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Suppressive CD8+ T Cells Arise in the Absence of CD4 Help and Compromise Control of Persistent Virus

Michael J. Molloy, Weijun Zhang, and Edward J. Usherwood

There is an urgent need to develop novel therapies for controlling chronic virus infections in immunocompromised patients. Disease associated with persistent γ-herpesvirus infection (EBV, human herpesvirus 8) is a significant problem in AIDS patients and transplant recipients, and clinical management of these conditions is difficult. Immune surveillance failure followed by γ-herpesvirus recrudescence can be modeled using murine γ-herpesvirus (MHV)-68 in mice lacking CD4+ T cells. In contrast with other chronic infections, no obvious defect in the functional capacity of the viral-specific CD8+ T cell response was detected. We show in this article that adoptive transfer of MHV-68–specific CD8+ T cells was ineffective at reducing the viral burden. Together, these indicate the potential presence of T cell extrinsic suppressive factors. Indeed, CD4-depleted mice infected with MHV-68 express increased levels of IL-10, a cytokine capable of suppressing the function of both APCs and T cells. CD4-depleted mice developed a population of CD8+ T cells capable of producing IL-10 that suppressed viral control. Although exhibiting cell surface markers indicative of activation, the IL-10–producing cells expressed increased levels of programmed death-1 but were not enriched in the MHV-68–specific compartment, nor were they uniformly CD44hi. Therapeutic administration of an IL-10R blocking Ab enhanced control of the recrudescent virus. These data implicate IL-10 as a promising target for the restoration of immune surveillance against chronic γ-herpesvirus infection in immunosuppressed individuals. The Journal of Immunology, 2011, 186: 6218–6226.

A variety of viruses such as hepatitis C virus (HCV), HIV, and EBV have the ability to escape the normally efficient immune response, instead persisting in the host with potentially dire consequences (1). Events leading to persistent infection remain poorly understood because T cell responses are generally competent at controlling intracellular pathogens such as viruses (2). One reason that these responses fail to control chronic infection is exhaustion of the antiviral T cell response (3–6). Chronic Ag stimulation of CD8+ T cells results in a gradual loss of functionality including the ability to proliferate, kill infected cells, and produce antiviral cytokines, but the degree of exhaustion varies according to the identity of the persisting pathogen and the inhibitory mechanisms involved in inhibiting the antiviral T cell response (6–11).

IL-10 has received particular attention for its correlation with persistent viral infections and for its ability to attenuate immune responses (12). Polymorphisms that cause increased IL-10 production have been associated with susceptibility to chronic HCV infection and severity of hepatitis B virus infection, whereas polymorphisms that reduce IL-10 expression associate with slower progression to AIDS (13–16). The effects of blocking the IL-10 pathway have been explored in several chronic virus infection models. Studies with lymphocytic choriomeningitis virus clone 13 and murine CMV indicated that Ab blockade or genetic removal of IL-10 signaling greatly improved the ability of mice to control infection through increased cytokine production and an increase in viral-specific T cells (17–20). IL-10 has also been implicated in persistent human infections, because CD4+ T cells from patients infected with HIV or HCV have enhanced responses on blockade of IL-10 signaling in vitro (21, 22). IL-10 affects a variety of APCs through alteration of MHC presentation and decreased inflammatory cytokine production (12). However, direct effects on CD8+ T cells have also been reported with Listeria monocytogenes infection (23). The source of IL-10 during chronic infections varies with the model studied because both myeloid and lymphocyte populations have been implicated in producing IL-10 (12).

Persistent infection of B lymphocytes with EBV is controlled mainly by CD8+ T cells, and patients are generally asymptomatic into advanced age (24). Conversely, immunosuppressed patients, such as those undergoing transplantation, can experience development of EBV-associated lymphoproliferative disease. In addition, the progression of HIV infection to AIDS can be accompanied by γ-herpesvirus–associated lymphomas (25). The development of AIDS is concomitant with declining CD4+ T cell numbers, and both human and mouse model data support the hypothesis that CD4 help is critical for controlling persistent viral infections (26, 27). However, the exact mechanisms by which CD4 T cell help promotes long-term immune surveillance and how immune surveillance of persistent infection breaks down in the absence of these cells remain unclear.

It is possible to dissect the interaction between CD4+ and CD8+ T cells in γ-herpesvirus infection through the use of the murine γ-herpesvirus (MHV) model, MHV-68. When mice lacking CD4+ T cells are infected with MHV-68, they control the initial viral burden with comparable kinetics to wild-type (WT) mice. However, control ultimately breaks down and the virus reactivates by day 40 postinfection (p.i.), whereas no detectable virus can be found in the lungs of WT animals. Interestingly, no decrease in Ag-specific CD8+ T cells or cytotoxicity has been reported for
mice that lack CD4 help (28–30). In addition, therapeutic vacci-
nation with recombinant vaccinia virus expressing an MHV-68
epitope did not reduce the viral burden despite dramatically
expanding the Ag-specific population (31). Several β- and
γ-herpesviruses such as human CMV and EBV have encoded IL-
10 homologs in their genomes (32, 33). Although MHV-68 does
not encode an IL-10 homolog, it does encode the M2 protein,
which induces IL-10–dependent B cell proliferation, perhaps fa-
cilitating the establishment of virus latency in memory B cells
(34). IL-10–deficient mice infected with MHV-68 have reduced
peak lytic viral levels compared with WT controls; however, they
also experience enhanced splenomegaly and lymphocytosis (35).
In addition, MHV-68–induced IL-10 production has been shown to
inhibit the ability of dendritic cells to fully activate T cell
responses (36).

Our studies aimed to explore the role of known mechanisms of
immune suppression on the MHV-68 CD8+ T cell response in
CD4-depleted mice. Interestingly, CD4-depleted mice produced
increased levels of IL-10 compared with intact animals and con-
tained a population of CD8+ T cells that were capable of pro-
ducing IL-10. Therapeutic blockade of IL-10R at late time points
improved control over the viral replication in the lungs of CD4–
depleted mice. These data indicate an important link between
CD4+ T cell help and IL-10 production from a subset of CD8+
T cells, which is an important factor limiting long-term control of
MHV-68 infection.

Materials and Methods

Mice and viruses infections

MHV-68 virus (clone G2.4) was originally obtained from Dr. A.A. Nash
(University of Edinburgh, U.K.). The virus was grown and titered using
NIH/HT3 cells as previously described (37). For MHV-68 infections, mice
were given 400 PFU intranasally under anesthesia with either ketamine/xylazine or isoflurane. C57BL/6 and congenic B6-Ly5.2-CR mice were
then purchased from the National Cancer Institute (Bethesda, MD). IL-10
Thy.1 reporter mice were obtained from Dr. Casey Weaver (University of
Alabama, Birmingham, AL). IL-10R-I-/- mice were obtained from Dr.
Joonsoo Kang (University of Massachusetts Medical Center, Worcester,
MA). MHC class II-/- mice were obtained from Taconic Laboratory. IL-
10+ mice were purchased from The Jackson Laboratory (Bar Harbor,
ME). All mice were bred and/or housed in the Dartmouth-Hitchcock
Medical Center mouse facility. The Animal Care and Use Program of
Dartmouth College approved all animal experiments.

CD4 depletion

For depletion of CD4+ T cells, mice were treated with 500 μg anti-CD4
(GK1.5) on days –1 and 0 relative to infection, followed by 250 μg on day
3 p.i. and twice weekly thereafter. Control mice were either untreated or
given rat IgG (Jackson ImmunoResearch, West Grove, PA).

Tissue preparation

Single-cell suspensions of spleens were prepared by passing through cell
strainers. Lungs were injected with 1 ml MEM containing 417.5 μM L-1-
Cerase CI and 200 μg/ml DNase I (Roche, Indianapolis, IN). Tissues
were minced with scissors and then incubated for 30 min at 37°C and
passed through cell strainers. Suspensions were resuspended in 80% iso-
tonic Percoll and subsequently overlaid with 40% isotonic Percoll. Sam-
ple were then centrifuged at 400 × g for 25 min. Cells at the 40/80
interface were collected, washed, and counted.

Tetramer, Ab staining, and flow cytometric analysis

MHC/peptide tetramers for the MHV-68 epitopes, ORF6,67/49/DB
and ORF61,324–333/K2, conjugated to APC were obtained from the National
Institutes of Health Tetramer Core facility (Emory University, Atlanta,
GA). Cells were incubated with anti-CD16/32 Ab for 10 min on ice. Cells
were washed and then stained for 1 h in the dark with tetramers as pre-
viously described (38), then stained with cell surface markers or the ap-
propriate isotype controls for 20 min on ice. Samples were analyzed using
FACSCalibur, FACSCanto, or Accuri Flow cytometers. Data were then
analyzed using either FlowJo or Accuri software.

Intracellular cytokine staining

Splenocytes were incubated with 1 μg/ml of the appropriate peptide plus
10 μg/ml brefeldin A in complete medium at 37°C for 5 h. Cells were then
stained with cell surface markers, fixed in 1% formaldehyde, and rendered
permeable with 0.5% saponin solution before staining with Abs against
IFN-γ, TNF-α, or granulocyte M, or the appropriate isotype control. The percentage of cytokine-producing cells was established by subtracting
from the no-peptide controls.

Ab blockade of inhibitory cytokines and receptors

Anti–glucocorticoid-induced TNFR-related protein Ab (clone ETA-1)
was provided by Dr. Mary Jo Turk (Dartmouth Medical School, Leb-
anon, NH). Mice were injected with 250 μg per injection on days 33 and
39 p.i. Anti–programmed death-1 (PD-1) (clone RM1-14) was provided by
Dr. Hideo Yagita (Juntendo University, Tokyo, Japan). Mice were injected
with 250 μg of the mAb given twice per week starting on day 33 p.i.
For anti–CTLA-4, clone 4F10 was purchased from the American Type
Culture Collection (Manassas, VA). Mice were given 200 μg twice per
week starting on day 33 p.i. IL-10R Ab (clone 1B1.3a) was purchased from
BioExcel (West Lebanon, NH). Mice were administered 250 μg
every 3 d. Anti–Gr-1 Ab (clone RB6-8C5) was given every day for 5 d.
Mice were given 250 μg per injection. All injections were performed via
the peritoneal route.

IL-10 ELISA

Serum was collected from mice infected at least 42 d p.i. by cardiac
puncture. Supernatants were collected from splenocytes in culture for 48 h.
The eBioscience mouse ready-SET-Go kit was used according to the
manufacturer’s protocol (eBioscience, San Diego, CA).

CFSE proliferation assay

Splenocytes from Thy.1 I-IL-10 reporter mice were incubated with 0.5 μg
CFSE at 1 × 10^6 cells/ml for 10 min, washed, and then added to a 96-well
plate coated with anti-CD3. Cells were incubated for 72 h at 37°C, and
CD8 T cells were analyzed for dye dilution by flow cytometry.

In vivo cytotoxicity assay

Naive C57BL/6 splenocytes were incubated with 1 μg/ml of the ORF6 or
ORF61 peptide, or no peptide for 5 h. Each population was then labeled
with 2.5, 0.25, or 0.025 μM CFSE (Molecular Probes, Eugene, OR). Cells
were then mixed at a 1:1:1 ratio, and 2 × 10^6 total cells were injected i.v.
Six hours later, mice were sacrificed and spleen cell suspensions were
incubated with 20 μg/ml 7-aminooctanoylmethyl D (7-AAD; Sigma-Aldrich,
St. Louis, MO) for 15 min at room temperature in the dark. Cells were then
analyzed by flow cytometry, and specific lysis was calculated using the
following formula: Specific killing = 100 – [(% peptide pulsed infected/
% peptide pulsed uninfected) × 100].

Generation of ORF61-specific CD8+ T cell line

Splenocytes were isolated from mice infected with MHV-68 for 60 d. A total
of 4 × 10^6 cells were plated in a volume of 2 ml with the ORF61 peptide
(1 ng/ml) and IL-2 (10 U/ml). After 6 d, the cells were cultured with ir-
radiated naive spleen cells with ORF61 peptide (1 ng/ml) and IL-2 (10 U/
ml) in a small cell culture flask. Media was changed every second day,
adding fresh IL-2 at 10 U/ml, and cultures were restimulated weekly with
peptide and IL-2 as described earlier, until p79 tetramer positive reached
>99% purity.

Bone marrow chimera generation

To generate mixed bone marrow chimeras, we lethally irradiated B6-Ly5.2
recipient mice with a split dose totaling 1000 rad and subsequently injected
them i.v. with a total of 10^8 bone marrow cells. For generation of the
CD8a-/-:WT, CD8a-/-::IL10-/-, and CD8a-/-::IL10-/- mixed
chimeras, bone marrow from CD8a-/- mice was mixed at a 80:20 ratio
with the additional population. Mice were then left to reconstitute for
50 d before infection.

Plaque assay

Lung tissue was disrupted and 10-fold serial dilutions were incubated on
3T3 monolayers for 1 h at 37°C. Two milliliters of a 1:1 mixture of
carboxymethyl-cellulose and DMEM was added and cultured for 5 d at
37°C. Media was then aspirated and monolayers were fixed with methanol.
at room temperature for 20 min and then were stained for 180 min with 8% Giemsa stain (Sigma-Aldrich). Plaques were counted microscopically.

**Quantitative PCR for viral transcripts**

Latent viral DNA was quantified by quantitative fluorescent PCR for the ORF50 gene as previously described (38).

**Statistical analysis**

The p values were calculated using either Student t test or one-way ANOVA with a Bonferroni posttest. A p value <0.05 was considered significant.

**Results**

**MHV-68–infected mice maintain functional capability in the absence of T cell help**

Previous work indicates that WT mice infected with MHV-68 are able to maintain control over the persistent infection. Depletion of CD4+ T cells does not alter the ability of the host to control the initial lytic infection. However, control is ultimately lost and the virus reactivates to high levels in the lungs within 6 wk p.i. (Fig. 1A) (28, 29). In contrast with chronic infections with other viruses such as lymphocytic choriomeningitis virus clone 13, we did not detect a decrease in the number of Ag-specific CD8+ T cells in either the spleen (Supplemental Fig. 1A) or the lung (Fig. 1B); in fact, the CD8+ T cell response was increased in CD4-depleted mice, consistent with previously published results (30). The proportion of virus-specific CD8+ T cells producing IFN-γ reflected the pattern seen by tetramer staining (Fig. 1C, 1D); however, a smaller proportion of cells coproduced IFN-γ and TNF-α, consistent with previous studies (28). The amount of IFN-γ produced by p56-specific cells was reduced in the CD4-depleted group; however, the p79-specific population was unaffected (Fig. 1C, 1D, Supplemental Fig. 1B, 1C). These changes did not impact on the in vivo cytolytic ability of the CD8+ T cells, which were unaffected in all cases (Fig. 1E). Overall, no consistent defects were apparent that would account for the loss of T cell-mediated control of MHV-68 in CD4-deficient mice.

**Adaptive CD8+ T cell therapy is inhibited in MHV-68–infected helpless mice during viral reactivation**

Postexposure vaccination with vaccinia virus expressing an MHV-68 epitope successfully induces robust expansion of Ag-specific CD8+ T cells; however, no effect can be seen on the virus load in the lungs of treated animals (31). Because these cells are primed in a CD4-deficient environment, they may carry an inherent defect in their ability to respond optimally to vaccination. To circumvent this issue, we generated an MHV-68–specific CD8+ T cell line from virus-infected WT mice. In brief, WT mice were infected with MHV-68, and splenocytes were isolated and cultured in vitro with the ORF61 peptide until >99% of the CD8+ T cells were specific for the epitope (Supplemental Fig. 2). A total of 1 × 10^6 CD8+ T cells were then adoptively transferred into MHV-68–infected hosts either at the time of infection or 60 d p.i., when reactivation is readily apparent. Transfer of cells during the primary response was able to reduce the viral burden in the lungs by >40-fold, whereas transfer of an identical number of cells during reactivation had no effect on the viral load (Fig. 2A). This suggests that transferred CD8+ T cells were being actively inhibited from controlling the virus in CD4-deficient mice. If the number of Ag-specific cells was increased to 5 × 10^6 or 1 × 10^7, the CD8+ T cells were able to overcome the putative suppressive environment and reduce the viral load (Fig. 2B). Taken together, the data indicate that despite having no obvious defect in the Ag-specific CD8+ T cell response, a suppressive environment likely exists in the absence of CD4 cell help that is capable of dampening the antiviral activity of both endogenous and adoptively transferred CD8+ T cells.

**Increased levels of IL-10 are produced in MHV-68–infected helpless mice**

We determined whether known suppressive mechanisms associated with other chronic infections were involved in inhibiting the CD8+ T cell response in CD4-depleted MHV-68–infected mice. To do so, we treated CD4+ T cell-depleted mice with Abs against PD-1,
glucocorticoid-induced TNFR-related protein, or CTLA-4 during late time points when virus had reactivated; however, no therapeutic value was observed (Supplemental Fig. 3A). In addition, we detected a dramatic increase in the number of CD11b+, Gr-1+ cells in the lungs and spleen of MHV-68–infected mice in the absence of CD4+ T cell help (Supplemental Fig. 3B). Myeloid cells with this phenotype are known to have suppressive capabilities in a variety of tumor models and to be induced by various infections (39–41). However, depletion of this population using anti–Gr-1 Ab had no effect on viral load in the lungs of CD4+ T cell-depleted mice (Supplemental Fig. 3C).

Interestingly, we observed an increase in both serum levels of IL-10 from helpless mice compared with WT mice, as well as from splenocytes cultured for 48 h ex vivo (Fig. 3A, 3B). To test whether IL-10 was contributing to the loss of long-term control of the virus in CD4-deficient animals, we used mice genetically deficient in the IL-10R1 chain. MHV-68–infected helpless mice lacking IL-10R1 had lower levels of virus reactivation in the lungs compared with WT mice (Fig. 3C), implicating a role for IL-10 in virus reactivation. To confirm that IL-10 was suppressing the immune response against MHV-68 in helpless mice, we generated virus-specific CD8+ T cell lines from WT or IL-10R1−/− mice and adoptively transferred identical numbers into CD4-deficient mice undergoing virus reactivation. Transfer of 2 × 10^6 cells from either population had no effect. However, although transfer of 4 × 10^6 WT cells had no effect on viral control, 4 × 10^6 IL-10R1−/− cells were able to reduce the viral burden (Fig. 3D).

**FIGURE 2.** Transfer of an ORF61-specific CD8+ T cell line in MHV-68–infected CD4-depleted mice. WT mice were infected with MHV-68 and splenocytes were isolated during maintenance of the memory population (day 60 p.i.) and cultured in vitro with the ORF61 peptide until >99% of the CD8+ T cells were specific for the epitope. A, A total of 1 × 10^6 CD8+ T cells were adoptively transferred into MHV-68–infected hosts either at the time of infection (top) or into CD4-depleted hosts 60 d p.i. (bottom), and viral titers were determined by plaque assay 6 d later. B, Various numbers of ORF61-specific CD8+ T cells were transferred into helpless mice at day 60 p.i., and viral titers were determined 6 d later. Each dot represents data from individual mice. Experiments were repeated at least two times with a minimum of four mice per group. *p < 0.05, **p < 0.01. n.s., not significant.

**FIGURE 3.** MHV-68–infected CD4-depleted mice have increased levels of IL-10 contributing to suppression of CD8+ T cell responses. A, Serum from intact and CD4-depleted mice infected for 42 d was analyzed for IL-10 by ELISA. B, Splenocytes were isolated from intact and CD4-depleted mice infected for 42 d and cultured at 37˚C for 48 h with the ORF61 peptide before supernatants were analyzed by ELISA for IL-10. C, WT and IL-10R1−/− mice were CD4 depleted and infected with MHV-68 for 60 d. Lungs were then analyzed for viral titer by plaque assay. D, CD4-depleted MHV-68–infected mice were infected for 60 d. Then either 0, 2 × 10^6, or 4 × 10^6 ORF61-specific WT or IL-10R1−/− CD8+ T cells were adoptively transferred. Six days later, the lungs were analyzed for viral titers by plaque assay. Each dot represents data from individual mice. Experiments were repeated at least two times with a minimum of four mice per group. *p < 0.05, **p < 0.01.
CD4-depleted mice develop a population of CD8\(^+\) T cells capable of producing IL-10

We next determined which cell populations were producing increased amounts of IL-10 in the absence of CD4 help on infection with MHV-68. To do so, we made use of Thy1.1–IL-10 reporter mice, which were genetically modified to include the Thy1.1 gene under control of the IL-10 promoter (42). Therefore, when the promoter of IL-10 is active, the cell expresses Thy1.1 on the cell surface. We examined intact and CD4-depleted mice infected with MHV-68 for 60 d for Thy1.1\(^+\) populations. No increases in Thy1.1 expression were found between the groups in CD11b\(^+\), CD11c\(^+\), Gr-1\(^-\), or CD19\(^+\) populations (Supplemental Fig. 4). A small population of CD8\(^+\) cells produced IL-10 in the intact but not CD4-depleted group, and further examination revealed these to be CD4 T cells (data not shown). Interestingly, we found a population of CD8\(^+\) T cells in both the spleen and lung that expressed the Thy1.1 marker only in the absence of CD4\(^+\) T cell help (Fig. 4A). The MHV-68–specific CD8\(^+\) T cell populations ORF6 and ORF61 were not enriched for Thy1.1\(^+\) cells in either the spleen or lung (Fig. 4B). To determine whether the Thy1.1\(^+\) cells were, in fact, capable of producing IL-10, we sorted CD8\(^+\), CD11c\(^-\) cells into Thy1.1\(^-\) and Thy1.1\(^+\) populations and cultured them for 24 h in either media alone or with agonistic anti-CD3 Ab. Although the Thy1.1\(^-\) cells failed to produce IL-10, Thy1.1\(^+\) CD8\(^+\) T cells produced IL-10 on stimulation (Fig. 4C). Anti-CD3 stimulation failed to induce proliferation, as well as robust IFN-\(\gamma\) and TNF-\(\alpha\) production from the Thy1.1\(^+\) cells compared with the Thy1.1\(^-\) cells (Fig. 4D, 4E). We next analyzed the CD8\(^+\) Thy1.1\(^+\) population using a broad array of cell surface differentiation markers. Thy1.1\(^+\) cells were found to be CD127\(^-\)/low, CD43\(^hi\), and had an increased percentage of CD62L\(^lo\) cells, characteristic of an activated phenotype (Fig. 5). The Thy1.1\(^+\) CD8\(^+\) cells also expressed increased levels of CD44 compared with the naive CD44\(^hi\) population; however, expression was decreased compared with Ag-experienced CD44\(^hi\) CD8\(^+\) T cells (Fig. 5).

Two populations of regulatory CD8\(^+\) T cells have recently been described in mice. One population expresses Foxp3 and suppresses via TGF-\(\beta\) (43). Another population capable of suppressing graft rejection through IL-10 has been reported to be separated from conventional CD8\(^+\) T cells by coexpression of CD122\(^+\) and PD-1 (44). When we analyzed the CD8\(^+\) population, we found that although the Thy1.1\(^+\) cells did not express any detectable Foxp3 (data not shown), they were, in fact, CD122\(^+\) and expressed increased levels of PD-1, indicating a phenotype similar to a previously described regulatory CD8\(^+\) T cell population (Fig. 5).

To determine whether this CD8\(^+\) population of IL-10–producing cells directly contributed to suppression of the MHV-68–specific immune response, we isolated the ability to either produce or respond to IL-10 to the CD8\(^+\) T cell compartment using mixed bone marrow chimeric mice. We reconstituted lethally irradiated mice with CD8\(\alpha\)^{-/} bone marrow mixed with either WT, IL-10\(^{-/-}\), or IL-10R1\(^{-/-}\) bone marrow at a 4:1 ratio. In this manner, we generated mice where the CD8\(^+\) compartment contained T cells that were exclusively either WT–, IL-10–, or IL-10R1–deficient, respectively, but the large majority of all other cells expressed these molecules. Chimeric mice were then CD4 depleted and infected with MHV-68 for at least 60 d; then the viral load was determined in the lungs. IL-10\(^{-/-}\) bone marrow chimeric mice had significantly lower levels of virus compared with WT mice in the absence of CD4\(^+\) T cell help, indicating that IL-10\(^{-/-}\) produced by CD8\(^+\) cells contributes to suppression of the MHV-68 immune response (Fig. 6A). IL-10R1\(^{-/-}\) bone marrow chimeric mice also had lower viral load compared with WT chimeras (Fig. 6A). The decrease was slightly less robust than that seen with the IL-10\(^{-/-}\)
chimeras, suggesting that both direct and indirect effects on the Ag-specific CD8+ T cell response were potentially responsible for improved viral control. Notably, two IL-10−/− chimaeras and three IL-10R1−/− mice had no detectable lytic virus in the lungs, whereas all WT chimera mice had high levels of reactivation.

Blockade of IL-10R during reactivation is able to reduce the viral burden in MHV-68–infected helpless mice

Although genetic disruption of the IL-10R1 gene was able to reduce the virus in reactivating “helpless” mice, this could be a result of altered kinetics during the primary response, because IL-10−/− mice infected with MHV-68 have been reported to have lower peak viral loads than WT mice (35). We therefore took a complementary approach to address the role of IL-10 in immune surveillance, by administering a blocking Ab at various times throughout the course of infection. Interestingly, blockade of IL-10R during the primary lytic infection (days 0–7), during reactivation (day 21+), or throughout the duration of the experiment all resulted in a reduction in viral load (Fig. 6B). Although IL-10R blockade improved control of the lytic virus, no effect was observed on the latent viral loads (Fig. 6C). For IL-10 blockade to be a clinically relevant therapy, it must be capable of restoring immune surveillance at late stages after virus reactivation has occurred. Therefore, CD4-depleted MHV-68 infected mice were left for 60 d, then given twice weekly injections of the anti–IL-10R Ab for 2 wk and virus titers were determined. Blockade of the IL-10R at these times resulted in a reduction of viral load compared with rat Ig-treated controls (Fig. 6D). Similar therapeutic efficacy was observed when class II−/− mice infected with MHV-68 were administered IL-10R blocking Abs (data not shown). This confirms the therapeutic value of IL-10 blockade even at late stages after virus reactivation. When the Ag-specific CD8+ T cell response was analyzed via bronchoalveolar lavage, enhanced effector responses were observed, as more TNF-α was produced on restimulation (Fig. 6E). A small but statistically significant increase in IFN-γ was also observed (data not shown). In addition, CD11c+ cells from the lung expressed increased levels of the costimulatory marker CD80 (Fig. 6E). Thus, disruption of IL-10 signaling allows for enhanced immune surveillance in immunocompromised MHV-68–infected mice.

Discussion

Previous studies have identified CD4+ T cells as being critical for the long-term control of MHV-68 infection (29). Our understanding of how the immune response against MHV-68 is suppressed in the absence of CD4 T cell help remains unclear because Ag-specific CD8+ T cell numbers, proliferative capability, and cytolytic activity of CD8+ T cells appear to be largely normal in helpless mice (Fig. 1, Supplemental Fig. 1) (28, 30, 31). A recent study indicated that class II−/− mice infected with MHV-68 express increased levels of PD-1 on Ag-specific CD8+ T cells, and that blockade of the PD-1 pathway improves viral control (10). Conversely, in our experiments, administration of PD-1 blocking Abs to CD4-depleted MHV-68–infected mice did not improve viral control. The exact reason for this discrepancy is unclear but could be a result of differences between using MHC class II−/− mice in the previous study versus depleting GK1.5 anti-CD4 Ab in this study. Our study aimed to explore various mechanisms to enhance control of MHV-68 in the immunocompromised state of a CD4-deficient host. We generated an ORF61-specific CD8+ T cell line in vitro and adoptively transferred the cells into MHV-68–infected mice that lack CD4+ T cell help. Although the T cell line was highly effective at reducing the viral load during the initial lytic infection, an identical number of cells was unable to

![FIGURE 5. Thy1.1+ CD8+ T cells express cell surface markers of activation. IL-10 Thy1.1 reporter mice were CD4 depleted for 60 d, and the expression of cell surface markers was analyzed by flow cytometry for CD8+ Thy1.1+ and Thy1.1− cells. Representative histograms and statistical analysis are shown. Solid histograms represent Thy1.1+; open histograms represent Thy1.1−. Error bars indicate SD. Experiments were repeated at least two times with a minimum of four mice per group. **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)

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provide therapeutic value if transferred at day 60 p.i. when MHV-68 has reactivated to high levels in the lungs (Fig. 2). This indicates that in addition to any potential CD8+ T cell intrinsic defects, a suppressive environment exists that is capable of inhibiting antiviral activity by the T cell line. Therefore, it was particularly relevant when we detected increased IL-10 secretion in helpless mice undergoing virus reactivation, because IL-10 has been implicated in the suppression of immune responses against a variety of acute and persistent infections (12, 45, 46). In contrast with other models, we observed an increased production of IL-10 only under conditions of CD4+ T cell depletion (Fig. 4), specifically by a population of CD8+ T cells (8, 17, 18).

The IL-10–producing CD8+ T cells expressed markers indicating Ag experience based on expression of surface markers. Interestingly, they were not enriched in the Ag-specific population recognizing the ORF6 or ORF61 epitopes, which may transmit different signals to the CD8 T cell, for example, the tetramer-positive population, suggesting these cells may be in a different stage of activation. IL-10 produced by CD8+ T cells contributed to suppression of the MHV-68 response as bone marrow chimeras where only CD8+ cells lacked the IL-10 gene had significantly lower viral loads than WT chimeras. In addition, mAb blockade of IL-10R at any stage of the infection enhanced control of the lytic virus, indicating an important role in subduing the T cell response throughout the infection.

During acute infection, MHV-68 induces expression of IL-10 from the dendritic cell compartment (36), which facilitates colonization of the host by the virus. In addition, IL-10 induction by the MHV-68 M2 protein facilitates induction of the latent state by promoting B cell proliferation and survival (34). Our results are distinct as we observe increased levels of IL-10 during chronic reactivation of MHV-68 only in the absence of CD4+ T cell help from a source distinct from that seen during the initial infection.

IL-10 production appears to be responsible, in part, for the breakdown of immune surveillance, allowing virus reactivation to occur. In other experiments, we have observed Thy1.1 expression from CD8+ T cells after infection of CD4-depleted IL-10 reporter mice with vaccinia virus, a virus that does not persist in the absence of CD4 help. Therefore, induction of the IL-10–producing CD8+ T regulatory population appears to be linked to the depletion of CD4+ T cells rather than virus persistence per se. One of the major roles associated with CD4+ T cell help is to appropriately “license” dendritic cells via CD40L–CD40 interactions (47, 48). These fully licensed dendritic cells then are capable of appropriately priming the CD8+ T cell response. In the absence of a suppressive environment, inhibitory molecules such as TRAIL are upregulated, limiting the memory capabilities of the CD8+ T cell population (49). As such, one hypothesis is that IL-10 production preferentially occurs in CD8+ T cells primed by unlicensed dendritic cells, which may transmit different signals to the CD8 T cell, for example, costimulatory signals or cytokines, compared with licensed APCs.

Recently, suppressive CD8+ T cells have been identified in a number of infectious diseases. Similar to CD4+ regulatory cells, CD8+ T cells can suppress through several mechanisms including IL-10, CTLA-4, and TGF-β (43). In mice, two distinct types of regulatory CD8+ T cells have been identified: CD25+ Foxp3+ cells that suppress via CTLA-4 and TGF-β, and CD122+ PD-1+ cells that suppress via IL-10 (43, 44, 50). Although the IL-10–producing cells identified in our experiments did not express Foxp3 (data not shown), they did express increased levels of PD-1 and CD122 (Fig. 5). With respect to human infection, regulatory CD8+ T cells have been identified in patients with HCV (51–53), HIV (54–56), and HSV-2 (57). Interestingly, HIV-specific CD8+ T cell production of IL-10 is correlated with declining CD4+ T cell numbers, increased HIV plasma RNA levels, and decreased cytolysis of infected cells (55, 56).
A study of EBV-infected patients revealed that CD8+ regulatory cells could be expanded from immunocompromised patients (58). Interestingly, the same population could not be expanded from EBV-infected healthy control patients, indicating the potential for preferential induction of regulatory CD8+ T cells in an immunocompromised state. The expanded regulatory CD8+ T cells produced both IFN-γ and IL-10, and required cell-to-cell contact to suppress CD4+ T cell proliferation. This is again reminiscent of the MHV-68 system because our data indicate production of IL-10 in an immunocompromised host. The IL-10 pathway is likely a key factor in the host–virus equilibrium in several herpesvirus infections, because many of these viruses, such as human CMV, EBV, and veterinary γ-herpesviruses, encode IL-10 homologs in their genomes (32, 33) and induce IL-10 production by the host. Therefore, disruption of the IL-10 pathway may provide a novel therapeutic for enhanced control of human γ-herpesvirus–induced disease in immunocompromised patients. In addition, it adds to our understanding of the host–virus equilibrium in persistent virus infections in general, and in particular the functions of CD4+ T cell help and the biology of IL-10 in these infections.

Disclosures

The authors have no financial conflicts of interest.

References


