Table 4 Comparison of the clinical and laboratory characteristics of the patients based on the presence or absence of at least a grade 3 dermatological adverse event

All patients $n = 89$	Non-grade 3 $n = 80$	Grade ≥3 $n = 9$	Univariate analysis P
Age (years)†	60 (19–73)	61 (48–65)	0.453
Sex (male/female)	40/40	8/1	0.027
Bodyweight (kg)†	62 (32–97)	64 (51–87)	0.593
Baseline white blood cell count (/μL)†	4900 (1500–9800)	4700 (3000–7000)	0.876
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)	14.4 (12.1–15.4)	0.196
Baseline platelet count ($\times 10^3$)†	16.0 (6.6–86.0)	13.5 (10.4–22.5)	0.605
Baseline ALT level (IU/L)†	40(15–300)	37 (23–87)	0.765
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.8 (0.6–0.9)	0.123
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.6 (3.2–7.6)	6.4 (5.7–7.1)	0.465
Initial telaprevir dose (1500/2250 mg)	62/18	7/2	0.675
Initial telaprevir/bodyweight (mg/kg)	33.7 (20–71.4)	30.0 (23.6–44.1)	0.563
Initial PEG IFN dose (1.5/<1.5 μg/kg)	66/14	9/0	0.198
Initial RBV dose (mg/kg)†	9.7 (2.2–15.5)	10.7 (7.7–12.9)	0.161
IL28B gene (rs8099917) (TT/non-TT/ND)	47/19/14	4/3/2	0.353
Core 70 a.a. mutation (wild/mutant/ND)	38/22/20	5/2/2	0.511
Previous treatment (naïve/relapse/NVR)	35/36/9	5/2/2	0.972
Onset of dermatological AE (days)	5 (1–75)	22 (1–60)	0.352

†Data are shown as median (range) values.
a.a., amino acid; AE, adverse event; ALT, alanine transaminase; Cr, creatinine; HCV, hepatitis C virus; IL28B, interleukin 28B; NVR, non-virological response; PEG IFN, pegylated interferon; RBV, ribavirin.

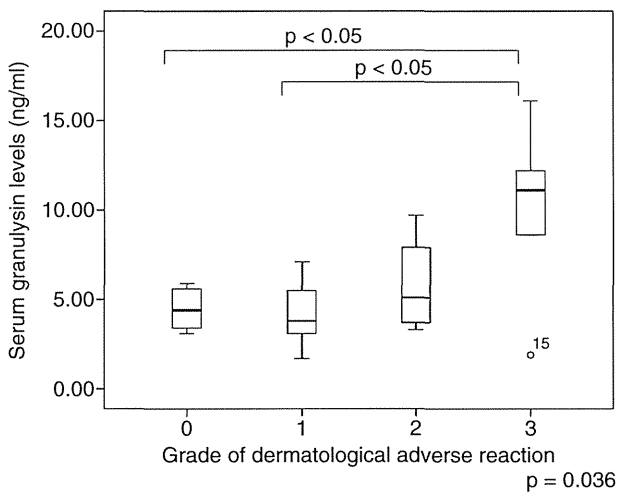

Figure 2 Association between dermatological adverse reaction severity and serum granulysin level. Serum granulysin levels were measured at the onset of dermatological reactions (i.e. within 3 days of onset); if the symptoms worsened, the time of worsening was adopted. In patients with no dermatological events, the highest serum granulysin level during treatment was adopted. $P < 0.05$, one-way ANOVA.

Recent genome-wide association studies have identified that genetic polymorphisms around the IL28B gene locus significantly associated with the outcome of PEG IFN and RBV combination therapy in HCV patients. Thus, PEG IFN and RBV combination therapy is ineffective in a subset of HCV-infected patients who have IL28B TG or GG genotypes, limiting the use of this therapy.[16] Therefore, novel drugs with different antiviral mechanisms were required. Accordingly, DAA were developed; they are mainly classified as NS3/4A protease inhibitors, or NS5B or NS5A inhibitors.[17] The NS3/4A serine protease inhibitor telaprevir, in combination with PEG IFN and RBV, has demonstrated the most promising results.[6–8] However, adverse events, especially severe dermatological reactions, develop more frequently in patients treated with telaprevir than those treated with only PEG IFN and RBV.

Little is known about the mechanisms of telaprevir-induced dermatological reactions. Reactions develop in patients treated with PEG IFN and RBV combination therapy[18,19] as well as telaprevir monotherapy.[20,21] It should be noted that the dermatological reactions in telaprevir monotherapy or PEG IFN and RBV therapy alone are generally mild.[7,8,20] However, dermatological

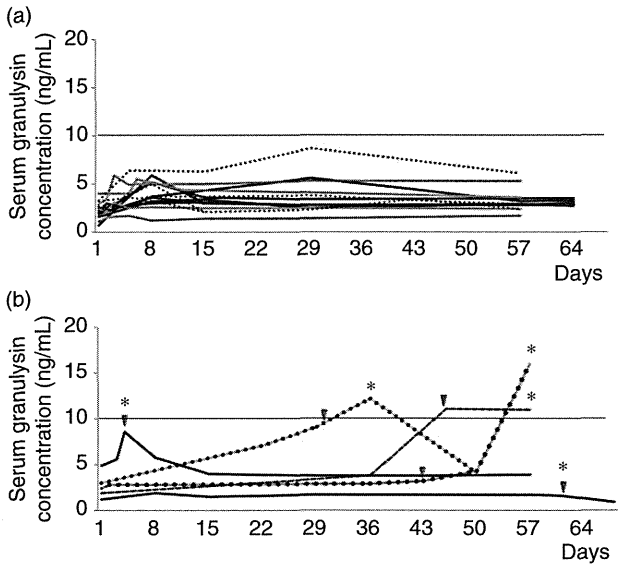

Figure 3 Association between time-dependent changes in serum granulysin levels and severe telaprevir-induced dermatological adverse reactions. (a) Time-dependent changes in serum granulysin levels patients with non-grade 3 dermatological reactions (three, five and six with grade 2, grade 1 and no reactions, respectively). The dashed line, gray line and black line indicate grade 2, grade 1 and no reaction, respectively. (b) Time-dependent changes in serum granulysin levels of five patients with grade 3 dermatological events. The dashed line indicates patients with severe systemic manifestations. Arrowheads indicate the onset of dermatological events and asterisks indicate the onset of grade 3 dermatological events.

reactions in telaprevir and PEG IFN/RBV combination therapy may be severe, indicating a synergistic effect. Severe dermatological events including SJS/TEN and DIHS have been reported in telaprevir-based triple therapy; these are life-threatening, and fatal cases have been reported.

The onset of grade 3 dermatological reactions tended to be later than non-grade 3 reactions, the same as in the study of Torii *et al.*[10] Taken together with the finding that male sex is a clinical risk factor, the results indicate that late-onset dermatological reactions in male patients treated with telaprevir-based triple therapy require more attention.

Roujeau *et al.* analyzed the risk factors for telaprevir-induced eczematous dermatitis and report that the incidence of telaprevir-related dermatitis was significantly higher age of more than 45 years, body mass index of less than 30 (kg/m^2), Caucasian ethnicity and treatment-naïve status.[9] While they analyzed the risk factors for telaprevir-induced eczematous dermatitis, the present study focused on the risk factors for severe telaprevir-induced dermatological reactions, because such reactions can affect treatment outcome (Table 2) and can be fatal. As mentioned above, male sex was significantly associated with grade 3 dermatological reactions. Sex is reported to be associated with the prevalence of some kinds of severe drug-induced dermatological events, although the underlying mechanism remains unknown.[22]

Fujita *et al.* report that serum granulysin levels are significantly elevated in SJS/TEN patients and thus may be a good predictive factor.[14] Therefore, we hypothesized that in telaprevir-based triple therapy for chronic hepatitis C patients, serum granulysin levels are associated with the severity of dermatological reactions and may thus be a predictive biomarker. However, Ogawa *et al.* report that serum granulysin levels also increase as a result of primary virus infections such as Epstein–Barr virus or parvovirus B19.[12] Thus, it remains unclear whether and how chronic viral infections, especially HCV, affect serum granulysin levels. In the present study, we compared serum granulysin levels between healthy volunteers and chronic hepatitis C patients; the results show that chronic HCV infection was not associated with serum granulysin levels (Fig. 1).

Chung *et al.* have reported that granulysin is the most highly expressed cytotoxic molecule in blisters of SJS/TEN and that massive keratinocyte death was induced by granulysin.[11] Fujita *et al.* reported that serum granulysin levels increased in the early stage of SJS/TEN caused by drugs including carbamazepine, imatinib and phenytoin.[14] Taken together with our results, we speculate that granulysin may be involved in the pathogenesis of early stage telaprevir-mediated dermatological adverse reactions possibly through induction of keratinocyte death.

Of five patients with grade 3 reactions, two patients without severe systemic manifestations did not have elevated serum granulysin of more than 10 ng/mL or did not have elevated levels before symptoms worsened. On the contrary, three patients with severe systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL, and the symptoms of two patients with serum granulysin levels already exceeding 8 ng/mL at onset and within 6 days worsened. Therefore, serum granulysin tests may predict grade 3 dermatological adverse reaction with systemic manifestations. Furthermore, if serum granulysin levels elevate more than 8 ng/mL, more attention should be paid.

In Western countries, the prevalence of dermatological reactions in patients treated with telaprevir-based and

PEG IFN/RBV therapy are reported to be approximately 55% and 33%, respectively;[9,23] meanwhile, in Japanese patients, the respective rates are 74.9% and 58.7%. Moreover, approximately 4% and 9% of patients in Western and Japanese patients develop grade 3 reactions, respectively;[10] this is almost the same as that in the present study (10%). The difference may be due to genetic or ethnic variation. Therefore, genome-wide association studies may have identified a gene locus associated with telaprevir-induced severe dermatological reactions.

A limitation of this study is that the number of patients with grade 3 dermatological reactions is relatively small. However, the serum granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of other patients. Also, in two of the three patients with severe dermatological reactions, the serum granulysin level elevated before symptoms worsened, which are novel findings. Further study is required.

Triple therapy with the second-generation protease inhibitor simeprevir is reported to result in a similar prevalence of adverse reactions as PEG IFN and RBV combination therapy.[24,25] However, simeprevir is not approved worldwide. Although simeprevir-based triple therapy is effective, only 36–53% of prior non-responders achieve SVR.[24] Shimada *et al.* recently reported that by extending PEG IFN and RBV therapy from 24 to 48 weeks, telaprevir-based triple therapy improves the SVR to up to 68% in prior null responders.[26] Thus, telaprevir is a therapeutic option for prior null responders.

In conclusion, the present study suggests that male sex is a significant risk factor for severe telaprevir-induced dermatological reactions. In addition, serum granulysin levels are significantly associated with the severity of dermatological reactions and thus may be a good predictor of severe dermatological reactions with systemic manifestations in patients treated with telaprevir-based triple therapy.

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Regulatory T Cells in Severe Drug Eruptions

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Abstract: Regulatory T cells (Tregs) are essential for limiting immunopathology and maintaining immune homeostasis. They represent a major barrier to aberrant and excessive immune responses to pathogens and allergens in infectious and allergic diseases, respectively. In this review, we describe our current understanding of the immunopathogenic mechanism behind protection against the development and exacerbation of severe drug eruptions, with special emphasis on regulatory T cells (Tregs). In this regard, our previous study demonstrated that the timing of the dysfunction of Tregs could determine the pathological phenotype and sequelae of severe drug eruptions. We also discuss the factors that abrogate Treg function and demonstrate that *Mycoplasma pneumoniae* is the only pathogen shown to cause a persistent loss of Treg function long after clinical resolution. A loss of Treg function observed at the different stages of severe drug eruptions would be a driving force in the subsequent development of autoimmune disease as long-term sequelae of severe drug eruptions.

Keywords: Drug-induced hypersensitivity syndrome, fixed drug eruption, herpesviruses, *Mycoplasma pneumoniae,* regulatory T cells, Stevens-Johnson syndrome, toxic epidermal necrolysis.

INTRODUCTION

Organs that are exposed to external environment, such as the skin and gastrointestinal tract, possess physical and biochemical barriers to microbial and chemical challenges and mechanical injury [1], and have evolved a variety of strategies to control inflammation and maintain immunological homeostasis. Although the skin is the primary organ targeted by the host immune response to drug, a delicate balance between the ability to react with some drugs and the ability not to react with other drugs is necessary to ensure the integrity of skin tissue. However, factors involved in disrupting this equilibrium existing in the skin are largely unknown, although both genetic and environmental factors have been implicated [2-5]. In this regard, viral and mycoplasmal infections are recognized as risk factors for the development of drug eruptions and as a major inducer of exacerbation of drug eruptions [6-8]. Immune responses in the context of such infections can thus have varying effects on the potential development of allergic inflammation [9], especially drug eruptions. Indeed, available evidence also strongly suggests that viral and mycoplasmal infections create a favorable milieu for the initiation and progression of adverse drug eruptions [10, 11], although the mechanisms whereby preceding viral or mycoplasmal infections induce or contribute to the development of drug eruptions are currently unknown. On the other hand, in order to maintain or restore a homeostatic environment, the anti-viral or anti-mycoplasmal immune responses could paradoxically create an environment that protects the host from excessive immune responses to these pathogens and allergens, which could in itself lead to greater pathological consequences than the invading pathogens and allergens themselves. Thus, generation and the subsequent activation of protective T cell responses that can ameliorate immune response-associated inflammation are a key component of immune reactions to drug.

$Foxp3^+CD4^+$ regulatory T cells (Tregs) represent a developmentally and functionally distinct T cell subpopulation that can suppress such aberrant and excessive immune responses [12, 13]. Evidence is recently accumulating that Tregs, either natural or induced, can inhibit the function of T effector cells (Teffs) at the site of microbial infections and allergic inflammation, thereby inhibiting severe immunopathology [14]. On the other hand, the Treg response is potentially harmful to the host in terms of infection control [15], because their activation may secure survival of invading pathogens for an extended period of time, thereby causing chronic infectious diseases. Numbers and functions of Tregs, therefore, should be controlled depending on the stage of infections and inflammation.

In this review, we describe our current understanding of the immunopathogenic mechanisms behind protection against the development and exacerbation of drug eruptions, with particular reference to severe drug eruptions, Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms (DiHS/DRESS).

Tregs in SJS/TEN

Because the most prevalent severe eruptions are thought to be mediated by drug-specific Teffs, the phenotype and functions of these Teffs are likely to determine the clinical picture of the disease. Alternatively, however, severe drug eruptions could be induced by a disbalance of the immune system caused by excessive activation of Teffs associated with an inadequately low function or number of Tregs that can limit immunopathology. In support of this possibility, Azukizawa *et al.* [16, 17] reported that in an animal model of TEN Tregs can prevent experimentally induced epidermal injury mimicking

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TEN, although the therapeutic effect cannot be observed. Their studies suggested the importance of Tregs in protecting susceptible patients from the development of TEN. Thus, drug-induced immunopathology in SJS/TEN is likely to be subject to control by Tregs. However, the degree to which Tregs truly played a role during the actual disease process in protecting the host's tissues from severe immunopathology in SJS/TEN was largely unknown.

The frequency of Tregs in total circulating $CD4^+$ T cells from patients with SJS/TEN at the acute stage was not significantly different from that in those from healthy controls [18]. Previous studies demonstrated that Tregs in healthy controls preferentially express chemokine receptors and adhesion molecules required for skin homing, such as CLA and CCR4 [19, 20]. This skin-homing phenotype was also found in Tregs in patients with SJS/TEN, although the frequency was slightly lower than healthy controls. In contrast, our immunohistochemical study showed that $Foxp3^+$ Tregs were rarely found in skin lesions, where multiple blisters developed: these results suggested their functional defects in patients with SJS/TEN. To examine their functional activity on a per-cell basis, we performed CD3-driven T-cell proliferation assay by coculturing FACS-sorted $CD4^+CD25^-$ Teffs with FACS-sorted $CD4^+CD25^{++}$ Tregs at different Tregs:Teffs ratios. Tregs obtained from patients with SJS/TEN at the acute stage were found to be profoundly defective in their capacity to suppress T cell proliferation (Fig. 1). The degree of functional defect was directly related to the severity of epidermal damage. Nevertheless, their defective capacity at the acute stage was returned to the presumed baseline of the patient before the onset of SJS/TEN upon clinical resolution [18]. These findings suggest the functionality of Tregs at the acute stage of SJS/TEN was impaired on a per-cell basis in agreement with our observation that severe epidermal damage can be seen in skin lesions of SJS/TEN patients, in whom circulating Tregs were present in normal frequency.

Could a functional defect in Treg recruitment into the inflammatory site accounts for the exacerbated pathology in SJS/TEN? To resolve this issue, we have to ask whether the skin immune system could limit the duration or intensity of the inflammatory response would rely on the capacity to direct Tregs to the site of action. We therefore compared the acute skin lesions of SJS/TEN with the corresponding lesions of a generalized bullous variant of fixed drug eruptions (gbFDE), whose clinical symptoms at the acute stage are indistinguishable from SJS/TEN. Despite such clinical similarities between gbFDE and SJS/TEN, subsequent evolution of the two conditions is quite different: the former resolves spontaneously upon discontinuation of the causative drug whereas the latter often results in full-thickness epidermal detachment, rapidly spreading to the whole body. Because the individual erythematous lesions in SJS/TEN form poorly defined macules rapidly extending to the perilesional uninvolved skin while the FDE lesions usually have well-defined border, the defect in regulatory mechanisms for preventing further disease progression to SJS/TEN could reside either within the cutaneous milieu in the inflammatory site, particularly in the periphery or within migrating Tregs themselves. Circulating Tregs obtained from gbFDE patients preferentially expressed CLA, CCR4 and CCR6 as demonstrated in healthy controls. The percentages of $CCR4^+$ cells and $CCR6^+$ cells in total $Foxp3^+$ Tregs were significantly lower in peripheral blood mononuclear cells (PBMCs) of SJS/TEN patients than those in PBMCs in gbFDE patients and healthy control. In addition, Tregs obtained from gbFDE patients were found to retain the suppressive capacity to inhibit CD3-driven proliferation of Teffs. Importantly, our immunohistochemical study on acute skin lesions of gbFDE and SJS/TEN showed that gbFDE lesions, especially in the periphery, were characterized by increased frequency of Tregs as compared with the corresponding SJS/TEN lesions. Particularly when the ratio

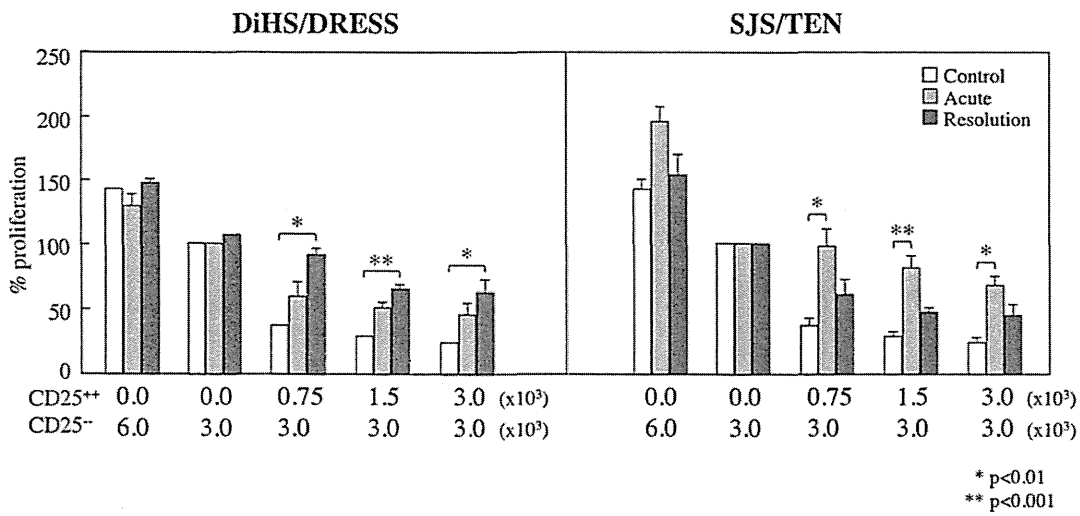

Fig. (1). Functional analysis of Tregs at the different stages of DiHS/DRESS and SJS/TEN. Highly purified $CD4^+CD25^{++}$ Tregs from patients with DiHS/DRESS or SJS/TEN or healthy controls were cocultured at different ratios with highly purified $CD4^+CD25^-$ Teffs from the same individuals in the presence of mitomycin C-treated allogeneic APCs and CD3 and CD28 mAbs. The results are expressed as the percent proliferation of Teffs in the absence of Tregs. (Modified from our previous study (18))[§].

[§]Mizukawa Y. *et al.* Mast cells contribute to preferential migration of regulatory T cells through the release of IL-16 in fixed drug eruption lesion. Manuscript in preparation

of Tregs to $CD8^+$ T cells was determined in individual samples, the ratio was the highest in the epidermis of the periphery of gbFDE lesions with the corresponding area of SJS/TEN lesions being the lowest (Mizukawa Y, *et al.* manuscript in preparation). Of note, $Foxp3^+$ Tregs preferentially accumulated beneath the epidermis and at the mid part of the dermis in the periphery of the gbFDE lesions, while those were sparsely distributed in the upper part of the dermis of the periphery of SJS/TEN lesions. These findings can be interpreted as indicating that timely and selective migration of Tregs into the periphery of gbFDE lesions could be crucial for preventing the excessive activation and recruitment of $CD8^+$ Teffs (Fig. 2). Our ongoing experiments clearly have suggested that mast cells abundantly residing in gbFDE lesions may facilitate such rapid and timely recruitment of Tregs to the inflammatory site although impaired recruitment of Tregs to SJS/TEN lesions could not solely be ascribed to low frequencies of mast cells. Thus, Treg recruitment to the inflammatory site could be controlled by local tissue-dependent factors, such as mast cells resident in the site, rather than an absolute, intrinsic homing ability of Tregs. Based on our data, IL-16 would be the key mast-cell-produced cytokine that can trigger Treg recruitment to the inflammatory site in gbFDE lesions, although IL-16 is not the only cytokine to have such functions [21, 22]. A rapid and proper localization of Tregs into the specific inflammatory site would serve to limit activation of potentially destructive $CD8^+$ Teffs, resulting in spontaneous resolution of gbFDE lesions upon withdrawal of the causative drug.

Treg Function in Infections with *Mycoplasma pneumoniae*

Mycoplasma pneumoniae (MP), a member of the smallest wall-less bacterial class, occurs as commensals or pathogens in animals and humans: *MP* is one of the most common causes of atypical pneumonia in pediatric and adult populations worldwide, while it causes asymptomatic infection in most humans [23, 24]. Although it can infect multiple organ systems, cutaneous symptoms can be seen in 20 to 25% of patients [25], some of which are maculopapular eruption and self-limited; however, more serious complications of *MP* infection, such as SJS/TEN, often occur both during and after active infection, although the mechanisms whereby *MP* infection might induce or contribute to the development of severe life-threatening drug eruptions in susceptible individuals are currently unknown. The main difficulty in assigning a pathogenic role to *MP* in these severe drug eruptions is that the majority of immunocompetent individuals infected with *MP* are asymptomatic and the infection will go unrecognized unless a serologic search is made to identify *MP*. We therefore explored the hypothesis that frequencies and function of Tregs could be specifically altered by *MP* infection depending on the stage of infection.

We evaluated the frequencies of Tregs in total PBMCs of patients with *MP* infection and those with other viral infection, such as varicella zoster virus (VZV) and parvovirus B19 (B19) infections, at their acute and resolution stages, respectively. No significant alterations, in the mean frequencies of Tregs were found in these patiens at

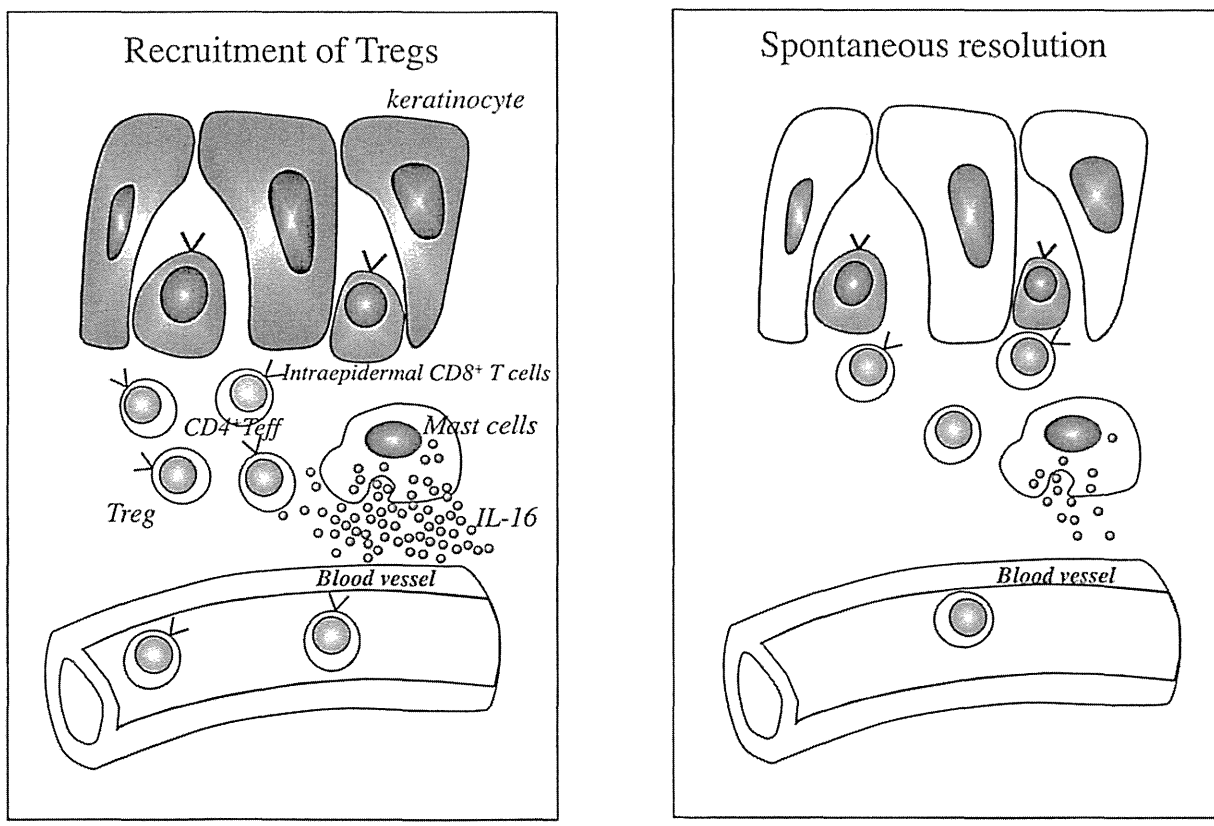

Fig. (2). Spontaneous resolution of FDE lesions induced by Treg recruitment to the inflammatory site, upon withdrawal of the causative drug.

all the time points examined, as compared with those of age-matched healthy controls. Patients with mild respiratory symptoms and slight erythema who were positive for *MP* IgM and showed a significant increase in PA and CF titers were enrolled in our study. Patients with primary VZV or B19 infection were also enrolled as those with viral infections. In humans, CCR6 expression on Tregs has been shown to reflect their functional migratory properties [26]. We therefore asked whether $CCR6^+$ Tregs could be altered during acute infection with *MP* or viruses. Frequencies of $CCR6^+$ Tregs were significantly decreased in patients with *MP* infection, regardless of the stage examined as compared with those in patients with viral infections and healthy controls (unpublished data).

We next examined whether the Tregs obtained from patients with *MP* infection are functionally defective by coculturing FACS-sorted $CD4^+CD25^-$ Teffs with FACS-sorted $CD4^+CD25^{++}$ Tregs obtained from the same patients. Tregs obtained from these patients with acute infection, either *MP* or viruses, exhibited a significantly impaired capacity to suppress CD3-driven, Teff proliferation, as compared with those from healthy controls (Fig. 3). The degree of functional defect in patients with acute viral and mycoplasmal infection was comparable to that in patients with SJS/TEN (Takahashi R, *et al.* Manuscript in preparation). Their impaired capacity at the acute stage of viral infection, however, had returned to a presumed baseline, which was indistinguishable from that of healthy controls, upon resolution. In contrast, functional activity of Tregs obtained from *MP* patients remained defective even after clinical resolution. Surprisingly, the impairment in suppressive function of Tregs remained detected even 1 year after clinical resolution, although the magnitude of the impairment became gradually less apparent (Fig. 3): this result was somewhat different from that observed in SJS/TEN, in which functional activity of Tregs was returned to the presumed baseline of the patient. These contradictory results suggest that there is more to be learned about the functional impairment of Tregs in the setting of SJS/TEN and MP infection not associated with SJS/TEN. We infer that systemic corticosteroids we used for the treatment of SJS/TEN would have served to rapidly restore the impaired function of Tregs because none of patients with MP infection had been treated with systemic corticosteroids. We also found that $CD4^+CD25^-$ Teffs from either the acute or resolution stage of *MP* infection were not resistant to suppression by Tregs from healthy controls, indicating that the defect in *MP* infection resides in the Tregs than in the Teffs (Takahashi R *et al.* Manuscript in preparation).

Thus, our study clearly demonstrates that *MP* infection persistently abrogates Treg function for an extended period of time, a finding never observed in infections with other pathogens including viruses. In view of the actions of Tregs, it makes good biological sense that a temporal limitation in Treg function or number is usually associated with subsequent better control of the acute infection by enhancing immune responses to the pathogens because suppression of the early immune response to infection would be harmful to the host. Nevertheless, restoration of Treg function is likely to occur at later time points in infections. Thus, a time-dependent balanced, rather than biased, Treg responses would be necessary for host protection and the resolution of infection. To date, as far as we tested, *MP* is the only pathogen shown to cause a persistent loss of Treg function even long after clinical resolution, while in other viral infections the defective Tregs regain their functional

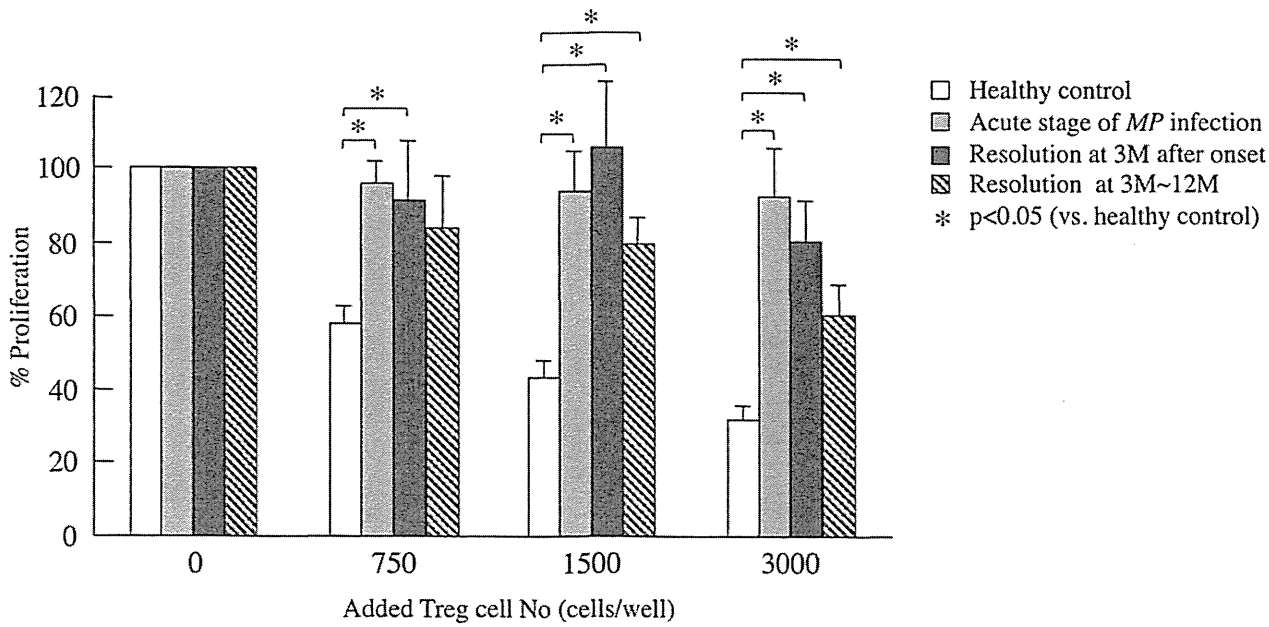

Fig. (3). Functional analysis of Tregs in patients with *MP* infections at the various time points after onset. Experiments are performed as described in the legend of Fig 1: graded numbers of Tregs as shown were added to Teffs°.

°Takahashi R. *et al.* Mycoplasma infection persistently abrogates the function of regulatory T cells. Manuscript in preparation.

competence upon clinical resolution. We can hypothesize that decreasing Treg function in patients with *MP* infection would serve to lower the activation threshold of drug-specific T cells, thus facilitating the development of drug eruptions. Although it remains unknown how *MP* infection persistently abrogates Treg function, ligation of Toll-like receptors (TLRs) by *MP* may be responsible for a persistent loss of Treg function. In this regard, recent studies have shown that ligation of TLR2 on Tregs by synthetic ligands temporarily abrogates their suppressive function [27] and that stimulation of 1α, 25VitD3-induced IL-10-secreting human Tregs with TLR9 ligands results in a loss of Treg function [28]. Given the ability of *MP* to stimulate TLR2 and 4 signaling pathway [29, 30], Tregs are likely to lose their suppressive capacity in response to engagement of TLRs.

Expansions of Tregs in DiHS/DRESS

DiHS/DRESS and SJS/TEN represent the opposite ends of a spectrum of severe drug eruptions. DiHS/DRESS offers a unique opportunity to elucidate the mechanism by which viral infections could affect the development of severe drug eruption, because previous studies established its strong association with human herpesvirus 6 (HHV-6) infection [31, 32]. This syndrome has several unique features that cannot be solely explained by a drug Ag-driven oligoclonal T-cell activation: the delayed onset in relation to the introduction of the causative drug is one of the important features of this syndrome that can be distinguished from other types of drug eruptions, which usually start 1-2 weeks after starting therapy: this syndrome typically occurs with fever and cutaneous lesions 3 weeks to 3 months after starting therapy with a limited number of drugs, mainly anticonvulsants [31-34]. Importantly, more severe reactions often occur 3-4 days after withdrawal of the causative drugs: this paradoxical worsening is also characteristic of DiHS/DERSS and may be erroneously labeled as severe infectious diseases. In addition, variable clinical symptoms, such as renal and liver symptoms, continue to deteriorate one after another even weeks or months after stopping the causative drug. Most erythematous macules do not evolve into blisters and no mucous membrane involvement is usually seen [33-35].

Despite such variable clinical presentations and courses, HHV-6 reactivations can be detected at a particular time point, 2-3 weeks after onset of rash in the vast majority of patients regardless of treatment [31, 33, 35]: a strong association between the magnitude of HHV-6 reactivations and the severity of this syndrome has been supported by a large number of independent groups over years in Japan [34, 36]. Recent studies of real-time measurements for viral loads during the course of DiHS/DRESS have demonstrated that not only HHV-6 but also other herpes viruses, such as Epstein-Barr virus (EBV), HHV-7 and cytomegalovirus (CMV), are reactivated in sequence as demonstrated in graft-versus-host disease (GVHD) [37]: the cascade of reactivation events initiated by HHV-6 or EBV would extend, with some delay, to HHV-7 as well, and eventually to CMV. These findings provide strong evidence to suggest the role of herpes viruses in the etiology of the disease, rather than a mere bystander, although reactivation of these viruses as a result of a transient immune dysfunction cannot be definitely excluded. Thus, immune systems of patients with DiHS/DRESS are characterized by inadequate control of herpes virus replication and highly variable waxing and waning nature of generalized immune activation.

Although it has been suggested that DiHS/DRESS is caused by an exaggerated cellular immune responses to either drugs, reactivated viruses or both [38], a link between sequential occurrence of herpes virus reactivations and Tregs has not been convincingly established. Given their potent suppressive capability, the role of Tregs in the clinical course of DiHS/DRESS could be either harmful or beneficial: they could be postulated to have a negative impact on herpes viruses infection by suppressing efficient anti-herpes virus-specific immune responses on the one hand, while they could be beneficial to the host by dampening excessive self-inflicted immune activation triggered by viruses or drug on the other. Their role would be different depending on the

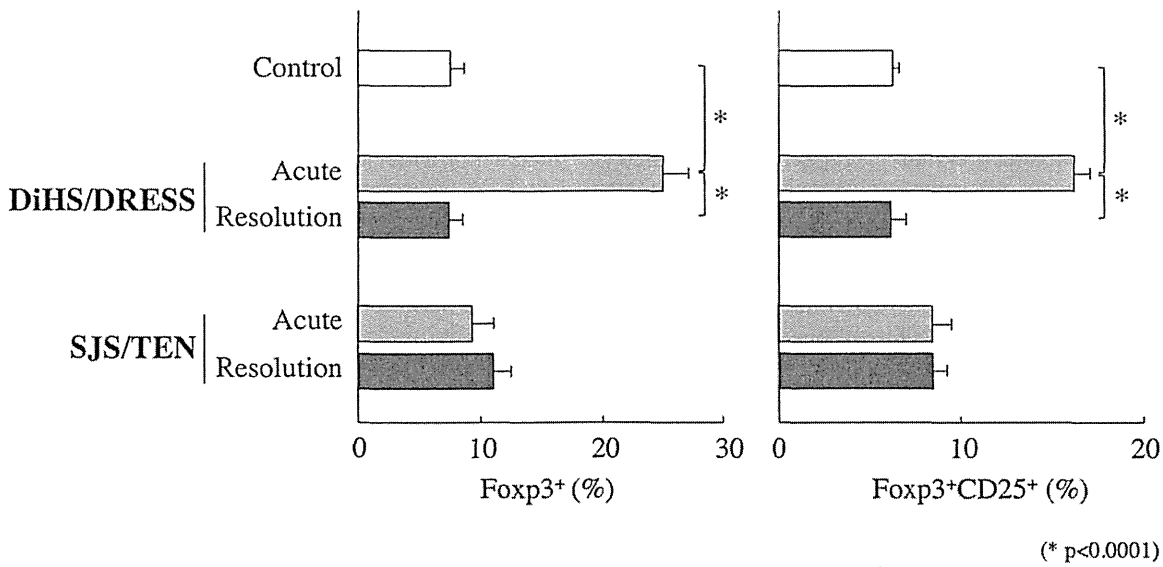

Fig. (4). Expansions of circulating Foxp3$^+$ Tregs at the acute stage of DiHS/DRESS but not of SJS/TEN. (Modified from our previous study [18]).

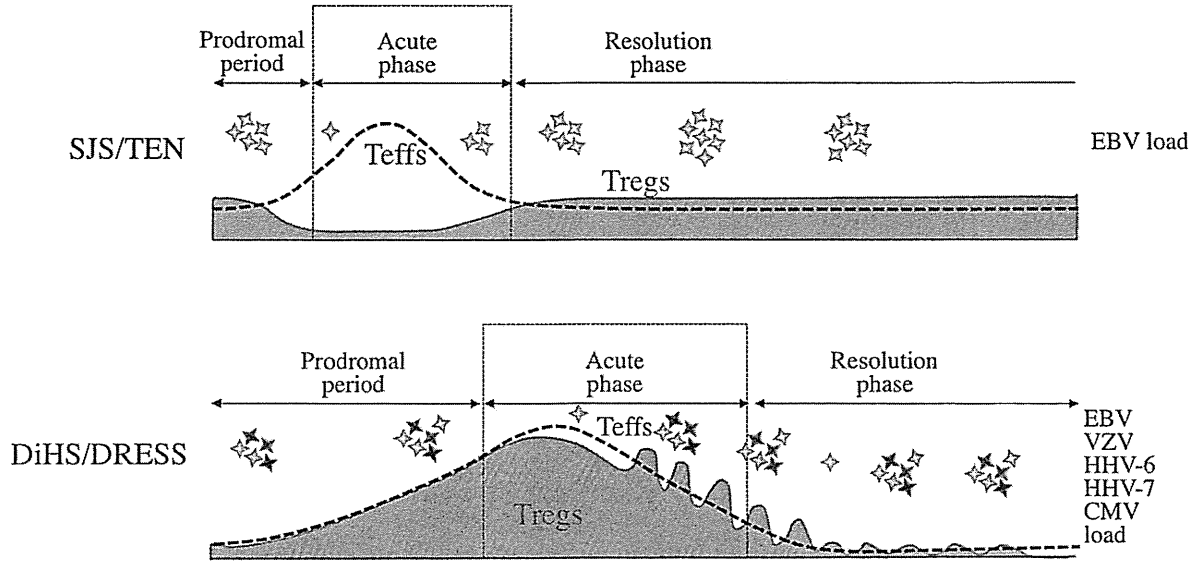

Fig. (5). Hypothetical model for the relationship among Tregs, Teffs and viral reactivation patterns in two types of severe drug eruptions. Systemic corticosteroids used for treatment of these severe drug eruption would serve to restore the functional impairment of Tregs.

stage of disease, the compartments in which Tregs are examined, and viral loads. According to our recent study [18], the acute stage of DiHS/DRESS is characterized by dramatic expansions of fully functional Tregs: the relative percentage of Tregs in circulating $CD4^+$ T cells was dramatically increased during the acute stage of DiHS/DRESS as compared with that in healthy controls (Fig. 4). Although it is difficult to precisely determine when Treg expansions occur during the course of disease, this expansion would occur far before onset of DiHS/DRESS, which would contribute to not only the delayed onset but also viral reactivation [34]. In order to counterbalance activating Teffs, expansions of Tregs are likely to be key for maintaining a healthy balance between protection and immunopathology. However, once the balance has been disturbed toward activation of Teffs, DiHS/DRESS ensues (Fig. 5). Thus, the expanded Tregs would also limit the severity of Teff-mediated immunopathology, which is reflected by the observation that epidermal damage can be rarely detected in the skin lesions of DiHS/DRESS.

A recent study [39] has indicated that Tregs can 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. **6A**). We therefore investigated whether Tregs expanded during the acute stage of DiHS/DRESS could represent the $CD45RA^-Foxp3^{++}$ iTregs phenotype. Although non-Tregs were also increased in the acute stage of DiHS/DRESS, iTregs were dramatically increased in the acute stage as compared with those in healthy controls (Fig. **6B**). This finding indicates that expanded Tregs in the acute stage of DiHS/DRESS are of the iTreg phenotype and that they could be induced in the periphery under specific conditions of cytokines and antigen, because iTregs can be produced from $CD4^+CD25^-$ T cells by culture with antigen and TGF-β or IL-10 and TGF-β, while IL-6 inhibits iTregs induction and promotes Th17 [40, 41]. Consistent with this view, our preliminary study shows that *in vitro* culture of PBMCs from DiHS/DRESS patients after resolution with the causative drug results in expansions of iTregs. In view of our observation that there is an overall increase in the total number of $CD4^+$ T cells in blood, this increase in Tregs during the acute stage of DiHS/DRESS could be actually much more than that in the relative percentage of Tregs. In addition, Tregs expanded in patients with DiHS/DRESS at the acute stage were found to retain the suppressive capacity indistinguishable from those in healthy controls [18]. Importantly, because not only Tregs but also Teffs are expanded during the acute stage of DiHS/DRESS (unpublished observation), it remains to be determined whether expanded Tregs indeed play a critical role for sequential reactivation of herpesviruses by blunting the immune responses to these herpesviruses. Further studies investigating cohorts followed up longitudinally would be required to address the precise role of Tregs in the modulation of herpesvirus-associated immune activation.

Thus, expansions of iTregs with immunosuppressive function provide potential mechanisms by which herpesviruse could be reactivated during the course of DiHS/DRESS. However, no satisfactory explanation for why only herpesviruses can be reactivated in sequence has been available; but some clues may come from studies of $CD16^+$ monocytes, which have been shown to mediate epidermal damage in SJS/TEN [42]. In contrast, they are not involved in the pathogenesis of DiHS/DRESS. Thus, monocyte migration to the inflammatory skin sites is likely the key to progression to SJS/TEN, but not to DiHS/DRESS. Indeed, because blood monocytes are shown to be major effectors involved in the innate responses to viral infections [43], their functional or numerical defects would leave the host vulnerable to severe viral infections. Human blood monocytes are heterogeneous populations and can be separated into three distinct subsets on

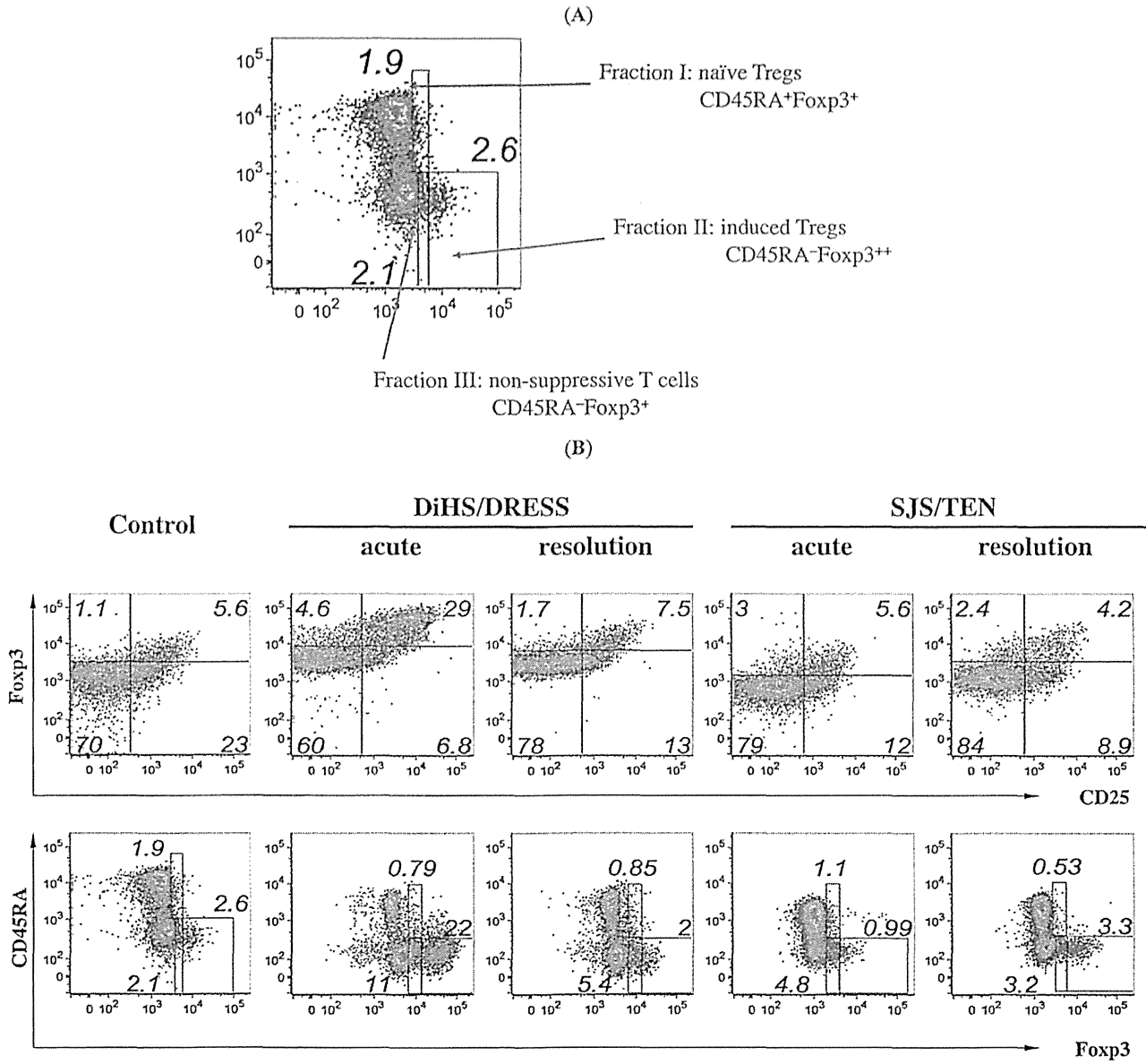

Fig. (6). Three functionally distinct subpopulations of Tregs. **A.** Three fractions in healthy controls. **B.** Expansions of Treg FrII in the acute stage of DiHS/DRESS.

the basis of their phenotypical and functional features: $CD14^{++}CD16^{-}$ classical monocytes (cMOs), $CD14^{+}CD16^{+}$ intermediate monocytes (iMOs), and $CD14^{dim}CD16^{+}$ nonclassical, proinflammatory monocytes (pMOs) [43, 44]. Among them, $CD14^{dim}CD16^{+}$ monocytes have received increasing attention over the last 4 years, because this population has been shown to patrol blood vessels and selectively detect virally infected cells to produce proinflammatory cytokines, TNF-α, IL-1β and CCL3, thus mediating anti-viral roles [43, 44]. These findings suggested that this population, pMOs, would be numerically or functionally impaired in patients with DiHS/DRESS who cannot control viral reactivations. We therefore investigated the dynamics of monocyte subsets in relation to those of Tregs in DiHS/DRESS and SJS/TEN. Surprisingly, pMOs have been depleted from the circulation and skin lesions in the acute stage of DiHS/DRESS, while the corresponding skin lesions of SJS/TEN were characterized by massive infiltrations of pMOs. This preferential depletion of pMOs was associated with expansions of Tregs in DiHS/DRESS: there was an inverse relationship between pMOs and Tregs (Fig. 7). More importantly, paired immunoglobulin-like type 2 receptor a (PILR-α) and herpesvirus entry mediator (HVEM), which can specifically bind to herpes simplex virus (HSV) envelope glycoprotein B (gB) and gD, respectively [45, 46], were preferentially expressed on pMOs. Because expansions of Tregs are only observed in the acute stage of DiHS/DRESS but not in SJS/TEN, it is logical to ask whether such alterations in monocyte subsets could be responsible for driving iTreg expansions in DiHS/DRESS. Our ongoing studies clearly show that cMOs have the most efficient capability to expand iTregs while pMOs have much less capability: consistent with the

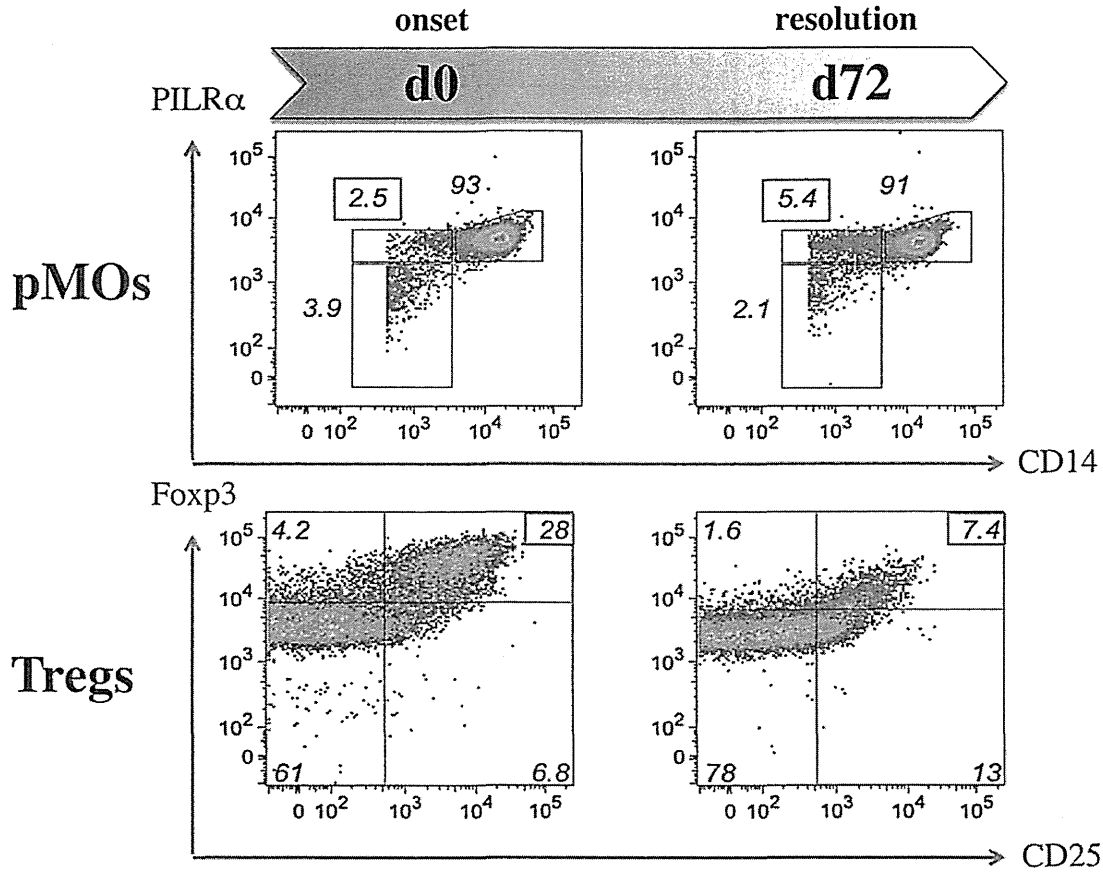

Fig. (7). Expansions of Tregs inversely associated with preferential depletion of pMOs expressing PILR-α during the clinical course of DiHS/DRESS.

result, a recent work has revealed that pMOs control the proliferation of Tregs in immune thrombocytopenia [47]. Our CFSE-based assays demonstrate that pMO-depleted MOs have the most potent capability to induce iTreg expansions and that *in vitro* stimulation of PBMCs from DiHS/DRESS patients with the causative drug induced selective proliferation of iTregs specific for drug (unpublished observation). These results indicate that preferential depletion of pMOs could function as a driving force behind expansions of Tregs and subsequent herpesvirus reactivations, eventually resulting in clinical disease (Fig. 7). This dynamic interaction between Tregs and MOs inferred from our data may be critical to initiation and exacerbation of a pre-existing anti-drug immune responses.

The role of Tregs in Autoimmune Sequelae of DiHS/DRESS

Viruses have been proposed repeatedly as triggering factors for development of autoimmune disease. However, a major difficulty in establishing a correlation between triggering viral infections and the actual autoimmune disease is that during the long prodromal period preceding the clinical onset of disease the virus involved at the early stage would have been eliminated by an anti-viral immune response, thereby making its direct identification at the lesion difficult later on when autoimmune disease has developed. In this regard, DiHS/DRESS is an excellent disease in which to directly observe the course of disease from viral reactivations to development of autoimmunity. Indeed, our observation study in our University hospital over ten years found a prevalence of autoantibodies, such as anti-nuclear antibody (ANA) and thyroglobulin antibody (Tg Ab), or autoimmune diseases in ~10% of patients with DiHS/DRESS [48]. In addition, our recent longitudinal analysis of Treg function during the acute stage and long after clinical resolution has demonstrated that size of Treg population contracts upon resolution of DiHS/DRESS and the remaining cells become functionally impaired over a prolonged period of time [18]: such a gradual loss of Treg function after resolution would be a driving force in the subsequent development of autoimmune disease. In contrast, although onset of SJS/TEN was associated with a functional defect of Tregs, this functional defect was eventually restored after clinical resolution.

We therefore asked whether serum autoAbs against epidermal proteins including periplakin could be detected in samples obtained at various time points including those during the acute stage and long after clinical resolution of SJS/TEN and DiHS/DRESS. Previous studies reported that sera obtained from the acute stage of SJS/TEN and erythema multiforme contain autoAbs against epidermal proteins which could be generated as a consequence of epidermal damage [49]; in the study, it has been suggested that these autoAbs might be involved during the process of epidermal

damage. In this regard, however, our recent studies clearly have shown that the existence of these autoAbs was not restricted to patients with SJS/TEN but was extended to those with DiHS/DRESS characterized by no epidermal damage. More importantly, these autoAbs were present in these patients beyond the time frame of the acute inflammatory response, particularly in patients with DiHS/DRESS. In some patients with DiHS/DRESS these autoAbs levels appeared to gradually increase with time. A likely interpretation of these findings is that the generation of autoAbs is neither a direct consequence of severe epidermal damage nor a primary cause of epidermal damage at least in patients with DiHS/DRESS. Because Treg function is severely impaired in the acute stage of SJS/TEN and after resolution of DiHS/DRESS, respectively, the defective Treg responses observed in the different stage of these drug eruptions provide an explanation for why autoimmune responses can be generated during the course of the disease of SJS/TEN and after clinical resolution of DiHS/DRESS [50], respectively.

CONCLUSION

We propose a model for the pathogenesis of severe drug eruptions that involves Tregs and MOs. With regard to the immune mechanisms, however, that drive the loss of Tregs or pMOs before, during, or after the development of severe drug eruptions, very little is known in patients, because the disease process would begin days or weeks before the development of clinically apparent eruptions. Therapies in severe drug eruptions should try to target the generation and maintenance of Tregs, particularly iTregs, to mediate complete resolution of the disease during very early disease development: however, the mechanisms they use to exert their suppressive function differ depending on the disease phenotype, stage and inflammatory status of the local environment. Further studies are needed to determine whether several therapeutic strategies currently used for the treatment of severe drug eruptions could maintain or enhance Treg function.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

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The dynamics of herpesvirus reactivations during and after severe drug eruptions: their relation to the clinical phenotype and therapeutic outcome

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cytomegalovirus; drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms; Epstein–Barr virus; human herpesvirus 6; Stevens–Johnson syndrome/toxic epidermal necrolysis.

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Abstract

Background: Drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms (DIHS/DRESS) and Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) represent contrasting poles of severe drug eruptions, and sequential reactivations of several herpesviruses have exclusively been demonstrated in the former. No previous studies, however, were extended beyond the acute stage. We sought to investigate whether herpesvirus reactivations could also be observed in SJS/TEN and beyond the acute stage of both diseases.

Methods: Patients with SJS (n = 16), SJS/TEN overlap (n = 2), TEN (n = 10), and DIHS/DRESS (n = 34) were enrolled. We performed a retrospective analysis of Epstein–Barr virus (EBV), human herpesvirus 6 (HHV-6), and cytomegalovirus (CMV) DNA loads sequentially determined by real-time polymerase chain reaction during a 2-year period after onset.

Results: Persistently increased EBV loads were detected in SJS during the acute stage and long after resolution, but not in others. In contrast, high HHV-6 loads were exclusively detected in DIHS/DRESS during the acute stage. The dynamics of herpesvirus reactivation varied in DIHS/DRESS according to the use of systemic corticosteroids: While EBV loads were higher in patients not receiving systemic corticosteroids, CMV and HHV-6 loads were higher in those receiving them.

Conclusions: Distinct patterns of herpesvirus reactivation according to the pathological phenotype and to the use of systemic corticosteroids were observed during the acute stage and follow-up period, which may contribute, at least in part, to the difference in the clinical manifestations and long-term outcomes. Systemic corticosteroids during the acute stage may improve the outcomes in DIHS/DRESS.

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are generally believed to be most severe adverse reactions to drug, characterized by the widespread destruction of the epithelium of the skin and mucous membranes (1, 2). Although the view that infectious agents caused SJS/TEN had seemed heretical 20 years ago, this view began to change 10 years ago, when some patients with SJS were found to be closely associated with *Mycoplasma pneumoniae* infection (3–5). Although a variety of infectious agents other than this organism, such as herpes simplex virus, have also been suggested to be linked to the development of SJS (6), it remains largely unknown whether these organisms could be the primary driving force in the pathogenesis or play a secondary part, fostering disease only in hosts with pre-existing abnormalities of the immune response to drug. Given their common properties to induce massive expansions of cross-reactive memory T-cell populations (7, 8) and their persistence in the host (9), herpesviruses are the most likely additional factors involved in the pathogenesis of severe drug eruptions. Indeed, we for the first time reported the sequential occurrence of herpesvirus reactivations during the course of drug-induced hypersensitivity syndrome (DIHS)/drug rash with eosinophilia and systemic symptoms (DRESS), representing contrasting poles of

severe drug eruptions (10, 11). This event stimulated many investigators to systematically search for herpesvirus reactivation during the course of other severe drug eruptions, SJS/TEN. Although many previous studies performed real-time quantitative polymerase chain reaction (PCR) to detect and quantify viral DNA using blood sample sequentially obtained from patients after onset of rashes, most of these studies were performed with small samples and not extended beyond the acute stage of the disease. Thus, these previous studies are not sufficient to explain the difference in clinical manifestations and long-term outcomes associated with these severe drug eruptions, some of which occurred after a disease-free interval of several months to years (12–17). In this regard, more detailed longitudinal studies of patients may provide additional insights into the role of virus reactivations in the pathogenesis of severe drug eruptions and their long-term sequelae.

In this report, we describe detailed longitudinal studies of patients with severe drug eruptions over a follow-up period of 2 years. Our results suggest that the viral reactivation events associated with severe drug eruptions extend both beyond a simple ability to handle specific herpesvirus and beyond the time frame of the acute stage and that distinct patterns of herpesvirus reactivations observed in these patients may contribute, at least in part, to the marked difference in clinical manifestations and long-term outcome.

Materials and methods

Patients and real-time polymerase chain reaction

Patients with severe drug eruptions who visited our hospital between 1999 and 2012 were enrolled. This study has been approved by the Institutional Review Board at Kyorin University School of Medicine.

The severe adverse drug eruptions were divided into four groups according to the clinical presentation, SJS (n = 16), SJS/TEN overlap (n = 2), TEN (n = 10), and DIHS/DRESS (n = 34). Patients with eczema/dermatitis (n = 17) and anaphylactoid purpura (n = 6) were enrolled as control groups.

Diagnosis of SJS, TEN, and DIHS/DRESS was made based on their criteria (18, 19; Table 1). According to the criteria for differentiating erythema multiforme major (EMM) from SJS (20), we excluded cases with the suspicion of EMM. The causative drugs were withdrawn when the diagnosis of drug reactions was made.

All patients with SJS/TEN were treated with systemic corticosteroids 0.8–1 mg/kg daily. In contrast, 15 of 34 patients with DIHS/DRESS were treated with systemic corticosteroids 0.8–1 mg/kg daily after admission, while two of 34 patients were treated with systemic corticosteroids 0.2–0.3 mg/kg daily before the first presentation to our hospital, without dosage increments after administration. The others were treated with supportive therapy alone.

Table 1 Characteristics of patients

	SJS	SJS/TEN overlap	TEN	DIHS/DRESS
Age, years*	57.3 ± 21.0	36.0 ± 5.6	45.7 ± 19.9	57.9 ± 16.9
Gender (M/F)	11/5	0/2	2/8	20/14
Skin detachment†	4.1 ± 0.9	18.5 ± 6.5	50.0 ± 8.9	
SCORTEN scale‡	1.1 ± 0.2	1.0 ± 0.0	2.1 ± 0.4	
Underlying illness (no. of patients)	Brain tumor (1) Chronic renal failure (1) COPD (1) Epilepsy (1) Hyperuricemia (1) Mycoplasma infection (1) Pneumonia (1) Psychological illness (1) Rectum carcinoma (1) Spondylopathy (1) Upper respiratory inflammation (1)	Psychological illness (1) Upper respiratory inflammation (1)	Arthropathy (1) Basedow's disease (1) Bronchial asthma (1) Cerebrovascular disease (1) Colon carcinoma (1) Diabetes mellitus (1) Hyperuricemia (1) Multiple sclerosis (1) Pneumonia (1) Spondylitis (1) Ulcerative colitis (1)	Brain tumor (1) Cerebrovascular disease (7) Epilepsy (7) Fibromyalgia (1) Hyperuricemia (3) Postherpetic neuralgia (1) Psychological illness (6) Rheumatoid arthritis (1) Spondylopathy (1)

The clinical criteria used for the diagnosis of Stevens–Johnson syndrome (SJS) were widespread erythematous macules or flat atypical targets and detachment below 10% of the body surface area; those for SJS/TEN overlap were widespread erythematous macules or flat atypical targets and detachment between 10% and 30% of the body surface area; those for TEN were widespread erythematous macules or flat atypical targets and detachment above 30% of the body surface area (18); and those for the drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms (DIHS/DRESS) were high fever, a widespread maculopapular and/or diffuse erythematous eruption, lymphadenopathy, leukocytosis with atypical lymphocytosis and/or eosinophilia, liver dysfunction, and human herpesvirus 6 (HHV-6) reactivation (19).

*Mean age ± SD.

†Mean percentage ± SEM.

‡Number of risk factors ± SEM.

Blood samples were obtained at or near the time of the initial presentation before starting therapy and thereafter on a biweekly basis during the course of the disease until resolution. Additional samples were also sequentially obtained on a several monthly basis for 2 years after onset. Real-time Epstein–Barr virus (EBV), HHV-6, and cytomegalovirus (CMV) DNA PCR was performed, as previously described (15). Blood samples obtained at the various time points were classified into five stages depending on the timing of sampling: days 1–10, days 11–30, days 31–100, days 101–300, and day 301 onward after the onset.

Statistical analysis

Data were analyzed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Differences in age and number of virus DNA genome copies in 10^6 peripheral leukocytes between the groups were analyzed by Welch's *t*-tests, while differences in gender and rate of patients with increased virus DNA loads were analyzed by Fisher's exact tests. Significance was defined as *P* value of 0.05 or less for all tests.

Results

Detection of EBV DNA at onset in patients with SJS

We initially determined EBV, HHV-6, and CMV DNA loads at or near the time of the initial presentation. Increased EBV DNA loads defined as >200 genome copies/10^6 leukocytes were detected within 10 days after the onset of rash in half of patients with SJS examined (40% in cases before systemic corticosteroid therapy), but in <20% of patients with TEN. In contrast, <10% of patients with DIHS/DRESS had increased EBV DNA levels in blood samples, while only a few control patients had low levels of EBV DNA in their blood (SJS *vs* DIHS/DRESS, $P < 0.05$ Fisher's exact tests; Fig. 1). As shown in Fig. 2, the median concentration of EBV DNA in the blood from patients with SJS at the acute stage was much higher than that in those with DIHS/DRESS. Importantly, EBV DNA in two patients with SJS was detected as early as day 4 of skin rashes, much earlier than in those with DIHS/DRESS. The increase in EBV DNA loads in patients with SJS was not correlated with symptom severity, white blood cell count, or other immunological parameters at the acute stage.

Distinct patterns of herpesvirus reactivation according to the pathological phenotype during the acute stage and follow-up period

As shown in Fig. 3A, the mean EBV DNA loads were approximately one log higher in patients with SJS than in those with DIHS/DRESS during the acute stage and remained increased for a prolonged period after clinical resolution. In contrast, small numbers of patients with DIHS/DRESS and TEN had EBV load levels comparable to those with SJS only late during the course of the disease, usually on days 31–100 (Fig. 3A).

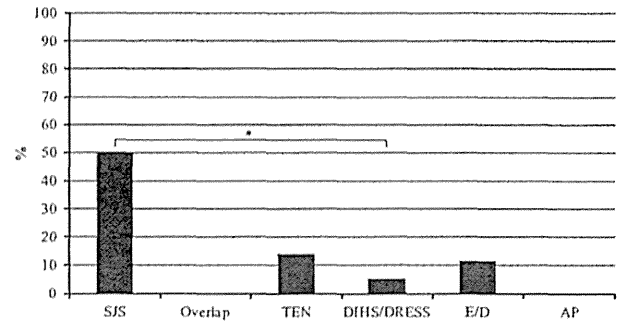

Figure 1 Frequencies of patients and controls with increased Epstein–Barr virus DNA loads, defined as more than 200 genome copies/10^6 leukocytes in their blood samples obtained within 10 days after the onset of rash. Abbreviations: AP, anaphylactoid purpura; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; E/D, eczema/dermatitis; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis. *$P < 0.05$ Fisher's exact tests.

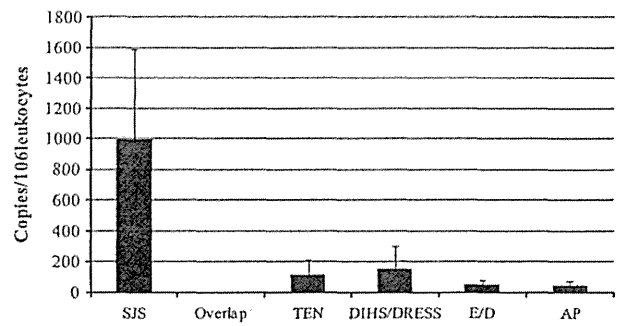

Figure 2 The mean values of Epstein–Barr virus DNA loads (genome copies/10^6 leukocytes) ± SEM in patients with severe drug eruptions and controls, whose blood samples were obtained within 10 days after the onset of rash. Abbreviations: AP, anaphylactoid purpura; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; E/D, eczema/dermatitis; SEM, standard error of the mean; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis. *P* = N.S. for all comparisons.

Cytomegalovirus reactivations occurred in 17.6% of patients with DIHS/DRESS and in 22.2% of those with SJS/TEN (Fig. 3B). During CMV reactivations, the patients showed a variety of clinical symptoms, including low-grade fever, rash, liver dysfunction, enterocolitis, hemorrhagic diarrhea, and pneumonia. In these patients with CMV reactivations, high levels of HHV-6 DNA were also detected prior to the detection of CMV DNA in the blood of patients with DIHS/DRESS. Patients who showed hemorrhagic diarrhea and enterocolitis were treated with ganciclovir.

Human herpesvirus 6 reactivations occurred in all patients with DIHS/DRESS at 2–4 weeks after onset, while no patients with SJS/TEN showed HHV-6 reactivations at any time point (Fig. 3C). During HHV-6 reactivations, the

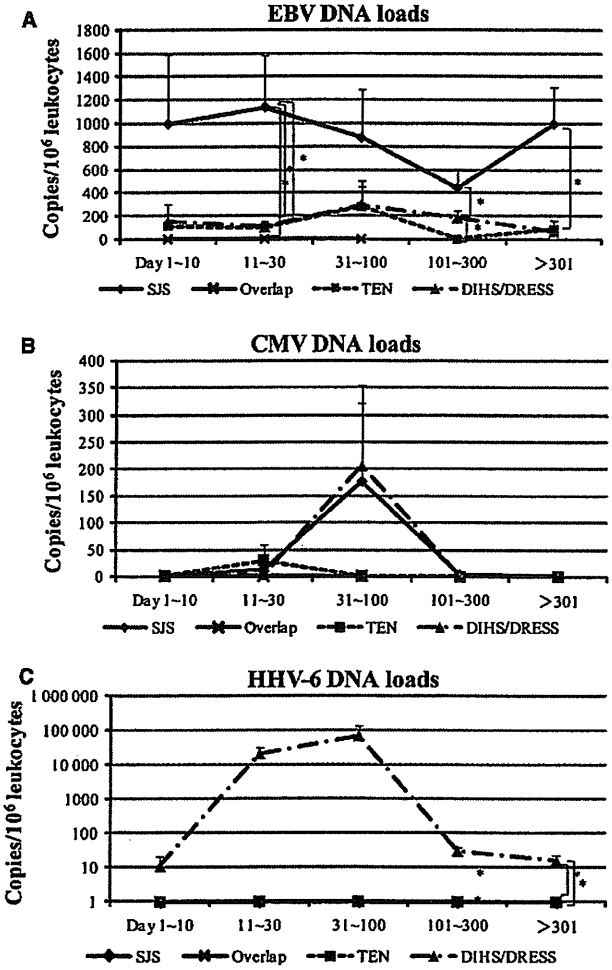

Figure 3 The mean values of Epstein–Barr virus (EBV; A), cytomegalovirus (CMV; B), and human herpesvirus 6 (HHV-6; C) DNA loads (genome copies/10^6 leukocytes) ± standard error of the mean (SEM) in patients with Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS)/drug rash with eosinophilia and systemic symptoms (DRESS) in the blood obtained at the various time points after onset are determined according to the timing of sampling as follows: days 1–10, days 11–30, days 31–100, days 101–300, and day > 301 after the onset. *$P < 0.05$ Welch's *t*-tests. Detailed data of this figure are shown in Table 2.

patients showed a variety of clinical symptoms, including low-grade fever, rash, and liver dysfunction; one patient developed limbic encephalitis.

The detailed data of Fig. 3 are shown in Table 2.

Effect of immunosuppressive drugs on virus reactivations

Because half of patients with DIHS/DRESS were treated with systemic corticosteroids and other half were not given any immunosuppressive agents, these patients were divided into two groups: steroid-treated and nontreated groups. These two groups were matched in age, and no statistical difference was seen in the severity at their initial presentation to our hospital: They included fever, body surface area, leukocyte and eosinophil count, levels of serum alanine aminotransferase, IgG, and C-reactive protein. The changes in EBV, CMV, and HHV-6 viral loads before, during, and following therapy were monitored for 2 years after onset, to investigate the effect of corticosteroids on viral reactivations. As shown in Fig. 4, the difference in viral loads and the duration of viral reactivations between patients treated with and without corticosteroids was clear. The EBV DNA loads were apparently lower in the steroid-treated group than in the nontreated group. In contrast, the blood of the steroid-treated group had more CMV and HHV-6 DNA than did the blood from the nontreated group. The mean duration of CMV and HHV-6 reactivations was also longer in the steroid-treated group than in the nontreated group. Systemic corticosteroids appeared to exert the opposite effects on EBV and HHV-6/CMV DNA loads, although the limitations of this study are mostly a result of its retrospective aspect.

The outcomes of these patients were as follows. One patient with DIHS/DRESS died of CMV enterocolitis 3 months after onset of rashes (15): His blood sample had shown the highest CMV DNA load detected in this study. In this patient, fatal CMV enterocolitis developed when he was placed on a gradual reducing dose of prednisone, from 40 to 30 mg. Three patients with DIHS/DRESS died several months after onset due to other infections or other complications. One patient with SJS died of complications subsequent to the development of diffuse large B-cell lymphoma (DLBCL) 2 years after onset, in whom EBV DNA loads persistently increased after resolution (21).

Discussion

No longitudinal studies of patients with severe drug eruptions have been performed despite sporadic case reports describing severe long-term sequelae, which developed after a disease-free interval of several months to years; several studies reported that autoimmune disorders could occur as a sequela of DIHS/DRESS (13, 14, 16), while short- or long-term complications of SJS/TEN are persistent ocular changes, such as severe dry eyes, vision loss, and bronchiolitis obliterans (22–25). Thus, different complications could develop at various times after clinical resolutions of DIHS/DRESS and SJS/TEN, respectively. Longitudinal studies could help determine whether an increase in viral loads occurs in association with the development of severe drug eruptions or whether individuals with increased viral loads are at greater risk of developing severe drug eruptions. Our real-time PCR analysis revealed a significantly higher level of EBV DNA in the blood from patients with SJS at onset than that from patients with other severe drug eruptions and other skin diseases: In contrast, only a fraction of patients with DIHS/DRESS had EBV DNA identified at onset. In addition, the EBV viral load observed during remission, long after clinical resolution, in many patients with SJS was in the same range as what was observed in these patients during the acute stage of SJS. In many patients with SJS, EBV DNA, although even

Table 2 Detailed data of figure 3

	Days				
	1–10	11–30	31–100	101–300	301–
SJS					
EBV DNA					
Mean*	994.8	1137.4	874.0	444.1	993.8
SE†	591.7	441.2	416.4	158.8	324.7
Max‡	3500.0	4100.0	3300.0	1000.0	1500.0
Freq (over 20)§	66.7	90.0	70.0	85.7	100.0
Freq (over 200)¶	50.0	60.0	50.0	57.1	75.0
*n***	6	10	10	7	4
Samples††	8	14	19	11	15
CMV DNA					
Mean*	0.0	13.8	176.8	4.0	0.0
SE†	0.0	9.5	145.3	4.0	0.0
Max‡	0.0	48.0	890.0	24.0	0.0
Freq (over 20)§	0.0	40.0	33.3	16.6	0.0
Freq (over 200)¶	0.0	0.0	16.7	0.0	0.0
n	3	5	6	6	1
Samples††	4	7	14	8	3
HHV-6 DNA					
Mean*	0.0	0.0	0.0	0.0	0.0
SE†	0.0	0.0	0.0	0.0	0.0
Max‡	0.0	0.0	0.0	0.0	0.0
Freq (over 20)§	0.0	0.0	0.0	0.0	0.0
Freq (over 200)¶	0.0	0.0	0.0	0.0	0.0
*n***	6	5	5	1	3
Samples††	7	6	7	1	4
Overlap					
EBV DNA					
Mean*	0.0	0.0	0.0		
SE†	0.0	0.0	0.0		
Max‡	0.0	0.0	0.0		
Freq (over 20)§	0.0	0.0	0.0		
Freq (over 200)¶	0.0	0.0	0.0		
*n***	2	2	2	0	0
Samples††	2	2	2	0	0
CMV DNA					
Mean*	0.0	0.0	0.0		
SE†	0.0	0.0	0.0		
Max‡	0.0	0.0	0.0		
Freq (over 20)§	0.0	0.0	0.0		
Freq (over 200)¶	0.0	0.0	0.0		
*n***	2	2	2	0	0
Samples††	2	2	3	0	0
HHV-6 DNA					
Mean*	0.0	0.0	0.0		
SE†	0.0	0.0	0.0		
Max‡	0.0	0.0	0.0		
Freq (over 20)§	0.0	0.0	0.0		
Freq (over 200)¶	0.0	0.0	0.0		
*n***	2	2	2	0	0
Samples††	2	2	2	0	0
TEN					
EBV DNA					
Mean*	114.3	98.5	276.6	0.0	85.6
SE†	98.6	54.5	223.4	0.0	74.2
Max‡	700.0	340.0	1800.0	0.0	380.0
Freq (over 20)§	28.5	50.0	25.0	0.0	40.0
Freq (over 200)¶	14.2	16.7	25.0	0.0	20.0
*n***	7	6	8	3	5
Samples††	9	6	14	3	5
CMV DNA					
Mean*	0.0	30.3	0.0	0.0	0.0
SE†	0.0	29.9	0.0	0.0	0.0
Max‡	0.0	120.0	0.0	0.0	0.0
Freq (over 20)§	0.0	25.0	0.0	0.0	0.0
Freq (over 200)¶	0.0	0.0	0.0	0.0	0.0
*n***	2	4	4	2	4
Samples††	3	5	12	2	4
HHV-6 DNA					
Mean*	0.0	0.0	0.0	0.0	0.0
SE†	0.0	0.0	0.0	0.0	0.0
Max‡	0.0	0.0	0.0	0.0	0.0
Freq (over 20)§	0.0	0.0	0.0	0.0	0.0
Freq (over 200)¶	0.0	0.0	0.0	0.0	0.0
*n***	2	3	3	1	5
Samples††	3	4	6	1	5
DIHS/DRESS					
EBV DNA					
Mean*	154.5	106.0	296.0	186.2	66.7
SE†	144.0	43.8	152.9	67.4	35.9
Max‡	2600.0	920.0	3300.0	1200.0	330.0
Freq (over 20)§	21.4	38.0	50.0	57.9	33.3
Freq (over 200)¶	5.6	23.8	36.3	31.6	16.7
*n***	18	26	22	19	12
Samples††	19	41	64	46	23
CMV DNA					
Mean*	0.0	4.6	205.7	0.0	0.0
SE†	0.0	2.5	147.2	0.0	0.0
Max‡	0.0	45.0	3400.0	0.0	0.0
Freq (over 20)§	0.0	12.0	26.1	0.0	0.0
Freq (over 200)¶	0.0	0.0	26.1	0.0	0.0
*n***	10	25	23	15	7
Samples††	14	40	66	34	14
HHV-6 DNA					
Mean*	10.7	20897.8	69707.1	30.0	15.9
SE†	10.7	12418.9	69558.8	9.8	8.2
Max‡	160.0	300000.0	1600000.0	110.0	96.0
Freq (over 20)§	5.3	56.7	47.8	46.7	30.7
Freq (over 200)¶	0.0	26.7	21.7	0.0	0.0
*n***	15	30	23	15	13
Samples††	21	55	67	38	24

CMV, cytomegalovirus; DIHS/DRESS, drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms; EBV, Epstein–Barr virus; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; HHV-6, human herpesvirus 6.

*Mean DNA load (copies/10^6 leukocytes).

†Standard error.

‡Maximum DNA load (copies/10^6 leukocytes).

§Rate of patients with positive DNA load determined over 20 copies/10^6 leukocytes.

¶Rate of patients with high DNA load determined over 200 copies/10^6 leukocytes.

**Number of studied patients.

††Number of studied samples.

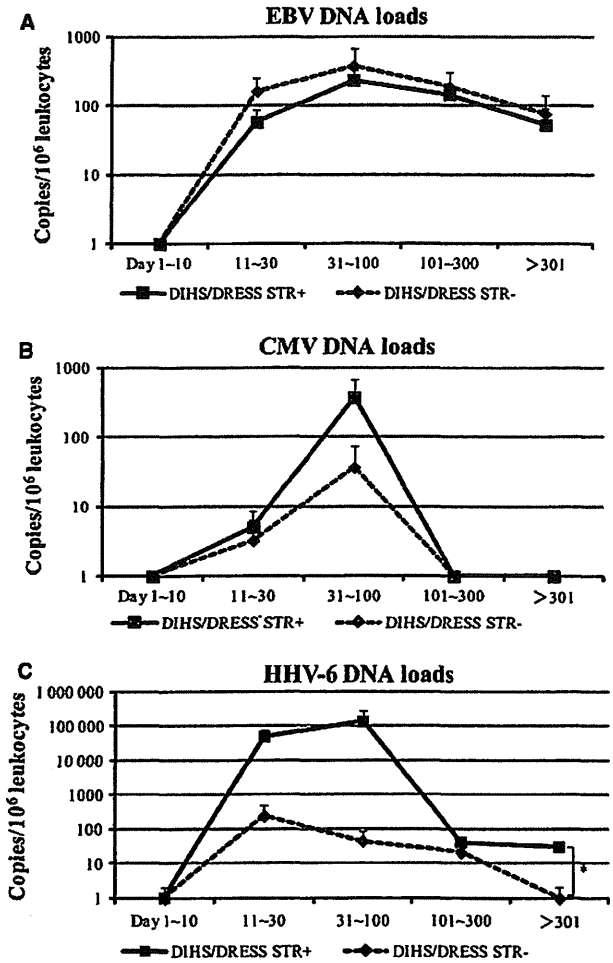

Figure 4 The effect of systemic corticosteroids on Epstein–Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 6 (HHV-6) viral loads in patients with drug-induced hypersensitivity syndrome (DIHS)/drug rash with eosinophilia and systemic symptoms (DRESS). Patients with DIHS/DRESS are divided into two groups: patients treated with systemic corticosteroid (STR+; $n = 15$) and supportive therapy alone (STR-; $n = 17$). Only patients without any detectable viral DNA loads at their initial presentation were used for this analysis to avoid the criticism of selection bias that may have been associated with the patients with increased viral loads before treatment. The mean values of EBV (A), CMV (B), and HHV-6 (C) DNA loads (genome copies/10^6 leukocytes) ± standard error of the mean (SEM) of these groups are shown. *$P < 0.05$ Welch's *t*-tests.

in very low titers in some patients, persisted for up to 2 years after successful therapy. These results suggest that patients with high EBV DNA loads are at risk of subsequently developing SJS, although we could not totally exclude the alternative possibility that the aggressive clinical course observed during the acute phase of SJS may be responsible for EBV reactivations: However, this alternative possibility is unlikely because the degree of the EBV viral loads in patients with SJS did not correlate with the severity of clinical symptoms and laboratory abnormalities during the acute stage (e.g., fever, body surface area, the SCORTEN score, and serum transaminase levels). Moreover, given the ability of corticosteroids to rather reduce the EBV viral loads in patients with DIHS/DRESS, EBV reactivation in patients with SJS is unlikely to be a direct consequence of corticosteroid therapy.

The higher EBV load specifically observed in patients with SJS may be caused by different factors. The most obvious explanation is that the observed increase in viral loads could reflect expansions of EBV-infected memory cells such as B cells. However, not consistent with this view, patients with SJS revealed a dramatic decrease in circulating T- and B-cell numbers at the acute stage (26). Alternatively, it is possible that the increase could be the result of destruction of EBV-specific $CD4^+$ and $CD8^+$ T cells during the active stage, thereby facilitating EBV persistence. This partly provides explanation for why some patients revealed higher EBV loads during the active stage. However, in view of persistence of high titers of EBV DNA observed even during remission in many patients with SJS, our finding could be interpreted as suggesting that SJS may develop in patients who are not capable of adequately mounting effective immune responses to the reactivating EBV.

Surprisingly, we noted that the vast majority of patients with TEN, except two, did not demonstrate increased EBV loads. In view of clinical similarities between SJS and TEN, differences in the pattern of the viral loads between them were surprising and could be interpreted as indicating the possibility that these two diseases may be distinct in the pattern of persistent EBV reactivation, although they share important common pathophysiologic processes. If so, we could hypothesize that increased EBV loads at the acute stage of SJS may have served to prevent further progression to TEN. Nevertheless, the predictive value of EBV loads for SJS must be regarded cautiously because it was derived from small numbers of patients in our study. Thus, important limitation of the present study was the relatively small numbers of patients that were available for analysis and is mostly a result of its retrospective aspect. Some patients were also lost to follow up after the treatment.

The persistently increased EBV loads observed in patients with SJS may be one factor that predisposes to the subsequent development of EBV-associated lymphoproliferative disease (27, 28). Indeed, we have recently seen a patient with SJS who subsequently developed DLBCL 2 years after complete resolution of SJS (21): This patient revealed persistently increased EBV loads during which the patient had remained symptom free after the resolution of SJS, suggesting that this patient may have had defects in long-term anti-EBV immunity.

Because HHV-6 and CMV viral loads were higher in patients with DIHS/DRESS receiving corticosteroids compared with those without corticosteroid therapy, the degree and duration of HHV-6 and CMV reactivation would be greatly influenced by the use of immunosuppressive drugs. Systemic corticosteroids, however, did not enhance EBV reactivation, contrary to our initial prediction. These findings indicate that the pattern of viral reactivations enhanced by immunosuppressive agents or regimens would be different