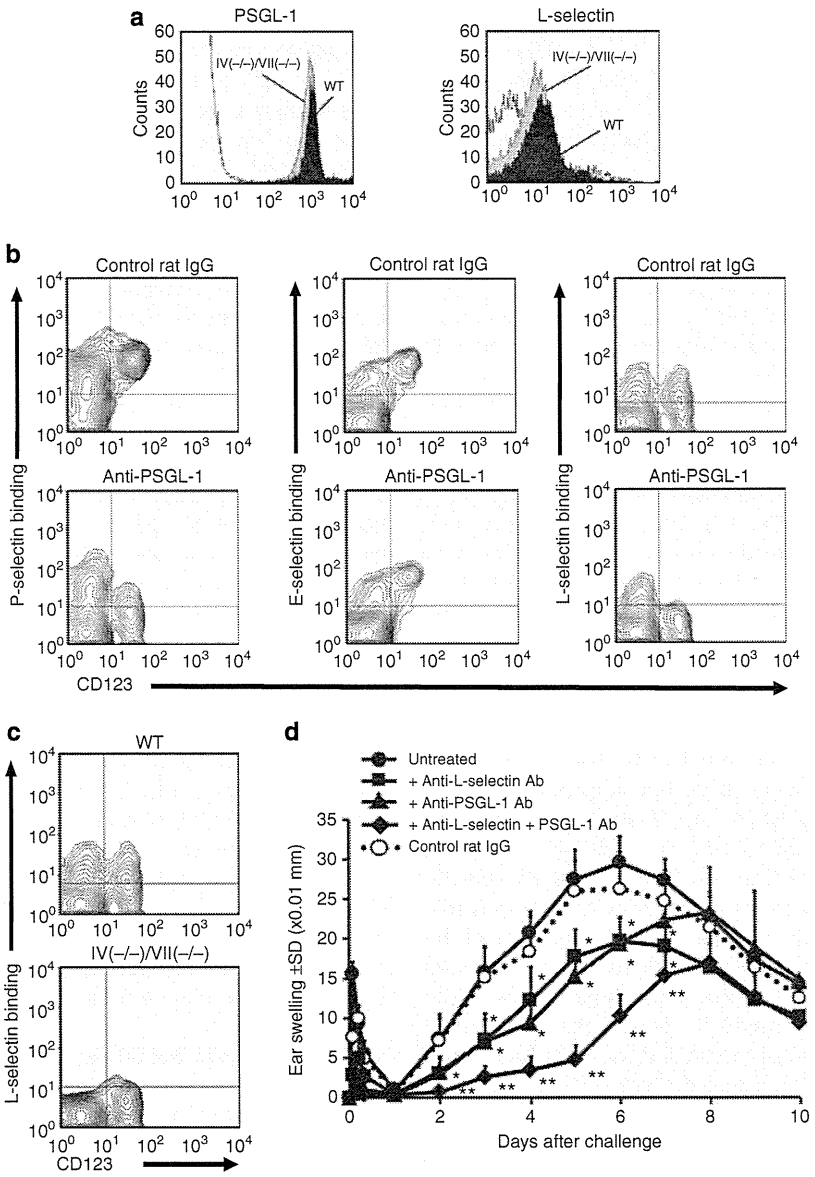

Figure 6. Basophil P-selectin glycoprotein-1 (PSGL-1)–L-selectin interaction involvement in IgE-mediated chronic allergic inflammation (IgE-CAI). (**a**) PSGL-1 and L-selectin expressions on basophils. (**b**) PSGL-1 Ab effect on selectin binding to primary basophils. (**c**) L-selectin binding to basophils from wild-type (WT) and α(1, 3) fucosyltransferase-IV (FT-IV)(−/−)/FT-VII(−/−) mice. (**d**) Effects of L-selectin and/or PSGL-1 Abs on IgE-CAI in WT mice. *$P<0.05$ compared with control IgG. **$P<0.05$ compared with L-selectin or PSGL-1 Ab alone.

of both the L- and P-selectins to the WT basophils (Figure 6b). Counter-receptor activity of PSGL-1 for L-selectin appears to be dependent on FT-VI/VII, as L-selectin failed to bind the primary basophils from FT-IV(−/−)/FT-VII(−/−) mice (Figure 6c). We then assessed whether blocking L-selectin could ameliorate IgE-CAI. As expected, the administration of the L-selectin blocking Ab (MEL-14, eBioscience, San Diego, CA) partly but significantly inhibited IgE-CAI. Similarly, PSGL-1 blocking Ab (4RA10) also inhibited IgE-CAI. When there was concomitant administration of these two Abs, further suppression of skin responses was also observed (Figure 6d).

DISCUSSION

Extravasation and recruitment of basophils to the skin are an essential step for the development of IgE-CAI (Mukai *et al.*, 2005; Obata *et al.*, 2007). This study examined the FT-IV/VII-dependent basophil recruitment and induction of IgE-CAI.

Although a single deficiency of the *FT-IV* or *FT-VII* genes did not affect IgE-CAI, greatly impaired skin responses were seen in the FT-IV(−/−)/FT-VII(−/−) mice. To elucidate the contribution of FTs in basophils during IgE-CAI, we transferred basophils into FcRγ(−/−) mice lacking FcεRI. Unlike those from WT mice, basophils from the FT-IV(−/−)/FT-VII(−/−)

mice failed to induce IgE-CAI in FcRγ(−/−) mice. These data confirm the critical contribution of basophils for the development of IgE-CAI (Mukai *et al.*, 2005) and suggest that impaired skin responses in FT-IV(−/−)/FT-VII(−/−) mice is largely due to the inability to recruit basophils into the skin.

Leukocytes other than basophils may also require selectins for their recruitment to the skin during IgE-CAI. Our results indicated that the transfer of WT basophils with basophil-depleted bone marrow cells (effector cells) from FT-IV(−/−)/FT-VII(−/−) mice were not able to fully develop IgE-CAI as compared with the WT basophils that were cotransferred with WT effector cells (Figure 4a). In addition, WT basophils themselves were not effectively recruited into the skin when in the presence of effector cells from FT-IV(−/−)/FT-VI(−/−) mice. Thus, it appears that some effector cells require FT-IV/VII-dependent modification of selectin ligands in order to be recruited to the skin. In addition, these cells appeared to increase the effectiveness of basophil recruitment to the skin. Once basophils are recruited into the skin, they can promote the accumulation of other effector cells. These cells, in turn, may then assist in further basophil recruitment into the skin.

Basophils from FT-IV(−/−)/FT-VII(−/−) mice did not show avidity to soluble E- and P-selectins, which indicates that these are dependent on the FT function (Figure 5b). However, IgE-CAI was unexpectedly not suppressed after the use of blocking Abs against P- and E-selectins, despite the inability of basophils from FT-IV(−/−)/VII(−/−) mice to induce IgE-CAI (Figure 3b). Conversely, blockade of L-selectin resulted in a moderate suppression of IgE-CAI. It is possible that PSGL-1 on basophils could be a counter-receptor of the basophil L-selectin. On the basis of our results that showed that WT basophils could successfully induce IgE-CAI in FT-IV(−/−)/FT-VII(−/−) mice, it appears that endothelial L-selectin ligands might not be essential for basophil recruitment. We demonstrated that L-selectin bound PSGL-1 *in vitro*, and this binding was dependent on the basophil FT-IV/VII. In addition, when we blocked PSGL-1, this alleviated IgE-CAI *in vivo*. These were similar to the level of suppression that was seen when using anti-L-selectin Ab. Thus, FT-mediated modification of basophil PSGL-1 and the binding to L-selectin appear to be one of the important steps required for the development of IgE-CAI.

Intriguingly, we also noted that coadministration of anti-PSGL-1 and L-selectin Abs was able to more efficiently inhibit IgE-CAI than the injection of a single Ab. Although we have not been able to completely assure that optimal doses of each antibody were used, this suggests that an adhesion pathway other than PSGL-1-L-selectin interaction might contribute to the development of IgE-CAI. Several lines of evidence have suggested that an L-selectin-dependent leukocyte–leukocyte interaction facilitates the subsequent direct interaction of leukocytes with endothelial selectins, which leads to the amplification of initial leukocyte recruitment (Alon *et al.*, 1996; Walcheck *et al.*, 1996; Sperandio *et al.*, 2003). In this respect, endothelial L-selectin ligands and P-selectin might assist in the capture and rolling of basophils and effector cells on the endothelial cells following the PSGL-1-L-selectin interaction, although the blocking of P-selectin alone is not sufficient for the inhibition of basophil recruitment and the development of IgE-CAI. The roles of E-, P-, and L-selectins in leukocyte capture and/or rolling on endothelial cells have been shown to be partially redundant, and these three selectins can also function synergistically (Ley *et al.*, 1993, 1995; Ley and Tedder, 1995; Lowe, 2002).

IgE-CAI offers a unique mouse model of skin inflammation, in that it is dependent on IgE and FcεRI of basophils, but independent of FcεRI of mast cells and other cells that usually have central roles in some human allergic inflammations (von Bubnoff *et al.*, 2003). In addition, the characteristics of mouse basophils differ from those of human basophils in many respects (Lee and McGarry, 2007). Another difference between humans and mice is seen in the regulatory functions of FTs. Human FT-VII, but not FT-IV, modifies PSGL-1 of leukocytes, leading to the expression of cutaneous lymphocyte–associated antigen, which acts as a functional selectin ligand and skin-homing receptor (Kieffer *et al.*, 2001). On the other hand, murine leukocytes express barely detectable levels of cutaneous lymphocyte–associated antigen epitope despite the expression of FT-VII, but still efficiently bind to E- and P-selectins. Murine FT-VII appears to fucosylate only a few quite specific glycans that interact preferentially with selectins (Kobzdej *et al.*, 2002). Thus, it would be difficult to consider the present findings for IgE-CAI in mice as directly applicable to human allergic skin diseases.

Collectively, basophil recruitment and development of IgE-CAI are entirely dependent on collaborative control by FT-IV and VII in the basophils. L-selectin binding to basophil PSGL-1 modified by the FTs could be a central event that ultimately leads to the subsequent inflammatory steps of IgE-CAI.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan). FcRγ chain(−/−) C57BL/6 mice (Takai *et al.*, 1994) were kindly provided by Dr Takai of Tohoku University, Japan. FT-IV(−/−) mice, FT-VII(−/−) mice, and FT-IV(−/−)/FT-VII(−/−) mice (Maly *et al.*, 1996; Homeister *et al.*, 2001) were originally established at the University of Michigan (Dr Lowe), with colonies maintained at Case Western Reserve University (Dr Myers), which provided animals to our department. The use of animals was in full compliance with the Committee for Animal Experiments of Tokyo Medical and Dental University.

Antibodies

Isotype-matched control Ab (rat IgG2aκ), rat anti-CD16/CD32 (2.4G2), biotinylated anti-CD49b (Dx5), and PE-labeled anti-PSGL-1 (P-selectin glycoprotein-1) (2PH1) Abs were from BD Bioscience Pharmingen. PE/Cy5-labeled anti-L-selectin Ab (MEL-14) was from BioLegend (San Diego, CA). FITC-conjugated anti-CD49b (Dx5), FITC- and PE-conjugated anti-FcεRI Ab (MAR-1), FITC- and PE-labeled anti-mouse CD123 (IL-3Rα), and PE-labeled anti-c-kit (ACK2), biotinylated anti-c-kit (2B8) Abs were purchased from eBioscience. Anti-CD3e (M-20) was from Santa Cruz Biotechnology

(Santa Cruz, CA). Anti-mouse mast cell protease-8 mAb (TUG8) (Ugajin *et al.*, 2009) was provided by Dr Karasuyama of Tokyo Medical and Dental University.

Cutaneous inflammatory reactions

Trinitrophenyl (TNP)-specific IgE was purified from ascites of BALB/c-*nu/nu* mice by intraperitoneal injection of the IGEL b4 B cell hybridoma (ATCC, Rockville, MD; TIB141) (Rudolph *et al.*, 1981). IgE-CAI was induced by passive immunization of mice with TNP-specific IgE (150 μg per mouse, intravenous) (Mukai *et al.*, 2005). Mice were challenged 24 hours later with TNP-OVA (10 μg per ear, Biosearch Technologies, Novato, CA) on each ear lobe.

CHS reactions were induced by application of 50 μl of 0.5% DNFB (Nacalai Tesque, Kyoto, Japan) in acetone:olive oil (4:1) onto the ventral skin on day 0. On day 5, each ear lobe was challenged with 20 μl of 0.2% DNFB in acetone:olive oil (4:1). Ear thickness was measured using a dial thickness gauge (Ozaki, Tokyo, Japan) before and after the challenges.

DTH to SRBCs was induced by subcutaneous immunization with 100 μl of 20% SRBCs on the back on days −1 and 0. On day 5, 20 μl of 20% SRBC was injected into the footpad. Footpad thickness was measured before and after the challenges. Each group consisted of at least four mice.

Cell preparation

Primary basophils were prepared by enrichment of CD49b (+) cells from freshly isolated bone marrow cells using the MACS system with biotinylated anti-CD49b and streptavidin microbeads. As determined by flow cytometric analysis for CD49b and CD123 expression, the CD49b (+) cells included ~20% basophils.

Bone marrow–derived basophils were prepared by culturing bone marrow cells in RPMI 1640 supplemented with 10% fetal calf serum and 10 ng ml^{-1} rIL-3 (R&D Systems, Minneapolis, MN) for 10 days, followed by isolation of the CD49b (+) cells using an MACS system.

Bone marrow–derived mast cells were obtained by culturing bone marrow cells in the presence of 10 ng ml^{-1} rIL-3 for 4 weeks, followed by isolation of the c-kit+ cells.

Basophil transfer

Basophil transfer for inducing IgE-CAI in FcRγ chain(−/−) mice was performed using a previously described method (Mukai *et al.*, 2005). Briefly, CD49b (+) basophil–enriched bone marrow cells (primary basophils; 6×10^6 cells per recipient) were transferred into irradiated FcRγ(−/−) mice (6 Gy) together with CD49b(−) bone marrow cells (effector cells) from naive FcRγ chain(−/−) mice. Four days later, mice were passively immunized with TNP-IgE followed by challenge with TNP-OVA.

In vitro selectin binding assay of basophils

CD49b (+) basophil–enriched bone marrow cells were suspended in phosphate-buffered saline containing 5% fetal calf serum, 0.1% NaN_3, 1 mmol l^{-1} Ca^{2+}, and 1 mmol l^{-1} Mg^{2+}, followed by incubation with 10 μg ml^{-1} of murine P-, E-, or L-selectin-human IgG Fc chimera or control human IgG1 Fc (R&D Systems) for 40 minutes at 4 °C. After washing the cells, they were incubated with PE-F $(ab')_2$ goat anti-human IgG Fc Ab (Rockland, Gilbertsville, PA) for 30 minutes at 4 °C. They were then counterstained with FITC- FcεRI Ab or FITC- CD123 Abs. Selectin binding was examined using flow cytometric analysis with FACS Calibur (BD Biosciences, Mountain View, CA).

Real-time PCR

Quantitative real-time reverse-transcriptase PCR was performed with reverse-transcribed RNA by real-time monitoring of the increase in fluorescence of the SYBR Green dye (Brilliant SYBR Green QPCR Master Mix, Stratagene, La Jolla, CA) using the Mx3000P Real-Time PCR system (Stratagene). The primers for PCR were 5′-TGTGTCCGTC GTGGATCTGA-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ for mouse GAPDH; 5′-CGCTGTGGGACCAATCTTGA-3′ and 5′-CCAGT GTTTGGCACCAGCA-3′ for mouse FT-IV; and 5′-AGATGCCCTGG TGGGCTTTAG-3′ and 5′-TCAGCCATGGGTCAAGGTAAGTC-3′ for mouse FT-VII.

Statistical analyses

A Student's *t*-test was used to assess statistical significance of the differences between the mean values. Analysis of the data for the time-course changes of the skin responses was performed by using the repeated measures analysis of variance test, followed by either a Student's *t*-test or Scheffe's F test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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

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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 $CD14^{dim}CD16^+$ 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

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The online version of this article contains supplemental material.

Abbreviations used in this article: AD, atopic dermatitis; ADEH, atopic dermatitis with eczema herpeticum; CLA, cutaneous lymphocyte–associated Ag; cMO, classical monocyte; EH, eczema herpeticum; iTreg, activated/induced regulatory T cell; non-Treg, nonsuppressive T cell; pMO, proinflammatory monocyte; rTreg, resting/naturally occurring regulatory T cell; SCORAD, SCORing Atopic Dermatitis; Treg, regulatory T cell; VZV, varicella zoster virus.

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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 $CD14^{dim}CD16^{+}$ 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 made 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 Abs to aeroallergen. 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 (RMO52, M5E2), CD16 (3G8), CD19 (Leu-12), CD25 (sA3), CD45RA (HI100), CCR4 (1G1), CD56 (Leu-19, HCD56), 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), FcεRIα (AER-37), IL-1β (H1b-98), IFN-γ (25723.11), TNF-α (6401.1111, 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-aminoactinomycin D, were purchased from BD Biosciences, eBioscience, Beckman Coulter, or BioLegend. For cell culture, Abs to human CD3 (HIT3a), CD28 (37407), TGF-β1 (19D8), CTLA4 (L3D10), and IL-10 (JES3-19F1) were purchased from eBioscience, R&D Systems, or BioLegend. For immunohistochemical staining, Ab to human CD8 (CD8/144B) and Abs to CD56 (123C3) and HSV (rabbit polyclonal, B0114) were purchased from DAKO. Ab to human varicella zoster virus (VZV; MAB8612) was purchased from Millipore, Ab to human Foxp3 (236A/E7) was purchased from eBioscience, and Ab to CD16 (2H7) was purchased from Novocastra. HSV-1gD protein was purchased from Abcam.

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 brefeldin 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.

Determination of HSV-1–specific $CD8^{+}$ and $CD4^{+}$ T cells

To determine HSV-1–specific $CD8^{+}$ and $CD4^{+}$ T cells, we performed an IFN-γ–induction assay using stimulation with HSV-1gD protein, which was established by previous researchers (11). Briefly, PBMCs were stimulated with 25 μg/ml HSV-1gD protein plus CD28/CD49d Abs for 24 h in the absence of brefeldin A. After 24 h of stimulation with HSV-1 protein, 10 μg/ml brefeldin A was added and then additional culture was done for 4 h. All cells were cultured in RPMI 1640 medium supplemented with 5% human AB serum (both from Sigma-Aldrich). 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

FACS-sorted $CD4^{+}CD25^{-}$ effector T cells (3,000 cells/well) 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 [^{3}H]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 $CD4^{+}CD25^{+}$ $CD127^{dim/-}$ 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 $CD14^{+}CD16^{-}$ and $CD14^{dim}CD16^{+}$ 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 $CD14^{dim}$ proinflammatory monocytes in the intracellular cytokine-production assay. Because a monocyte population characterized by low levels of CD14 and the absence of FcεRI expression was found to be identical to the $CD14^{dim}CD16^{+}$ pMOs, gating on the $CD14^{dim}FcεRI^{-}$ 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), $CD14^{dim}CD16^{+}$ pMOs, and $CD14^{dim}CD16^{-}$ monocytes (Fig. 7A).

Cocultures of Tregs and monocytes

For coculture studies, $CD14^{dim}$ pMOs were purified by sorting out the $CD14^{dim}$ (purity > 93 ± 1.5%). The FACS-sorted $CD14^{dim}$ pMOs (12,500 cells) were cocultured with allogeneic purified $CD3^{+}$ T cells (1.25×10^{5} 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 + 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, CD8, 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 Histofine Simple Stain AP (Nichirei Bioscience). Immunoreactivity was detected using an Alkaline Phosphatase Substrate Kit (VECTOR BLUE III, Vector Laboratories). To deactivate primary Abs, sections were boiled in an autoclave at 121°C for 10 min. After cooling down, specimens were washed with PBS and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Then, the sections were incubated with 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 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.

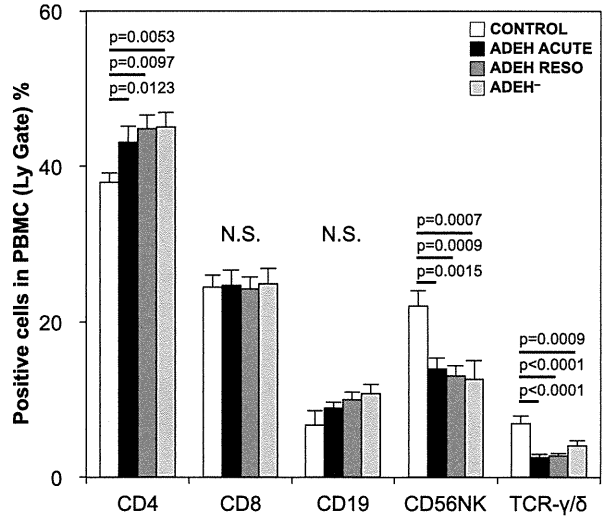

FIGURE 1. The percentages of $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and TCR-γ/δ^+ T cells in PBMCs from ADEH patients, $ADEH^-$ patients, and healthy controls. Thirty-nine ADEH patients, 17 $ADEH^-$ patients, and 34 healthy controls were analyzed as described in *Materials 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.

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 $\gamma\delta^+$ 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 $ADEH^-$ and in healthy controls. The frequencies of CLA^+ and $CCR4^+$ Tregs at the onset of disease were significantly higher than those after resolution of EH. Given the potent ability of $CCR4^+$ Tregs to preferentially migrate into the skin (16), these findings can be interpreted as indicating that the increased Tregs at the onset of EH exhibit a skin-homing phenotype similar to those in $ADEH^-$ and in healthy controls. Thus, selective expansion of Tregs with the potent skin-homing potential is likely to occur at disease onset, probably prior to onset of EH in AD patients. As shown in Fig. 2B, the frequencies of $Foxp3^+$ $CTLA\text{-}4^+$, $Foxp3^+CD127^{dim/-}$, $Foxp3^+CD39^+$, $Foxp3^+HELIOS^+$, and $Foxp3^+Ki67^+$ Tregs were increased significantly at the onset of EH. A recent study (17) indicated that $Foxp3^+CD4^+$ T cells could be classified into functionally distinct subpopulations based on CD45RA and Foxp3 expression levels: $CD45RA^+Foxp3^+$ resting/natural occurring Tregs (rTregs) and $CD45RA^-Foxp3^{++}$ activated/induced Tregs (iTregs), both of which have suppressive function in vitro, and $CD45RA^-Foxp3^+$ nonsuppressive T cells (non-Tregs) (Fig. 2C). Therefore, we investigated whether $Foxp3^+$ Tregs that expanded during the acute stage of ADEH could represent the $CD45RA^-Foxp3^{++}$ iTreg phenotype. As shown in Fig. 2D, although rTregs and non-Tregs were slightly increased in the acute stage of ADEH, iTregs were dramatically increased in the acute stage of EH compared with those in $ADEH^-$ and those in healthy controls. This finding indicates that expanded Tregs at onset of EH are of the iTreg phenotype and that they could be due to the division of existing cells rather than de novo induction of this phenotype in their precursors. After resolution of EH, the frequencies of Tregs decreased to values similar to those in healthy controls, suggesting that Tregs could be reduced upon clinical resolution and that EH can only be recovered by a timely decrease in Treg frequencies. These considerations prompted us to investigate a correlation between clinical symptoms of EH and Treg frequency.

Table I. Clinical and biological characteristics of ADEH, $ADEH^-$, and control samples

Clinical and Biological Characteristics	ADEH	*p* Value[a]	$ADEH^-$	*p* Value[a]	Healthy Control
No. of samples	39		17		34
Age (y; mean ± SEM)	27.6 ± 2.2	0.001	32.8 ± 2.5	0.797	33.6 ± 1.1
SCORAD (mean ± SEM)[b]	40.0 ± 3.6		36.1 ± 2.2		NA
Serum IgE (IU/ml; mean ± SEM)[b]	9758.3 ± 1810.4	0.049	10382.7 ± 3909.5	0.043	118.9 ± 47.1
Serum anti-HSV IgG (EIA unit; mean ± SEM)[b]	73.98 ± 19.2	0.033	117.46 ± 22.7	0.002	5.3 ± 1.6

[a]Versus healthy control.
[b]Data for SCORAD, serum IgE, and serum anti-HSV IgG titers in ADEH are at onset of disease.
EIA, Enzyme immunoassay; NA, not applicable.

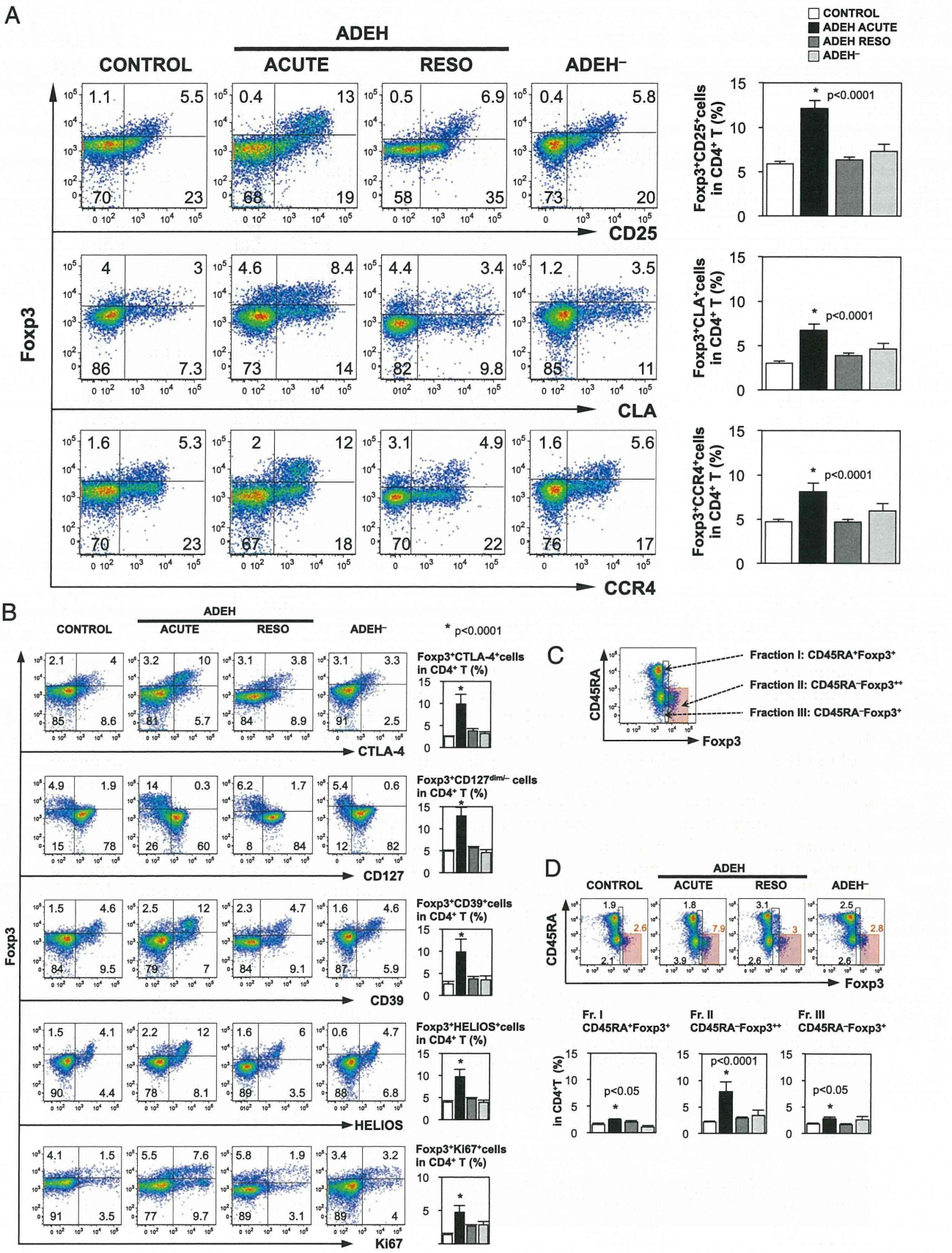

FIGURE 2. Expansion of skin-homing Foxp3$^+$CD25$^+$CTLA-4$^+$CD127 $^{dim/-}$CD39$^+$HELIOS$^+$ Tregs during the acute stage of ADEH. (**A**) Representative flow cytometry dot plots showing the expression of Foxp3 versus CD25, Foxp3 versus CLA, and Foxp3 versus CCR4 in CD4$^+$ T cells from ADEH, ADEH$^-$, 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$^+$CD127$^{dim/-}$, *(Figure legend continues)*

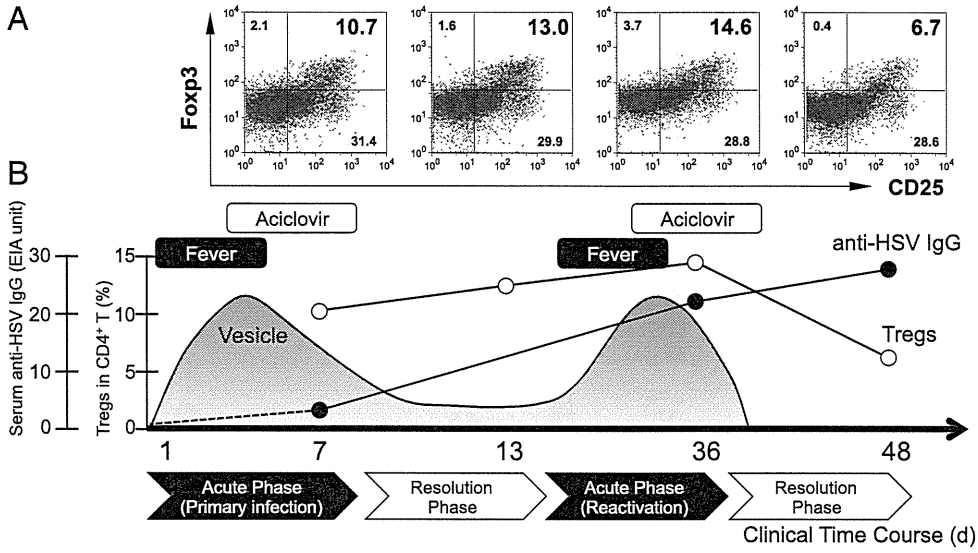

FIGURE 3. A sequential analysis of Treg frequency, serum HSV IgG level, and clinical course in a single AD patient with EH induced by a primary infection with HSV. (**A**) Flow cytometry dot plots showing the expression of Foxp3 versus CD25 in $CD4^+$ T cells. (**B**) Clinical course of symptoms in relation 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: $CD45RA^+Foxp3^+$ rTreg, $CD45RA^-Foxp3^{++}$ 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.

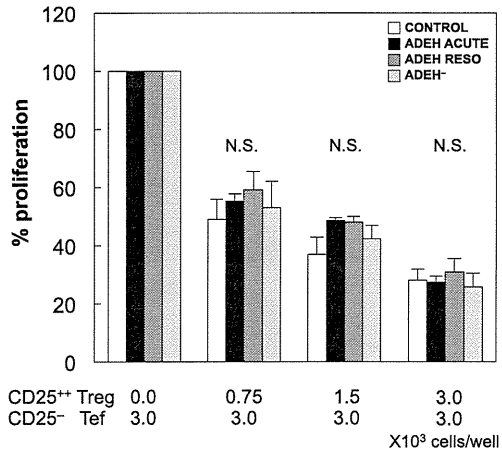

FIGURE 4. Functional analysis of Tregs at the different disease stages of ADEH, ADEH$^-$, and healthy controls. Highly purified CD4$^+$CD25^{++} Treg populations from patients with EH at either the acute or resolution stage, ADEH$^-$, or healthy controls were cocultured at different ratios with highly purified CD4$^+$CD25$^-$ effector T cell populations from the same stage or from healthy controls in the presence of mitomycin C–treated allogeneic APCs and anti-CD3 and anti-CD28 Abs. Proliferation was assessed by a [^{3}H]thymidine-incorporation assay. The results are expressed as the percentage proliferation of CD4$^+$CD25$^-$ effector 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 CD14^{dim}CD16$^+$ pMOs and alteration of cytokine production

Recent studies reported that CD14^{dim}CD16$^+$ 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),

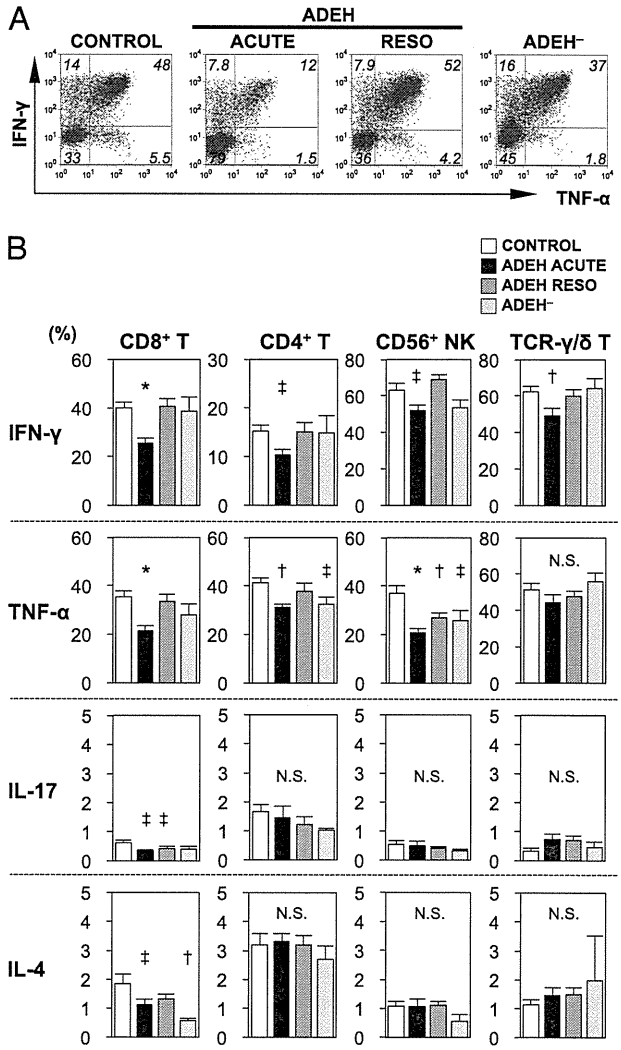

FIGURE 5. Intracellular expression of IFN-γ, TNF-α, IL-17, and IL-4 by CD8$^+$ T, CD4$^+$ T, CD56$^+$ NK, and TCR-γ/δ$^+$ cells from ADEH patients, ADEH$^-$ patients, and healthy controls. (**A**) Representative experiment showing intracellular IFN-γ versus TNF-α dot plots in CD8$^+$ T cells from ADEH and ADEH$^-$ patients and healthy controls. (**B**) Mean percentage of IFN-γ$^+$, TNF-α$^+$, IL-17$^+$, or IL-4$^+$ cells ± SEM in each subset. ADEH (acute stage, n = 9–17; resolution [RESO] stage, n = 9–21), ADEH$^-$ (n = 7), and healthy controls (n = 14–35). $^*p < 0.001$, $^\dagger p < 0.01$, $^\ddagger p < 0.05$ versus healthy controls, Student t test.

the CD14^{dim}FcεRI$^-$ 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

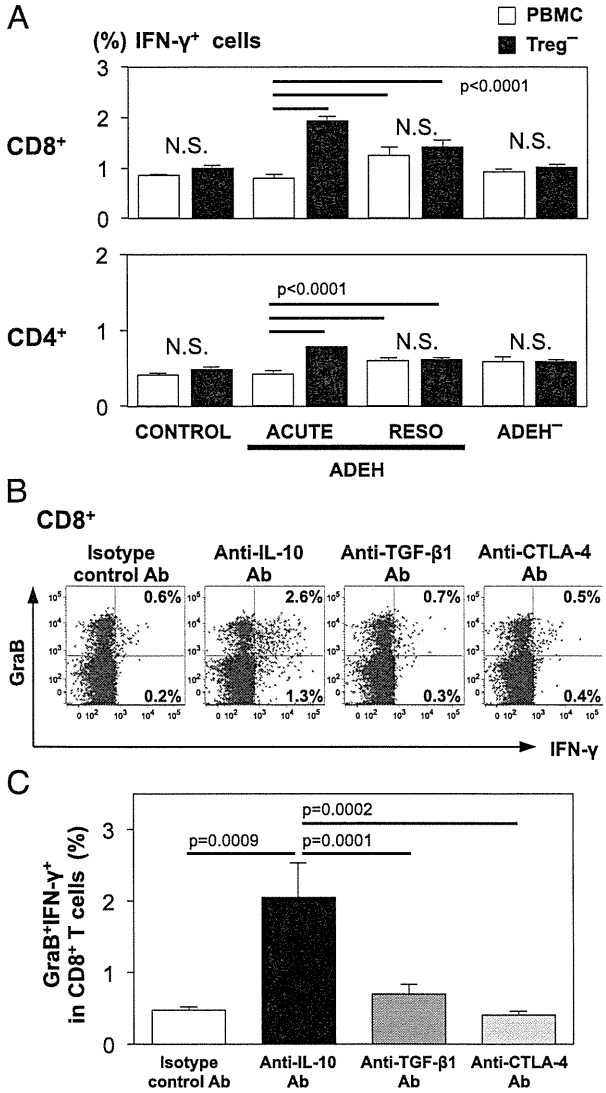

FIGURE 6. Restoration of IFN-γ production by HSV-1–specific $CD8^+$ and $CD4^+$ T cells obtained during the acute stage of disease from ADEH patients by Treg depletion. (**A**) PBMCs were obtained from ADEH patients, $ADEH^-$ patients, and healthy controls, and $CD4^+CD25^+CD127^{dim/-}$ Tregs were depleted from the PBMCs ($Treg^-$) as described in *Materials and Methods*. PBMCs and $Treg^-$, respectively, were stimulated with 25 μg/ml HSV-1gD protein or medium for 24 h with CD28/CD49d Abs. After 24 h of culture, brefeldin A was added, and culture continued for an additional 4 h. Intracellular cytokine was detected by FACS. The results are expressed as the average (± SEM) percentage of $IFN\text{-}\gamma^+$ cells in $CD8^+$ and $CD4^+$ T cells from PBMCs and Treg-depleted PBMCs, respectively. ADEH ($n = 16$), $ADEH^-$ ($n = 15$), and healthy controls ($n = 16$). (**B**) Restoration of IFN-γ production from $CD8^+$ T cells by anti–IL-10 Ab. Representative flow cytometry dot plots showing the expression of granzyme B (GraB) versus IFN-γ in $CD8^+$ T cells from the acute stage of ADEH. (**C**) Average (± SEM) percentage of $GraB^+IFN\text{-}\gamma^+$ cells in $CD8^+$ T cells from PBMCs ($n = 8$). The p values were determined using the Student t test.

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 replications, 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 generation and IFN-γR expression that may contribute to an impaired immune response to HSV. Although their study was restricted to European American and African American subjects, our results suggest that similar genetic defects may be observed in Asian subjects. Thus, an important implication of our results is that such impaired IFN-γ production would be restored to the normal level by a timely decrease in Treg frequency occurring at the late stage of EH, which is the key to successful resolution of an infection. Our Treg-depletion experiments showed that IFN-γ responses were more sensitive to the action of Tregs than were TNF-α responses, which are in accordance with the recent data in mice showing that acute shutoff effector cytokine production by Tregs was selective for IFN-γ but not TNF-α (28). If so, therapeutic inactivation of Tregs may provide a greater benefit for protective immunity to HSV in a setting of 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-

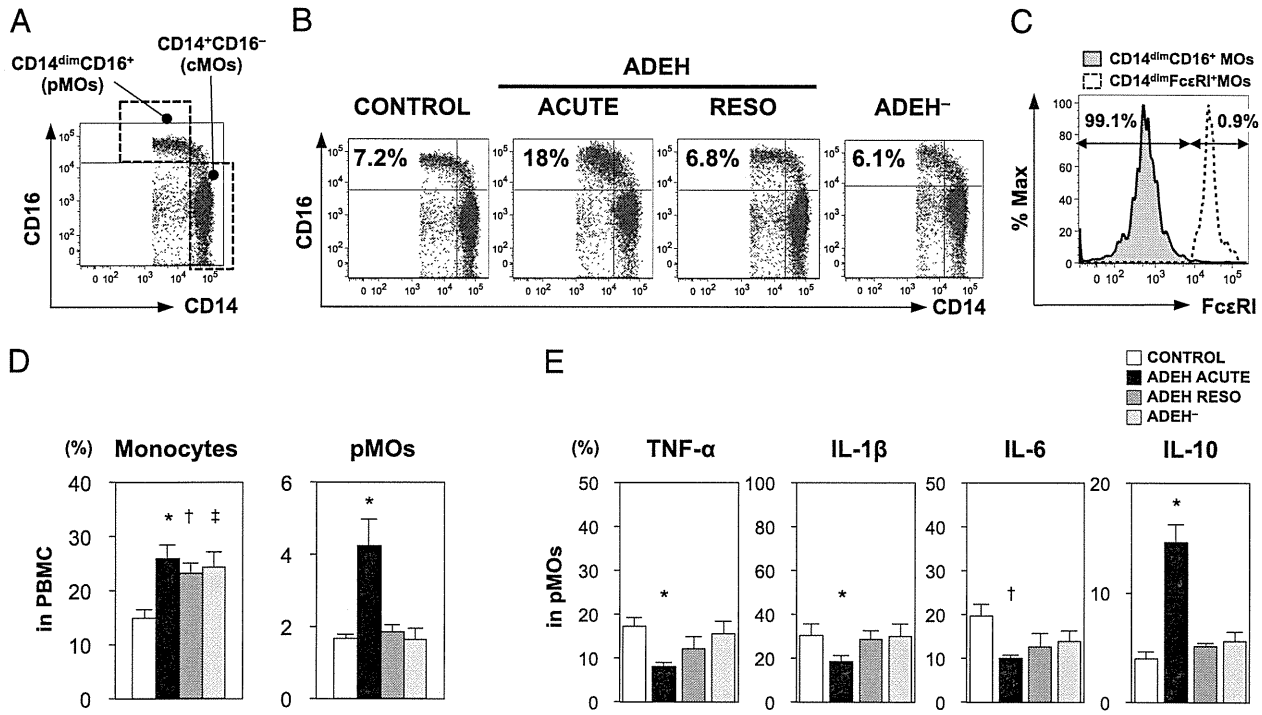

FIGURE 7. Expansion of $CD14^{dim}CD16^{+}$ pMOs with the potent ability to produce IL-10 upon TLR-2 ligand stimulation in ADEH patients at onset of EH. (**A**) PBMCs were stained by anti-CD14 Ab and anti-CD16 Ab. The result of flow cytometric analysis of cell surface expression of CD14 and CD16 is shown. Monocytes can be classified into distinct populations based on the CD14 and CD16 expression, $CD14^{dim}CD16^{+}$ pMOs and $CD14^{+}CD16^{-}$ cMOs in healthy controls. (**B**) Representative dot plots showing these subsets in ADEH patients, $ADEH^{-}$ patients, and healthy controls. (**C**) Representative graph showing the expression of FcεRI on $CD14^{dim}CD16^{+}$ pMOs in healthy controls. The vast majority of $CD14^{dim}CD16^{+}$ pMOs are negative for FcεRI. (**D**) The percentages of monocytes and pMOs in PBMCs from ADEH patients, $ADEH^{-}$ patients, and healthy controls. (**E**) Mean percentage of TNF-α^{+}, IL-$1\beta^{+}$, IL-6^{+}, or IL-10^{+} cells ± SEM in pMOs. ADEH (acute stage, n = 8–19; resolution [RESO] stage, n = 7–15), $ADEH^{-}$ (n = 5–6), and healthy controls (n = 8–15). $^{*}p < 0.001$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.05$ versus healthy controls, Student t test.

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 the remission of clinical symptoms of EH with antiviral treatment (~day 13). In this patient, long-term remission was induced only after Treg frequencies were dramatically decreased, suggesting that increased frequencies of Tregs are not a mere consequence of HSV reactivation but are a cause. Also, contrary to that 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 rather impaired (R. Takahashi, manuscript in preparation).

If increased frequencies of Tregs are responsible, in part, for the HSV reactivation observed in recurrent EH, it is logical to ask what factors are responsible for driving Treg expansion in AD patients. To address this issue, we have to ask how a situation favoring the expansion of Tregs can be created before the onset of EH. $Foxp3^{+}$ Tregs were shown to expand quickly during 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

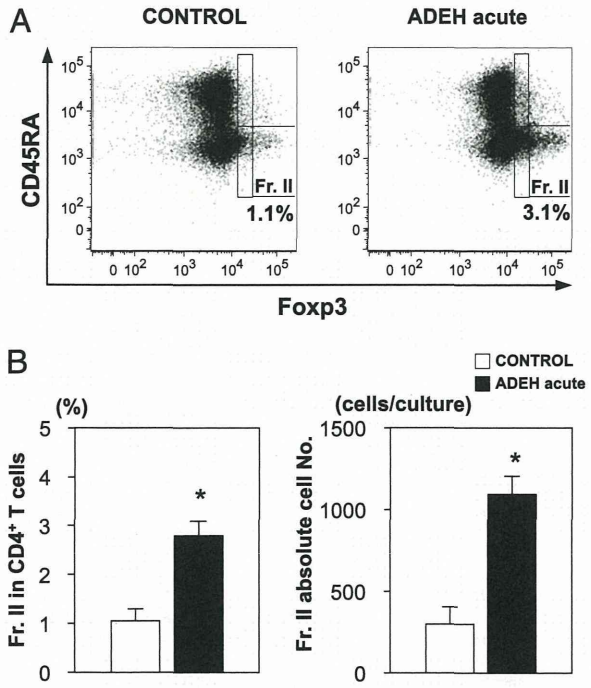

FIGURE 8. Expansion of iTregs by pMOs from the acute stage of disease in ADEH patients. Purified $CD14^{dim}$ monocytes from ADEH patients at the acute stage of disease or healthy controls were cocultured with allogeneic $CD3^+$ T cells from a healthy volunteer in the presence of anti-CD3 Ab + CD28 Ab for 7 d. (**A**) $CD45RA^-Foxp3^{++}$ activated/iTregs (fraction II) were analyzed. Representative flow cytometry dot plots showing the expansion of iTregs induced by pMOs from ADEH patients at onset of disease and from healthy control. Numbers indicate the frequency of $CD45RA^-Foxp3^{++}$ iTregs (Fr. II). (**B**) Average percentage and absolute cell numbers per culture. Mean and SEM are shown. ADEH (acute stage, $n = 4$) and healthy controls ($n = 5$). Student t test. $^*p < 0.05$ versus healthy controls. Fr., Fraction.

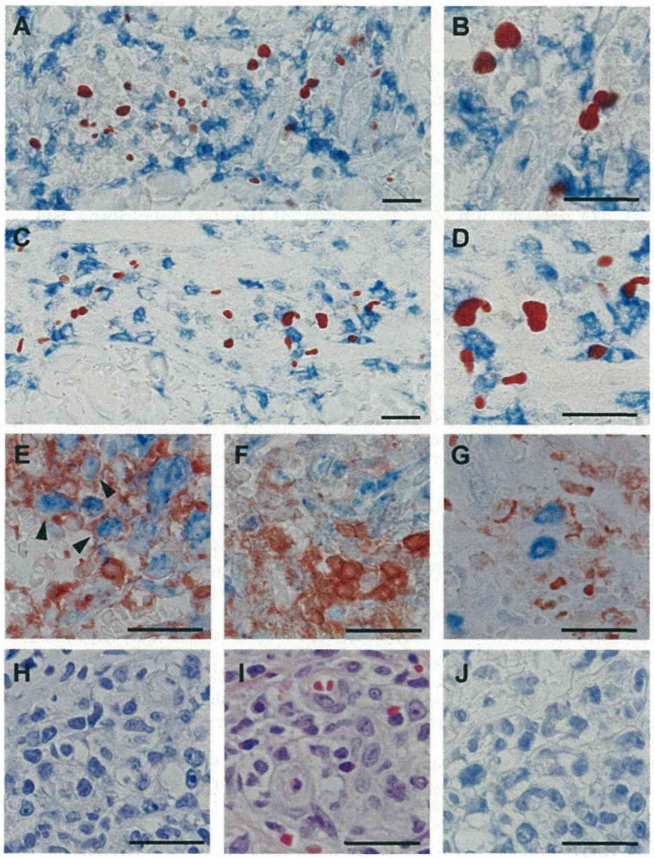

FIGURE 9. Immunohistochemical analysis of ADEH lesions during the acute stage of disease. $Foxp3^+$ Tregs are frequently detected in ADEH lesions, where they are located near $CD8^+$ T cells (**A**, **B**) and $CD16^+$ monocytes (**C**, **D**). (**E**) HSV Ag is detected in the infiltrated $CD16^+$ monocytes (arrowheads). The percentage of HSV^+ cells in the infiltrated $CD16^+$ cells was 18.8 ± 2.5% in the lesional skin site. (**F**) CD8 T^+ cells are located in the vicinity of the HSV^+ cells. (**G**) VZV Ags are also found in $CD16^+$ cells. (**H** and **I**) $CD56^+$ NK cells and neutrophils are not found in the skin lesion. (**J**) Isotype control staining shows no background staining. In (A) and (B), Foxp3 (red) and CD8 (blue). In (C) and (D), Foxp3 (red) and CD16 (blue). In (E), CD16 (red) and HSV (blue). In (F), CD8 (red) and HSV (blue). In (G), CD16 (red) and VZV (blue). In (H), CD56 (red) with hematoxylin. In (I) H&E. In (J), isotype control with hematoxylin. Scale bar, 25 μm.

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 $CD14^{dim}$ pMO subset, with their relatively high capacity to synthesize IL-10, rather than with the Tregs themselves. Indeed, our data showed that purified $CD14^{dim}$ 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.

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Ⅴ．班会議プログラム・議事録・抄録

難治性疾患等克服研究事業
重症型扁平苔癬の病態解析及び診断基準・治療指針の確立　研究班

第1回班会議

会　場：パシフィコ横浜　会議センター　415
日　時：6月15日（土）　15:30～18:00

【議　題】

1、　委員長、各委員紹介
2、　扁平苔癬の定義、扁平苔癬の診断基準作製のための審議
3、　役割分担等
　◎ ガイドライン原稿分担（案）
　　-背景、概念（井川、横関）
　　-分類、診断基準（塩原（皮膚）、魚島（口腔内））
　　-病態（小豆澤、片山）
　　-疫学（井川）
　　-症状（西澤（皮膚）、魚島（口腔内））
　　-検査（西澤）
　　-治療法のアルゴリズム（佐藤（皮膚）、魚島（口腔内））
　　-治療法のEBMに基づいた検討（案）
　　　ステロイド外用剤の有効性（井川）
　　　免疫抑制剤外用剤の有効性（西澤）
　　　ステロイド剤全身投与の有効性（井川）
　　　免疫抑制剤全身投与の有効性（西澤）
　　　光線療法の有効性（佐藤）
　　　レチノイドの有効性（小豆澤）
　　　グリセオフルビンの有効性（塩原）
　　　歯科金属除去の有効性（魚島）
4、　疫学調査の検討（西澤、井川）
5、　各個研究について
6、　次回委員会日程決定

難治性疾患等克服研究事業
重症型扁平苔癬の病態解析及び診断基準・治療指針の確立　研究班

平成25年度第1回班会議録

出席者：　佐藤貴浩、平井亜衣子、小豆澤宏明、魚島勝美、横関博雄、井川　健、
西澤　綾
会　場：　パシフィコ横浜　会議センター　415
日　時：　6月15日（土）　15:30～18:00

① 班長挨拶、班員自己紹介
日常生活に支障をきたすような重症型扁平苔癬について、実態を把握すると同時に、しっかりとした診断、治療を可能とすることを目的とすること。
各班員の自己紹介をおこなった。

② 扁平苔癬の定義、診断基準作製に関する審議
-「何項目以上で扁平苔癬」というような診断基準については、本疾患の場合は適当ではないのでは、という意見が多く、「定義」をしっかりとするということとなった。また、皮膚所見としての定義、粘膜所見としての定義を両方記載することとなった。
-すでにOral Lichen Planusにおいては、Modified World Health Organization diagnostic criteria of OLP and OLL (2003)を参考とする。
***別紙（定義）の内容についてご検討いただければ幸いです。よろしくお願いいたします。**

③ 疫学調査票についての審議
-あいさつ文において、扁平苔癬の「定義」を記載すること。
-重症型扁平苔癬の定義を調査票に記載すること。
-その上で、調査は扁平苔癬、重症型扁平苔癬にわけて記載してもらうようにすること。
となった。
***別紙（調査票）の内容についてご検討いただければ幸いです。よろしくお願いします。**

④ ガイドライン原稿分担
後ほどの検討となった。

⑤ 各個研究

1）金属アレルギーと扁平苔癬の検討-発生部位との関連について（東京医科歯科大学皮膚科　西澤　綾先生）
過去16年間にわたる、東京医科歯科大学皮膚科における扁平苔癬と金属アレルギーの関連についての統計データをもとに、発生部位との関連などについてさらに詳細に検討を加える。

2）Age-dependent breakdown of peripheral tolerance to the epidermal autoantigen（大阪大学皮膚科　小豆澤宏明先生）

Thymoma-associated multiorgan autoimmunity と考えられた臨床例、また同様の疾患で thymoma との関連が見られなかった症例の提示あり。

K5-mOVA マウスを利用して、表皮自己抗原に対する末梢免疫寛容が経時的に破たんしていくメカニズムについて検討をする。

3）扁平苔癬（皮膚）と OLP に共通する特異的発現遺伝子の検出　（新潟大学口腔健康科学　魚島勝美先生）

それぞれの組織から mRNA を抽出してきて、micro array による検討を行う予定である。

（文責：井川　健）

難治性疾患等克服研究事業
重症型扁平苔癬の病態解析及び診断基準・治療指針の確立　研究班

第2回班会議議事録

会　場：ハイアットリージェンシー東京　5F「足柄」
日　時：8月10日（土）　12:30～14:00
出席者（順不同、敬称略）片山一朗、佐藤貴浩、塩原哲也、三橋善比古、種井良二、
濱崎洋一郎、横関博雄、井川　健

１、　扁平苔癬の定義について
話し合いの結果、別紙（扁平苔癬定義）に示すような定義となった。
「項目いくつ以上で扁平苔癬の診断」、といった診断基準ではなく、この定義をもって扁平苔癬の診断を行う、ということになった。

２、　実態調査票について
なるべく簡単、かつ、必要な情報を得るという目的から、項目の削減ならびに回答方法の変更（マークシートタイプなど）が必要であろうということになった。別紙（扁平苔癬実態調査　案）のような内容とさせていただいた。また、過去3年間の新規患者を対象とすることとなった。

３、　原稿役割分担について
原稿の分担について、別紙（原稿分担　案）の内容にてお願いすることとなった。

４、　次回委員会日程決定
次回委員会日程については、11月の日本皮膚アレルギー学会会期中に行う予定となった。

扁平苔癬診療ガイドライン委員会のお知らせ

日時：平成２５年１１月２９日（金）　１１：３０～１２：３０

場所：第 43 回日本皮膚アレルギー・接触皮膚炎学会総会学術大会　会場

ホテル日航金沢　 5 階　梅の間

審議事項

１．ガイドライン原稿についての検討