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〈上田真由美　外園千恵〉

■ Stevens-Johnson 症候群（SJS）と中毒性表皮壊死症（TEN：Toxic Epidermal Necrolysis）は同一スペクトラムに属する皮膚粘膜疾患であり，突然の高熱，咽頭痛，結膜炎につづいて，全身の皮膚・粘膜にびらんと水疱を生じる．眼科的に両疾患は同じ眼所見を呈し，いずれも著しい視力障害が後遺症となりうる．急性期の全身状態が重篤であるほど眼には関心がいきにくいが，発症時から眼科的治療を行うことが大変重要であり，初期の全身および眼局所治療が視力予後を決定する[1,2]．

■ 発症時，皮疹・粘膜疹とほぼ同時または先行して，両眼性に急性結膜炎を生じる．結膜全体に及ぶ高度な充血，眼瞼の発赤腫脹，眼脂がみられる．皮疹がでる前に眼科を受診して，ウイルス性結膜炎と診断される患者も多い[3]．眼表面炎症が高度であることを示す非特異的所見として偽膜形成（膜様分泌物）がみられ，さらに SJS/TEN の特徴的所見として角結膜上皮欠損，睫毛の脱落が認められる（図1）．急性期の治療は，皮膚科でステロイドの大量全身投与や血漿交換が行われるが，ステロイドパルスは眼表面の消炎に大変効果的である．著者の施設において発症後4日以内にステロイドパルスを施行できた5症例は，全例で良好な視力予後を得た[4]．皮疹が順調に軽快しても眼表面炎症が遷延することが多いため，ステロイドの減量は皮膚所見だけではなく眼所見も考慮して行う．具体的には，角結膜上皮欠損の改善を得ることができてから，ゆっくりと全身と局所のステロイド量を減量していく．

■ 眼障害を伴う SJS/TEN では眼局所のステロイド投与が必須であり，ベタメタゾンの点眼ならびに眼軟膏を1日各4回程度で投与する．手技的には点眼のほうが簡単であるが，眼軟膏は点眼液と比較して眼表面での滞留時間が長く，少ない回数で高い効果を期待できる．ただし眼軟膏は，点入後しばらく見えにくくなるという欠点がある．SJS/TEN 急性期には眼瞼腫脹や Nikolsky 現象のために点眼あるいは眼軟膏の点入が困難なことが多いが，できるだけ眼表面全体に作用するように，仰臥位にて下まぶた（もしくは上まぶた）を引っ張り，点眼なら1，2滴，眼軟膏は0.5 cm 程度を点入する（表）．点眼か眼軟膏かまたは併用かは，眼瞼皮膚の状態（腫脹や Nikolsky 現象の程度），瞬目するかどうかなど患者の状態を考慮し，効果のあらわれ方をみながら眼科医が判断して，決定する．筆者らは，眼瞼腫脹が強く，眼局所の投薬回数を少なくしたい場合には眼軟膏を主体とし（例：ベタメタゾン点眼2回／日，ベタメタゾン眼軟膏6回／日），瞬目が十分できて全身状態が比較的良好な場合には点眼を主体としている（例：ベタメタゾン点眼8回／日，ベタメタゾン眼軟膏2回／日）．

■ ステロイド投与中は感染症に十分注意せねばな

表　点眼液ならびに眼軟膏の使用方法

点眼液	① 手を石鹸で洗う ② 下まぶたを軽く引っ張り容器を眼の真上に持ってきて，1滴点眼する（容器の先がまぶたの縁やまつげに触れないように注意） ③ 点眼後は眼を静かに閉じ，目頭を軽くおさえる ④ 目の外にあふれた点眼液は清潔なガーゼやティッシュで拭き取る
眼軟膏	① 手を石鹸で洗う ② 下まぶたを軽く引っ張り，チューブを少し押して下まぶたの内側に薬を0.5〜1cmつける （チューブの先がまぶたやまつげ，眼球に触れないように注意） ③ 目を閉じ，軟膏が溶けて全体に拡がるまで少し時間をおく ④ 目の外にあふれた眼軟膏は清潔なガーゼやティッシュで拭き取る
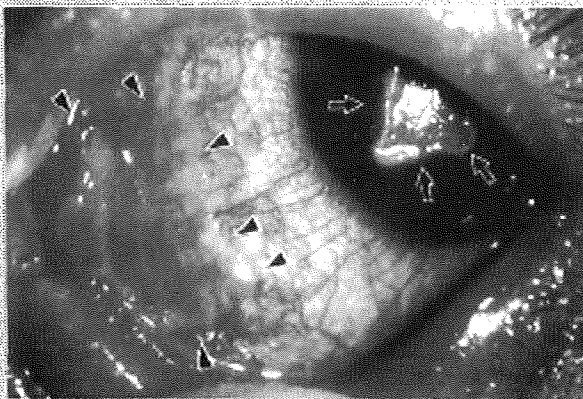
Stevens-Johnson 症候群の急性期の眼所見
著明な充血と，角膜上皮欠損（➡）ならびに結膜上皮欠損（▷）を認める．

眼軟膏を塗った後に液体の点眼薬をさすと，点眼薬がはじかれて吸収されにくくなるため，眼軟膏と点眼薬を同時に使用するときは，点眼薬を先にさしてから眼軟膏を入れる．

らない。ベタメタゾン局所投与に加えて，広域スペクトルを有するキノロン系抗菌点眼薬を併用する。抗菌点眼薬の使用にもかかわらず，MRSA もしくは MRSE 眼感染症を生じることがあるため，定期的に結膜嚢培養を行い，眼表面常在細菌の有無や種類を把握しておく。

■ 急性期に消炎が十分に行われず角膜上皮幹細胞が消失すると，角膜は厚い不透明組織で覆われて著しい視力障害を生じる。一方，角膜上皮欠損を生じても十分に消炎でき角膜上皮幹細胞が残存した場合には，上皮欠損は角膜上皮により修復され良好な視力を維持すること可能となる。このように急性期の十分な消炎は，角膜上皮幹細胞の残存を可能にし，視力予後を良好にする[1, 2]。

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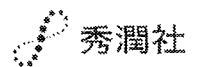うえた・まゆみ，そとその・ちえ（京都府立医科大学眼科）
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Viral connection between drug rashes and autoimmune diseases: How autoimmune responses are generated after resolution of drug rashes

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ARTICLE INFO

ABSTRACT

Viral infections are most likely triggering factors of autoimmune diseases, although a single vial infection is not sufficient to cause clinically evident autoimmune diseases. Any disease that profoundly alters the immune system may cause perturbed viral infections, thereby rendering otherwise refractory patients susceptible to autoimmune diseases. In this regard, drug-induced hypersensitivity syndrome (DIHS), a drug rash characterized by sequential reactivations of herpesviruses and the subsequent development of autoimmune diseases, offers a unique opportunity to investigate the mechanism of how autoimmunity is elicited after viral infections. Indeed, several autoimmune diseases have been reported to occur at intervals of several months to years after clinical resolution of DIHS. Two representative cases who developed autoimmune diseases three to four years after DIHS are shown. Our recent analyses of the kinetics of a developing disease have shown that fully functional FoxP3$^+$ regulatory T (Treg) cells are expanded at the acute stage thereby allowing viral reactivations but lose their suppressive function coincident with their contraction upon clinical resolution. The functional defect of Treg cells would be responsible for the subsequent development of autoimmune diseases. Patients with DIHS need close monitoring because of possible progression to autoimmune diseases even after the complete resolution.

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Contents

1. Introduction

Although many different mechanisms have been invoked, it is still not precisely clear how autoimmunity is elicited. All individuals appear to have potentially autoreactive lymphocytes, but these cells reside in the immune system throughout life in a resting state without causing autoimmune diseases unless excessively activated. Thus, activation and clonal expansions of such autoreactive lymphocytes is a critical step in the pathogenesis of autoimmune diseases. In experimental animal models, infectious agents have been shown to be possible

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candidates for the development of autoimmune diseases. There is a major difficulty, however, in establishing a correlation between triggering infectious agents and the actual autoimmune disease, particularly in humans, because of the usually long prodrome period preceding clinical onset of disease, in which immune responses are either normal or only mildly disturbed. Nevertheless, a variety of viruses have been identified as either linked to or causative of autoimmune diseases in many clinical and laboratory studies: they include systemic lupus erythematous (SLE) [1–3], Sjögren syndrome [4], rheumatoid arthritis [5], insulin-dependent diabetes mellitus (IDDM) [6] and autoimmune thyroiditis [7] [Fig. 1]. Because only a minority of infected individuals develops autoimmune diseases while sparing the vast majority, it is clear that other factors, including inherited abnormalities in the response to the virus, contribute to the pathogenesis of the disease. Because there is no evidence for disease-specific strains of the virus, it is unlikely that there is anything different about the virus. Therefore, the logical conclusion is that any disease that profoundly alters the immune system may cause perturbed viral infections, thereby rendering otherwise refractory patients susceptible to autoimmune diseases.

In this regard, drug-induced hypersensitivity syndrome (DIHS) offers a unique opportunity to link viral infections with the subsequent development of autoimmune disease, because this disease is characterized by sequential reactivations of various herpesviruses during the acute stage, similar to graft-versus-host disease (GVHD) [8,9], and the development of autoimmune diseases after clinical resolution of the disease [10–13]. In this review, we will discuss how patients with this disease can develop autoimmune diseases over a prolonged period of time after clinical resolution.

2. Viral infections as a triggering factor of autoimmune disease

Viral infections are important triggering factors for autoimmune diseases, but it remains largely unknown how viral infections result in the subsequent development of autoimmune diseases. Among a variety of viruses, Epstein–Barr virus (EBV) is most closely associated with autoimmune diseases, such as SLE. Patients with SLE have higher titers of anti-EBV Abs than control populations [1,14], and have elevated frequencies of infected cells in the blood, similar to those seen in immunosuppressed organ transplant recipients [15]: these

Fig. 1. The time interval between DIHS and the subsequent autoimmune diseases. The interval varies depending on the disease from a few months to 4 years.

infected cells are predominantly memory B cells. In addition, SLE patients have a 40-fold higher EBV viral load in peripheral blood leukocytes than control populations due to poor cytotoxic T cell responses [16]. Patients with active lupus flares have more infected cells than do patients with quiescent disease irrespective of treatment. These findings suggest that EBV infection has a causative role in the development of SLE, although they cannot be interpreted per se supporting a causative role in SLE: criteria are required to establish a causative role for the virus in the disease process. They include direct isolation of the virus from patients with the disease and detection of IgM Abs to the virus, and their absence in control populations. In chronic disease such as SLE, which have been suggested to be caused by a ubiquitous, persistent pathogen in humans, such as EBV, it is difficult to meet this criteria. In this regard, however, experimental studies provide support for a role of EBV in induction of SLE. Some Abs generated by EBV infection in infectious mononucleosis have been shown to cross-react with nuclear Ags [17,18], suggesting that molecular mimicry may be involved in the generation of certain autoantibodies. In addition, autoantibodies from patients with SLE bind a shared sequence of ribonucleoprotein Smith (SmD) and EBV-encoded nuclear antigen, EBNA-1 [19]. Immunization of mice with plasmids for in vivo expression of EBNA-1 peptides can elicit the production of anti-dsDNA and anti-Sm Abs [20]. These experimental results strongly suggest that EBV-driven immune responses could be responsible for the development of SLE but not merely an epiphenomenon of disrupted immune function by EBV infection. In view of the unique ability of EBV to infect, activate and latently persist in B cells, EBV-infected B cells including autoreactive B cells are likely to persist in the body and promote progression of autoimmune disease. Because analysis of diversity in T-cell receptors of CD8$^+$ T cells markedly expanded during the acute phase of infectious mononucleosis showed that the majority of these cells are antigen specific [21] and up to 40% of the CD8$^+$ cytotoxic T cells are directed against a single viral epitope [22], the impaired elimination of EBV-infected B cells and increased EBV load observed in SLE patients would be a consequence, not a cause, of a defect in the generation of EBV-specific CD8$^+$ cytotoxic T cells. Indeed, T cells from patients with SLE have decreased ability to control infected B cells from normal individuals or patients [23]. Therefore, these results might imply that the increased frequency of infected memory B cells in the blood of SLE patients is due to a defect in EBV-specific CD8$^+$ cytotoxic T cells that can control memory B cell homeostasis.

Many studies have shown an increased IL-10 production by the peripheral blood B cells and monocytes from SLE patients and this increase correlates with disease activity [24]. IL-10 is produced by Th2 cells, some CD8$^+$ T cells, natural and antigen-induced regulatory T (Treg) cells, B cells, mast cells and macrophages, and a pleiotropic cytokine that possess both immunosuppressive and immunomodulatory properties. Some viruses evade immune attack by taking advantage of the immunomodulatory effects of IL-10: indeed, EBV-infected cells express a viral homolog of IL-10 [25]. Viral IL-10 promotes B cell growth directly and inhibits dendritic cell (DC) maturation and function [26]. Viral IL-10 also inhibits production of IFN-γ by T cells and production of IL-12 by macrophages. Thus, by this mechanism, viral IL-10 may enhance survival of EBV in the host.
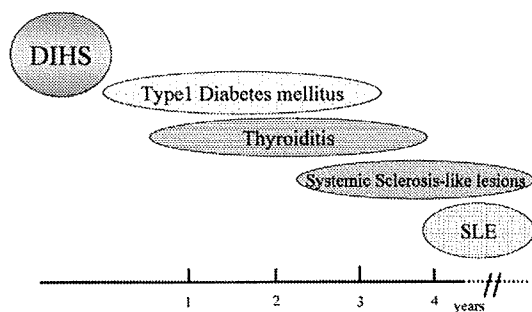
Taken together, these data demonstrate a strong association between preceding infection with EBV and the subsequent development of SLE, although the mechanisms by which EBV might promote autoimmune development remain elusive. It is likely that EBV is required but not alone sufficient. It is possible that infections with certain other viruses can give the final touch that tips the balance in an individual predisposed by EBV toward the development of autoimmune diseases.

3. Clinical manifestations of DIHS and sequential reactivations of herpesviruses

Infection with EBV itself is clearly not sufficient for the development of autoimmune diseases and prior or post infections with other viruses may greatly alter the outcome of an acute EBV infection in a host. Among various viruses, the herpesvirus family is the most likely candidate that can greatly influence immune responses to EBV, because the herpesviruses can induce and maintain a potent memory T-cell response due to their common properties of ubiquitous prevalence in human populations and the capacity to grow in lymphoid cells [27]. In addition, these herpesviruses have been shown to be reactivated in a sequential manner in bone marrow transplant (BMT) patients, coincident with clinical symptoms of graft-versus-host disease (GVHD) [28]: a sequential occurrence of herpesvirus reactivation observed in the setting of GVHD has been suggested to not only augment responsiveness to particular alloantigens but also contribute to the eventual development of autoimmune diseases.

Interestingly, similar sequential reactivations of these herpesviruses can be detected during the acute phase of DIHS, coincident with various clinical symptoms [29]. As a disease model in which to study the association of drug eruptions and viral reactivations, DIHS has been studied extensively for more than 10 years [29–32] and has provided a detailed understanding of the role of herpesviruses in the pathogenesis. DIHS is a life-threatening multiorgan system reaction, characterized by rash, fever, lymphadenopathy, hepatitis, and leukocytosis with eosinophilia [33,34]. Although the precise incidence of DIHS remains to be determined, the disease is much more common than that of Stevens–Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN), another extreme of a spectrum of severe drug eruptions, with an incidence ranging from 1.2 to 6 per million person-years. Although the clinical entity of DIHS has become widely recognized in Japan based on the diagnosis criteria established by our Japanese consensus group, many cases in other countries may go unrecognized and be frequently misdiagnosed as other idiopathic skin diseases such as erythroderma, because of its variable presentation and diverse clinical features and laboratory abnormalities. Although Orientals are more likely to be affected than are Caucasians, it remains unknown whether a racial predilection could exist. There is no age or sex predilection: generally adults are more affected than are children.

DIHS typically occurs with fever or cutaneous lesions 3 weeks to 3 months after starting therapy with a limited number of drugs: they include carbamazepine, phenytoin, phenobarbital, zonisamide, dapsone, salazosulfapyridine, mexilletine, allopurinol, and minocycline [32,33]. Significant differences exist among these drugs with regard to the potential to cause DIHS depending on race: in Japan, minocycline rarely causes DIHS while mexiletine frequently does, and this is precisely opposite of what happens in Caucasians. This delayed onset in relation to introduction of the causative drug is one of the important features of DIHS that can be distinguished from other types of drug eruptions, which typically begin 1 to 2 weeks after starting therapy. Fever usually precedes the rash by days and usually is followed by a maculopapular rash, which is pruritic and is associated with variable degrees of lymphadenopathy. The temperature ranges from 38 °C to 40 °C with spikes that often generates a concern of an underlying infection. The face, upper trunk and upper extremities are initially affected and followed by involvement of lower extremities. Periorbital and facial edema and erythema with pinhead-sized pustules are one of the characteristic features of early cutaneous lesions in DIHS. In many if not all patients, a dramatic paradoxical deterioration of the clinical symptoms can be specifically observed 3 to 4 days after withdrawal of the causative drug: the fever and rash often persist or become worse despite discontinuation of the causative drug. These often cause the diagnostic delay. Most of erythematous macules do not evolve into blisters and no mucous membrane involvement is usually seen, both of which help distinguish DIHS from SJS/TEN. Tender lymphadenopathy can be seen in >70% of patients early in the illness, affecting predominantly cervical nodes. In many cases, several flare-ups can be seen even for weeks or months after stopping the causative drug. Such variability in the presentation and course of clinical symptoms may be mistaken for severe infectious diseases by physicians, who may not have seen patients with DIHS, and unnecessary empirical antibiotic therapy may be started, which increases the risk of developing additional drug rashes. Unexplained cross-reactivity to unrelated multiple drugs with different structures is frequently seen in patients with DIHS: besides this, cross-reactivity among carbamazepine, phenytoin and phenobarbital has been frequently reported, because they are metabolized to hydroxylated aromatic compound and arene oxides are suggested intermediates in the reaction [34,35].

Leukocytosis with atypical lymphocytes and eosinophilia of various degrees is a prominent feature of DIHS. Nevertheless, leukopenia or lymphopenia often precedes leukocytosis, although this is not usually recognized because this occurs several days before the initial presentation of patients. Eosinophilia can be seen in 60–70% of the patients. Liver abnormalities occur in up to 70% of patients and are characterized by a marked increase in serum alanine aminotransferase value. Elevations in liver enzymes in most cases of DIHS resolve within 3 weeks without sequelae, but some patients have a prolonged course characterized by multiple exacerbations and remissions of elevations in liver enzymes. Although the hepatitis is usually anicteric, the development of severe hepatitis with jaundice increases the risk of reported mortality. Renal involvement can be seen, in particular in patients receiving allopurinol. The mortality from DIHS can be approximately 10–20% and has been correlated with the degree of renal involvement, rather than hepatic involvement. Depending on the sites and severity of organ damages, various clinical symptoms would develop at

various time points after onset: they include pneumonitis, coronary artery thrombosis, thyroiditis, and limbic encephalitis [36]. Their development may be recognized only months or years later. A dramatic decrease in serum IgG, IgA, IgM levels is typically observed at onset and the lowest levels are usually detected several days to weeks after withdrawal of the causative drug. Although their levels at onset may be within normal range, their levels are indeed decreased when compared with those after full recovery. The overshoot in the Ig levels is transiently observed after the nadir in the decrease, but they finally return to normal and full recovery. Because the causative drugs of DIHS have in common immunomodulatory effects on B cells [30], a decrease in Ig levels at onset could be induced in susceptible patients in which B cells might be harmfully affected by protracted administration of the drugs.

Unique features of DIHS, such as the delayed onset, unexplained cross-reactivity to unrelated multiple drugs, and frequent deterioration and several flare-ups of clinical symptoms despite discontinuation of the causative drug, are not consistent with a drug etiology. Rather, other factors that greatly modulate immune responses to drug could be involved in the pathogenesis of DIHS. In support of this possibility, we previously demonstrated that human herpesvirus 6 (HHV-6) can be specifically reactivated in patients with DIHS: HHV-6 reactivation as evidenced by the rise in HHV-6 IgG titers and HHV-DNA levels is found to occur 2 to 3 weeks after onset of the symptoms; this reactivation event specifically occurs over a predictable time course in the vast majority of patients with DIHS despite its high variability of clinical manifestations [30,31]. Because HHV-6 reactivation can only be detected in DIHS but not in other drug eruptions, this is a gold standard test for identifying patients with DIHS. Although in the earlier studies HHV-6 was thought to be the only virus reactivated during the course of DIHS, recent studies have demonstrated that not only HHV-6 but also other herpesviruses, such as HHV-7, EBV, and cytomegalovirus (CMV), can be reactivated during the course of DIHS. Although the order of herpesviruses sequentially reactivated was not exactly the same in patients with DIHS examined, our results of real-time PCR analyses of viral loads in blood samples showed that various herpesviruses sequentially reactivate during the course of DIHS in most patients regardless of therapy: the cascade of reactivation events initiated by HHV-6 or EBV extends, with some delay, to HHV-7 as well, and eventually to CMV in patients with DIHS. Interestingly, this cascade of sequential herpesvirus reactivations observed in DIHS is similar to that observed in GVHD [37]. In view or the similarity between DIHS and GVHD with regard to the clinical manifestations and viral reactivations, frequent deterioration or several flare-ups of clinical symptoms occurring despite discontinuation of the causative drug in DIHS could be explained by sequential reactivation of the herpesviruses. Our clinical studies demonstrated that reactivations of these herpesviruses can be detected coincident with onset of clinical symptoms in some patients while they are not associated with evidence of overt clinical symptoms in other patients. However, in considering that the symptoms of DIHS would be mediated by the immune responses to viruses rather than gene products of these herpesviruses per se and that the appearance of viral DNA in the blood would be

transient, it is quite difficult to more precisely quantify viral genome by conventional weekly sampling of blood. The virus would have been eliminated due to intense anti-viral immune responses, making its direct identification at the clinical onset of symptoms virtually impossible. Thus, in order to determine which herpesvirus can initially trigger the cascade of reactivation events and whether reactivations of herpesviruses could occur during the prodromal stage preceding clinical onset, a frequent sampling of peripheral blood prior to onset of disease in those patients may be necessary, although extremely difficult. Additional difficulty in identifying the overall status of viral reactivations using blood samples is that we cannot exclude the possibility that different herpesviruses may reactivate in other organs, such as spleen and liver, in sequential order totally independent of that occurring in the blood.

Our recent studies of real-time measurement for EBV, CMV, HHV-6 and HHV-7 DNA loads in the blood demonstrated that high HHV-6 DNA loads at or near the time of the initial presentation were only detected in a few out of > twenty patients with DIHS. High EBV DNA loads were also detected in some patients with DIHS, but at later time points when compared with HHV-6 loads.

Although various clinical symptoms develop at various time points after onset despite withdrawal of the causative drugs, patients with DIHS can eventually recover after undefined period (months). Oral corticosteroids have been the main therapeutic options for treating DIHS. Rapid resolution of rashes and fever occurs within in several days after starting systemic corticosteroids: the usual dosage is 1–1.5 mg predonisolone/kg/day [2–4].

4. Autoimmune diseases as sequelae of DIHS

Several autoimmune diseases have been reported to occur as sequelae of DIHS [10–13,32]: they include type 1 diabetes mellitus, autoimmune hypothyroidism, sclerodermoid GVHD-like lesions and SLE [Fig. 1]. Because they appeared for several months to years after the acute illness was resolved, it is difficult to find a link between DIHS and the subsequent autoimmune diseases unless special attention is given to factors that trigger the development of autoimmune diseases. Importantly, viral infections have been also implicated in these autoimmune diseases, which provide a strong basis for virus involvement. In these conditions, a biphasic pattern of disease has been suggested to occur in which an early acute viral stage is followed by a later autoimmune phase. Therefore, they cannot necessarily occur immediately after viral infections, but rather occur after a disease-free interval of several months to years. This is reminiscent of type 1 diabetes after congenital rubella syndrome: infections take place in utero and diabetes occurs 5–20 years later [38]. Thus, a major difficulty in establishing a correlation between triggering events, such as DIHS or a drug rash associated with viral reactivations, and the actual autoimmune disease is the long prodromal period preceding clinical onset of disease.

In this regard, we have recently experienced two interesting cases who developed sclerodermoid GVHD-like lesions and SLE, respectively, three to four years after resolution of DIHS [12,13]. The former case is a 46-year-old woman who developed systemic sclerosis-like lesions 3 years after DIHS [13]. She

presented with multiple brownish, indurated plaques with xerosis on the extremities with diffuse alopecia on the scalp. She had had zonisamide-induced DIHS 4 years previously. Along with HHV-6 reactivation, various manifestations such as fever, liver dysfunction, and generalized rash occurred. Consistent with this finding, Caballero et al. reported that generalized sclerodermatous changes appeared between day 332 and 876 in a group of patients after BMT. Importantly, in this case, antinuclear antibody (ANA) was negative during the course of DIHS and became detectable (1:40) coincident with the development of alopecia: a dramatic increase in ANA (1:5120) was found at her initial presentation to our department because of sclerodermoid GVHD-like lesions, indicating that the disease process of DIHS may act as a trigger for the development of autoimmune diseases. These findings could be interpreted as an indication that sequential reactivations of herpesviruses and/or immune responses to them occurring during the disease process of DIHS may render otherwise refractory individuals susceptible to autoimmune disease.

The latter case initially presenting with Kikuchi–Fujimoto disease (KFD) evolved into clinically evident SLE within 2 weeks: the patient had had an episode of DIHS 4 years previously [13]. This patient (a 36-year-old Japanese man) presented with bilateral painless enlarged lymph nodes of the neck, with high-grade fever and malaise. Two weeks after the onset of lymphadenopathy, non-itchy erythematous macules developed on the ears, face, and neck. His past medical history revealed that he had been diagnosed as having DIHS due to carbamazepine in our Department: on that occasion, not only HHV-6 but also EBV reactivation was confirmed during the course of DIHS, and he had completely recovered after 3 months. He remained asymptomatic for 4 years until he presented with cervical lymphadenopathy. Clinical manifestations and histology of the lymph node at his presentation with the lymphadenopathy were consistent with a diagnosis of KFD. However, 1 week later his erythematous macules dramatically deteriorated, evolving to dusky red, well-demarcated, slightly infiltrated plaques, suggestive of LE. Laboratory work-up revealed leukopenia, positive ANA (1:80) and decreased serum C3 and C4 levels. Renal biopsy specimens showed moderate diffuse mesangial proliferation, consistent with lupus nephritis. Histologic examination of the plaque showed vacuolar changes of basal layer and a moderate lymphocytic infiltrate in the upper dermis: lupus band test was positive. By in situ hybridization, expression of EBV-encoded RNA (EBER) was detected in the lymph node. The presence of EBV DNA was also confirmed by PCR in the lymph node, but not in the blood. Based on these findings, we made a diagnosis of KFD evolving into clinically evident SLE.

In view of the fact that EBV reactivations were confirmed on two occasions in this patient, DIHS and KFD, we reason that EBV reactivations are pathologically involved in the pathogenesis of SLE subsequent to KFD. If so, one would expect that EBV reactivations occurring during the episode of DIHS could profoundly influence the sequelae of DIHS: EBV repeatedly reactivated during the episode of DIHS and at the time of the lymhadenitis might have contributed to the induction of SLE. Reactivations of the herpesviruses, which cannot be determined in this patient but may in turn serve to reactivate EBV, could subclinically occur during the disease-

free period that is the prodromal period, and eventually cause flare-up of a previously dormant disease state through a process of enhanced T- and B-cell activation. This long prodromal period that precedes the clinical onset of disease makes detection of the causative viruses difficult later on, when autoimmune disease occurred. These considerations prompted us to investigate whether autoimmune responses could have developed during or after the development of DIHS and if so, the investigation into mechanisms of how autoimmune responses are generated during and after the disease process in patients with DIHS could give an important clue as the pathogenesis of autoimmune diseases developing after clinical resolution of DIHS.

5. Regulatory T cells at the acute and resolution stages of DIHS

Treg cells are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. They express high levels of CD4, CD25, and the transcription factor, FoxP3 [39]. Given the capacity of Treg cells to prevent autoimmune diseases, we can hypothesize that Treg cells may be functionally altered at the acute and resolution stages of DIHS. To date, however, the role of Treg cells in drug eruptions has not been extensively studied. As a consequence, the degree to which Treg cells truly played a protective role during the actual disease process is largely unknown. We, therefore, sought to investigate the frequency and function of Treg cells both during the acute stage and again long after clinical resolution of DIHS.

Surprisingly, dramatic expansions of fully functional $CD4^+$ $CD25^+FoxP3^+$ Treg cells were specifically found in the acute stage of DIHS irrespective of whether patients were treated with immunosuppressive agents (Takahashi R, et al. Manuscript submitted). In view of the observation that sequential reactivations of herpesviruses is specifically observed in the acute stage of DIHS, this expanded Treg population would serve to prevent the activation and expansion of anti-viral T cells, thereby allowing latent herpesvirus to reactivate in an uncontrolled fashion. Most of Treg cells expanded at the acute stage can be characterized by increased expression of CCR4 and CLA, indicating a skin-homing phenotype. Consistent with this finding, FoxP3$^+$ Treg cells were frequently detected in the skin lesions of DIHS. The expanded Treg cells with the skin-homing phenotype, however, were eventually contracted upon resolution of the acute illness and their preferential expansion of CCR4 and CLA returned to normal. The sampling at the resolution stage was performed after immunosuppressive treatment was withdrawn. We also investigated whether the functional activity of Treg cells could be altered depending on the stage of the disease examined. FoxP3$^+$ Treg cells expanded at the acute stage of DIHS were found to retain the suppressive capacity. However, their suppressive capacity became defective after clinical resolution of DIHS: Treg cells lose their ability to inhibit cytokine production and proliferation of effector T cells isolated from the same patients despite their normal frequencies in the blood, coincident with their contraction upon clinical resolution. Importantly, no patients with DIHS showed any sign suggestive of the development of autoimmunity when samples at the resolution stage were obtained. These results clearly
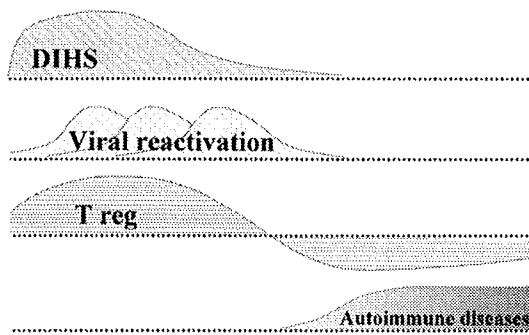
Fig. 2. Potential roles of dysfunctional Treg cells from patients with DIHS in the development of autoimmune diseases. At the acute stage, fully functional Treg cells are expanded, thereby allowing sequential reactivations of latent herpesviruses. Sequential reactivations of herpesviruses could in turn elicit repeated and excessive activation of anti-viral T cell responses that can cross-react with the drug, leading to severe drug-induced immunopathology. To limit excessive activation of anti-viral T cell responses, Treg cells could be repeatedly activated. Treg cells thus excessively activated might be eventually exhausted at the resolution stage. The degree of exhaustion is dependent on the severity and duration of viral reactivations and the resultant activation of anti-viral T cell responses. A resultant loss of their suppressive function would enable autoreactive T cells to be liberated from Treg cell control, thereby increasing the risk of autoimmunity triggered by viruses.

indicate that a functional defect of Treg cells would be responsible for the subsequent development of autoimmunity [Fig. 2]. Probably the best explanation for this progressive dysfunction of Treg cells is that Treg cells might be exhausted owing to repeated and excessive activation of effector T cells including anti-viral T cells driven by a high viral load in hosts: Treg cells could be overloaded and be driven eventually to exhaustion.

6. Conclusion

Our case series indicate that ~10% of patients with DIHS eventually develop autoimmune diseases or phenomena after disease-free intervals of several months to years. We are somewhat surprised to find that autoimmune diseases after resolution of DIHS have not extensively been reported despite the high frequency in our case series. We emphasize, however, that a functional defect of Treg cells, the resultant activation and proliferation of effector T cells, and an increase in autoantibody titers are often asymptomatic and that these abnormalities may go unrecognized unless special attention has been focused on this point in patients recovered from DIHS. We can presume that autoimmune diseases as sequelae of DIHS are much more frequent than actually reported. To ensure an early diagnosis of possible development of autoimmune diseases, patients with DIHS and those suggestive of viral reactivations should be carefully followed up even long after complete resolution of clinical symptoms has occurred. Even for physicians who may not have been familiar with DIHS, a history of a clinical illness compatible with DIHS should be sought in all patients presenting with lupus-like manifestations.

Take-home messages

• There is a strong association between preceding infection with EBV and the subsequent development of SLE. Because

EBV is likely to be required but not alone sufficient to elicit autoimmunity, infections with other viruses may give the final touch that tips the balance in an individual predisposed by EBV toward the development of autoimmunity.

• DIHS, a drug rash characterized by sequential reactivations of herpesviruses and the subsequent development of autoimmune diseases, offers a unique opportunity to link viral infections with the subsequent development of autoimmune diseases.

• Several autoimmune diseases, such as type 1 diabetes mellitus, autoimmune thyroiditis, and SLE, have been reported to occur at intervals of several months to years after clinical resolution of DIHS.

• FoxP3⁻ T cells lose their ability to inhibit cytokine production and proliferation of effector T cells, coincident with their contraction upon clinical resolution of DIHS. This functional defect of Treg cells would be responsible for the subsequent development of autoimmunity.

Acknowledgements

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Subtypes of multiple sclerosis and microarray patterns

Multiple sclerosis is an autoimmune disease characterized by the production of autoantibodies. In an elegant article, Quintana et al. (PNAS 2008;105:18889–94) have analyzed pattern of antibody reactivity in multiple sclerosis serum against a panel of central nervous system protein and lipid autoantigens and heat shock protein. The authors found specific patterns that could distinguish multiple sclerosis from healthy individuals as well as from other neurological and autoimmune disorders (Alzheimer's disease, adrenoleukodystrophy and systemic lupus erythematosus). Moreover, relapsing-remitting multiple sclerosis sera had auto-antibodies to heat shock protein which were not observed in other clinical subtypes of this sickness. Antibodies to lipids and central nervous system-derived peptides were also correlated to brain biopsy specimens. This study suggests that the unique autoantibody patterns in multiple sclerosis using microarray technique.

Natural autoantibodies in rheumatoid arthritis

Natural autoantibodies are present in healthy individuals and their role is not completely known. In a recent paper, Gyorgy et al., (Arthritis Res Ther 2008;10:R110) have evaluated the presence of natural antibodies against glycosaminoglycans using ELISA in 66 sera from rheumatoid arthritis patients, 11 samples from umbilical cords and 54 healthy controls. The authors found antibodies to glycosaminoglycans in sera from rheumatoid arthritis patients and healthy individuals, with higher titers in the first group. On the other hand, sera from umbilical cords did not exhibit these antibodies. Using six different types of aminoglycans, this study observed cross-reactivity among these carbohydrates. Moreover, natural antibodies also reacted against bacterial peptidoglycans and fungal polysaccharides. In an interesting way, IgM anti-chondroitin sulphate C antibody serum levels were correlated to disease activity of rheumatoid arthritis and C-reactive protein. This study brings additional knowledge regarding to natural antibodies and the pathophysiology of rheumatoid arthritis and suggests their association with disease activity.

Defective Regulatory T Cells In Patients with Severe Drug Eruptions: Timing of the Dysfunction Is Associated with the Pathological Phenotype and Outcome[1]

Ryo Takahashi,[2]* Yoko Kano,[†] Yoshimi Yamazaki,[†] Momoko Kimishima,[†] Yoshiko Mizukawa,[†] and Tetsuo Shiohara*[†]

Toxic epidermal necrolysis (TEN) and drug-induced hypersensitivity syndrome (DIHS) represent two ends of a spectrum of severe drug eruptions: DIHS is unique in that severe epidermal damage seen in TEN is absent, sequential reactivations of herpesviruses occur, and autoimmunity often ensues. To investigate whether changes in regulatory T (Treg) cell function would contribute to variability in the clinical manifestations, we examined the frequency, phenotype, and function of Treg cells both during the acute stage and again long after clinical resolution of both diseases. Dramatic expansions of functional Treg cells were found in the acute stage of DIHS. In contrast, Treg function was profoundly impaired in TEN, although present in normal frequency. Skin homing addressins were more preferentially expressed on Treg cells in DIHS than in TEN. Indeed, Treg cells were more abundantly present in the skin lesions of DIHS. Surprisingly, Treg cells contracted upon resolution of DIHS became functionally deficient, whereas their functional defects in TEN were restored upon recovery. These findings indicate that a transitory impairment in their function during the acute stage of TEN may be related to severe epidermal damage, while a gradual loss of their function after resolution of DIHS may increase the risk of subsequently developing autoimmune disease. *The Journal of Immunology*, 2009, 182: 8071–8079.

D rugs often induce various cutaneous adverse reactions of different severity, ranging from simple, uncomplicated papulomacular to potentially fatal, severe eruptions, such as Stevens-Johnson syndrome (SJS)[3] and toxic epidermal necrolysis (TEN), in susceptible patients. Although many factors that cause variability in the clinical course have been suggested, it remains unknown which factors are predominantly involved in the process. Because the most prevalent severe drug eruptions are thought to be mediated by drug-reactive T cells (1–4), the phenotype and functions of these effector T cells are likely to determine the clinical picture of the disease. However, investigators need to be made aware of the alternative view that severe drug eruptions are due to a dysbalance of the immune system caused by excessive activation of effector T cells and an inadequately low function or number of T cells that can limit immunopathology. In this regard, regulatory T (Treg) cells are most likely candidates to search for

inadequate down-regulation in severe drug eruptions. Indeed, Azukizawa et al. (5) reported that in an animal model of TEN Treg cells can prevent experimentally induced epidermal injury mimicking TEN (6), although the therapeutic effect cannot be observed. Thus, drug-induced immunopathology is likely to be subject to control by Treg cells. To date, however, data are not available on the presence and function of Treg cells in TEN: the degree to which Treg cells truly played a protective role during the actual disease process leading to severe immunopathology is largely unknown.

Much less attention has been focused on the role of Treg cells in the pathogenesis of drug-induced hypersensitivity syndrome (DIHS) or drug rash with eosinophilia and systemic symptoms representing another end of a spectrum of severe drug eruptions: this syndrome is characterized by delayed onset, multiorgan involvement, sequential reactivations of various latent herpesviruses (7–9), and hypogammaglobulinemia (10) during the active phase and autoimmune manifestations occurring as short-term or long-term sequelae of the disease, such as type 1 diabetes mellitus (11), autoimmune thyroiditis and systemic sclerosis-like manifestations (12), findings never reported in TEN. In view of a large body of evidence indicating the roles of viruses (13) and Treg cells (14) in several autoimmune diseases, this syndrome offers a unique opportunity to encompass viral infections, drug eruptions, and the subsequent development of autoimmune diseases. Given the capacity of Treg cells to prevent organ-specific tissue injury (15) and autoimmune diseases (14), we can hypothesize that Treg cells play a critical role in the disease process of the acute stage of both diseases.

In this regard, 26 years ago, Dosch et al. (16) provided interesting insight into the workings of hypogammaglobulinemia observed in patients with DIHS. They described an increased frequency of circulating suppressor T cells capable of inhibiting Ig production in these patients with transient Ab deficiency. Based on

FIGURE 1. Expansion of Foxp3$^+$ CD25$^-$ Treg cells at the acute stage of DIHS but not of TEN and maculopapular drug eruption. *A*, Representative flow cytometry dot plots showing the expression of Foxp3 and CD25 in CD4$^+$ T cells in DIHS, TEN, and maculopapular drug eruption patients and in healthy controls. Numbers in each quadrant indicate the frequency of Foxp3$^+$CD25$^-$, Foxp3$^+$CD25$^+$, and Foxp3$^-$CD25$^-$ cells. *B*, The mean frequency of Foxp3$^+$, Foxp3$^+$CD25$^+$ Treg cells, and CD25$^+$ T cells in CD4$^+$ T cells from patients with DIHS, TEN, and maculopapular drug eruption and in healthy controls. Results are expressed as the mean ± SEM. DIHS (acute stage, n = 8; resolution stage, n = 6), TEN (acute stage, n = 4; resolution stage, n = 5), and maculopapular drug eruption (acute stage, n = 5) and in healthy controls (n = 10). *, p < 0.0001 (Student's t test).
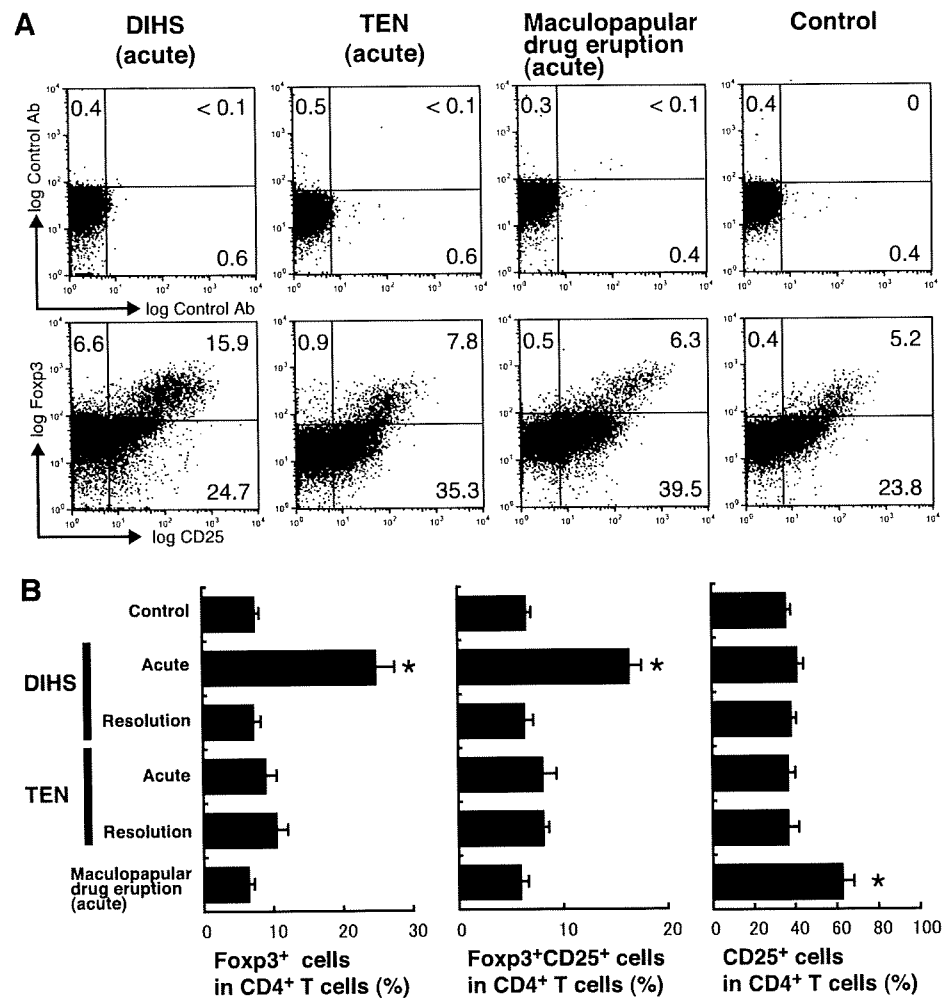
this finding and our recent observation that in vitro activation of patients' lymphocytes with the relevant drug resulted in profoundly decreased responsiveness in the acute stage but not in the resolution stage (17), we sought to compare the frequency and function of Treg cells in the peripheral blood at different time points after onset between patients with DIHS and those with TEN induced by the same drugs. Our study demonstrates that Treg cells with suppressive function were expanded at the acute stage of DIHS and that contraction of the Treg cell subset occurred coincident with resolution of the disease. In contrast, onset of TEN was associated with a functional defect of Treg cells, but this defect was eventually restored after clinical resolution. However, in patients with DIHS, a functional defect in the Treg cell population became evident upon clinical resolution despite their normal frequencies before the development of autoimmunity.

Materials and Methods

Subjects

Three groups of patients were included in this study: patients with DIHS (n = 12), patients with TEN (n = 11), patients with maculopapular drug eruption (n = 5), and healthy controls (n = 10). DIHS is characterized by high fever, a widespread erythematous eruption, lymphadenopathy, leukocytosis with atypical lymphocytosis and/or eosinophilia, and liver dysfunction (18): the diagnosis of DIHS was made based on the criteria established by a Japanese consensus group. In all patients with DIHS, HHV-6 reactivation as evidenced by the rise in serum HHV-6 IgG titers and HHV-6 DNA levels in the blood occurred commonly 2–3 wk after the onset of the

illness. Criteria for the diagnosis of TEN were high fever, severe mucocutaneous lesions, and detachment of epidermal sheets above 30% of the body surface area and patients with areas of epidermal detachment between 10 and 30% were initially defined as SJS/TEN overlap syndrome (19). In this study, however, patients with overlap SJS/TEN were included into a "TEN" category, because there were no significant differences between the two in the frequency and function of Treg cells. The ages of patients ranged from 14 to 74 years (mean age, 46.8 ± 4.7 years in DIHS; 49.1 ± 5.0 years in TEN; and 45.2 ± 9.7 years in maculopapular drug eruption). Informed consent was obtained from each patient. These studies were approved by the review board at Kyorin University and followed the guidelines for the ethical conduct of human research. The causative drugs, most of which were either carbamazepine (CBZ) or phenobarbital (PB), were withdrawn when the diagnosis of DIHS, TEN, or maculopapular drug eruption was made.

Blood and serum samples were obtained from these patients on or near the day of the initial presentation before starting treatment, and additional samples were subsequently obtained from these patients on a monthly basis. A late follow-up sample (>6 mo after onset) was also collected in patients with DIHS and those with TEN after withdrawal of immunosuppressive agents for the treatment. For these studies, healthy control subjects, matched for sex and age, were selected for comparisons of frequencies, phenotype, and functions of Treg cells (n = 10; mean age, 40.6 ± 5.2). Biopsy specimens were obtained from skin lesions in each patient near the day of the initial presentation before starting treatment.

Abs and reagents

mAbs to human CD4 (SK3), CD8 (Leu-2a), CD25 (2A3), CCR4 (1G1), cutaneous lymphocyte-associated Ag (CLA; HECA-452), the isotype controls to these Abs, and 7-amino-actinomycin D were purchased from BD Biosciences. Goat F(ab')$_2$ anti-human IgG Fc was purchased from
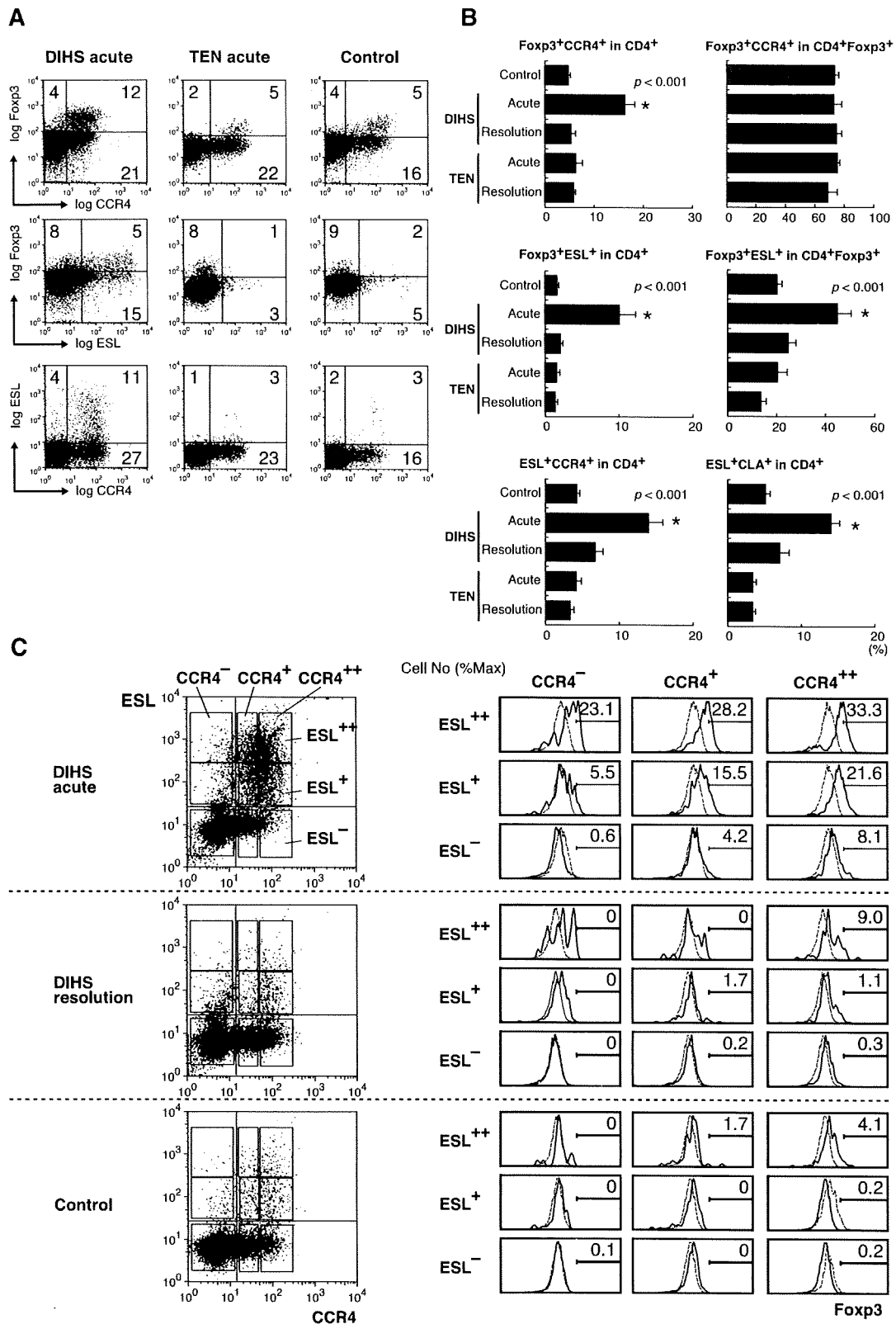
FIGURE 2. Expression of skin-homing addressins on Treg cells expanded at the acute stage of DIHS. *A*, Representative flow cytometry dot plots showing the expression of Foxp3, CCR4, and ESL in CD4$^+$ T cells in DIHS, TEN, and healthy controls. *B*, The mean frequency of Foxp3$^+$CD4$^+$ Treg cells coexpressing CCR4 or ESL and ESL$^+$CCR4$^+$CD4$^+$ or ESL$^+$CLA$^-$CD4$^+$ T cells in the acute and resolution stages of DIHS, TEN, and healthy controls. Results are expressed as the mean ± SEM. DIHS (acute stage, n = 8; resolution stage, n = 6), TEN (acute stage, n = 4; resolution stage, n = 5), and healthy controls (n = 10) (Student's t test). *C*, Preferential expression of Foxp3 in the CCR4^{++}ESL^{++} fraction obtained at the acute stage of DIHS. Purified CD4$^+$ T cells were stained by CCR4, ESL, and Foxp3 and then analyzed by FACS. Nine gates were set based on the intensity of ESL and CCR4 expression. The percentage of Foxp3$^+$ cells differentially expressing ESL and CCR4 is shown in the gated subsets indicated. Results of one representative experiment from three independent experiments are shown.

Beckman Coulter. mAbs to human CD3 (HIT3a), forkhead box P3 (Foxp3; PCH101), and the isotype controls to Foxp3 were purchased from eBioscience. Human IgG Fc was purchased from Valeant Pharmaceuticals. Recombinant E-selectin-IgG Fc chimera and mAb to human CD28 (37407) were purchased from R&D Systems. mAbs to mouse IgG Fc (Alexa Fluor 488 and Alexa Fluor 355 labeled) was purchased from Invitrogen.

Cell isolation from peripheral blood

PBMCs were prepared by density gradient separation (Lymphoprep; Axis-Shield) of peripheral blood of healthy donors, DIHS patients, and TEN patients. CD4 T cells were isolated using magnetic beads (CD4 T cell isolation kit II; Miltenyi Biotec). CD4 T cells thus isolated were incubated with anti-CD4-allophycocyanin and anti-CD25-FITC Abs, and subpopulations CD4$^+$CD25^{++} and CD4$^+$CD25$^-$ of CD4 T cells were isolated by using FACSAria (BD Biosciences).

To prepare T cell-depleted APCs, CD3$^-$ T cells were depleted from PBMCs using CD3 magnetic beads (Miltenyi Biotec) according to the manufacturer's recommendations, and then the T cell-depleted APCs were incubated with mitomycin C (Kyowa Hakko Kogyo) at 37°C for 45 min. To prepare Treg cell-depleted PBMCs for cytokine assay, CD25$^+$ cells were depleted from PBMCs using CD25 magnetic beads (Miltenyi Biotec) according to the manufacturer's recommendations.

T cell proliferation assay

FACS-sorted CD4$^-$CD25$^-$ effector T cells (3,000 cells/well) were cocultured with FACS-sorted Treg (CD4$^+$CD25^{++}) cells (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 (20–22). Cells were also cocultured at different Treg:effector cell ratios of 0.25:1 and 0.5:1. The cells were stimulated with 0.1 μg/ml soluble anti-CD3 (HIT3a) and soluble anti-CD28. All cells were cultured in triplicate and in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% human AB serum (Sigma-Aldrich). After 4 days of culture, 1 μCi of [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 percent proliferation resulting from the addition of CD4$^+$CD25^{++} T cells to the culture.

Flow cytometric analysis

To simultaneously detect surface chemokine receptors and intracellular Foxp3 expression, fluorescence-conjugated anti-CD4-FITC, anti-chemokine receptor Abs, and 7-aminoactinomycin D was added to PBMCs and incubated for 30 min at room temperature. To simultaneously detect surface E-selectin ligand (ESL) and intracellular Foxp3 expression, CD4$^+$ T cells isolated from PBMCs using a CD4 Isolation kit were stained with anti-CD25-PE and ESL detection protocol, as described below. To detect intracellular Foxp3 expression, an allophycocyanin anti-human Foxp3 staining set (eBioscience) was used according to the manufacturer's instruction. Cells were analyzed with a FACSCalibur (BD Bioscience).

Detection of ESL

In this study, the ESL epitope is defined as a site specifically bound to rE-selectin-IgG Fc chimera. The detection of ESL was performed by FACS analysis as described previously (23).

Measurements of cytokine levels in culture supernatants

TNF-α and IFN-γ levels were measured using cytometric bead arrays (BD Biosciences) according to the manufacturer's protocol.

Detection of Foxp3$^+$ T cells in skin lesions by immunofluorescence

Immunofluorescent detection of Foxp3 expression was performed as follows. The biopsy specimens were frozen immediately in liquid nitrogen, embedded in Sakura Tissue-Tek OCT Compound 4583 (Sakura Finetek) and stored at −80°C until used. Frozen sections 5-μm thick were air dried, fixed in acetone at 4°C for 10 min, and then followed with 4% paraformaldehyde at 4°C for 15 min. After Ag retrieval and incubation in blocking solution, samples were stained with anti-Foxp3 mAb at 4°C overnight and then followed by anti-mouse IgG/Fc-Alexa Fluor 488 or anti-mouse IgG/Fc-Alexa Fluor 355 at 37°C for 30 min. Finally, samples were stained by anti-human CD8 mAb-PE or anti-human CD4 mAb-FITC and anti-human CD8 mAb-PE.
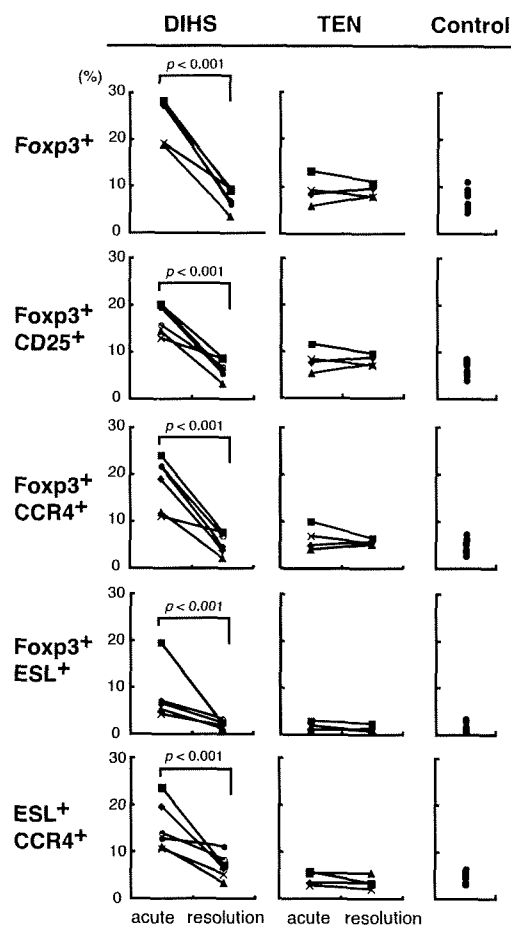

FIGURE 3. Longitudinal analysis of Treg cell levels at the acute and resolution stages of DIHS and TEN. Longitudinal monitoring of indicated subsets was performed in the same patients (DIHS, $n = 6$; TEN, $n = 4$; control, $n = 10$; Student's t test). Shown is the frequency of indicated subsets in CD4$^+$ T cells.

Statistics

Data are expressed as mean ± SEM and were determined using Student's t test or Dunnett's test.

Results

Preferential expansion of Treg cells in PBMCs from patients with DIHS

We initially assessed the frequencies of CD4$^+$CD25$^+$Foxp3$^+$ T cells in total PBMCs of patients with DIHS and those with TEN at their acute and resolution stages, respectively. The frequency of Treg cells in total CD4$^+$ T cells from patients with TEN at the acute stage was not significantly different from that in those from healthy controls (Fig. 1). In contrast, patients with DIHS at the acute stage showed significantly increased frequencies of Treg cells in total CD4$^+$ T cells compared with healthy controls (Fig. 1). Similar expansions of Treg cells were not observed in other types (maculopapular) of drug eruption (Fig. 1), indicating that increased frequencies of Treg cells are not a general phenomenon in patients with drug eruptions. Nevertheless, it was also possible that the increased frequency of Treg cells observed in DIHS merely reflects the overall status of activation of T cells, because recent studies on human Treg cells have demonstrated that TCR stimulation alone with anti-CD3 and anti-CD28 induces Foxp3 expression without acquisition of suppressive function (21, 24, 25). This
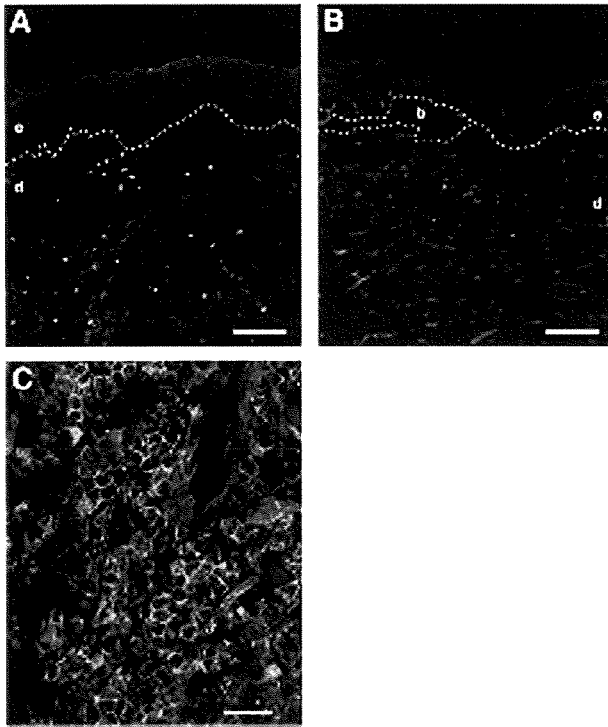
FIGURE 4. Localization of Foxp3^{+} Treg cells in the skin lesion of DIHS and TEN. *A* and *B*, Dual staining of Foxp3 (green) and CD8 (red). Foxp3^{+} Treg cells are clearly visible with green nuclei. Foxp3^{−} Treg cells are abundantly found in the dermis of DIHS lesions (*A*). In contrast, Treg cells are rarely found in the blisters and dermis of TEN lesions while CD8^{+} T cells are frequently detected in the upper dermis (*B*). In the mid-dermis, the diffuse nonspecific staining of collagen fibers distinct from Treg cells is seen. *C*, In triple-color immunofluorescence staining, Foxp3^{+} Treg cells (blue) are found in close proximity to CD8^{+} T cells (red) and CD4^{+} T cells (green) in the dermis of DIHS lesions. d, Dermis; e, epidermis; b, blister. Bar: *A* and *B*, 100 μm and *C*, 50 μm.

possibility is unlikely, however, because the total frequencies of CD25^{+} T cells were only increased in patients with maculopapular drug eruption but not in those with DIHS and TEN, compared with healthy controls. Thus, selective expansions of Treg cells in peripheral blood are likely a common feature in patients with DIHS at the acute stage.

Trafficking receptors expressed on the expanded Treg cells

Previous studies demonstrated that Treg cells preferentially express chemokine receptors and adhesion molecules required for skin homing, such as CLA and CCR4 (26, 27). We therefore investigated the expression profile of previously described chemokine receptors and adhesion molecules associated with Treg cells. In addition, we assessed whether the Treg cells could be able to bind rE-selectin-IgG chimera, as described in our previous studies on skin-homing T cells (23). Most of Treg cells expanded at the acute stage of DIHS expressed CCR4 (73.3 ± 5.4%, *n* = 10) (Fig. 2*A*, Foxp3 vs CCR4; *B*, Foxp3^{+}CCR4^{+} in CD4^{+}), indicating a skin-homing phenotype. Nevertheless this skin-homing phenotype was also preferentially found in Treg cells in patients with TEN and healthy controls (Fig. 2*B*, Foxp3^{+}CCR4^{+} in CD4^{+}Foxp3^{+}). A substantial proportion of Treg cells expanded at the acute stage of DIHS coexpressed CCR4 and ESL, as assessed by the ability to bind rE-selectin-IgG chimera. The frequency of ESL^{+} cells in Treg cells was significantly higher in patients with DIHS at the acute stage than
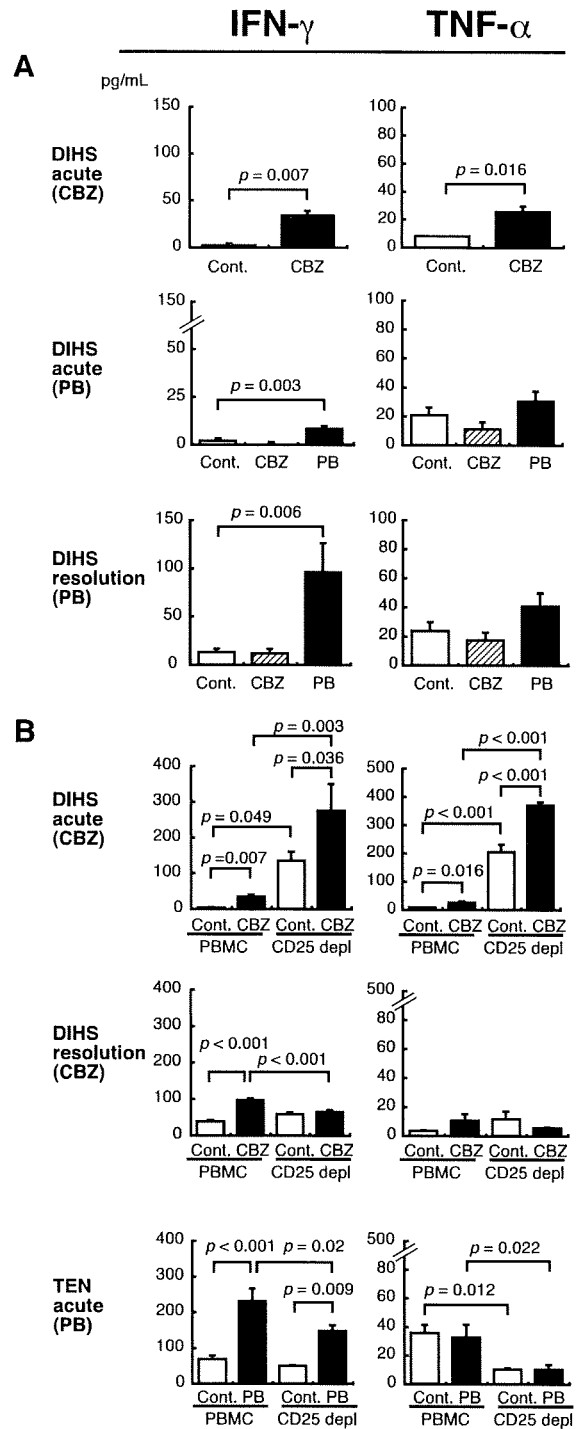
FIGURE 5. The effects of Treg cell depletion on cytokine production by effector T cells stimulated with drug Ag in vitro. *A*, PBMCs were obtained at the different stages from patients with DIHS who were hypersensitive to CBZ or PB and were stimulated with CBZ or PB without exogenous IL-2. Supernatants were collected on day 5 of cultures and cytokine concentrations were determined by cytometric bead array (see *Materials and Methods* for details). *B*, Cytokine production by CD25-depleted PBMCs (CD25 depl) from patients with DIHS or those with TEN obtained at the different stages was also studied and compared with those by PBMCs before CD25 depletion. The data are expressed as the mean ± SEM (*n* = 3–5; Student's *t* test).

in those with TEN and healthy controls (Fig. 2*B*, Foxp3^{+}ESL^{+} in CD4^{+}Foxp3^{+}). Most of the Treg cells expanded at the acute stage of DIHS can be characterized by increased expression of ESL and

A
□ Control
▨ DIHS acute
■ DIHS resolution

B
□ Control
▨ TEN acute
■ TEN resolution

C
□ Control
▨ DIHS (CD25⁻: acute, CD25⁺⁺: acute)
■ DIHS (CD25⁻: acute, CD25⁺⁺: resolution)

D
□ Control
▨ DIHS (CD25⁻: resolution, CD25⁺⁺: acute)
■ DIHS (CD25⁻: resolution, CD25⁺⁺: resolution)

FIGURE 6. Functional analysis of Treg cells at the different stages of DIHS and TEN. A, Highly purified CD4$^+$CD25^{++} Treg cell populations from patients with DIHS at either the acute or resolution stage, or healthy controls were cocultured at different ratios with highly purified CD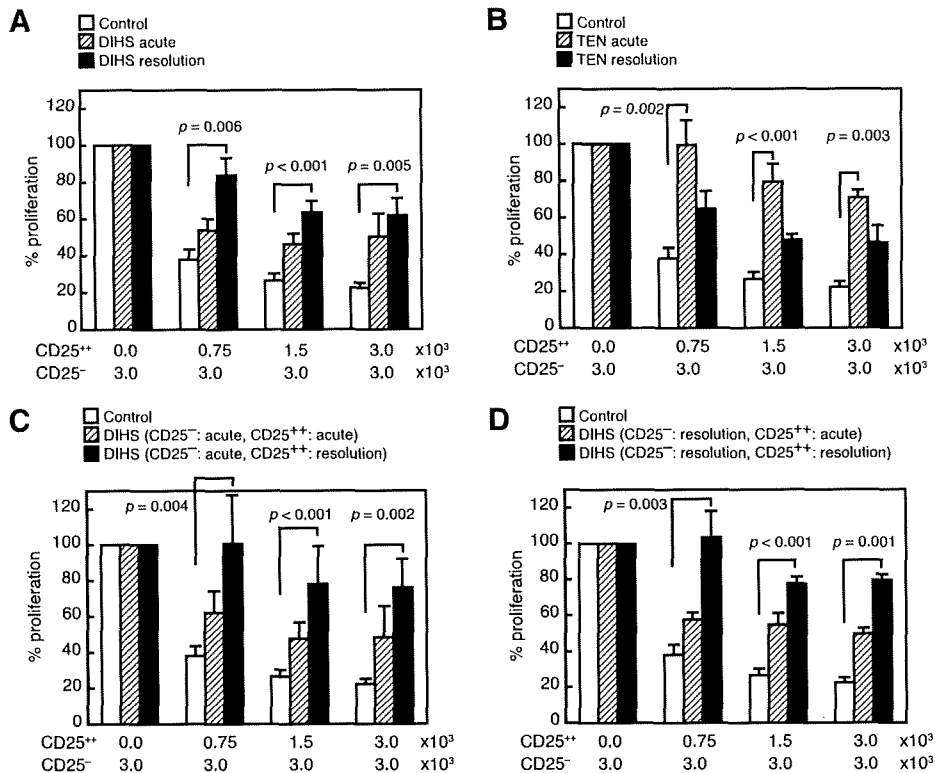4$^+$CD25$^-$ effector T cell populations from the same patients at the same stage, or from healthy controls in the presence of mitomycin C-treated allogeneic APCs and anti-CD3 and anti-CD28 mAbs. Proliferation was assessed by a [^{3}H]thymidine incorporation assay. The results are expressed as the percent proliferation of CD4$^+$CD25$^-$ effector T cells in the absence of CD4$^+$CD25^{++} Treg cells. B, Similarly, CD4$^+$CD25^{++} Treg cell populations from TEN patients were cocultured with CD4$^+$CD25$^-$ effector T cell populations from the same patients at the same stage. C, Highly purified CD4$^+$CD25^{++} Treg cell populations from patients with DIHS at either the acute or resolution stage were cocultured with a highly purified constant CD4$^+$CD25$^-$ effector T cell population from the same patients at the acute stage. D, Highly purified CD4$^+$CD25^{++} Treg cell populations from patients with DIHS at either the acute or resolution stage were cocultured with a highly purified constant CD4$^+$CD25$^-$ effector T cell population from the same patients at the resolution stage. Mean (n = 3–7) and SEM are shown. One-way ANOVA followed by Dunnett test was used to determine significant differences between patients and controls.

CCR4 (Fig. 2C), but the preferential expression of ESL on Treg cells was no longer observed in patients with DIHS after clinical resolution (Figs. 2, B and C). Thus, in patients with DIHS at the acute stage, a larger fraction of Treg cells are likely to have more potent ability to migrate into the skin, as compared with those observed under other conditions.

In six patients with DIHS and four patients with TEN, frequencies of Treg cells and their CCR4 and ESL expression were reexamined >6 mo after the first sampling. As shown in Fig. 3, when compared with the frequency of Treg cells from the same patients at the resolution stage, three patients with DIHS at the acute stage showed a >3-fold expansion of Treg cells in peripheral blood CD4$^+$ T cells. In contrast, four patients with TEN sampled twice on both occasions 6 mo apart showed identical values for both samples. Thus, the expanded Treg cells were contracted upon resolution in patients with DIHS and their preferential expression of ESL and CCR4 returned to normal.

Treg cells in skin lesions of DIHS and TEN

The most obvious difference in the skin lesions found between the two forms of drug reactions is the presence of a massive destruction of the epidermis: full-thickness epidermal necrosis typically observed in TEN is not detected in the majority of skin lesions of DIHS (7, 18, 28). To directly assess the relative contribution of Treg cells to the control of drug-induced immunopathology in

these skin lesions, we investigated whether the expanded Treg cells could migrate into the inflammatory sites to help limit epidermal damage caused by vigorous T cell activation. As shown in Fig. 4A, Foxp3$^+$ T cells were frequently detected in the skin lesions of DIHS by immunofluorescence. Generally, Foxp3$^+$ T cells were frequently detected close to where many CD8$^+$ T cells were infiltrated (Fig. 4C). On the other hand, Foxp3$^+$ T cells were much less frequently detected in skin lesions of TEN, where multiple blisters developed (Fig. 4B).

Alterations in suppressive function of Treg cells depending on the stage in DIHS and TEN

We next investigated whether the functional activity of Treg cells from patients with DIHS and TEN could be altered depending on the stage examined. Before determining the functionality of Treg cells, we examined whether effector T cells in PBMCs from these patients at the acute and resolution stages of DIHS could have the ability to make inflammatory cytokines in response to relevant Ag stimulation. As shown in Fig. 5A, effector T cells at either the acute or resolution stage produced detectable quantities of inflammatory cytokines only when stimulated with relevant drug Ag in vitro, although those at the resolution stage had a more potent ability. To test the suppressive function of these Treg cells, we initially analyzed the effects of depletion of Treg cells on cytokine production by drug Ag-specific effector T cells from these patients. In PBMCs

obtained from patients with DIHS at the acute stage, Treg cell depletion induced a dramatic increase in IFN-γ and TNF-α production under either basal or Ag-stimulated conditions (Fig. 5B). In contrast, in those obtained from the same patients with DIHS at the resolution stage, Treg cell depletion did not induce a significant increase in the cytokine production under either basal or Ag-stimulated conditions. In the acute stage of TEN, Treg cell depletion from PBMCs did not result in a significant increase in cytokine production, as observed in the resolution stage of DIHS.

We next asked whether Treg cells obtained at the different stages could have the suppressive capacity to inhibit activation of effector T cells. To this end, we conducted a T cell proliferation assay using allogeneic APCs. As shown in Fig. 6A, on a cell-cell basis, Treg cells in patients with DIHS at the acute stage were found to retain the suppressive capacity, although less than that of healthy controls. However, surprisingly, their suppressive capacity became defective after clinical resolution of DIHS.

In contrast, Treg cells obtained from patients with TEN at the acute stage were found to be profoundly defective in their capacity to suppress T cell proliferation (Fig. 6B); the degree of functional defect in TEN was directly related to the severity of epidermal damage. Nevertheless, their defective capacity at the acute stage was restored upon clinical resolution (Fig. 6B). To exclude the possibility that subtle changes in Treg cell function were exaggerated in our T cell proliferation assay because the CD25$^-$ effector population was also potentially changing depending on the stage examined, add-back experiments using the CD4$^+$CD25$^-$ effector cells from the constant stage and the CD25^{++} Treg cells from the different stages were performed for some patients with DIHS: these populations at the different stages were obtained from the same patients. As shown in Figs. 6, C and D, the impairment in suppressive function of Treg cells was specifically observed at the resolution stage regardless of whether the effector population at either the acute (Fig. 6C) or resolution (Fig. 6D) stage was used.

Discussion

In this study, we for the first time demonstrated that CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are expanded at the acute stage of DIHS while such expansions are never observed in patients with other types of drug eruptions such as TEN and maculopapular drug eruption: in the acute stage of TEN, although Treg cells can be present in normal numbers in the blood, their capacity to migrate into the skin and to suppress the activation of effector T cells is profoundly impaired, thereby allowing drug-specific T cells to function in an uncontrolled fashion, as demonstrated in experimental animals (5, 6, 29). A marked increase in the frequency of Treg cells at the acute stage of DIHS raises the question of whether a simple accumulation of Treg cells in the peripheral blood has occurred as a consequence of their defective migration to the inflammatory skin sites. However, this possibility is unlikely, because this cell compartment expanded in the acute stage of DIHS is further characterized by an increase of Treg cells coexpressing ESL and CCR4, well-known markers associated with skin-homing T cells (23, 27, 30). Indeed, our immunohistochemical study showed that Foxp3$^+$ Treg cells migrated to the skin lesions of DIHS at much higher frequencies than those in the lesions of TEN. An influx of Treg cells with suppressive properties into the skin lesions of DIHS would serve to alleviate lesion severity. However, the "dark side" of reduced lesion severity could be reactivation of viruses persisting in a latent state, which is typically observed in patients with DIHS as well as those with graft-versus-host disease (7–9, 31). Thus, this expanded Treg cell population might serve to prevent the activation and expansion of antiviral T cells, thereby reducing

lesion severity and possibly allowing latent herpesviruses to reactivate in an uncontrolled fashion.

Our in vitro Treg cell depletion study and T cell proliferation study clearly demonstrated that the expanded Treg cells displayed a strong inhibitory function on cytokine production and proliferation of effector CD4$^+$CD25$^-$ T cells on a per-cell basis. These results indicate that the expanded CD4$^+$CD25$^+$Foxp3$^+$ T cells were fully functional Treg cells and not effector T cells. Although their suppressive function was not assessed using an Ag-driven T cell proliferation or cytokine production assay due to a limited number of Treg cells available, we reasoned that the T cell proliferation assay using allogeneic APCs is sufficient to assess the function of Treg cells to suppress Ag-driven T cell activation at a single-cell level. Given an increased frequency of these cells in peripheral blood, the overall inhibitory effect of the expanded Treg cells would be even more pronounced in vivo, indicating that these T cells would limit the severity of a T cell-mediated immunoinflammatory response to drug. These findings provide an explanation for why severe epidermal damage cannot be detected in the skin lesions of DIHS, unlike TEN lesions, why the onset of this disease is delayed in relation to the introduction of the causative drug, and why proliferation of drug-specific T cells can only be detected at the resolution stage of DIHS, but not at the acute stage, whereas this can be detected at the acute stage of other types of drug eruptions, such as TEN, as evidenced by the results of lymphocyte transformation tests (17, 31). Hypogammaglobulinemia and a profound decrease in B cell numbers specifically observed at the onset of DIHS (10) may be related to expansions of functional Treg cells, because Treg cells have been shown to have the ability to induce B cell death (32).

In contrast, Treg cells detected in the resolution stage of DIHS were found to be functionally impaired in their in vitro suppressive activity despite their normal frequencies, although all of the patients examined had not developed autoimmune diseases at the time of sampling. Consistent with this finding, some recent studies have demonstrated the existence of Treg cells with a reduced in vitro suppressive function in patients with type 1 diabetes and other autoimmune diseases (33–35); interestingly, some of which were reported to develop as long-term sequelae of DIHS (11, 12, 36, 37). An important question to be resolved is whether the expanded Treg cells in patients with DIHS could be driven eventually to exhaustion during the disease course or replaced by other subsets of Treg cells with lower functional activity.

The studies on an animal model of TEN suggested the importance of Treg cells in protecting susceptible patients from the development of TEN (6), although their protective role has never been examined during the actual disease process. In this regard, our data show that the functionality of Treg cells at the acute stage of TEN was impaired, on a per-cell basis, in agreement with the present finding that severe epidermal damage can be seen in skin legions of TEN patients, in whom circulating Treg cells are present in normal frequency. These data suggest that the major difference in Treg cells between patients with TEN and healthy controls is qualitative and not quantitative. It should be noted, however, that our data do not provide an answer to the important question of how Treg cells become functionally impaired at the onset of TEN. Given the ability of Treg cells to sense pathogens directly through TLR such as TLR2 and consequently modify their function (38), along with the fact that TLR2 is critical in the initiation of innate immune responses to herpesviruses and *Mycoplasma* (39, 40), it is reasonable to speculate that the suppressive function of Treg cells

might be temporarily impaired during the acute phase of the infection. In support of this possibility, infections with these pathogens have been implicated in the pathogenesis of severe drug eruptions (41). Addressing this question will require longitudinal numerical/functional analyses of Treg cells at different stages of infection.

Similar expansion of Treg cells with the antiproliferative activity during the acute phase and their subsequent contraction upon resolution have been also reported to occur in patients with sarcoidosis (42). Interestingly, the clinical features of both DIHS and sarcoidosis fit within the spectrum of immune reconstitution syndrome (IRS), which is characterized by a paradoxical deterioration of clinical symptoms or laboratory parameters attributable to restoration of a valid immune response against previously unrecognized pathogens (31, 43, 44). Although the IRS was originally defined in HIV-infected patients, it was necessary to broaden the concept of IRS to include diverse infectious or autoimmune diseases such as herpes zoster and Graves' disease that occur following the tapering or discontinuation of immunosuppressive drugs (31, 41, 42). Our kinetic analyses of circulating lymphocyte count in patients with DIHS showed that a lymphopenic state immediately preceded lymphocytosis, a finding typically seen at the onset of DIHS, and that Treg cells were expanded during the recovery from lymphopenia, coincident with the onset (data not shown). Indeed, reconstitution of T cell-deficient mice by an adoptive transfer of mature peripheral lymphocytes was accompanied by rapid expansions of Treg cells that facilitated CD4^{+} T cell clonal anergy induction probably to prevent the development of overt autoimmunity in hosts recovering from lymphopenia (45).

In summary, our findings suggest that the timing of a dysfunction of Treg cells in individuals with drug-specific T cells in their T cell repertoire is associated with the pathological phenotype and outcome of T cell-mediated drug eruptions, although it remains unclear whether a dysfunction in Treg cells is causative in the induction of the disease. Expansions of Treg cells at the acute stage of DIHS may reflect an attempt to limit collateral tissue damage induced by activation and migration of effector T cells while allowing latent herpesviruses to reactivate in a sequential manner. However, a resultant loss of their suppressive function would increase the susceptibility of these patients to autoimmune diseases. In contrast, their functional defect at the acute stage of TEN would have detrimental effects on epidermal damage induced by excessive activation of effector T cells. Information regarding Treg cells during the disease process will help clinicians in terms of assessing the risk of eventually developing autoimmune diseases as well as the efficient management of these patients with therapeutic strategies to restore defective Treg cell functions.

Disclosures

The authors have no financial conflict of interest.

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Diagnosis and Treatment of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis with Ocular Complications

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Purpose: To present a detailed clarification of the symptoms at disease onset of Stevens-Johnson syndrome (SJS) and its more severe variant, toxic epidermal necrolysis (TEN), with ocular complications and to clarify the relationship between topical steroid use and visual prognosis.

Design: Cross-sectional study.

Participants: Ninety-four patients with SJS and TEN with ocular complications.

Methods: A structured interview, examination of the patient medical records, or both addressing clinical manifestations at disease onset were conducted for 94 patients seen at Kyoto Prefectural University of Medicine. Any topical steroid use during the first week at the acute stage also was investigated.

Main Outcome Measures: The incidence and the details of prodromal symptoms and the mucosal involvements and the relationship between topical steroid use and visual outcomes.

Results: Common cold-like symptoms (general malaise, fever, sore throat, etc.) preceded skin eruptions in 75 cases, and extremely high fever accompanied disease onset in 86 cases. Acute conjunctivitis and oral and nail involvements were reported in all patients who remembered the details. Acute conjunctivitis occurred before the skin eruptions in 42 patients and simultaneously in 21 patients, whereas only 1 patient reported posteruption conjunctivitis. Visual outcomes were significantly better in the group receiving topical steroids compared with those of the no-treatment group ($P<0.00001$).

Conclusions: Acute conjunctivitis occurring before or simultaneously with skin eruptions accompanied by extremely high fever and oral and nail involvement indicate the initiation of SJS or TEN. Topical steroid treatment from disease onset seems to be important for the improvement of visual prognosis.

Financial Disclosure(s): The author(s) have no proprietary or commercial interest in any materials discussed in this article. *Ophthalmology 2009;116:685–690 © 2009 by the American Academy of Ophthalmology.*

Stevens-Johnson syndrome (SJS) and its more severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory disorders that affect the skin and mucous membranes.[1-4] Although the incidence of SJS and TEN is very low, approximately 0.4 to 1 case per 1 million persons and 1 to 6 cases per 1 million persons, respectively, both can affect anybody at any age, usually as a consequence of adverse drug reactions.[5-7] A variety of drugs including antibiotics, nonsteroidal anti-inflammatory drugs, and antiepileptic medications, that is, any of the popularly used drugs, have been reported to cause severe drug reactions and to induce SJS or TEN.

The mortality rates for SJS and TEN are high: 1% to 5% and 25% to 35%, respectively.[8,9] Ocular complications occur in more than 50% of the patients, and ocular surface inflammation develops rapidly at the acute stage.[10,11] Extensive inflammation of the ocular surface often is accompanied by pseudomembranous formation and corneal or conjunctival epithelial defects, or both. The common pathway after the acute stage includes persistent epithelial defects, ulceration, and perforation, finally developing into corneal cicatricial changes such as neovascularization, opacification, keratinization, and symblepharon.[12,13] Even after the acute-stage impairments subside, permanent visual impairment or blindness remains and conjunctival inflammation prolongs at the chronic stage.[14] Patients with SJS or TEN require life-long management for ocular discomfort and morbidity. Stevens-Johnson syndrome or TEN accompanied by ocular complications, at both the acute and chronic stage, are 2 of the most devastating ocular surface diseases, and both are extremely difficult to treat.

The loss of corneal epithelial stem cells, which are located in the limbal region,[15-18] evidenced by the loss of palisades of Vogt, is the most common ocular feature of SJS.[13] As soon as the corneal epithelial stem cells are lost at the acute stage of SJS or TEN, the corneal epithelium does not regenerate, thus resulting in conjunctival epithelial invasion into the cornea (conjunctivalization) and cicatricial changes of the ocular surface. In contrast, the regeneration of the epidermis develops rather smoothly at the remission of the diseases.

Penetrating keratoplasty (PK) generally is contraindicated for eyes with SJS or TEN because PK does not supply the limbal region of the eye with corneal epithelial stem

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