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*J Immunol* 2006; 176:3342-3349; doi: 10.4049/jimmunol.176.6.3342

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In Vitro Suppression of CD8\(^+\) T Cell Function by Friend Virus-Induced Regulatory T Cells\(^1\)

Shelly J. Robertson, Ronald J. Messer, Aaron B. Carmody, and Kim J. Hasenkrug\(^2\)

Regulatory T cell (Treg)-mediated suppression of CD8\(^+\) T cells has been implicated in the establishment and maintenance of chronic viral infections, but little is known about the mechanism of suppression. In this study an in vitro assay was developed to investigate the suppression of CD8\(^+\) T cells by Friend retrovirus (FV)-induced Tregs. CD4\(^+\)CD25\(^+\) T cells isolated from mice chronically infected with the FV suppressed the development of effector function in naive CD8\(^+\) T cells without affecting their ability to proliferate or up-regulate activation markers. In vitro restimulation was not required for suppression by FV-induced Tregs, correlating with their high activation state in vivo. Suppression was mediated by direct T cell-T cell interactions and occurred in the absence of APCs. Furthermore, suppression occurred irrespective of the TCR specificity of the CD8\(^+\) T cells. Most interestingly, FV-induced Tregs were able to suppress the function of CD8\(^+\) effector T cells that had been physiologically activated during acute FV infection. The ability to suppress the effector function of activated CTLs is likely a requisite role for Tregs in limiting immunopathology by CD8\(^+\) T cells during antiviral immune responses. Such activity may also have adverse consequences by allowing viruses to establish and maintain chronic infections if suppression of antiviral immune responses occurs before virus eradication. The Journal of Immunology, 2006, 176: 3342–3349.

During the last decade, a subset of CD4\(^+\) T cells has been identified that induces peripheral tolerance to self-Ags and prevents autoimmune diseases (1–3). These cells, termed “natural” regulatory T cells (Tregs),\(^3\) constitutively express the IL-2R \(\alpha\)-chain (CD25) (4, 5), and their suppressive activity depends on expression of the transcriptional repressor Foxp3 (6–9). It has been reported that there is a significant pool of natural Tregs that are continuously stimulated by self-Ags in vivo, thereby producing a basal level of T cell suppression to maintain self tolerance (10). In vitro assays developed to assess the suppressive activity of the natural Treg require in vitro activation or reactivation of the Treg to achieve suppression (11, 12). Thus, it appears that constant antigenic stimulation may be required for natural Treg suppression. Numerous studies have demonstrated that a predominant suppressive effect of natural Tregs in vitro is to inhibit the proliferative responses of CD4\(^+\) (12, 13) or CD8\(^+\) T cells (14–16).

More recently, Tregs have been implicated in the down-regulation of immune responses to foreign Ags (17–21). These induced Tregs are thought to minimize the immunopathological consequences of T cell responses to microbial infection (5, 19, 22). For example, in a mouse model of ocular infection with HSV, depletion of Tregs results in more severe immunopathological lesions (23). Not surprisingly, infectious organisms have evolved mechanisms to subvert the immunosuppressive properties of Tregs to establish and/or maintain chronic infections. For example, mice infected with Leishmania major undergo an expansion of natural Tregs at the site of infection that prevents eradication of the pathogen (20). Thus, pathogen-specific Tregs may arise from the pool of natural Tregs.

In studies of Friend retrovirus (FV) (24–26) infection of mice, it was found that immunosuppressive CD4\(^+\) T cells (17) facilitated virus persistence by inhibiting CD8\(^+\) T cell responses. Interestingly, inhibition was not due to diminished CD8\(^+\) T cell proliferation or activation, but rather to the inhibition of effector functions (27). In this study, we describe an in vitro assay used to investigate the molecular mechanisms of FV-induced Treg suppression of CD8\(^+\) T cell function. These experiments focus primarily on the subset of CD4\(^+\)CD25\(^+\) T cells because it contains the most potent suppressive activity in vitro. Findings from this assay closely duplicate the known activity of FV-induced Tregs in vivo and provide new insights into the properties and mechanisms of action of these cells. This assay demonstrates suppression through cell-to-cell interactions between Tregs taken directly ex vivo and activated CD8\(^+\) T cells. Furthermore, because there is no requirement for in vitro activation of the Treg, the cellular responses probably reflect the physiological state more closely than assays that use artificial ligands such as anti-CD3 to stimulate suppressive activity.

Materials and Methods

Mice

Experiments were conducted using 12- to 24-wk-old female (C57BL/10 \(\times\) A.BY)\(_F\) mice bred at the Rocky Mountain Laboratories. The relevant FV resistance genotype of these mice is H-\(^2\)-\(^{2/6}\), Fv\(^{1/9}\), Fv\(^{2/8}\), and Rf\(^{3/9}\). FV-specific TCR transgenic (Tg) mice, which carry a transgene for a TCR that recognizes the Gag leader peptide of FV (28, 29), were also bred at Rocky Mountain Laboratories. For in vivo activation studies, (FV-specific TCR \(\times\) B6.GFP)\(_F\) mice were bred to obtain FV-specific TCR Tg CD8\(^+\) T cells that express the GFP (30). OT-I TCR Tg mice that carry a TCR transgene specific for OVA residues 257–264 (31) were purchased from...
The Jackson Laboratory. Mice were treated in accordance with the regulations and guidelines of the Animal Care and Use Committee of the Rocky Mountain Laboratories and the National Institutes of Health.

**Virus and virus infection**

The FV stock used in these experiments was uncloned FV complex containing a B-tropic Friend murine leukemia helper virus and a polycythemia-inducing, spleen focus-forming virus (32). Mice were infected by i.v. injection of 0.5 ml of phosphate-buffered balanced salt solution containing 2% FBS and 1500 spleen focus-forming units of FV complex. Mice that had recovered from FV-induced splenomegaly were considered chronically infected at 8 wk postinfection (33). Virus levels stabilized at 60 h and assayed for IFN-γ content by ELISA. For assessment of proliferation, purified FV-specific TCR Tg CD8+ T cells were labeled with CFSE (Molecular Probes) before coculture as described previously (27, 35).

**IFN-γ ELISA**

IFN-γ ELISA plates were prepared by coating 96-well Immunol II plates (Thermo Lab Systems) with 2 μg/ml purified anti-mouse IFN-γ capture Ab (R4-6A2) (BD Pharmingen) in sodium carbonate buffer (pH 9.6). Culture supernatants diluted in PBS containing 5% FBS were added to the wells and incubated for 2 h at room temperature. Plates were washed four times with PBS containing 0.5% Tween 20 and incubated for 2 h at room temperature with biotinylated anti-mouse IFN-γ (XMG1.2) (BD Pharmingen). Bound Ab was detected with HRP-conjugated streptavidin (Calbiochem) and developed with tetramethylbenzidine substrate. All culture supernatants were assayed in duplicate, and concentrations of IFN-γ were determined based on a standard curve using purified mouse IFN-γ (BD Pharmingen).

**Preactivation and purification of virus-specific CTLs**

FV-specific GFP+ CD8+ T cells were purified using anti-CD8α MACS beads. To activate the purified FV-specific GFP+ CD8+ T cells, 8 × 10^5 cells in 0.5 ml of PBS solution containing 15 U/ml heparin were adoptively transferred by i.v. injection into mice infected with FV for 2 days. For 4 days, activated GFP+ CD8+ T cells were resorted from the spleens of adoptively transferred mice by first enriching for CD8+ T cells with anti-CD8α MACS beads. Enriched cells were then stained with PE-anti-CD44 and allophycocyanin-anti-CD8 and sorted for GFP-positive CD8+ T cells in 0.5 ml of PBS solution containing 15 U/ml heparin were adoptively transferred by i.v. injection into mice infected with FV for 2 days. After 4 days, activated GFP+ CD8+ T cells were resorted from the spleens of adoptively transferred mice by first enriching for CD8+ T cells with anti-CD8α MACS beads. Enriched cells were then stained with PE-anti-CD44 and allophycocyanin-anti-CD8 and sorted for GFP-positive CD8+ T cells. Purified cells were then cocultured with CD4+ CD25+ T cells from either naive or chronically infected mice in the presence of D^*GagL MHC class I tetramer (Beckman Coulter) (36).

**Intracellular cytokine staining**

For intracellular cytokine staining, cocultured cells were incubated at 37°C and 5% CO2 with brefeldin A (10 μg/ml) for 6 h at room temperature. Plates were washed four times with PBS containing 0.5% Tween 20 and incubated for 2 h at room temperature with biotinylated anti-mouse IFN-γ (XMG1.2) (BD Pharmingen). Bound Ab was detected with HRP-conjugated streptavidin (Calbiochem) and developed with tetramethylbenzidine substrate. All culture supernatants were assayed in duplicate, and concentrations of IFN-γ were determined based on a standard curve using purified mouse IFN-γ (BD Pharmingen).

**Statistical analysis**

Statistical significance was determined using two-tailed unpaired t tests except for Fig. 2A, where a paired t test was used. Values of p < 0.05 were considered statistically significant.

**Results**

Chronic FV infection is associated with increased numbers of CD4+ CD25+ T cells and changes in their phenotype

The IL-2R α-chain (CD25) is expressed on a subset of CD4+ T cells that exhibit suppressive activity, and this marker has commonly been used to identify natural Tregs (2). To determine whether chronic FV infection resulted in changes in this cell population, we assessed the proportion and absolute numbers of CD4+ CD25+ T cells in infected mice compared with naive control mice. Flow cytometric analysis of splenocytes from FV-infected and naive mice revealed no significant difference in the percentage of lymphocytes coexpressing CD4 and CD25 (Fig. 1A) or in the percentage of CD4+ T cells that coexpressed CD25 (Fig. 1B). However, chronically infected mice had 2- to 3-fold higher numbers of CD4+ CD25+ T cells compared with naive mice because of their enlarged spleens (Fig. 1A).

Additional markers have been associated with Tregs and include Foxp3 (6, 7, 37), GITR (34, 38), CD152 (CTLA-4) (39), CD103 (40, 41), lymphocyte activation gene (LAG) 3 (42), CD62L (10,
induced Treg suppress activated CD8\(^+\) T cells

CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells from mice chronically infected with FV do not suppress Ag-induced CD8\(^+\) T cell proliferation

To investigate the means by which FV-induced Tregs suppress CD8\(^+\) T cells, an in vitro coculture assay was developed that examined the effects of Tregs on CD8\(^+\) T cell proliferation, activation, and effector function. CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells were purified from the spleens of both naive and chronically infected mice. With no in vitro stimulation, these CD4\(^+\) T cell subsets were then cocultured with FV-specific CD8\(^+\) T cells from mice carrying a TCR transgenic specific for a FV epitope (D\(^b\), GagL). The FV-specific CD8\(^+\) T cells were stimulated with peptide-pulsed APCs. To assess the effects on CD8\(^+\) T cell proliferation, these cells were labeled with the fluorescent dye CFSE before coculture. CFSE content was measured by flow cytometry following 60 h in culture. As shown in Fig. 2A, neither CD25\(^+\) nor CD25\(^-\) subsets of CD4\(^+\) T cells from FV-infected mice affected the proliferation of stimulated CD8\(^+\) T cells.

CD4\(^+\)CD25\(^+\) T cells from mice chronically infected with FV suppress IFN-\(\gamma\) production by both virus-specific and nonspecific CD8\(^+\) T cells

Production of IFN-\(\gamma\) is an important mechanism by which activated CD8\(^+\) T cells mediate antiviral responses, including FV infections (48–50). As a measure of whether FV-induced Tregs influence CD8\(^+\) T cell effector function, we next examined the production of IFN-\(\gamma\) by CD8\(^+\) T cells in the cocultures. Stimulation of FV-specific CD8\(^+\) T cells with peptide-pulsed APCs induced IFN-\(\gamma\) expression that was suppressed in a dose-dependent manner by CD4\(^+\)CD25\(^+\) T cells from FV-infected mice, but not by the same subset of cells from naive mice (Fig. 2B). Suppression of CD8\(^+\) T cell IFN-\(\gamma\) production was not associated with decreased expression of the early activation marker CD69 (46) or the activation-associated glycoform of CD43 (51) (Fig. 2C). Neither the CD25\(^+\) subset of CD4\(^+\) T cells nor CD4\(^+\) spleen cells from chronically infected mice suppressed IFN-\(\gamma\)-expression in vitro (data not shown). Moreover, because the vast majority of chronic virus is harbored in the CD4\(^+\) subset (52), suppression could not be attributed to virus infection within the cocultures. These results indicated that the immunosuppressive activity in mice chronically infected with FV was predominantly contained within the CD4\(^+\)CD25\(^+\) T cell subset. Thus, subsequent studies were focused on the CD4\(^+\)CD25\(^+\) T cell subset, which will be referred to as FV-induced Treg.

To determine whether FV-induced Tregs could suppress non-FV-specific CD8\(^+\) T cells in vitro, coculture experiments were conducted as described above, except that responses by OVA-specific (OT-1) TCR Tg CD8\(^+\) T cells were analyzed. As observed with FV-specific CD8\(^+\) T cells, CD8\(^+\) CD25\(^+\) T cells from chronically infected but not from naive mice significantly reduced the production of IFN-\(\gamma\) by stimulated OVA-specific CD8\(^+\) T cells, even at a 1:1 CD4 to CD8 ratio (Fig. 2D). Thus, in vitro suppression of IFN-\(\gamma\) production by FV-induced Tregs was independent of the specificity of the CD8\(^+\) T cell.
**FV-induced Tregs suppress granzyme B expression by CD8+ T cells**

CD8+ T cell effector function was also measured by intracellular staining of granzyme B, an important molecule in the CD8+ T cell-mediated killing of FV-infected cells in vivo (53). Stimulation of FV-specific CD8+ T cells by peptide-pulsed APCs induced granzyme B production (Fig. 3A) and granzyme B levels were similar upon addition of CD4+CD25+ T cells from naive mice (Fig. 3B). In contrast, the overall granzyme B signal in CD8-gated cells was significantly lower in cocultures containing CD4+CD25+ T cells from chronically infected mice (Fig. 3B). Suppression of granzyme B expression was not observed with the CD25+ subset of T cells from either naive or persistently infected mice (Fig. 3C). Thus, FV-induced Tregs suppressed expression of both IFN-γ and granzyme B.

**Suppressive activity of FV-induced Treg requires cell-to-cell contact**

Mechanisms of T-regulated suppression have been proposed that include both soluble factors, such as IL-10 and TGF-β, and cell-to-cell contact-dependent interactions (54, 55). To determine whether FV-induced Treg suppression of CD8+ T cell was mediated by soluble factors or required direct cell-to-cell contact, in vitro suppression assays were performed using a Transwell system in which the Tregs were physically separated from CD8+ T cell targets by a permeable membrane. Separation of Treg from stimulated CD8+ T cells completely abolished suppression (Fig. 4A). Furthermore, supernatants from standard cocultures did not transfer suppressive activity (Fig. 4B), nor was suppression in cocultures blocked by the addition of anti-TGFβ and anti-IL-10R Abs (data not shown).

**FV-induced Treg inhibit effector function of activated CD8+ T cells in the absence of APCs**

Because the in vitro suppression assay contained peptide-pulsed APCs to activate the CD8+ T cells, it was not known whether suppression was occurring via direct interactions between the Tregs and the CD8+ T cells, or whether it required the presence of APCs. Furthermore, although the in vitro assay described above...
revealed that FV-induced Treg prevented complete activation of naive CD8\(^+\) T cells, it did not address whether Treg could suppress previously activated CD8\(^+\) T cells. Suppression of activated cells might be critical if the function of the virus-induced Treg in vivo was to limit immunopathology during an ongoing immune response. To address both questions, we assessed the effects of FV-induced Tregs on in vivo-activated TCR Tg CD8\(^+\) T cells and modified the assay such that APC were no longer needed for in vitro activation. To obtain activated FV-specific CD8\(^+\) T cells, TCR Tg CD8\(^+\) T cells that were marked with a GFP transgene were adoptively transferred into mice acutely infected with FV, a context in which the cells proliferate and differentiate into effector cells normally (27). After 4 days, donor cells were repurified from host spleens by sorting for GFP-positive CD8\(^+\) T cells. Ninety-eight percent or more of the transferred CD8\(^+\) T cells were activated as evidenced by the high expression of CD44 (Fig. 5A). Activation of the CD8\(^+\) T cells was maintained in vitro by the addition of FV-specific MHC class I tetramers, thereby eliminating the need for APCs. Intracellular staining demonstrated that the majority of CD8\(^+\) T cells from cocultures containing naive CD4\(^+\)CD25\(^+\) T cells expressed IFN-\(\gamma\) (Fig. 5B). In contrast, CD8\(^+\) T cells cocultured in the presence of CD4\(^+\)CD25\(^+\) T cells from chronically infected mice expressed significantly lower levels of IFN-\(\gamma\) (Fig. 5B). Virtually none of the IFN-\(\gamma\)-produced in these cocultures came from the CD4\(^+\) T cells (Fig. 5C). Thus, FV-induced Treg cells directly suppressed IFN-\(\gamma\)-production by activated CD8\(^+\) T cells in the absence of APCs.

**Discussion**

One of the most significant findings from the current study is that FV-induced Tregs suppress the effector function of naturally expanded and activated CD8\(^+\) T cells. This ability is integral to the hypothesized function for Tregs in preventing CD8\(^+\) T-cell-mediated immunopathology during the course of a viral infection (22, 23, 56). Most viral infections elicit potent CD8\(^+\) T cell responses that are characterized by a preset program of rapid proliferation coupled with development of effector functions that include secretion of IFN-\(\gamma\) and formation of cytolytic granules containing granzyme B (57, 58). The expansion and differentiation of CD8\(^+\) T cells is not only essential for virus clearance, but the degree of expansion also correlates with the pool size of memory cells necessary for protection from future infections (58, 59). This expansion and differentiation process leads to circulation of large numbers of activated CTLs that have the potential to cause significant collateral damage due to cross-reactivity or bystander killing. Thus, in order for Treg to play a role in preventing immunopathological damage from these cells without disrupting immunity, they must have the capacity to suppress at a postproliferative stage of the process. Our data demonstrate that FV-induced Tregs indeed possess this capacity and operate by suppressing activated and differentiated CD8\(^+\) T cells at the level of effector function.

The beneficial aspect of protection from immunopathology is counterweighted by the ability of some pathogens to subvert the immunoregulatory system to assist in establishing and maintaining chronic infection. Since the initial description of Tregs in chronic FV infections of mice was reported (17), Tregs have also been shown to suppress immune responses in humans chronically infected with HIV (60–63), the hepatitis C virus (18, 64), and EBV (65). It is notable that these are all viruses that cause chronic infections, and evidence suggests that immunosuppressive activity by Tregs before complete eradication of the infectious agent contributes to their evasion of host immune responses. Another adverse consequence of virus-induced Tregs could be generalized immunosuppression due to the nonspecific nature of Treg-mediated suppression (66–68). Consistent with this hypothesis, we...
show that FV-induced Treg suppress CD8+ T cells in vitro regardless of the TCR specificity of the CD8+ T cell. This result is in keeping with previous in vivo experiments showing that FV-induced Tregs suppressed CD8+ T cell-mediated rejection of both FV-induced tumors and tumors not expressing FV Ags (17). Indeed, HIV patients have an increased risk of developing certain malignancies, especially those associated with viruses (69–71). Of course, HIV patients are subject to other forms of virus-induced immunosuppression such as CD4+ T cell depletion, but virus-induced Treg cells may be a contributing factor in increased susceptibility to cancer.

The ability to inhibit CD8+ T cell effector function rather than proliferation distinguishes FV-induced Treg from natural Tregs, which generally inhibit at the level of proliferation in vitro. Another notable difference between natural Tregs and virus-induced Tregs is that natural Tregs typically require stimulation in vitro to exhibit suppressive activity (11, 12). In our in vitro assay, FV-induced Tregs demonstrated suppressive activity directly ex vivo. This finding is consistent with studies of the hepatitis C virus (64) and the feline immunodeficiency virus (72) showing that virus-induced immunosuppression such as CD4+ T cell depletion, but virus-induced Treg cells may be a contributing factor in increased susceptibility to cancer.

In conclusion, the results from the current in vitro study are consistent with the salient features of in vivo Treg function in mice with chronic FV infections. For example, both in vivo and in vitro FV-induced Tregs inhibit virus-specific CD8+ T cell effector functions without inhibiting proliferative responses or the up-regulation of activation markers (27). The in vitro assay also duplicates the finding that FV-induced Treg suppression of CD8+ T cells occurs without regard to the specificity of the CD8+ T cells (17). Although little is known about the mechanisms of virus-induced Treg suppression in vivo, the in vitro assay has enabled us to determine that direct cell-to-cell interactions between Treg and CD8+ T cells mediate suppression with no involvement of APCs. This finding indicates that distinct cell surface interactions mediate inhibitory signaling events. Thus, this assay provides us with an opportunity to discover the molecules involved in Treg-mediated suppression of CD8+ T cells and investigate their function. Such information is critical for the development of therapeutics to modulate Treg function by blocking the receptor-ligand interactions or the downstream signaling pathways.

Acknowledgments

We thank Robert Heinzen, Jeff Shannon, Brandon Walter, and Sonja Best for critical review of the manuscript, and Anita Mora and Gary Hettrick for assistance with graphics.

Disclosures

The authors have no financial conflict of interest.

FIGURE 5. FV-induced Tregs suppress in vivo-activated CD8+ T cells in the absence of APCs. A, The activation of adoptively transferred GFP+ FV-specific CD8+ T cells, as indicated by CD44 expression, was determined by flow cytometry. Histograms depict the relative expression of CD44 in the in vivo-activated CD8+ T cells (thick line; MFI = 11,413) compared with naive FV-specific CD8+ T cells before transfer (shaded; MFI = 44.2). In vivo-activated, GFP-positive, FV-specific CD8+ T cells (3 x 105) were stimulated with a specific tetramer and cocultured with CD4+ CD25+ T cells (6 x 105) from either naive or chronically infected mice. After 48 h, cells were stained for intracellular IFN-γ and analyzed by flow cytometry. B and C, Representative histograms of CD8-gated (B) or CD4-gated (C) T cells from cocultures containing CD4+ CD25+ T cells from chronically infected (thick line) or naive mice (shaded) are shown. Data were confirmed by ELISA and showed significant suppression of IFN-γ production from two independent experiments (p = 0.0128 and 0.014). The MFI values for IFN-γ staining in CD8+ T cells (B) were 67.0 for naive and 18.5 for chronically infected mice. For CD4+ T cells (C) the MFI values were 6.0 for naive and 6.2 for chronically infected mice.
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