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Pathological Role of Regulatory T Cells in the Initiation and Maintenance of Eczema Herpeticum Lesions

Ryo Takahashi,* Yohei Sato,† Maiko Kurata,‡ Yoshimi Yamazaki,‡ Momoko Kimishima,‡ and Tetsuo Shiohara*†

It remains unknown why the occurrence of eczema herpeticum (EH) caused by an extensive disseminated cutaneous infection with HSV-1 or HSV-2 is associated with the exacerbation of atopic dermatitis lesions after withdrawal of treatment. Although regulatory T cells (Tregs) limit the magnitude of HSV-specific T cell responses in mice, their role in the induction and resolution of EH has not been defined. We initially investigated the frequencies, phenotype, and function of Tregs in the peripheral blood of atopic dermatitis with EH (ADEH) patients at onset and after clinical resolution, atopic dermatitis patients without EH, and healthy controls. Tregs with the skin-homing phenotype and the activated/induced phenotype were expanded at onset and contracted upon resolution. Treg-suppressive capacity was retained in ADEH patients and, the expanded Tregs suppressed IFN-γ production from HSV-1–specific CD8+ and CD4+ T cells. The increased frequency of CD14dimCD16+ proinflammatory monocytes (pMOs) was also observed in the blood and EH skin lesions. Thus, pMOs detected in ADEH patients at onset were characterized by an increased ability to produce IL-10 and a decreased ability to produce proinflammatory cytokines, unlike their normal counterparts. Our coculture study using Tregs and pMOs showed that the pMOs can promote the expansion of inducible Tregs. Tregs were detected in ADEH patients at onset and after clinical resolution, atopic dermatitis patients without EH, and healthy controls. Tregs with the skin-homing phenotype and the activated/induced phenotype were expanded at onset and contracted upon resolution. Treg-suppressive capacity was retained in ADEH patients and, the expanded Tregs suppressed IFN-γ production from HSV-1–specific CD8+ and CD4+ T cells. The increased frequency of CD14dimCD16+ proinflammatory monocytes (pMOs) was also observed in the blood and EH skin lesions. Thus, pMOs detected in ADEH patients at onset were characterized by an increased ability to produce IL-10 and a decreased ability to produce proinflammatory cytokines, unlike their normal counterparts. Our coculture study using Tregs and pMOs showed that the pMOs can promote the expansion of inducible Tregs. Tregs were detected frequently in the vicinity of HSV-expressing and varicella zoster virus–expressing CD16+ monocytes in the EH lesions. Expansions of functional Tregs, together with pMOs, initially required for ameliorating excessive inflammation occurring after withdrawal of topical corticosteroids could, in turn, contribute to the initiation and progression of HSV reactivation, resulting in the onset of EH. *The Journal of Immunology, 2014, 192: 969–978.

Eczema herpeticum (EH), caused by an extensive disseminated cutaneous infection with HSV-1 or HSV-2, is the most commonly recognized viral complication in patients with atopic dermatitis (AD) (1–3). EH can present in a primary form or a recurrent form, and the primary infection is generally considered to be more severe with greater cutaneous involvement (3). However, patients with recurrent EH often develop disseminated vesicular lesions that are accompanied by systemic symptoms, such as fever, malaise, and lymphadenopathy, findings that are indistinguishable from the primary infection. In addition, given that these recurrent EH lesions often occur in the previously involved site, the dissemination is likely not true autoinoculation derived from the original infection site, but it may represent reactivation from viral latency at the site. These results, together with the finding that severe, untreated AD lesions have EH develop more easily than in patients with well-controlled disease (1), suggest that AD patients with uncontrolled eczematous lesions are at greater risk for the development of this viral complication.

Protective immunity to HSV in humans depends on the CD4+ and CD8+ T effector cell populations that recognize viral Ags; in contrast, damage to the skin is also initiated by these effector T cell populations (4). Because CD4+ regulatory T cells (Tregs) are known to suppress such excessive adaptive immune responses to HSV (5), thereby limiting immunopathology, it has been tempting to speculate that an imbalance in the proportion of effector T cells to Tregs may contribute to the development of EH. A number of previous studies (6, 7) yielded conflicting findings on the frequency of circulating Tregs in patients with AD. However, because no previous studies assessed alterations in the frequency and functional properties of circulating Tregs, depending on the clinical symptoms in patients with AD, particularly those associated with viral infections, it remains unknown how alterations in Treg frequency and function could contribute to the initiation and resolution of EH lesions. Thus, the occurrence of EH often associated with the exacerbation of AD lesions may have resulted from alterations in the number and function of Tregs.

In recent studies, Leung and colleagues (8) found that AD patients susceptible to EH have a unique phenotype characterized by more severe disease, early age of onset, more frequent history of other atopic disorders, greater Th2 polarity, allergen sensitization, and more frequent skin infections with other microbes. However, Tregs in AD patients with recurrent episodes of EH have not been studied extensively during the course of the illness, particularly at the acute stage and after clinical resolution. To elucidate the role of Tregs in the development and resolution of
EH, sequential analyses of Tregs at different stages of the illness are needed. In this study, we were interested in investigating the role of Tregs in controlling excessive immune responses associated with HSV infections during the course of the illness and examining differences in Treg number, phenotype, and function between AD patients complicated with EH and those without EH. We also explored the role of CD14dimCD16+ proinflammatory monocytes (pMOs) in inducing Treg expansions. Our results indicated that the increased frequencies of fully functional Tregs, together with pMOs at or just prior to the onset of EH, compromise the efficacy of antiviral immune responses, thereby allowing reactivation of latent HSV and the induction of illness.

Materials and Methods

Patients

Thirty-nine patients with adult-type AD according to the criteria of Hanifin and Rajka (9), who developed EH were enrolled in this study of atopic dermatitis with eczema herpeticum (ADEH). A diagnosis of EH was clinically based on the appearance of disseminated vesicles, pustules, or erosion on the face and other sites of the body, such as the trunk and arms. The clinical diagnosis was confirmed by either commercial immunofluorescence tests to identify HSV-infected cells, PCR for HSV-DNA, or serology. All patients had long-lasting mild-to-severe disease with typical eczematous skin lesions, elevated serum IgE levels (<1000 IU/ml), and specific serum IgE Ab to aeroallergens. The severity of disease was assessed by the SCORing Atopic Dermatitis (SCORAD) (10) index at the time of enrollment. Clinical characteristics are presented in Table I. All patients with EH were positive for HSV-IgG and had recurrent HSV infection but with or without a history of EH: they were defined as recurrent EH. Seventeen AD patients who were positive for HSV-IgG but had no episode of EH were defined as ADEH. Healthy volunteers who had no history of asthma, allergic rhinitis, or AD were enrolled in this study and served as healthy controls. Blood samples were obtained from these patients on or near the day of the initial presentation before starting antiviral treatment, and additional samples were subsequently obtained from these patients ≥2 wk after clinical resolution. In the patients with primary HSV infection, blood samples were sequentially obtained on several occasions 1–2 wk apart after onset. Informed consent was obtained from all subjects before entry into the study, and the Institutional Review Board at Kyorin University School of Medicine approved this study. None of the patients or control subjects received medication, such as corticosteroids or cyclosporine A, or any systemic therapy, including UV light therapy, within ≥4 wk before study entry. Topical corticosteroids were withheld for ≥2 d before blood collection. Peripheral blood was obtained on informed consent in accordance with the Declaration of Helsinki under protocols approved by the Institutional Review Board at Kyorin University School of Medicine.

Abs and reagents

For FACS analysis, Abs to human CD4 (SK3), CD8 (Leu-2a, OKT8), CD14 (RM052, MSEE), CD16 (3G8), CD19 (Leu-12), CD25 (sA3), CD45RA (HI100),CCR4 (1G1), CD56 (Leu-19, HCDC6), TCR-γ-δ (11F2, B1), cutaneous lymphocyte–associated Ag (CLA; HECA-452), Foxp3 (PCH101), Helios (22F6), CTLA-4 (BNI3), CD127 (HIL-7R-M21), CD39 (eBioA1), Ki67 (B56), FcRlO (AER-37), IL-1β (H1b-98), IFN-γ (25723.11), TNF-α (601.1111, Mab11), IL-17A (eBio64DEC17), IL-4 (3010.211), IL-6 (AS12), and the isotype controls to these Abs, as well as BD FastImmune CD28/ CD49d costimulatory reagent and 7-aminocoumarin D, were purchased from BD Biosciences, eBioscience, Beckman Coulter, or BioLegend. For cell culture, Abs to human CD3 (HI38), CD28 (37407), TGF-β1 (1D8), CD40L (L3D10), and IL-10 (JE3-19F1) were purchased from eBioScience, and Ab to human AB serum (both from Sigma-Aldrich). After stimulation, intracellular staining was done as described below.

Stimulation of T cells and monocytes

PBMCs were stimulated with 25 ng/ml PMA plus 1 μg/ml ionomycin or 10 μg/ml Pam3Cys for 4 h (PMA and Pam3Cys for T cells and monocytes, respectively) in the presence of 10 μg/ml brefeneldin A (Sigma–Aldrich) or for 15 h (Pam3Cys for IL-10 production from monocytes) in the presence of BD GolgiStop (BD Biosciences). All cells were cultured in RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% FCS. After stimulation, intracellular staining was done as described below.

Flow cytometric analysis

PBMCs were stained with a combination of labeled Abs and measured by flow cytometry. To detect intracellular Foxp3 expression, an Allophycocyanin Anti-Human Foxp3 staining set (eBioscience) was used according to the manufacturer’s instructions. For intracellular staining, cells harvested from stimulation cultures were incubated in lysing solution and permeabilizing solution (BD Biosciences) and then incubated for 30 min with anti-IFN-γ, anti–TNF-α, anti–IL-17, anti–IL-4, anti–IL-6, or anti–IL-1β Ab. To detect intracellular IL-10 expression, Fixation/Permeabilization buffer (eBioscience) was used. The samples were analyzed on a FACSCalibur or FACSCanto II flow cytometer (BD Biosciences). For control, Abs were replaced with nonreactive isotype-matched Abs.

T cell–proliferation assay

PBMCs from patients were cocultured with FACS-sorted Tregs (CD4+CD25++; 3,000 cells/well) in the presence of allogeneic APCs (25,000 cells/well obtained from healthy adult volunteers) in U-bottom 96-well plates (Corning), as described previously (12, 13). Cells were also cocultured at Treg/effector cell ratios of 0.25:1, 0.5:1, and 1:1. The cells were stimulated with 0.1 μg/ml soluble anti-CD3 (HIT3a) and anti-CD28. All cells were cultured in triplicate and in RPMI 1640 medium supplemented with 5% human AB serum (both from Sigma–Aldrich). After 4 d of culture, 1 μCi [3H]thymidine (GE Healthcare) was added to each well. The cells were harvested after 16 h, and radioactivity was measured using a scintillation counter (MicroBeta; PerkinElmer Life Sciences). The proliferative response of CD4+CD25+ T cells in the absence of CD4+CD25− T cells was normalized to 100% to calculate the percentage of proliferation resulting from the addition of CD4+CD25+ T cells to the culture.

Treg-depletion assay

Depletion of Tregs from PBMCs was performed using MACS CD4+CD25+ CD127dim− regulatory T cell isolation kit II (Miltenyi Biotec), according to the manufacturer’s instructions.

Identification of monocyte subpopulations

PBMCs were gated on the putative monocyte fraction, including a portion of the adjacent lymphocytes, as demonstrated previously (14). Monocyte populations can be divided phenotypically, based on the surface expression of CD14 and CD16, into CD14+CD16−, CD14+CD16+, and CD14−CD16− cells. However, because our previous study (14) demonstrated that cell surface expression of CD16 was dramatically downregulated after Pam3Cys stimulation in vitro, CD16 expression was not used for identifying the CD14+CD16− mononuclear monocytes in the intracellular cytokine-production assay. Because a monocyte population characterized by low levels of CD14 and the absence of FcRII expression was found to be identical to the CD14+CD16− pMOs, gating on the CD14+CD16− pMOs for the CD14dimFcrRII− population was used as pMOs (Fig. 7A, 7C) for the intracellular cytokine-production assay. Thus, monocytes were divided into three subpopulations: CD14+CD16− classical monocytes (cMOs), CD14+CD16+ pMOs, and CD14−CD16− monocytes (Fig. 7A).

Cocultures of Tregs and monocytes

For coculture studies, CD14dim pMOs were purified by sorting out the CD14+ pMOs (purity ∼ 93 ± 1.5%). The FACS-sorted CD14dim pMOs (12,500 cells) were cocultured with allogeneic purified CD4+ T cells (1.25 × 105 cells), obtained from a healthy volunteer, in a 5–ml BD Falcon polystyrene round-bottom tube with RPMI 1640 medium supplemented with 5% AB
serum in the presence of anti-CD3Ab and anti-CD28Ab for 7 d, after which all cultured cells in the tube were analyzed by FACS.

Two-color immunohistochemical staining

Double immunohistochemical staining was performed by using Abs to Foxp3, CD16, CD19, HSV, and VZV Ag, and positive cells were identified in the skin biopsy specimens from EH lesions. Briefly, after deparaffinization, 5-μm sections were treated with target retrieval solution (Dako; S1699) and boiled in an autoclave at 121°C for 10 min. After cooling down, the sections were incubated with primary Abs at 4°C overnight. After washing out unbound primary Abs with PBS, the tissue sections were incubated with EnVision + Dual Link (Dako; K4063). Immunoreactivity was detected using an AEC Liquid Substrate Chromogen (Dako; K3464). Microscope images were taken with a Canon Eos 5D mark II digital single-lens reflex camera attached to an Olympus BX51 microscope. HSV Ag–expressing CD16+ cells in the dermis were counted in five high-power fields.

Statistical analysis

Data are expressed as mean ± SEM and were determined using the Student t test or the Dunnett test.

Results

Frequency and phenotype of Tregs in PBMCs at onset of EH

We first examined the frequency and phenotype of Tregs in PBMC obtained from patients enrolled. The clinical and biological characteristics of patients at the time of obtaining blood are summarized in Table I. As shown in Fig. 1, a significant decrease in the frequencies of γδ T cells and CD56+ NK cells was found in AD patients, irrespective of EH, compared with healthy controls, a finding consistent with our previous report (15). In contrast, there were no significant differences in the frequencies of CD8+ T cells. We next investigated whether the frequency and phenotype of circulating Foxp3+ Tregs could be altered depending on the acute and resolution stages of EH. As shown in Fig. 2A, AD patients with EH had a significantly higher frequency of CD4+CD25+ Foxp3+ Tregs in PBMCs at the onset of disease compared with after disease resolution: the mean frequencies of Tregs at the onset of EH were higher than those in ADEH2 patients, suggesting that Tregs could be reduced upon secondary Abs at 4°C overnight. After washing out unbound primary Abs with PBS, the tissue sections were incubated with EnVision + Dual Link (Dako; K4063). Immunoreactivity was detected using an AEC Liquid Substrate Chromogen (Dako; K3464). Microscope images were taken with a Canon EOS 5D mark II digital single-lens reflex camera attached to an Olympus BX51 microscope. HSV Ag–expressing CD16+ cells in the dermis were counted in five high-power fields.

Table I. Clinical and biological characteristics of ADEH, ADEH−, and control samples

<table>
<thead>
<tr>
<th>Clinical and Biological Characteristics</th>
<th>ADEH</th>
<th>p Valueb</th>
<th>ADEH−</th>
<th>p Valuec</th>
<th>Healthy Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>39</td>
<td>17</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y; mean ± SEM)</td>
<td>27.6 ± 2.2</td>
<td>0.001</td>
<td>32.8 ± 2.5</td>
<td>0.797</td>
<td>33.6 ± 1.1</td>
</tr>
<tr>
<td>tSCORAD (mean ± SEM)b</td>
<td>40.0 ± 3.6</td>
<td>NA</td>
<td>36.1 ± 3.2</td>
<td>NA</td>
<td>31.9 ± 1.3</td>
</tr>
<tr>
<td>Serum IgE (IU/mL; mean ± SEM)b</td>
<td>9758.3 ± 1810.4</td>
<td>0.049</td>
<td>10382.7 ± 3909.5</td>
<td>0.043</td>
<td>118.9 ± 47.1</td>
</tr>
<tr>
<td>Serum anti-HSV IgG (EIA unit; mean ± SEM)b</td>
<td>73.98 ± 19.2</td>
<td>0.033</td>
<td>117.46 ± 22.7</td>
<td>0.002</td>
<td>5.3 ± 1.6</td>
</tr>
</tbody>
</table>

77x138 Versus healthy control.

87x368 Data for SCORAD, serum IgE, and serum anti-HSV IgG titers in ADEH are at onset of disease.

EIA, Enzyme immunoassay; NA, not applicable.

FIGURE 1. The percentages of CD4+ and CD8+ T cells, CD19+ B cells, CD56+ NK cells, and TCRγδ+ T cells in PBMCs from ADEH patients, ADEH patients, and healthy controls. Thirty-nine ADEH patients, 17 ADEH patients, and 34 healthy controls were analyzed as described in Materials and Methods. Results are expressed as the mean percentage of each subset ± SEM in PBMCs (in a lymphocyte gate). The p values were determined using the Student t test. RESO, Resolution.
FIGURE 2. Expansion of skin-homing Foxp3+CD25+CD127dim/2CD39+HELIOS+ Tregs during the acute stage of ADEH. (A) Representative flow cytometry dot plots showing the expression of Foxp3 versus CD25, Foxp3 versus CLA, and Foxp3 versus CCR4 in CD4+ T cells from ADEH, ADEH−, and healthy controls. Numbers in each quadrant indicate the frequency of each fraction. The mean frequency of Foxp3+CD25+, Foxp3+CLA+, and Foxp3+CCR4+ Tregs in CD4+ T cells in the patients and healthy controls is shown (right panels). ADEH acute stage (n = 19), ADEH resolution stage (RESO; n = 23), ADEH+ (n = 16), and healthy controls (n = 34). Data are mean ± SEM. (B) Phenotypic analysis of CD4+Foxp3+ Tregs obtained from the patients and healthy controls. Representative flow cytometry dot plots showing the expression of Foxp3 versus CTLA-4, Foxp3 versus CD127, Foxp3 versus CD39, Foxp3 versus HELIOS, and Foxp3 versus Ki67 in CD4+ T cells. The mean frequency of Foxp3+CTLA-4+, Foxp3+CD127dim/2, Foxp3+CD39+, and Foxp3+HELIOS+ Tregs in CD4+ T cells is shown (right panels). Fraction I: CD45RA+Foxp3*, Fraction II: CD45RA−Foxp3*, Fraction III: CD45RA−Foxp3+.

(Figure legend continues)
FIGURE 3. A sequential analysis of Treg frequency, serum HSV IgG level, and clinical course in a single AD patient with EH induced by a primary infection with HSV. (A) Flow cytometry dot plots showing the expression of Foxp3 versus CD25 in CD4+ T cells. (B) Clinical course of symptoms in relation to treatment. Treg frequency, and serum HSV IgG titers.

**Temporal relationship between clinical course and Treg frequency**

We undertook a sequential analysis of Treg frequency in an AD patient with EH induced by a primary infection of HSV over the ensuing 48-d period (Fig. 3). This patient provided blood samples twice during an episode of EH and twice during remission. The frequency of Tregs observed at onset was in the same range as what we observed in other AD patients with recurrent EH induced by reactivation of HSV. However, in contrast to what was observed in the recurrent form of disease, the frequency of Tregs after clinical resolution did not correlate with the improvement in clinical symptoms, and it was even increased after resolution on day 13 compared with that at onset in this patient. Symptomatic flares (on day 36), although subsequently followed by clinical improvement of EH, were observed during this period in this patient, despite a persistent elevation in circulating Tregs. However, after the frequency of Tregs was profoundly decreased (on day 48), symptomatic flares of EH were no longer seen. These results indicate that expansion of Tregs is not a consequence of HSV infection but may be a causal factor.

**Functional activity of Tregs in EH**

Because our previous studies demonstrated that the functional activity of Tregs was profoundly altered depending on the stage examined in severe drug eruptions such as Stevens-Johnson syndrome and toxic epidermal necrolysis (18), we next investigated whether changes in Treg frequency were associated with alterations in Treg function. As shown in Fig. 4, Tregs obtained from AD patients with EH, either at the onset of disease or after its resolution, retained the suppressive capacity to inhibit proliferation of effector T cells, similar to healthy controls. Similar results were seen, even when CD4+CD25++ Tregs and CD4+CD25− effector T cells obtained from the different stages in either the same or different patients were cocultured (data not shown). These results suggest that the net inhibitory effects of Tregs during the acute stage of EH could be maximal because of their increased frequency at this time.

To further investigate which inhibitory mediators or molecules from Tregs could be responsible for inhibition of effector T cell proliferation, FACS-sorted CD4+CD25− and CD4+CD25++ cells were cocultured with allogeneic APCs, obtained from healthy adult volunteers, in the presence of anti-CD3 + CD28 Abs, and the blocking Abs to TGF-β1, CTLA-4, or IL-10 or isotype-control Ab were added to the culture. As shown in Supplemental Fig. 1, the suppressive activity of Tregs was abrogated when an anti–TGF-β1 or anti–CTLA-4 Ab was used. These results indicate that Tregs could inhibit proliferation of effector T cells via either TGF-β or CTLA-4.

**Inhibition of IFN-γ and TNF-α production by CD8+ T cells, CD4+ T cells, and CD56+ NK cells associated with expansion of Tregs**

Because Tregs were shown to inhibit IFN-γ and TNF-α production (18, 19), we postulated that their production could be impaired by the increased Tregs. To monitor IFN-γ, TNF-α, IL-17, and IL-4 production at a single-cell level in these patients at onset of disease and after its resolution, we analyzed the cytokine-expression profile of various lymphocyte subsets after a short in vitro stimulation with PMA plus ionomycin. As shown in Fig. 5, IFN-γ and TNF-α production by CD8+ T cells, CD4+ T cells, and CD56+ NK cells was impaired in AD patients with EH at onset of disease compared with after its resolution. However, following the decrease in Tregs after the resolution of disease, the impaired ability of these cells to produce IFN-γ and TNF-α was restored to levels comparable to those in healthy controls.

**Depletion of Tregs restores HSV-1–specific CD8+ and CD4+ T cell IFN-γ production**

We next asked whether HSV-specific immune responses could be impaired by expanded Tregs at the onset of EH. To demonstrate that

Foxp3+CD39+, Foxp3+HELIOS+, and Foxp3+Ki67+ Tregs in CD4+ T cells from the patients and healthy controls is shown (right panels). (C) CD4+Foxp3+ cells can be divided into three functionally distinct subpopulations defined by Foxp3 and CD45RA expression: CD45RA−Foxp3+ rTregs, CD45RA+Foxp3+ iTregs, and CD45RA− Foxp3+ non-Tregs. (D) Increased CD4+CD45RA− Foxp3+ iTregs are seen during the acute stage of ADEH but not during the resolution stage or in ADEH+ patients. Representative flow cytometry dot plots showing the expression of CD45RA versus Foxp3 in CD4+ T cells. Fraction II is indicated by red boxes. The mean frequency of each fraction in CD4+ T cells from the patients and healthy controls is shown (lower panels). In (B) and (D): ADEH (acute stage, n = 9; resolution stage [RESO], n = 12), ADEH− (n = 6), and healthy controls (n = 8). The p values were determined using the Student t test (versus healthy controls). Fr., Fraction.
Tregs could be responsible for inhibition of HSV-1–specific CD8+ T cells produced in CD14+ cMOs (20, 21) and that they can patrol the absence of HSV-1–specific CD8+ T cells in the presence of mitomycin C–treated allogeneic APCs and anti-CD3 and anti-CD28 Abs. Proliferation was assessed by a [3H]thymidine-incorporation assay. The results are expressed as the percentage proliferation of CD4+CD25++ T cells in the absence of CD4+CD25++ Tregs. Mean (n = 3) and SEM are shown.

IFN-γ production by HSV-specific CD8+ and CD4+ T cells could be abrogated by expanded Tregs at the onset of disease, we analyzed the effects of the depletion of Tregs on IFN-γ production by HSV-1 Ag–specific CD8+ and CD4+ T cells from these patients. As shown in Fig. 6A, IFN-γ production by HSV-1–specific CD8+ and CD4+ T cells was restored upon depletion of Tregs from PBMCs obtained from AD patients with EH at onset of disease; surprisingly, Treg depletion resulted in a 2-fold increase in the frequency of HSV-1–specific CD8+ T cells producing IFN-γ. TNF-α production by HSV-1–specific CD8+ T cells was not restored by Treg depletion (data not shown).

To further confirm whether IL-10, TGF-β1, and CTLA-4 from Tregs could be responsible for inhibition of HSV-1–specific CD8+ T cell IFN-γ production, the blocking Abs to IL-10, TGF-β1, or CTLA-4 or isotype-control Abs were added to the culture. As shown in Fig. 6B and 6C, IFN-γ production by HSV-1–specific granzyme B+ CD8+ T cells increased significantly when anti–IL-10 Ab was added to the culture. These results indicate that Tregs expanded at the initial stage of HSV infection may hamper the anti–HSV-1–specific immune responses required for clearance of HSV and, thereby, diminish viral control.

**Expansion of CD14dimCD16+ pMOs and alteration of cytokine production**

Recent studies reported that CD14dimCD16+ pMOs can produce less IL-10 and more inflammatory cytokines, such as IL-1β and TNF-α, in response to viruses or microbial stimuli than those produced in CD14+ cMOs (20, 21) and that they can patrol the whole body for signs of inflammation and infection (22), suggesting their important role in antiviral immunity. Because pMOs were reported to increase in frequency in the settings of systemic inflammation and infection (23), we investigated whether they also could be increased at the onset of EH and whether their production of cytokines could be altered depending on the stage of disease. As shown in Fig. 7B and 7D, pMOs were dramatically increased at the onset of EH. Because the vast majority of pMOs were negative for FcεRI expression in healthy controls (Fig. 7C), the CD14dimFceRI− population was defined as pMOs for intracellular cytokine analysis. As shown in Fig. 7E, the selective impairment of TNF-α, IL-1β, and IL-6 production was observed in the pMOs from ADEH patients at the onset of EH, and it was restored to the levels similar to those in healthy controls upon clinical resolution, whereas their IL-10 production was dramatically increased at the onset of EH. These results suggest that, at the onset of EH, the pMO subset may have a positive effect on Treg development.

**pMO-mediated iTreg expansion at onset of EH**

Recently, Zhong et al. (24) reported that pMOs can control peripheral Treg development in immune thrombocytopenia. Because we demonstrated in this study that pMOs in ADEH have the ability to produce more IL-10 and less proinflammatory cytokines than their normal counterparts, we investigated whether pMOs derived from AD patients at the onset of EH could induce iTreg expansion, unlike their counterparts in thrombocytopenia. As shown in Fig. 8, we found that pMOs obtained from ADEH...
patients at the onset of EH induced significant increases in the frequencies of iTregs compared with those from healthy controls. These results indicate that the pMOs derived from patients with ADEH can promote Treg development via their increased IL-10 production.

Selective accumulation of Tregs in EH lesions associated with HSV and VZV reactivation

Recent studies (25) demonstrated that HSV, as well as VZV, could be reactivated in EH lesions. To assess the relative contribution of Tregs to the reactivation of HSV or VZV at the preferential sites of virus replication, such as EH lesions, we used immunohistochemistry to investigate whether abundant Foxp3+ Tregs could be detected in the vicinity of CD8+ T cells and HSV- and VZV-expressing cells. As shown in Fig. 9, Foxp3+ Tregs were detected frequently in the EH lesions where CD8+ T cells and CD16+ cells were infiltrated. Foxp3+ Tregs also appeared to be spatially associated with HSV- and VZV-expressing keratinocytes and CD16+ monocytes, suggesting that accumulation of Tregs in EH lesions could contribute, in part, to the lack of an effective defense against viral reactivation. In contrast, neither NK cells nor neutrophils were found in the EH lesions, which makes it unlikely that Treg-dependent inhibition of NK cell activity or innate immune responses is the preferred mechanism of HSV reactivation in EH lesions. Interestingly, HSV-1 protein and VZV protein were detected in the cytoplasm of CD16+ pMOs, which were abundant in EH lesions (Fig. 9E, 9G). The results further indicate that the abundance of CD16+ monocytes with increased IL-10–producing ability in EH lesions may favor HSV reactivation, either directly or by inducing Treg expansion that can impair the immune defense against viral infections.

Discussion

HSV-specific CD4+ and CD8+ T cells were shown to play a critical role in the control and resolution of HSV disease (4), and their defects are more closely associated with severe HSV disease (26) rather than humoral immune defects. In contrast, because the cytotoxic protective T cell responses cause damage to infected host cells, the balance between the magnitude and the timing of this effector T cell response and those of Treg responses determines whether immune protection (i.e., elimination of the virus) or viral reactivation will predominate. Indeed, studies (5) using a murine model of neonatal HSV-2 infection demonstrated that Tregs suppress T cell effector responses to HSV, which could contribute to increased virulence of this virus.

In our study, we found that increased frequencies of functionally equivalent Foxp3+ Tregs correlated positively with disease activity in EH, suggesting that they participate in the regulation of antiviral effector responses whose dysregulation results in viral reactivation. In accordance with our results, Leung et al. (27) demonstrated that patients with ADEH have genetic defects in IFN-γ production by Tregs was selective for IFN-γ responses. If homeostasis in the skin immune system of ADEH patients is seen as a “balance” between genetically impaired IFN-γ responses. If homeostasis in the skin immune system of ADEH patients is seen as a “balance” between genetically impaired IFN-γ responses and Treg-mediated immunosuppression, one can imagine how the balance could be tipped by withdrawal of immunosuppressive therapy, such as corticosteroids.

Although the induction of Tregs is most likely a mechanism by which immunopathological damage is prevented, the immuno-
suppressive properties of Tregs can also allow some viruses to escape elimination by both the innate and acquired immune system, thereby allowing these latent viruses to reactivate. However, we could not totally exclude the possibility that an increase in the frequencies of Tregs is a secondary event that requires and follows the reactivation of HSV or clinical exacerbation. Consistent with this possibility, a number of microbial pathogens, including HSV, were shown to increase the frequency of Tregs in infected tissues or lymphoid organs of adult mice and humans (5). These findings suggested that the increase in the frequencies of Tregs that we observed at the onset of EH might be a consequence, not a cause, of viral reactivation or a clinical exacerbation. However, this possibility is unlikely because our sequential analysis of Treg frequencies in a patient with recurrent episodes of EH during the 48-d period after a primary infection demonstrated that the Foxp3+ Treg frequency remained higher than 10% by day 36, regardless of viral reactivation or a clinical exacerbation. Consistent with this possibility, our ongoing studies demonstrated that an increase in Treg frequency was never observed at the time of acute infection with VZV and parvovirus B19, during which the function of Tregs was impaired (R. Takahashi, manuscript in preparation).

If increased frequencies of Tregs are responsible, in part, for the course of immune reconstitution; thus, these expanded Tregs could act to prevent lymphopenia-associated autoimmunity in individuals recovering from lymphopenia in mice (29). Importantly, similar expansions of fully functional Tregs during the acute phase and the subsequent contraction upon resolution of disease as observed in EH also were reported to occur in patients with sarcoidosis or drug-induced hypersensitivity syndrome (30, 31), both of which fit within the spectrum of immune reconstitution syndrome (32, 33). In a recent retrospective analysis (1) of 100 cases of EH, 36% of the patients had noted a severe exacerbation of their AD that typically had started ∼2 wk before the onset of EH, and the majority of the patients had not received any corticosteroid therapy in the 4 wk before the onset of EH. In interpreting these findings, one must also appreciate that EH often develops in a lymphopenic state. A likely interpretation of these observations, in consideration of the data presented in this article, is that an abrupt shift in host immune responses from an immunosuppressive state to a robust pathogenic inflammatory state, an immune reconstitution syndrome–like phenomenon, would occur upon withdrawal of immunosuppressive agents, such as topical corticosteroids, long before the onset of EH, which could be manifested as an exacerbation of clinical symptoms. To counterbalance these overshooting inflammatory responses, functional Tregs that are key for maintaining a healthy balance between protection and immunopathology would expand, thereby allowing latent HSV to be reactivated in a uncontrolled fashion. Thus, the increased frequency of Tregs initially required as a counter-regulatory mechanism directed at preventing excessive inflammation could, in turn, contribute to the initiation and progression of HSV reactivation. Because this ordered sequence of events is thought to be orchestrated by interactions among innate and acquired immune cells, cytokines, and...
latent viruses, clear-cut documentation in an in vivo setting would be difficult to obtain. Altogether, our data propose a dual role for Tregs, either harmful or beneficial, by dampening the magnitude of antiviral immune responses at the site of infection depending on how and when they are expanded. We speculate that the magnitude and duration of Treg expansion exceed those required for protection and result, instead, in a net pathological, rather than protective, immune response: thus, a timely downregulation of Treg responses is crucial for the effective control of virus infections. Considering the dual role of Tregs in EH, therapeutic interventions on the expanded Tregs seem complicated and warrant additional studies.

With regard to how pMOs affect the Treg compartment, a few recent studies (24, 34) revealed their inhibitory effect on Treg expansion, contrary to our current data. In the current study, we demonstrated that pMOs detected in the acute stage of EH were characterized by the potent ability to expand Tregs, probably through their increased production of IL-10 and their decreased production of IL-1β, TNF-α, and IL-6, which were quite different from those detected in healthy controls, ADEH+ patients, and ADEH patients at the resolution stage. In view of the finding that this cytokine production pattern of pMOs in ADEH was quite similar to that of CD14+ cMOs that have the potent ability to expand Tregs, the failure of ADEH patients to develop efficient anti-HSV immune responses may reside fundamentally in the CD14+ pMO subset, with their relatively high capacity to synthesize IL-10, rather than with the Tregs themselves. Indeed, our data showed that purified CD14+ pMOs from ADEH patients have a potent ability to expand Tregs isolated from healthy volunteers. If so, the ability of anti–IL-10 Ab to restore HSV-1–specific CD8+ T cell IFN-γ production in ADEH patients could be attributed to its effect on the pMOs but not on Tregs. Given the preferential expression of HSV-1gD Ag in pMOs in EH lesions, HSV-1 infection of the pMOs may direct them toward a distinct phenotype characterized by increased IL-10 production and decreased proinflammatory cytokines, which, in turn, promotes the expansion of Tregs; this may represent an important HSV-1 immune-evasion strategy. If so, we cannot exclude the possibility that the expansion of Tregs in the acute stage of EH is a consequence of high levels of HSV replication. Thus, pMO-derived IL-10 might serve to expand Tregs and, thereby, interfere with the HSV immune responses. Rather, our results support that pMOs might be the most efficient in controlling anti-HSV immune responses. Thus, the specific profile of cytokines generated by pMOs during viral infection could shape the nature of the ongoing inflammatory responses.

In conclusion, increased and sustained Treg responses may be harmful to AD patients by blunting a proinflammatory response.
critical for HSV clearance, especially during the process of immune reconstitution after withdrawal of immunosuppressive agents. Our results suggest that therapeutic strategies that inhibit the expansion of Tregs may improve control of HSV reactivation in AD patients with a history of EH. Pharmacologic approaches aimed at maintaining an appropriate inflammatory response required for HSV clearance, while inhibiting collateral damage to tissues, are needed for AD patients who are frequently at risk for developing EH.

Disclosures
The authors have no financial conflicts of interest.

References