induced tumor necrosis factor receptor) 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⁺ (lymphocyte activation gene-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- β 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 PBMC of immunocompromised transplant patients produce both IL-10 and IFN- γ , 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), HCV (35, 36) and lymphocytic choriomeningitis virus (LCMV) clone13 (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 the 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 National Cancer Institute (Frederick, MD). 10BiT mice (with Thy1.1 gene under the control of IL-10 promoter) were provided by Dr. Casey Weaver (University of Alabama, Birmingham, AL). CD25^{-/-} mice (B6.129S4-Il2ra^{tm1Dw/J}) and Ebi3^{-/-} mice (B6.129X1-Ebi3^{tm1Rsb/J}) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred or housed in 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 titered by using NIH 3T3 cells as previously described (4). For infection, mice were intranasally (i.n.) given 400 PFU of MHV-68 under anesthesia with isoflurane.

Generation of mixed bone marrow chimeric mice

B6-Ly5.2 recipient mice were lethally irradiated with 1000 rads (500 rad twice given 24h apart) and subsequently injected i.v. with a total of 4.5×10^6 bone marrow (BM) cells containing a mixture of B6-Ly5.2 (CD45.1⁺) and CD25^{-/-} (CD45.2⁺) cells at a 1:2 ratio. Reconstitution of the BM was checked 50 d post-BM transfer by staining blood cells for the congenic markers.

Depletion of CD4⁺ T cells and blocking of CD40-CD40L pathway

Mice were administered i.p. 500 µg of anti-CD4 Ab (GK1.5) for depletion of CD4⁺ T cells and anti-CD40L Ab (MR-1, BioXcell, West Lebanon, NH) for blocking of CD40-CD40L pathway, respectively, at days -1 and 0 of infection, followed by 250 µg twice weekly thereafter until the mice were sacrificed at days 14 to 105 for all the experiments. Control mice were either untreated or given RatIgG (Jackson ImmunoResearch, West Grove, PA) or HamIgG (Rockland, Gilbertsville, PA).

Preparation of splenocytes

Single cell suspensions were prepared by passing spleens through cell strainers and red blood cells 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, Foxp3 Staining Buffer Set (eBioscience) was used. Abs used for flow cytometric analysis were as follows: CD8a PerCP-eFluor 710 (53–6.7, eBioscience, San Diego, CA), Thy1.1

APC (OX-7, Biolegend, San Diego, CA), CD45.1 APC (A20, Biolegend), CD45.2 APC (104, eBioscience), LAG-3 Alexa Fluor 488 (C9B7W, AbD Serotec, Raleigh, NC), GITR PE (DTA-1, Miltenyi Biotec, Cambridge, MA), PD-1 Alexa Fluor 488 (RMP1-30, AbD Serotec), CD122 PE (TM- β 1, Biolegend), IL-10R PE (1B1.3a, Biolegend), gp130 PE (125623, R&D systems, Minneapolis, MN), CD25 PE (PC61, Biolegend), IL-10 PE and APC (JES5-16E3, eBioscience), IFN- γ APC (XMG1.2, Biolegend), granzyme B PE (GB11, Invitrogen, Carlsbad, CA), TNF- α PE (MP6-XT22, Biolegend), IL-2 PE (JES6-5H4, Biolegend), CTLA-4 PE (UC10-4B9, Biolegend) and Foxp3 PE (FJK-16s, eBioscience). Samples were analyzed using FACSCanto or Accuri flow cytometers in the Dartlab core facility. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) or Accuri software (Ann Arbor, MI).

Intracellular cytokine/molecule staining

Splenocytes were restimulated with 50 ng/ml PMA (Sigma-Aldrich, Milwaukee, WI) and 1 μ g/ml ionomycin calcium salt from *Streptomyces conglobatus* (Sigma-Aldrich) in complete medium with 10 U/ml rIL-2 and 10 μ g/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/K^b (TSINFVKI) epitope conjugated to APC were obtained from the NIH 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 cells, splenocytes were restimulated with 50 ng/ml PMA and 1 μ g/ml ionomycin in complete medium with 10 U/ml rIL-2 and 10 μ g/ml brefeldin A at 37°C for 5 h. Splenocytes were then stained with ORF61₅₂₄₋₅₃₁ 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 days, and were stained with anti-Thy1.1 and anti-CD8 α Abs. Thy1.1⁺CD8⁺ T cells were sorted using a FACSARIA cell sorter. Target cells for cytotoxicity assay were prepared from spleens of naïve C57BL/6 mice and incubated with or without ORF61₅₂₄₋₅₃₁ peptide (1 μ g/ml) in complete medium at 37°C for 2 h. The ORF61₅₂₄₋₅₃₁ pulsed and unpulsed splenocytes (10⁷/ml) were labeled with 2 μ M and 0.2 μ M CFSE (Sigma-Aldrich), respectively, in HBSS at RT for 10 min. The target cells (4 $\times$ 10⁴) were cultured with Thy1.1⁺ or Thy1.1⁻ CD8⁺ T cells at an effector to target (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 ORF61₅₂₄₋₅₃₁ specific CD8⁺ T cells among Thy1.1⁺CD8⁺ T cells was 6.7% (Supplementary Fig. 2). Cells were then stained with 10 μ M 7-Aminoactinomycin D (7-AAD, BD Bioscience, San Jose, California) 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; % specific lysis = [1 - (ratio of Thy1.1⁺ or Thy1.1⁻ CD8 group / ratio of target only group)] $\times$ 100%.

Suppression assay

Splenocytes were prepared from 10BiT mice depleted of CD4⁺ cells and infected with MHV-68 for 42 days, and Thy1.1⁺CD8⁺ T cells were sorted. Responder CD8⁺ T cells were prepared from spleens of naïve B6-Ly5.2 mice, and purified using EasySep Mouse CD8⁺ T Cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada). Responder cells (5×10^4) were labeled with 0.5 μ M 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 μ g/ml anti-CD3 ϵ 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 μ g/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 μ g/ml anti-CD3 ϵ 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 days. The splenocytes were labeled with 0.5 μ M CFSE, and then cultured in 96 well plate coated with/without 5 μ g/ml anti-CD3 ϵ Ab in complete medium at 37°C for 72 h. After staining with anti-Thy1.1 and anti-CD8 α 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 μ g/ml agonistic Abs to OX-40 (OX-86, BioXcell) or CD27 (RM27-3E5, provided by Dr. Hideo Yagita, Juntendo University, Tokyo, Japan), 20 μ g/ml IL-10R blocking Ab or 20 μ g/ml RatIgG were added to the culture medium at the beginning of the culture.

Statistical analysis

Student's t tests were performed using GraphPad Prism 5 (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 C57BL/6 (WT) mice and CD4⁺ cell-depleted (α CD4) mice were infected with MHV-68. At different time points post-infection (pi), splenocytes were prepared and IL-10 production was measured by intracellular cytokine staining. During the acute phase of infection (d14 pi), 1.1% of CD8⁺ T cells produced IL-10 in the WT mice, whereas the proportion was approximately half of that in the α CD4 mice (Fig. 1A). During the chronic phase of infection (d42 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 α 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 d42 pi, and then declined but remained at significantly higher levels until d105 pi compared to 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 α 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 d14 pi, and 7.4% and 6.1% at d42 pi for WT and α 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 up-regulated 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 bone marrow chimeric mice containing both CD25^{+/+} (CD45.1⁺) and CD25^{-/-} (CD45.2⁺) cells which could be distinguished by staining of 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 d14 pi, the frequency of IL-10 producing cells was similar in CD25^{+/+} and CD25^{-/-} CD8⁺ T cells (Fig. 2B). In contrast, at d40 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 (d14 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 to intact mice (Fig. 3A). In contrast, during the chronic phase (d42 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 Epstein-Barr virus-induced gene 3 (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 d14 and d55 pi, the absence of the Ebi3 did not affect the tendencies observed in the WT and α CD4 mice (Supplementary Fig. 1A and B), 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-2R β or IL-15R β), 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 majority of CTLA-4 is not expressed on the surface but is sequestered intracellularly (54). We used the 10BiT 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 d14 pi (Fig. 4A) and d42 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 d14 pi (Fig. 4A), and this proportion declined further at d42 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 acute (13.7% vs 34.6%) and chronic phase (7.8% vs 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- γ and TNF- α after anti-CD3 stimulation compared to 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⁺CD8⁺ T cells producing IFN- γ , granzyme B, TNF- α and IL-2 were 95%, 74%, 57% and 20% at d14 pi (Fig. 5A) and 94%, 52%, 58% and 6% at d42 pi (Fig. 5B), respectively. Comparing the two time points, similar frequencies of IL-10⁺CD8⁺ T cells produced IFN- γ and TNF- α , whereas higher frequencies of IL-10⁺CD8⁺ T cells from d14 pi produced granzyme B and IL-2 compared to the cells from d42 pi (Fig. 5C). These results indicated that most of the IL-10 producing CD8⁺ T cells could produce IFN- γ and about half of them could produce granzyme B and TNF- α .

Since 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 d42 pi were cultured with ORF61₅₂₄₋₅₃₁ pulsed target cells at different ratios. At a 30:1 of E:T ratio, the specific lysis by the Thy1.1⁺ and Thy1.1⁻CD8⁺ T cells were 80% and 40%, respectively (Fig. 5D, E). This difference of lysis by Thy1.1⁺ and Thy1.1⁻ cells was likely due to the lower frequency of ORF61₅₂₄₋₅₃₁ specific CD8⁺ T cells in the Thy1.1⁻CD8⁺ T cells (2.8%) compared to the frequency in the Thy1.1⁺CD8⁺ T cell population (6.7%) (Supplementary 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 naïve 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 naïve mice and labeled with CFSE, an intracellular fluorescent dye whose intensity reduces by half after every cell division. When the responder cells were cultured alone or with 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 44% of the responder cells proliferated, indicating that the Thy1.1⁺CD8⁺ T cells suppressed the proliferation of naïve 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 which 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 to 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.

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⁺CD8⁺ T cells was 5.5% at d59 pi (data not shown). Since approximately 95% of the IL-10⁺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 d14 pi, 80% of Thy1.1⁺CD8⁺ T cells and 97% of Thy1.1⁻CD8⁺ T cells proliferated (Fig. 7A, B). At d42 and d105 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, B). 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 TNF receptor family, has also been shown to restore CD4⁺CD25⁺ Treg proliferation *in vitro* (60). Signaling through CD27, another TNF receptor

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, B). OX-40 and CD27 agonistic Abs had no effect on proliferation of these cells (Fig. 8A, B). 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. 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 (Fig. 2 and 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 (supplementary 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-27 receptor (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 to non-IL-10 producing CD8⁺ T cells in both acute and chronic phases of the infection. On the other hand, 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 naïve CD8⁺ T cells *in vitro* in a cell contact-independent manner (Fig. 6A, B). 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 an 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, 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 the effector molecules such as IFN- γ , TNF- α and granzyme B (Fig. 5A, B, C), and exhibited cytotoxicity against target cells loaded with an MHV-68 epitope (Fig. 5D, E), 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 convert from an effector to a regulatory phenotype or vice versa.

Tregs have been shown to be non-proliferative *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-2 receptor consists of IL-2R α (CD25), β (CD122) and γ (CD132) subunits, which form a high-affinity trimeric IL-2R. In the absence of CD25 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 naïve 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 naïve 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 regulatory T cell 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Kinetics of IL-10 producing CD8⁺ T cells in intact and CD4⁺ T cell deficient mice infected with MHV-68. Intact (WT) and CD4⁺ cell-depleted (αCD4) C57BL/6 mice were infected with 400 PFU MHV-68 i.n.. Spleens were harvested at d14 to d105 post-infection (pi) and splenocytes were stimulated with PMA/ionomycin, and stained with anti-IL-10 Ab intracellularly. (**A** and **B**) Representative FACS plots were gated on CD8⁺ T cells. The value shown is the mean of the percentages of IL-10⁺ cells within CD8⁺ T cell population. Each point on the graph represents a single mouse. (**C**) Kinetics of the frequencies and total number of IL-10⁺ cells within CD8⁺ T cell population. Error bars represent SEM. (**D**) Representative FACS plots were gated on IL-10⁺CD8⁺ T cells. The values represent the frequency of ORF61-specific cells within IL-10⁺CD8⁺ T cells. Data are representative of five (**A**), three (**B**), two to five (**C**) and three (**D**) independent experiments with three to seven mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ when comparing the corresponding values of WT and αCD4 groups.

Figure 2.

Deficiency in IL-2 signaling is partially responsible for the increase in IL-10 producing CD8⁺ T cells during the chronic phase of infection. (A) Experimental design of generation and infection of WT:CD25^{-/-} mixed bone marrow chimeric mice. Splenocytes were prepared from intact and CD4⁺ cell-depleted chimeric mice at d14 and d40 pi. (B) The frequencies of IL-10 producing CD25^{+/+} or CD25^{-/-} T cells within CD8⁺ T cells. Top graphs: each point on the graph represents a single mouse. Bottom graphs: CD25^{+/+} and CD25^{-/-} cells from individual mice are connected with a line. Data are representative of two independent experiments with four to seven mice per group. **p* < 0.05, ***p* < 0.01, ****p* < 0.0001.

Figure 3.

Role of CD40-CD40L pathway in generation of IL-10 producing CD8⁺ T cells. Intact (WT), CD4⁺ cell-depleted (α CD4) and CD40L-blocked (α CD40L) C57BL/6 mice were infected. Splenocytes were prepared and intracellular staining of IL-10 was performed. Representative FACS plots were gated on CD8⁺ T cells. The value shown is the mean of the percentages of IL-10⁺ cells within CD8⁺ T cell population at d14 pi (A) and d42 pi (B). Each point on the graph represents a single mouse. Data are representative of three independent experiments with three to seven mice per group. ** $p < 0.01$, *** $p < 0.0001$.

Figure 4.

Phenotypes of IL-10 producing CD8⁺ T cells. Splenocytes were prepared from CD4⁺ cell-depleted and infected 10BiT mice at (A) day 14 and (B) day 42 pi. 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

Figure 5.

IL-10 producing CD8⁺ T cells can produce effector cytokines/molecules and have cytotoxicity against peptide pulsed target cells *in vitro*. For intracellular cytokine/molecules assay, splenocytes from CD4⁺ cell-depleted and infected 10BiT mice were stimulated with PMA/ionomycin and co-stained with anti-IL-10 Ab and anti-IFN- γ , -granzyme B, -TNF- α or -IL-2 Ab. **(A and B)** Representative FACS plots were gated on CD8⁺ T cells and histograms were gated on IL-10⁺CD8⁺ T cells. The value shown is the mean of percentages of cells producing effector molecules. **(C)** Graphs were based on the data pooled from two independent experiments and show the mean of percentages of cells producing effector molecules within IL-10⁺CD8⁺ T cells. For *in vitro* cytotoxicity assay, splenocytes from CD4⁺ cell-depleted and infected 10BiT mice at d42 pi were pooled and Thy1.1⁺ or Thy1.1⁻CD8⁺ T cells were purified for the culture with ORF61₅₂₄₋₅₃₁ peptide pulsed and unpulsed target cells. **(D)** Representative histograms of live target cells. The values are total numbers of non-pulsed (left) and ORF61₅₂₄₋₅₃₁-pulsed (right) live cells. **(E)** Specific lysis of target cells by Thy1.1⁺ or Thy1.1⁻CD8⁺ T cells was calculated based on the pooled data from three independent experiments. Error bars on the graphs represent SEM. Each experiment was performed with three to four mice per group. * $p < 0.05$, *** $p < 0.0001$.

Figure 6.

IL-10 producing CD8⁺ T cells suppress naïve 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 days and splenic Thy1.1⁺ or Thy1.1⁻CD8⁺ T cells were purified. Responder CD8⁺ T cells were purified from naïve 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-CD3e 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 are inserted to compare CFSE dilution between plots and percentages are frequency 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-CD3e Ab, while the top wells contained either 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 RatIgG. 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 days. 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-CD8 α 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-CD3 ϵ Ab, and the control (filled) was CD8⁺ T cells from non-stimulated 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 d14 pi, five for d42 and two for d105 with three to four mice per group. *** $p < 0.0001$.

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 days. Splenocytes were labeled with CFSE and cultured with anti-CD3 stimulation in the presence of either 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-CD8α Abs and proliferation of Thy1.1⁺ and Thy1.1⁻ CD8⁺ T cells were 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. *** $p < 0.0001$.