EL27—2　重症薬疹のトピックス

ウイルス感染と重症薬疹

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はじめに

ウイルス感染がアレルギーの発症や経過に影響をおよぼすことは以前から知られている．たとえば，EBウイルスによる伝染性単核症にアンピシリンを投与すると，しばしば薬疹が出現するが，この現象はアンピシリン疹として有名である．近年，多臓器障害を伴う重症型薬疹のひとつであるdrug-induced hypersensitivity syndrome（DIHS）において，HHV-6などのヒトヘルペスウイルスの再活性化がみられることが明らかとなってきた[1,2]．即ち，薬疹は投与された薬剤によるアレルギー反応と考えられてきたが，その発症にウイルス感染が広く影響をおよぼしている可能性を示すデータが集積しつつある．本講演では，ウイルス感染症との関係が強く疑われている薬疹について代表的なものを取り上げて，ウイルス感染がその発症に果たす役割について解説する．さらに，造血幹細胞移植後のgraft versus host disease（GVHD）におけるHHV-6の再活性化についてわれわれの知見を紹介し，DIHSとの類似性についても言及する．

ウイルス感染症が関与する薬疹

ウイルス感染症と薬疹との関わりは大きく2つのグループに大別される．一つはウイルス感染が先行し引き続き薬疹を生じるタイプで，このグループに属するものとしては，伝染性単核症に併発するアンピシリン疹が有名であるが，そのほかヒト免疫不全ウイルス（HIV）感染症患者にみられる薬疹などもあげられる．もう一つは，薬剤によりウイルスの再活性化が引き起こされるタイプで，このグループの代表がDIHSである．

1. ウイルス感染が薬疹に先行するタイプ

●HIV感染症と薬疹

HIV感染症の患者では，CD4＋T細胞数が減少し，免疫不全症状がみられる．その一方で，薬疹の多発や虫刺症の著明な増悪などのアレルギー症状が目立つようになる．アモキシシリンやサルファ剤に対する薬疹は，非感染者より約10倍多いとされる[3]．カリニ肺炎の予防・治療に用いられるST合剤に対しては，約50％の感染者が薬疹を生ずる．薬疹のタイプは紅斑丘疹型が多いが，toxic epidermal necrolysis（TEN）型の頻度も健常群と比べて高率にみられる（図1）[4]．興味深いことに，病期が進みCD4＋T細胞が減少する程，薬疹の頻度や重症度が増すことが知られている[5]．その理由として，HIVにより免疫を制御しているCD4＋T細胞（Regulatory T cell：Treg）の減少や機能低下が著しく起こり，その結果，アレルギー反応がむしろ増強するとの報告がみられる[6]．また，HIV感染患者では，EBV，サイトメガロウイルス，HHV-6，HHV-7などの様々なウイルスの再活性化がみられ，それらのウイルスの薬疹発症への関与も疑われている．その他，HIV感染患者では，薬物代謝に関わるグルタチオンの欠乏や薬物のアセチル化の遅延[7]が高頻度にみられるため，薬物の中間代謝産物が毒性を発揮したり，アレルギー反応を引き起こすことも一因ではないかと考えられている．

2. 薬剤によりウイルスの再活性化が引き起こされるタイプ

●DIHSとHHV-6

DIHSとは，薬剤投与開始から3週～6カ月で遅発性に発症し，多臓器障害を伴う重症型薬疹のひとつである．皮疹は，紅斑丘疹型（時に多形紅斑型）に始まって紅皮症となることが多い．皮疹だけでなく，リンパ節腫脹，発熱，異型リンパ球の出現や好酸球の増多，肝障害などの症状を認め，しばしば原因薬剤の中止後も，皮疹や臓器障害が遷延する．近年，発症後2～4週後にHHV-6の再活性化を伴うことが判明し，薬剤アレルギーとウイルス感染症の複合した新たな病態として認識されるようになった．

われわれは最近，DIHS急性期に血清TARC値が著しく高値（平均20,000 pg/ml以上）を示すことを見出した（図2）[8]．TARCはTh2細胞を誘導するケモカイ

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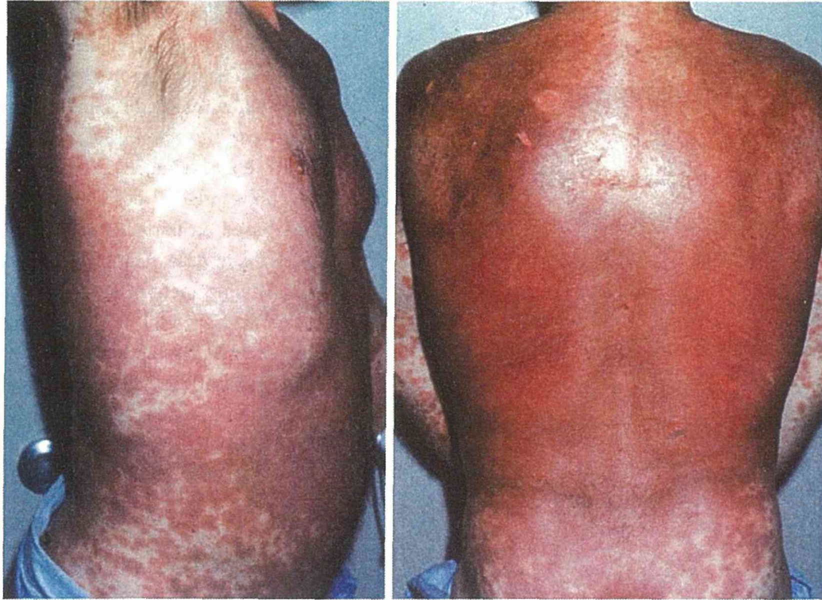

図1　ST合剤によるTEN型薬疹.

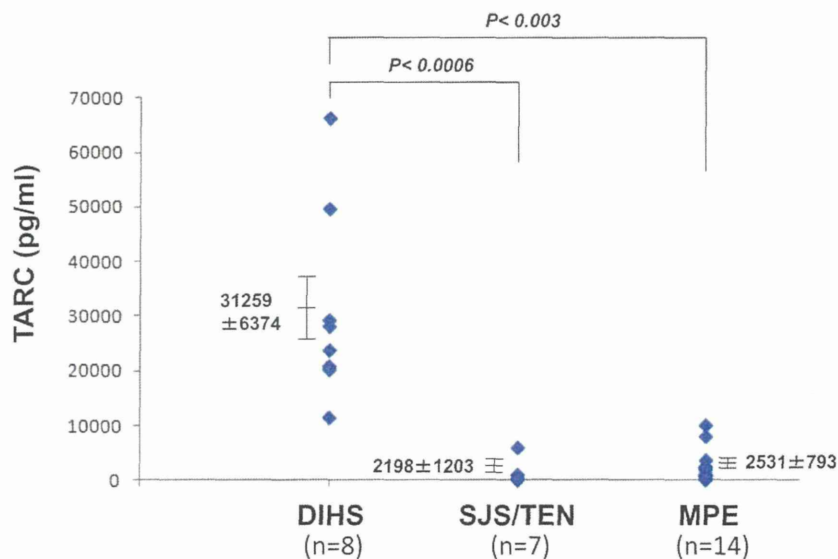

図2　DIHS, SJS/TEN, MPE（紅斑丘疹型薬疹）の急性期における血清TARC値の比較.

ンの一つで, 現在, アトピー性皮膚炎の重症度マーカーとして広く使用されている. TARCの上昇はDIHSに特徴的で, 一般の紅斑丘疹型薬疹やStevens-Johnson/TENでは中等度の上昇を示すのみである（平均2,000 pg/ml）. さらに, HHV-6再活性化を伴わないDIHS類似の薬疹と比べても, HHV-6再活性化を伴う真のDIHSにおいて有意に高値を認めたことから, HHV-6の再活性化に関わっている可能性が推測される.

GVHDにおけるHHV-6の再活性化—DIHSの病態モデル？

われわれは, GVHDとヒトヘルペスウイルス再活性化との関連性を調べる目的で, 移植前ならびに移植後経時的に, 末梢血中のヒトヘルペスウイルス（HHV-6, HHV-7, EBV, CMV）DNAの定量を行い, GVHDとの関係を検討した[9]. その結果, 移植患者15例中, GVHDを発症した10例全例にHHV-6の再活性化を認め, そのうち8例では発疹の出現・消退と血中HHV-6 DNAレベルとの間に相関がみられた. 一方, GVHDを発症しなかった5例の内HHV-6 DNAが検

出されたのは1例のみで，また，HHV-6以外のヒトヘルペスウイルスとGVHDとの相関はみられなかった．さらに，発疹の出現に一致して，血清中IL-10と可溶性IL-2受容体の上昇も認められた．以上のことから，造血幹細胞移植後のGVHDの発症にはHHV-6の再活性化とIL-10産生T細胞の活性化が密接に関わっているものと考えられた．

以上の結果から，GVHDとDIHSとの間には，皮膚症状，発熱，臓器障害，HHV-6再活性化，IL-10産生T細胞の活性化など，多くの共通点があり，GVHDがDIHSの病態モデルとなり得る可能性が示唆された．

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Increased ratio of FoxP3+ regulatory T cells/CD3+ T cells in skin lesions in drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms

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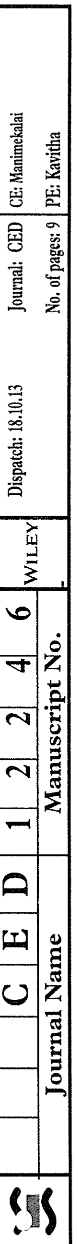

Summary

Background. Drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic symptoms (DIHS/DRESS) is a severe drug eruption accompanied by multiorgan disorders. Several unique aspects of DIHS/DRESS, including reactivation of herpesvirus, liver dysfunction and hypogammaglobulinemia, have similarities to graft-versus-host disease (GVHD).
Aim. In this study, we focused on the dynamics of regulatory T cells (Tregs) infiltrating into the skin lesions of DIHS/DRESS and GVHD.
Methods. Skin biopsies were taken from patients with DIHS/DRESS, GVHD, or maculopapular drug eruption. Tregs were detected using immunostaining with anti-FoxP3.
Results. The ratio of FoxP3+ T cells to CD3+ T cells was significantly higher in the skin lesions of DIHS/DRESS than in those of patients with GVHD and was positively correlated with the number of days from disease onset in the acute phase.
Conclusions. The dynamics of Tregs in skin lesions are different between DIHS/DRESS and GVHD, despite there being many similarities between these conditions.

Introduction

Drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic symptoms (DIHS/DRESS) is a severe drug eruption accompanied by multiorgan disorders.[1] It may be related to reactivation of human herpesvirus (HHV), especially HHV-6,[2–4] and to mild epidermal injury, in contrast to other severe adverse cutaneous drug reactions such as toxic epidermal necrosis (TEN) and Stevens–Johnson syndrome (SJS). However, the mechanisms of HHV reactivation and development of drug rashes are currently unknown. DIHS/DRESS has several notable features, such as delayed onset, worsening of clinical symptoms even after withdrawal of the causative drug, hypogammaglobulinemia,[5] reactivation of latent HHV during the acute stage of the disease, and autoimmune complications developing as short-term or long-term sequelae, such as autoimmune thyroiditis, positive reaction of antinuclear antibodies and fulminant type 1 diabetes mellitus.[6,7] Many aspects of this syndrome suggest close similarities between DIHS/DRESS and graft-versus-host disease (GVHD). We and other researchers have also revealed a relationship between HHV-6 reactivation and rash/GVHD after allogeneic stem cell transplantation.[8,9] Various complications frequently occurring in GVHD, such as autoimmune disease,[10,11] are frequently observed during the course of DIHS/DRESS, even long after its clinical resolution. However, there are clinical and histological differences

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between DIHS/DRESS and GVHD; for example, interface dermatitis and apoptotic keratinocytes can be observed in both DIHS/DRESS and GVHD, but are more severe in the latter.

Recently, much attention has been focused on regulatory T cells (Tregs) and their roles in drug eruption/GVHD. However, the dynamics of Tregs in the skin lesions in DIHS/DRESS and GVHD are not fully understood. In this study, we focused on the dynamics of Tregs infiltrating into the skin, one of the major target organs in DIHS/DRESS and GVHD, to examine the involvement of Tregs in the development of DIHS/DRESS and GVHD skin lesions.

Methods

The study was approved by the medical ethics committee of Nara Medical University, and all patients gave informed consent.

Patients and samples

Our study consisted of three groups of patients: patients with DIHS/DRESS ($n = 12$), patients with acute GVHD ($n = 12$) and patients with maculopapular drug eruption (MDE) ($n = 18$). The eliciting drugs had been withdrawn by the time of diagnosis of DIHS/DRESS or drug eruption in all patients.

The DIHS/DRESS group consisted of 12 patients (5 men, 7 women; median age 59 years, range 13–75) who were enrolled consecutively during the period April 2003. The profiles of these patients are shown in Table 1. Diagnosis of DIHS/DRESS was based on criteria established by a Japanese consensus group[12] and by RegiSCAR (European Registry of Severe Cutaneous Adverse Reactions).[13] Reactivation of HHV, including HHV-6 and HHV-7, was demonstrated by an increase in the titre of the specific serum IgG antibody and/or DNA levels in whole blood as detailed below. Skin biopsies were also taken from areas of maculopapular erythema in this group.

Table 2 details the characteristics of 12 consecutive patients with clinical signs of acute GVHD (3 men, 9 women; median age 52 years, range 7–66) who received allogeneic stem cell transplantation for haematological malignancy during the period November 2002 to August 2011. All 12 patients had received standard prophylaxis (ciclosporin in 10 patients and mycophenolate mofetil in 2 patients) prior to transplantation. Skin biopsies were taken from areas of erythematous maculopapular rash in all 12 patients, which were clinically graded according to standard criteria.[14]

The final group consisted of 18 patients (10 men, 8 women; median age 61 years, range 32–81). Skin biopsies were also taken from areas of cutaneous rash of patients without allografts or DIHS/DRESS ($n = 18$) that was clinically and histopathologically considered to be an MDE.

Assessment of herpesvirus DNA

DNA levels were assessed by PCR. DNA was extracted from whole blood using a commercial kit (QIAamp DNA Blood Mini-kit; Qiagen Inc., Tokyo, Japan) in accordance with the manufacturer's instructions, and then used for PCR. For assessment of HHV-6 and HHV-7 DNA levels in peripheral blood, real-time PCR was performed as described in a previous report,[15] and results expressed as viral DNA genome equivalents per 1 mL of whole blood. In DIHS/DRESS, HHV-6 DNA is usually detected during days 14–21 after the onset of skin eruption, whereas it is usually increased in accordance with the skin eruption in GVHD, as described previously.[9]

Immunohistochemistry

Tissues were fixed in formalin, embedded in paraffin wax, and cut into sections 4 μm thick. Immunostaining was performed using anti-CD3 (code A0452; Dako, Glostrup, Denmark) polyclonal antibody, anti-FoxP3 (clone 236 A/E7; BD Biosciences Inc., San Jose, CA, USA), and anti-CD4 (NCL-CD4-368, clone 4B12), anti-CD8 (NCL-C8-295, clone 1A5) (both Novocastra Ltd, Newcastle upon Tyne, UK) monoclonal antibodies as primary antibodies. Biotinylated antimouse IgG was used as secondary antibody, and bound antibody was evaluated using streptavidin-biotinylated peroxidase complex. After washing, sections were exposed to the chromogen and counterstained with haematoxylin. The numbers of immunostained cells in the dermis were counted in five high-power fields (HPF) and expressed as the mean number. The ratios of FoxP3+ Tregs, CD4+ T cells, and the ratio of CD8+ T cells to CD3+ T cells in the dermis were then calculated.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using the Student *t*-test. Pearson correlation coefficient was used to evaluate the correlation between the FoxP3+ Treg/CD3+ T-cell ratio in lesional skin and the number of days from onset. $P < 0.05$ was considered statistically significant.

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Table 1 Characteristics of patients with drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic symptoms.

Patient	Age, years/sex	Causative drug	Viral reactivation	Viral DNA loads* (in whole blood) or titres	Time from disease onset to skin biopsy, days	Immunosuppressive treatments at the time of skin biopsy	Time between skin biopsy and viral reactivation, days	FoxP3+/CD3+ cells in skin lesions, %	Skin rash	Eosinophils, per μL	Liver dysfunction, IU/L
1	62/M	Carbamazepine	HHV-7	1.2×10^4	5	None	−1	14.7	Maculopapular erythema	980	286/723
2	68/F	Carbamazepine	HHV-6	8.8×10^3	15	None	2	17	Maculopapular erythema	1560	34/45
3	75/F	Allopurinol	HHV-6, HHV-7	1.3×10^3 (HHV-6)	13	None	3	27.2	Maculopapular erythema, purpura	1200	57/84
4	61/F	Salazosulfapyridine	HHV-6	7.2×10^4	13	Prednisolone 10 mg/day	4	23.8	Erythroderma	1200	49/107
5	64/F	Mexiletine	HHV-6	3.4×10^5	10	Betamethasone 1.0 mg/day	5	17.3	Maculopapular erythema, purpura	3000	100/182
6	44/M	Carbamazepine	HHV-6, HHV-7	7.4×10^3 (HHV-6)	11	Betamethasone 1.0 mg/day	6	14.7	Erythroderma, pustules	7300	91/130
7	62/M	Lamotrigine	HHV-7	IgG (1 : 20) (day 15); IgG (1 : 1280) (day 29)	13	None	7	13.3	Maculopapular erythema	700	28/104
8	32/M	Allopurinol	HHV-6	4.8×10^3	8	None	9	9.9	Maculopapular erythema	2200	52/304
9	56/M	Cyanamide	HHV-6	2.4×10^4	4	None	10	15.6	Maculopapular erythema	3200	101/119
10	57/F	Salazosulfapyridine	HHV-6	2.6×10^3	10	Prednisolone 10 mg/day	10	7.5	Erythroderma, pustule	5800	257/383
11	13/F	Carbamazepine	HHV-6	2.0×10^4	2	None	13	7.8	Maculopapular erythema	2100	124/295
12	36/F	Lamotrigine	HHV-6	1.4×10^5	6	None	13	6.2	Erythroderma	2400	40/108

*Loads are number of virus copies/mL. HHV, herpesvirus. Maximum value in the category of eosinophil and AST/ALT during the course of DIHS/DRESS.

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Table 2 Profiles of patients with graft-versus-host disease after allogenic stem cell transplantation.

Underlying disease	Transplant type	Pre-transplant conditioning	Viral reactivation	Viral DNA loads (of whole blood)	Time from disease onset to skin biopsy, days	FoxP3+/CD3+ cells in skin lesions,%	Grade of GVHD
ALL	CBCT	TBI, FLU, BU	HHV-6	1.6×10^4	7	9.5	I
MDS	PBSCT	TBI, FLU, CPA, Mesna	HHV-6, CMV	5.2×10^3	3	4.2	I
MDS	CBCT	TBI, FLU, CPA, Mesna	HHV-6, CMV	8.0×10^4	3	3	I
AML	PBSCT	TBI, FLU, BU	HHV-6	9.2×10^3	2	2.5	IV
MDS	PBSCT	FLU, BU	CMV	4.4×10^3	5	4.8	II
ALL	CBCT	TBI, CPA, VP-16	HHV-6	1.2×10^3	6	8.2	II
ALL	PBSCT	FLU, BU	ND	ND	4	0.7	IV
ALL	BMT	TBI, CPA, BU, Mesna	ND	ND	29	3.3	III
ALL	BMT	TBI, L-PAM	ND	ND	27	0.6	IV
MM	PBSCT	L-PAM, BTZ	HHV-6	3.4×10^3	6	4.2	II
CML	BMT	TBI, FLU, BU, ATG	HHV-7	8.4×10^3	3	9.8	I
AML	CBCT	FLU, BU, Ara-C	HHV-6	7.2×10^3	4	6.7	I

*Loads are number of virus copies/mL. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; Ara-C, cytosine arabinoside; ATG, antithymocyte globulin; BMT, bone marrow transplantation; BTZ, bortezomib; BU, busulfan; CBCT, cord blood cell transplantation; CLL, chronic lymphoblastic leukemia; CML, chronic myeloid leukemia; CPA, cyclophosphamide; FLU, fludarabine; GVHD, graft-versus-host disease; L-PAM, L-phenylalanine monohydrochloride; MDS, myelodysplastic syndrome; MM, multiple myeloma; ND, no data; PBST, peripheral blood cell stem cell transplantation; TBI, total body irradiation; VP-16, etoposide.

Results

Histopathological examination

Histopathological examination of skin biopsies obtained from the erythematous maculopapular rashes of patients with DIHS/DRESS showed perivascular lymphocytic infiltration with eosinophils (8 cases; 66.7%), interface dermatitis with vacuolar degeneration (2 cases; 16.7%) and spongiotic dermatitis with vacuolar degeneration (2 cases; 16.7%). Skin biopsies from rashes in patients with acute GVHD were graded according to the criteria by Lerner *et al.*[15,16] and showed vacuolar degeneration (histological grade I; 6 cases; 50%) and spongiosis with apoptotic cells (histological grade II; 6 cases; 50%). None of the cases showed a cleft between the epidermis and dermis (histological grade III or IV). Tissue from MDE mainly exhibited perivascular lymphocytic inflammation, occasionally with eosinophils.

Increased FoxP3+ Treg/CD3+ T-cell ratio in the skin lesions of DIHS/DRESS

The FoxP3+ Treg/CD3+ T-cell ratio was significantly higher in DIHS/DRESS rashes than in GVHD and MDE tissue (Figs 1 and 2), but the ratio in GVHD was not significantly different from that in MDE. In skin biopsy specimens from GVHD rashes and MDEs, we found small numbers of FoxP3+ Tregs. By contrast, CD4+/CD3+ and CD8+/CD3+ T-cell ratios in the skin lesions were similar for the three groups (Figs 1 and 2). The numbers of CD3+ T cells per 5 high-power fields in skin biopsies of those patients were also not significantly different.

Relationships between FoxP3+ Tregs/CD3+ T cells and the period from onset

Figure 3 shows the relationships between the ratio of FoxP3+ Tregs/CD3+ T cells in the lesional skin and the number of days from disease onset. All patients with DIHS/DRESS in this study had received no major treatment such as high-dose corticosteroid before the skin biopsies were taken. The FoxP3+ Treg/CD3+ T-cell ratio was positively correlated with the number of days from disease onset during the acute phase in DIHS/DRESS, but there was no correlation in either GVHD or MDE.

Discussion

Although DIHS/DRESS and GVHD can have similar presentations, there are a some clinical and histologi-

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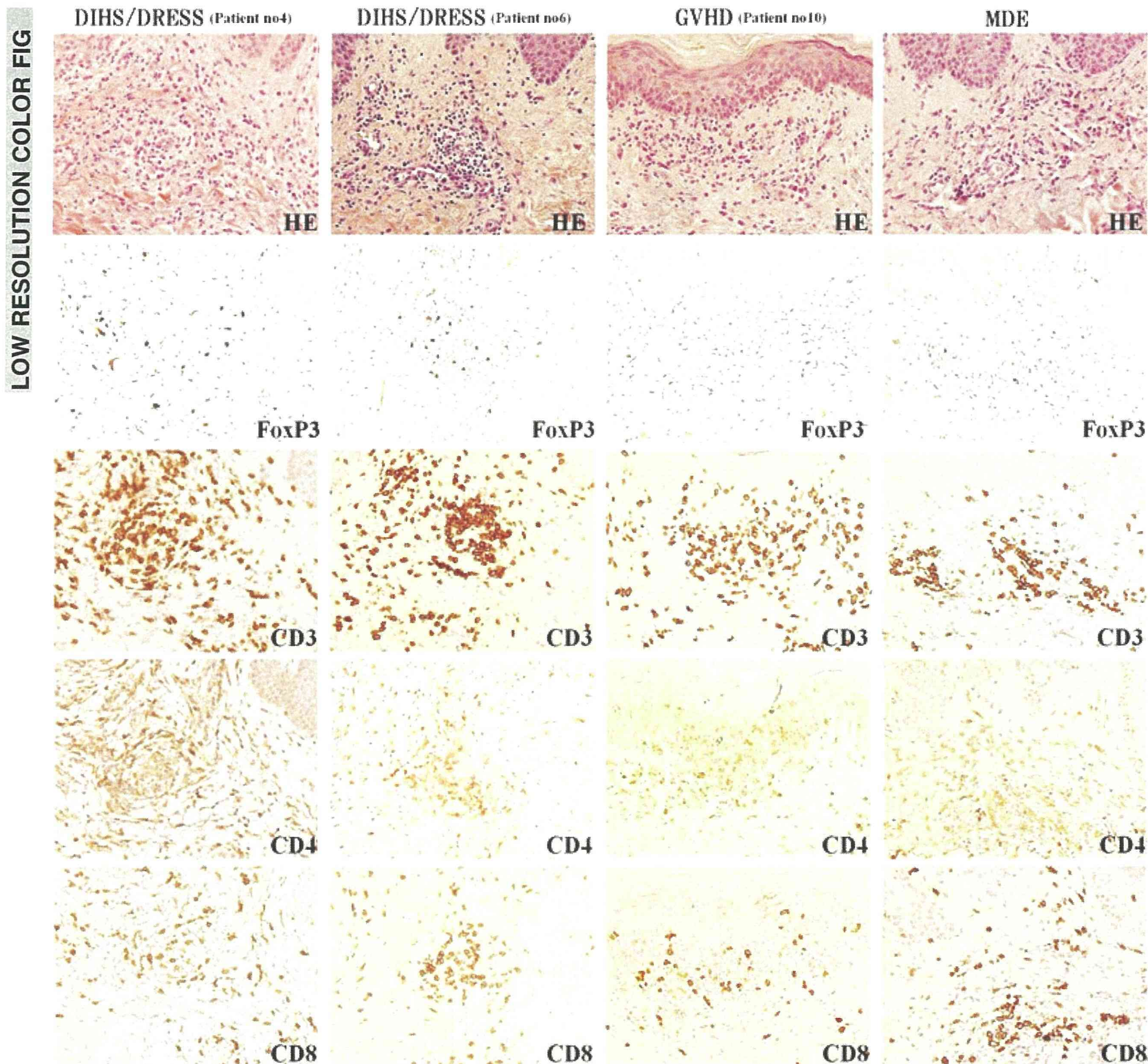

Figure 1 Expression of FoxP3+, CD3+, CD4+ and CD8+ T cells in drug-induced hypersensitivity syndrome (DIHS/DRESS), graft-versus-host disease (GVHD) and maculopapular drug eruption (MDE). Skin biopsies from patients with DIHS/DRESS showed a high number of FoxP3+ T cells in the epidermal-dermal junction and upper dermis compared with those in GVHD and MDE. Sections were counter-stained with haematoxylin, and images show representative serial sections from the same lesion of a patient with each disease (original magnification × 200). Patient numbers correspond with those in the tables.

cal differences between them. The cutaneous presentation of DIHS/DRESS often involves a maculopapular rash or erythroderma, but not blister formation or erosion. The common pathological findings of DIHS/DRESS are superficial perivascular lymphocytic infiltration with extravascular eosinophils, but histologically, severe liquefaction degeneration of the basal layer or epidermal necrosis is rarely found. By contrast, GVHD often presents with blister formation and erosion, and histologically shows lichenoid reaction with epidermal necrosis and/or epidermolysis.

Previous research on the dynamics of skin-infiltrating Tregs in GVHD showed that a decreased number of skin-infiltrating Tregs was associated with severity of GVHD[17]; however, another study showed that Tregs increased with degree of inflammation and grade of GVHD.[18]

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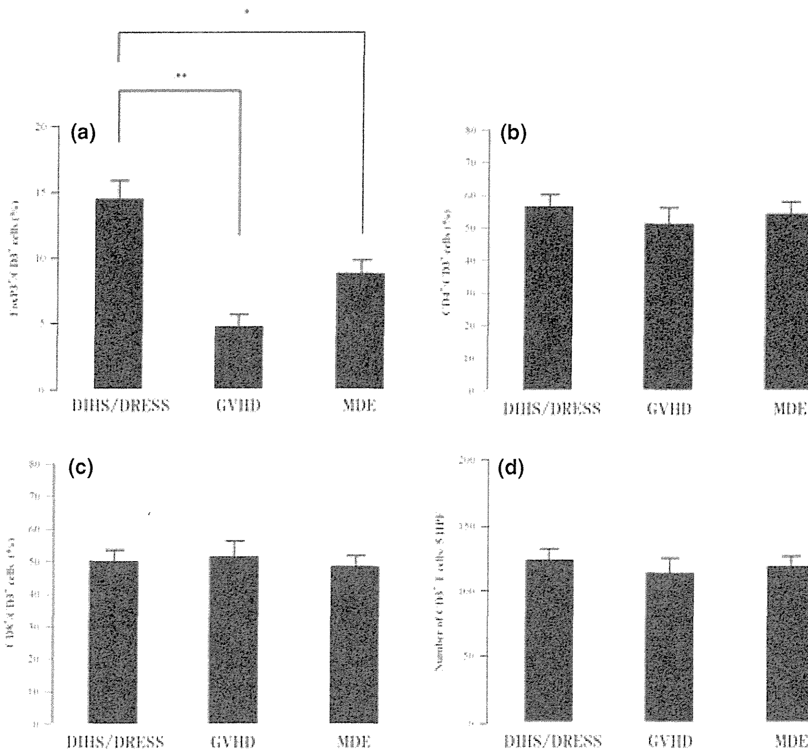

Figure 2 Ratios of FoxP3+ regulatory T cells, CD4+ T cells and ratio of CD8+ T cells to CD3+ T cells in paraffin wax-embedded biopsies taken from patients with drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic symptoms (DIHS/DRESS; $n = 12$).), graft-versus-host disease (GVHD; $n = 12$) and) and maculopapular drug eruption (MDE; $n = 18$) are shown. (a) In DIHS/DRESS, a high ratio of FoxP3+ T cells per 100 CD3+ T cells was observed. (b, c) The ratios of CD4+/CD3+ and CD8+/CD3+ T cells infiltrating into the lesional skin of DIHS/DRESS were not statistically different from those in GVHD and MDE. (d) Numbers of infiltrating CD3+ T cells were quite similar in DIHS/DRESS, GVHD and MDE (* $P < 0.05$, ** $P < 0.01$).

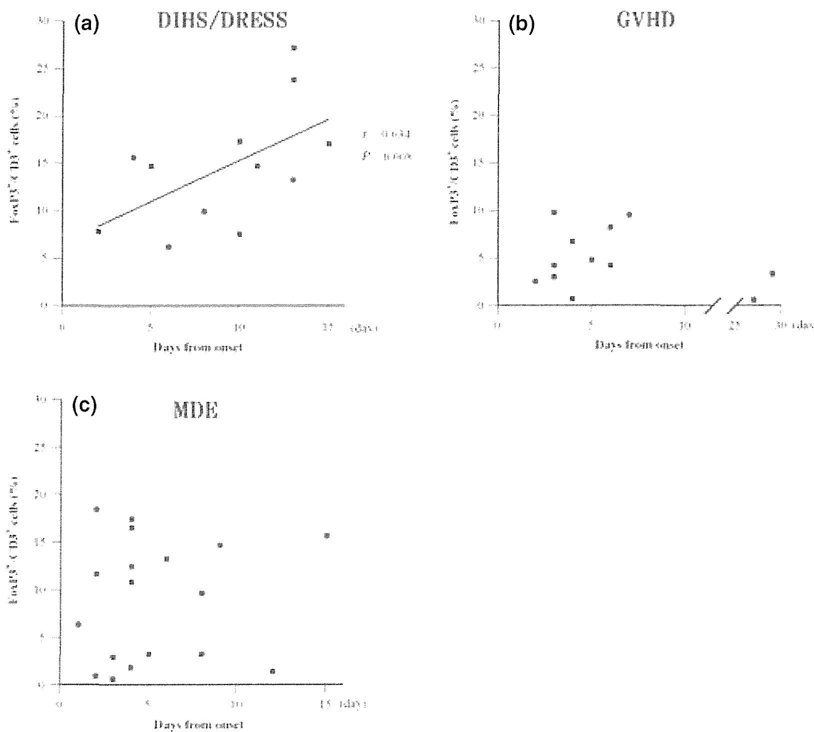

Figure 3 There was a correlation between the FoxP3+ Treg/CD3+ T-cell ratio and the time from disease onset in skin biopsies from patients with drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic.

Patients with DIHS/DRESS in the acute stage were found to exhibit increased frequencies of Tregs and gradual loss of their function after resolution in peripheral blood mononuclear cells (PBMCs).[19] However, there have been no studies about the dynamics of skin-infiltrating Tregs in DIHS/DRESS. Therefore, we

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focused on the dynamics of infiltrating Tregs in the skin lesions of these diseases, and found considerable differences between DIHS/DRESS and GVHD.

In the current study, the FoxP3+ Treg cell/CD3+ T-cell ratio was significantly higher in lesions from DIHS/DRESS than in those from GVHD and MDE, whereas the numbers of CD3+ T cells infiltrating into the skin lesions were similar in all three conditions (Figs 1 and 2). We also found that the ratio was positively correlated with the number of days from disease onset during the acute phase of DIHS/DRESS (Fig. 3). However, each dot in Fig. 3 represents the FoxP3+/CD3+ ratio from different patient samples, so the data does not show sequential data from individual patients, and thus results must be interpreted with caution. By contrast, the ratios of CD4+CD3+ T cells and CD8+CD3+ T cells in cutaneous lesions were similar for DIHS/DRESS, GVHD and MDE (Fig. 2). These findings suggest that clinical and histological differences between DIHS/DRESS and GVHD may result from differences in the frequency of FoxP3+ Tregs infiltrating into the skin lesions of these diseases. Tregs play a significant role in suppression of various diseases, including allergic responses, autoimmune and infectious disease, and cancers.[20,21] Accordingly, it is likely that an increased number of FoxP3+ T cells infiltrating into DIHS/DRESS skin lesions can protect the epidermis from severe damage compared with that in GVHD skin lesions.

Conclusion

In conclusion, the present study suggests that, despite many similarities, the dynamics of Tregs are different between DIHS/DRESS and GVHD in skin lesions, and that this difference may exert a considerable influence on the development of skin presentations in the two diseases.

What's already known about this topic?

- There are close similarities between DIHS/DRESS and GVHD, including HHV-6 reactivation, skin eruption, and autoimmune disease-like complications.
- However, there are also some clinical and histological differences between these two conditions.
- There are conflicting reports about the dynamics of skin-infiltrating Tregs in GVHD: severity of disease has been associated with both a decreased and an increased number of skin-infiltrating Tregs.
- Patients with DIHS/DRESS patients exhibit increased frequencies of Tregs in PBMCs at the acute stage; however, the dynamics of skin-infiltrating Tregs in DIHS/DRESS are currently unknown.

What does this study add?

- In the current study, levels of FoxP3+ Tregs were significantly higher in the skin lesions of DIHS/DRESS than in those of GVHD.
- The FoxP3+ Treg cell/CD3+ T-cell ratio was positively correlated with the number of days from disease onset during the acute phase of DIHS/DRESS, but not in GVHD or MDE.

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CPD questions

Learning objective

To provide up-to-date information about the immunopathological conditions in drug-induced hypersensitivity syndrome/drug rash with eosinophilia with systemic symptoms (DIHS/DRESS) and graft-versus-host disease (GVHD).

Question 1

Which type of lymphocyte is specifically increased in the skin lesions in DIHS/DRESS?
a) CD4+ T cell.
b) CD8+ T cell.
c) Regulatory T cell.
d) NK cell.
e) B cell.

Question 2

Which of the following diseases often shows epidermal necrosis?
a) DIHS.
b) GVHD.
c) Maculopapular drug eruption.
d) Urticaria.
e) Contact dermatitis.

Question 3

Which type of virus is commonly reactivated in DIHS/DRESS?
a) HSV.
b) VZV.
c) HHV-6.
d) HHV-8.
e) EBV.

Question 4

Which of the following statements about regulatory T cells is true?
a) CD8+.
b) CD20+.
c) CD56+.
d) FoxP3+.
e) Enhance allergic responses.

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Question 5

Which of the following features is not seen in DIHS/DRESS?

a) Fever.
b) Eosinophilia.
c) Penicillin allergy.
d) Lymph-node swelling.
e) Delayed onset.

Instructions for answering questions

This learning activity is freely available online at http://www.wileyhealthlearning.com/ced.

Users are encouraged to

- Read the article in print or online, paying particular attention to the learning points and any author conflict of interest disclosures
- Reflect on the article
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Basophils are required for the induction of Th2 immunity to haptens and peptide antigens

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The relative contributions of basophils and dendritic cells in Th2 skewing to foreign antigen exposure remain unclear. Here we report the ability of basophils to induce Th2 polarization upon epicutaneous sensitization with different antigens using basophil conditionally depleted Bas TRECK transgenic mice. Basophils are responsible for Th2 skewing to haptens and peptide antigens, but not protein antigens *in vivo*. Consistent with this, basophils cannot take up or process ovalbumin protein in significant quantities, but present ovalbumin peptide to T cells for Th2 differentiation via major histocompatibility complex class II. Intriguingly, basophils promote Th2 skewing upon ovalbumin protein exposure in the presence of dendritic cells. Taken together, our results suggest that basophils alone are able to induce Th2 skewing with haptens and peptide antigens but require dendritic cells for the induction of Th2 for protein antigens upon epicutaneous immunization.

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The induction of adaptive cellular immunity in the skin is initiated by antigen-presenting cells (APCs) such as dendritic cells (DCs), which provide signals through the peptide-major histocompatibility complex (MHC), costimulatory molecules, and instructive cytokines to naive T cells upon antigen encounter[1–3]. Distinct subsets of $CD4^+$ T helper (Th) cells are then generated to control different types of protective immunity[4]. Th2 cells are crucial for the clearance of parasites, such as helminths, via expansion and activation of innate effector cells, including eosinophils and basophils[5]. The induction of Th2 immune responses was previously considered to depend mainly on DCs[6]. However, this dogma has recently been challenged as basophils might also have a pivotal role in this process[7–10].

It has been demonstrated that $CD49b^+$ $Fc\varepsilon RI^+$ $c\text{-}Kit^-$ basophils migrate into the draining lymph nodes (LNs) from the site of helminth infection or papain injection and act as APCs by taking up and processing antigens[7,9,10]. In addition, basophils express MHC class II and costimulatory molecules and secrete interleukin (IL)-4 and thymic stromal lymphopoietin (TSLP), which are critical for Th2 development. Thus, basophils alone are considered to induce Th2 polarization from naive T cells without requiring DCs in certain conditions. On the contrary, another group has found that IL-4-producing basophils were recruited to the mediastinal LNs upon first house dust mite exposure and contributed to the strength of the Th2 response in the lung, but that basophils could not present antigens or express the chaperones involved in antigen presentation[11]. These authors, therefore, proposed that DCs are necessary and sufficient for the induction of Th2 immunity to house dust mites in the lung without the requirement of basophils. Consistent with this, it has been reported that Th2 responses were severely impaired either after *Schistosoma mansoni* egg injection or during active *S. mansoni* infection by depletion of $CD11c^+$ cells, but not by depletion of basophils using anti-$Fc\varepsilon RI\alpha$ antibody[12]. Therefore, it is of great importance to determine whether or not, and under which conditions, basophils induce Th2 skewing to foreign antigen exposure.

Recently, we demonstrated that basophils use a specific 4 kb enhancer fragment containing the 3′-untranslated region and HS4 elements to regulate *Il4* gene expression[13]. Utilizing this system, we have generated mice that express human diphtheria toxin receptor under the control of HS4, which are named basophil-specific enhancer-mediated toxin receptor-mediated conditional cell knock-out (Bas TRECK) transgenic (Tg) mice[14–16]. In Bas TRECK Tg mice, basophils are specifically and conditionally depleted by diphtheria toxin (DT) treatment. Using these mice, we examined whether basophils induce Th2 polarization upon stimulation by different antigens such as haptens, peptides and protein antigens. We herein demonstrate that basophils are necessary for the induction of cutaneous Th2 immunity against haptens and peptide antigens but are dispensable for protein antigens.

Results

Impaired induction of IgG1 to peptide antigens. We first confirmed that basophils in the bone marrow (BM) were completely depleted without affecting T cell, mast cell or DC population in Bas TRECK Tg mice after an intraperitoneal injection of DT (Fig. 1a, and see Supplementary Figs S1A–D,S2). To test whether basophils are involved in the induction of cutaneous Th2 responses against protein antigens, we pretreated wild-type C57BL/6 (B6) and Bas TRECK Tg mice with DT and immunized them with ovalbumin (OVA) protein via a cutaneous patch to induce a Th2-type cutaneous immune response[17,18].

In this model, DT-treated Bas TRECK Tg (basophil-depleted) mice exhibited similar clinical manifestations to B6 mice (Fig. 1b,c). Basophils were accumulated in the skin lesion of B6 mice but absent in that of basophil-depleted mice (see Supplementary Fig. S2). In addition, the levels of serum OVA-specific IgG1 in basophil-depleted mice were comparable to those in B6 mice (Fig. 1d), whereas the production of Th1-dependent serum IgG2a was not induced by cutaneous application of OVA proteins (Fig. 1d, right panel). To evaluate the T-cell stimulatory capacity of basophils upon protein antigen exposure, the skin draining LN cells from B6 and basophil-depleted mice after cutaneous OVA protein application were challenged with OVA protein *in vitro*. The incorporation of ^{3}H-thymidine in the presence of OVA protein was comparable between basophil-depleted mice and B6 mice (see Supplementary Fig. S3). The above results suggest that basophils were not essential to Th2-type immune responses induced by cutaneous application of protein antigens.

Previous studies have reported that basophils, but not DCs, functioned as APC for peptide antigen-induced Th2 *in vitro*[9]. As such, we next compared the *in vivo* Th2-induction capacity of basophils upon intraperitoneal exposure to OVA protein or peptide (amino acids 323–339) mixed with alum as a strong adjuvant of Th2 responses. In this model, a significantly decreased OVA-specific IgG1 level was observed in basophil-depleted mice with OVA peptide immunization, but not with OVA protein immunization (Fig. 1e). At the same time point, antigen-specific IgE was undetectable in both groups due to the genetic background of these mice as B6 (refs 19,20); therefore, OVA-specific IgG1 has been used as a marker of Th2-dependent immunoglobulin in this model[19]. In addition, we examined that IgG1 production by anti-CD40 is enhanced by IL-4 (Supplementary Fig. S4), which also supports the rationale that IgG1 can be used as a marker of Th2 induction. We also found that the frequency of IL-4^+ cells in spleen $CD4^+$ T cells from basophil-depleted mice was significantly lower than that from B6 mice upon OVA peptide intraperitoneal immunization, but not upon OVA protein immunization (Fig. 1f,g). The frequency of IL-4^+ cells in non-T cells was also comparable between these two groups (Supplementary Fig. S5).

In addition, after immunized with OVA peptide, the numbers of eosinophils, $CD4^+$, $CD4^+CD44^+CD62L^+$ central memory, and $CD4^+CD44^+CD62L^-$ effector memory T cells in splenocytes from basophil-depleted mice were significantly decreased compared with those from B6 mice (Supplementary Fig. S6A). Consistently, upon immunization with OVA peptide, the messenger RNA (mRNA) levels of *IL-4* and *IL-13* in the mesenteric LNs in basophil-depleted mice were significantly decreased compared with those in B6 mice (Supplementary Fig. S6B).

To further evaluate the role of basophils on T-cell differentiation after immunization with OVA protein or OVA peptide, splenocytes from B6 and basophil-depleted mice were re-challenged in the presence or absence of each antigen *in vitro*. Markedly decreased incorporation of ^{3}H-thymidine and the levels of IL-4, IL-5 and IL-13 in the culture supernatant were examined in basophil-depleted mice upon OVA peptide immunization, but not upon OVA protein immunization (Supplementary Fig. S7A,B).

We next evaluated Th2 induction under the condition where both OVA protein and OVA peptide are used at the same time with alum. The levels of serum OVA-specific IgG1 (Supplementary Fig. S8A–C), the frequency of IL-4^+ cells in $CD4^+$ T from basophil-depleted mice was similar to that from B6 mice in all *mol* ratio (protein:peptide = 2:1, 1:1, and 1:2) (Supplementary Fig. S8D,E).

Attenuation of inflammation by repeated hapten application. Haptens are one of external antigens via cutaneous penetration. A previous report demonstrated that repeated elicitation with

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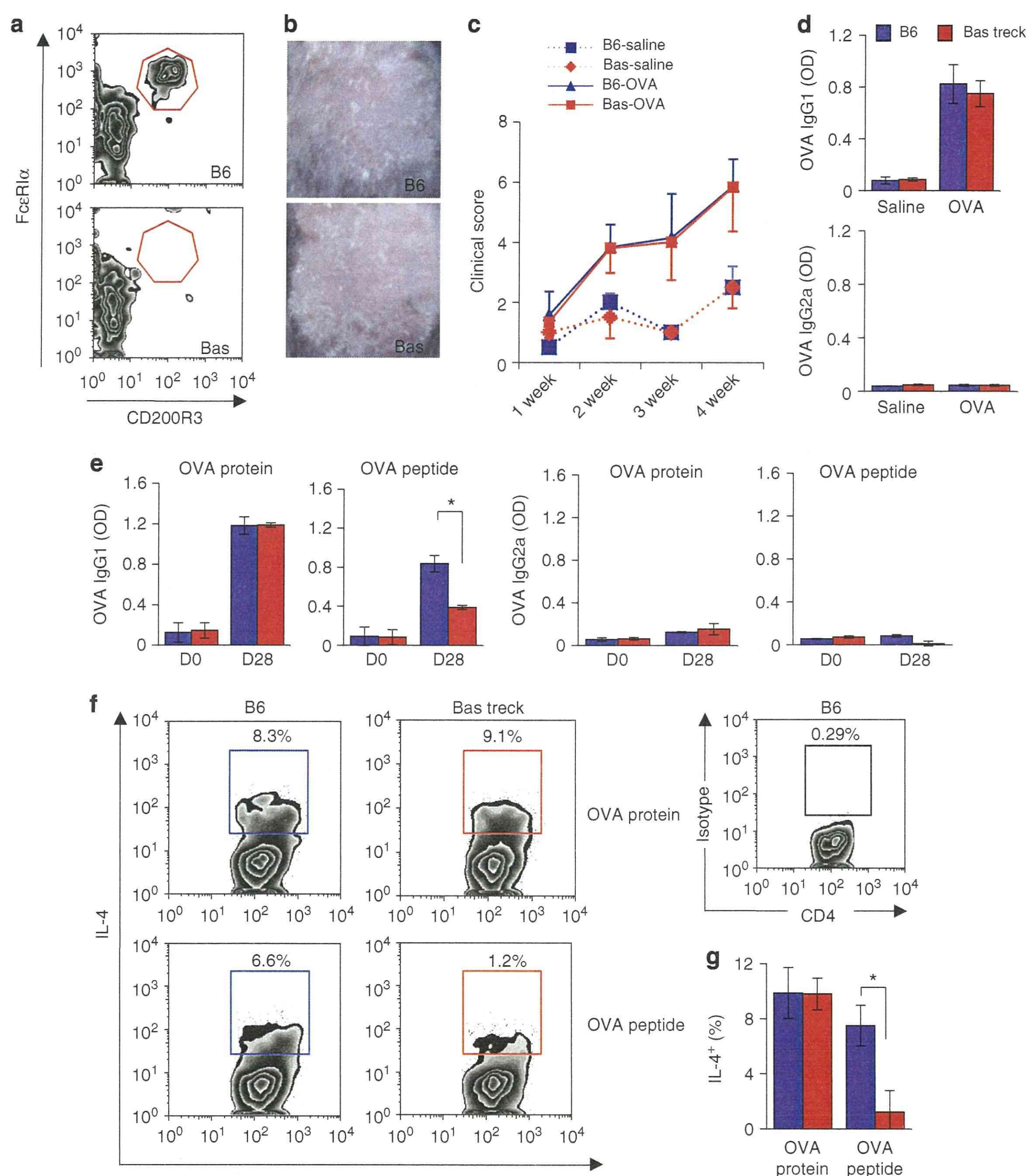

Figure 1 | Impaired induction of IgG1 to peptide antigens in the absence of basophils. (**a**) Basophils in BM in B6 and Bas TRECK mice after an intraperitoneal injection of diphtheria toxin. (**b**,**c**) Clinical manifestations (**b**) and clinical scores (**c**) of Th2-type cutaneous immune response model in DT-treated B6 and DT-treated Bas TRECK mice ($n=10$ per group). (**d**) Serum OVA-specific IgG1 levels and IgG2a in DT-treated Bas TRECK mice and DT-treated B6. (**e**) OVA-specific IgG1 and IgG2a responses in DT-treated B6 mice and DT-treated Bas TRECK mice in the model of immunization with OVA protein or peptide (amino acids 323-339) on day 0 and 28 mixed with alum, a strong promoter of Th2 responses. (**f**,**g**) Intracellular cytokine staining for IL-4 (**f**) and the frequency of IL-4$^+$ cells (**g**) in CD4$^+$ T cells. All data are presented as the mean ± s.d. and are representative of three experiments. *$P<0.05$, Wilcoxon signed-rank test versus corresponding groups.

hapten results in a shift from Th1- to Th2-mediated cutaneous inflammation, which mimics atopic dermatitis[21]. Therefore, to examine the role of basophils in hapten-induced Th2-type inflammation in the skin, we performed repeated cutaneous application of oxazolone to B6 mice and basophil-depleted mice.

Although Th1-mediated delayed-type hypersensitivity as manifested by the ear swelling response to a single elicitation of oxazolone was similar between B6 and basophil-depleted mice (Fig. 2a), the repeated application-induced immune reaction in basophil-depleted mice was much less than that in B6 mice (Fig. 2b). Immediate-type hypersensitivity and late phase reaction, as manifested by the ear swelling responses 1 and 6 h after the last hapten application in basophil-depleted mice was significantly attenuated compared with those in B6 mice (Fig. 2c). Histological examination revealed attenuated epidermal thickening and cell infiltrations in the dermis of basophil-depleted mice (Fig. 2d,e, Supplementary Table S1). In addition, serum oxazolone-specific IgG1 levels in basophil-depleted mice were significantly decreased compared with those in B6 mice (Fig. 2f, left panel). Furthermore, the oxazolone-specific IgG2a

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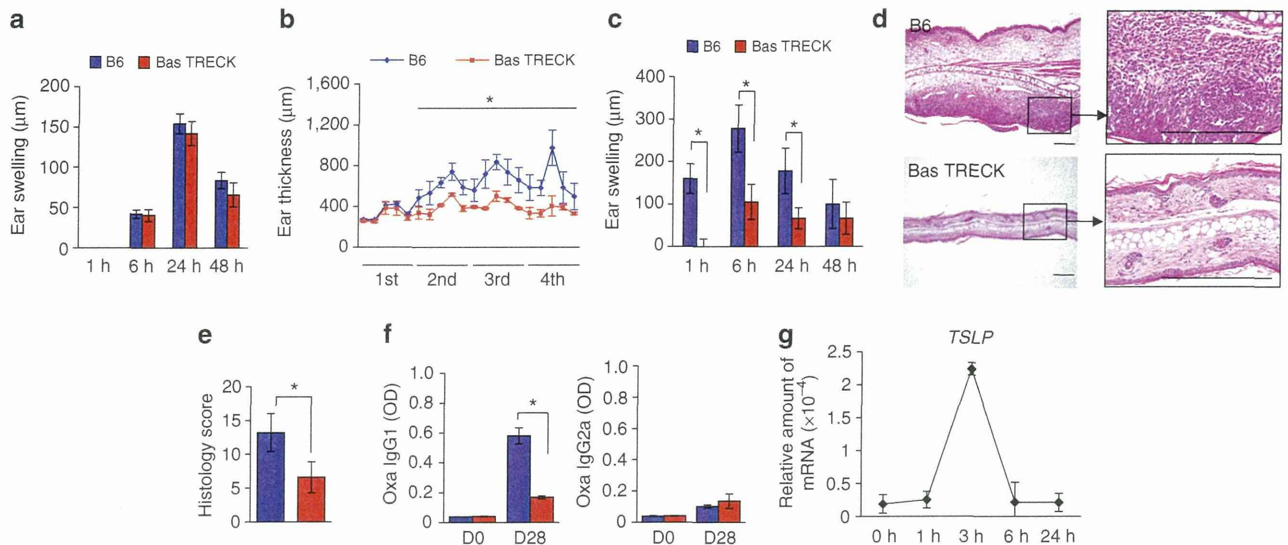

Figure 2 | Attenuation of ear inflammation by repeated hapten application in the DT-treated Bas TRECK mice. (**a**) Th1 immune response to single application of oxazolone of DT-treated B6 and DT-treated Bas TRECK (Bas -) mice. (**b**) Time course of repeated hapten application in these mice, (**c**) On fourth application, the ear swelling of these mice in the acute phase. (**d**) Histological examination (Scale bar, 100 μm), and scores (**e**). (**f**) Serum oxazolone-specific IgG1 and IgG2a levels in DT-treated B6 or DT-treated Bas TRECK (Bas -) mice on day 0 and 28. (**g**) The mRNA level of *TSLP* in the skin lesion after repeated hapten application. All data are presented as the mean ± s.d. ($n > 3$) and are representative of three experiments. $^*P < 0.05$ (Wilcoxon signed-rank test) versus corresponding groups.

levels in basophil-depleted mice were the same as those in B6 mice (Fig. 2f, right panel). The mRNA level of *TSLP* in the skin lesion was increased 3 h after the last challenge in the repeated hapten application model (Fig. 2g). These results suggest that basophils are responsible for hapten-induced Th2 skin reaction and IgG1 induction.

Basophils express MHC class II and costimulatory molecules. Next, we examined whether basophils express MHC class II, costimulatory molecules and chemokine receptors in draining LNs with repeated cutaneous hapten application. As reported previously, basophils were not detected in draining LNs in the steady states; however, after repeated hapten application, a significant number of $CD49b^+$ $Fc\varepsilon RI^+$ IgE^+ $CD200R^+$ c-Kit^- basophils were detected (Fig. 3a). These basophils expressed MHC class II, CD40, CD80, CD86, CD62L, CXCR4, and CCR7, but not CCR4 (Fig. 3b), which suggest their potential to enter lymphoid tissues.

Basophils produce the Th2-inducing cytokine IL-4 and express TSLP after stimulation with papain *in vitro* and *in vivo*[10]. However, it remains unclear whether basophils express IL-4 in the repeated hapten application model. After repeated hapten application, a fraction of basophils produced IL-4 in draining LNs using an intracellular staining assay without any stimulation *in vitro* (Fig. 3c). As TSLP is known as a critical inducer and activator of basophils, the induction of basophils in the draining LNs (Fig. 3a) and their activation (Fig. 3b,c) may depend on the induction of TSLP in the draining LNs (Fig. 2h). In addition, basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs after the hapten application model (Fig. 3d). Therefore, basophils accumulated in the draining LNs may be a source of IL-4 for the induction of Th2 response in the repeated hapten application model.

Basophils stimulate T cells with OVA peptide. Next, we examined whether basophils activate T cells *in vitro*. To address this issue, we used Fluo-8 for intracellular Ca^{2+} staining and monitored the influx of Ca^{2+} as an indicator of T-cell activation. Firstly, we confirmed that sorted basophils exhibited their characteristic multilobed nuclei by cytology (Fig. 4a). We incubated Fluo-8-labelled OVA-specific naive $CD4^+$ T cells from DO11.10 T-cell antigen receptor Tg mice and CellTracker Red-labelled BM-derived basophils (BMBs) with OVA protein or OVA peptide. When an intracellular Ca^{2+} level of T cells is increased, green fluorescence intensity is induced in this system. Mixing with OVA peptide induced a prominent Ca^{2+} increase in T cells as shown in green fluorescence (Fig. 4b,c and see Supplementary Movie 1). However, mixing with OVA protein did not increase intracellular Ca^{2+} concentrations in T cells (Fig. 4d). On the other hand, DCs induced a prominent Ca^{2+} increase in T cells irrespective of the presence of OVA protein or OVA peptide (Fig. 4e), while basophils can activate T cells only in the presence of OVA peptide (Fig. 4d). The capacity for inducing a calcium response in T cells in the presence of peptide is comparable between DCs and basophils (Fig. 4d,e).

Basophils are unable to process protein antigens. Our studies demonstrated that basophils have a role in Th2 induction with peptide antigens, but not with protein antigens. One possible reason for this is that basophils are unable to take up and process protein antigens. To explore this, we examined whether basophils take up and process OVA protein into OVA peptide. To address this issue, we used dye quenched (DQ) ovalbumin (DQ-OVA), a fluorogenic substrate for proteases, which becomes fluorescent upon hydrolysis of the DQ-OVA to single peptide by proteases. Using this system, we tested BMBs for their ability to take up and process OVA and found that $CD49b^+$ $Fc\varepsilon RI^+$ $CD11c^-$ BMBs could hardly take up and process OVA protein compared with bone marrow-derived dendritic cells (BMDCs) (Fig. 5a).

Next, we used *in vitro* systems for Th2 differentiation and T-cell stimulatory capacity with purified OVA-specific $CD4^+$ T cells from 4get × DO11.10 double Tg mice or from CFSE-labelled DO11.10 mice. Co-culture of BMBs and $CD4^+$ T cells together in the presence of antigens led to IL-4-enhanced green fluorescence

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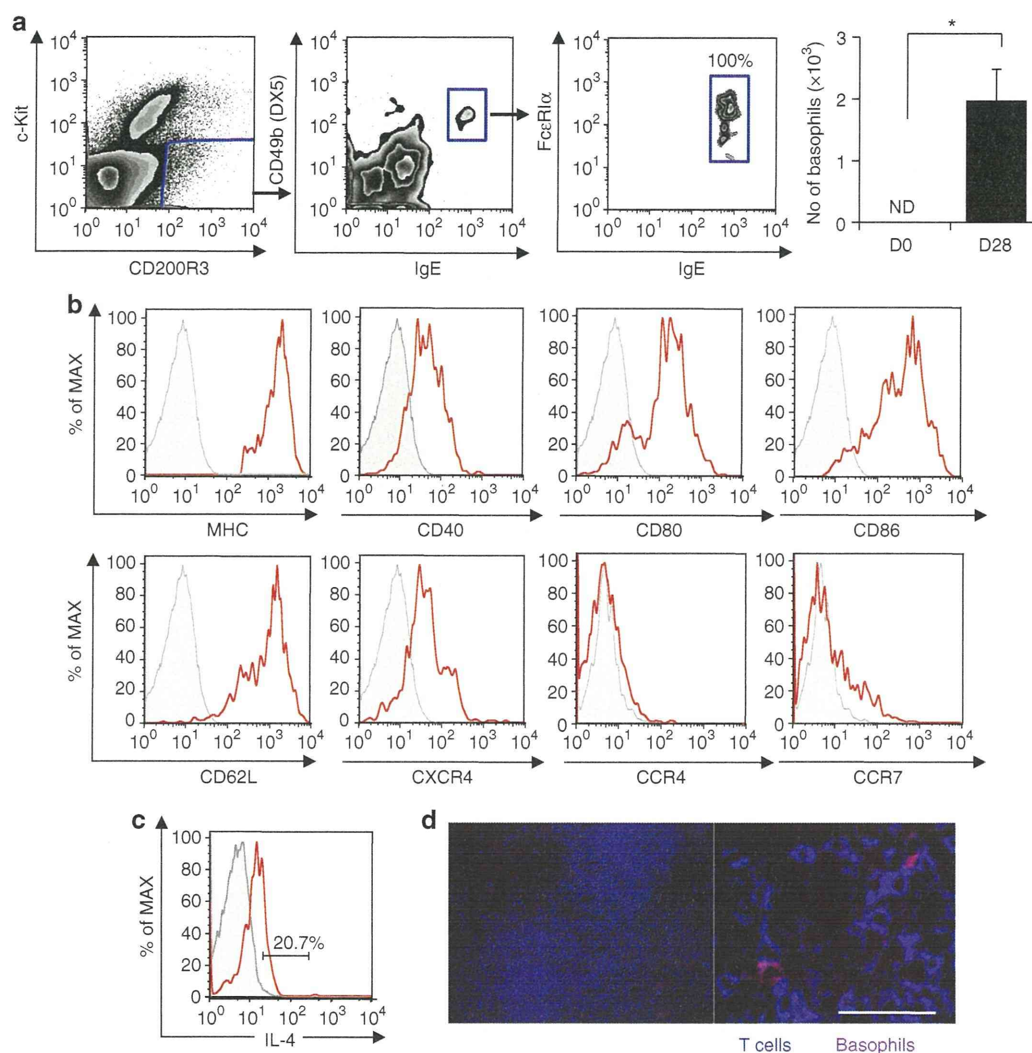

Figure 3 | Basophils expressed MHC class II and costimulatory molecules in the repeated hapten application model. (**a**) The number of CD49b$^+$ FcεRI$^+$ IgE$^+$ CD200R$^+$ c-Kit$^-$ basophils as gated (left panels), before (D0) and 28 days after repeated hapten application. (**b**) Expression of MHC class II, CD40, CD80, and CD86, CD62L, CCR4, CXCR4 and CCR7, on basophils (shaded histogram represents isotype-matched control antibody). (**c**) IL-4 production in basophils in draining LNs by means of an *in vivo* intracellular staining assay after repeated hapten application. (**d**) Basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs after the hapten application model. All data are presented as the mean ± s.d. and are representative of three experiments. Scale bar, 50 μm.

protein (eGFP) expression in CD4$^+$ T cells as reported previously[8]. Although OVA peptide induced IL-4 induction in CD4$^+$ T cells in a dose-dependent manner, OVA protein did not (Fig. 5b,d). We also performed intracellular cytokine staining for IL-4 to measure IL-4 production at the protein level. Although OVA peptide induced IL-4 production in CD4$^+$ T cells, OVA protein did not (Supplementary Fig. S9A,B). Moreover, induction of Th2 responses by basophils was IL-4 dependent using neutralizing anti-IL-4 antibody (Supplementary Fig. S9C). T-cell stimulatory capacity was measured by a CFSE-diluted assay after incubation for 1 (Fig. 5c) or 3 days (Supplementary Fig. S10). T-cell proliferation was enhanced by basophils in the presence of OVA peptide but not in the presence of OVA protein (Fig. 5c,e, and Supplementary Fig. S10).

As basophils cannot take up or process protein antigens (Fig. 5a), other cells are required to prepare peptide to be presentable. One of the candidates is DCs as it has been reported that the Th2 response to cysteine proteases requires the cooperation of DCs and basophils via reactive oxygen species-mediated signalling[22]. Therefore, we examined whether DCs support basophil-dependent Th2 induction upon exposure to protein antigens. To assess this issue, we evaluated Th2 differentiation by culturing BMBs with OVA protein in the presence or absence of BMDCs. Basophils significantly increased the frequency of IL-4-eGFP-positive CD4$^+$ T cells upon OVA protein exposure in the presence of DCs (Fig. 5f). In addition, we demonstrated that the addition of a blocking antibody to MHC class II abrogated the basophil-induced Th2 responses, which rules out the possibility that the antigen has a direct effect on the T cells (Fig. 5g).

It has recently been shown that basophils are a heterogeneous population that can be driven by either IL-3 or TSLP[15]. The number of BMBs driven by IL-3 after a repeated hapten application model was high and that after OVA cutaneous patch was rather low when compared with control mice (Fig. 6b, left panel). Intriguingly, the numbers of BMBs driven by TSLP after both repeated hapten application and OVA cutaneous patch models were high when compared with control mice (Fig. 6b, right panel). These results suggest that delivery of haptens promotes both IL-3 and TSLP responses, while delivery

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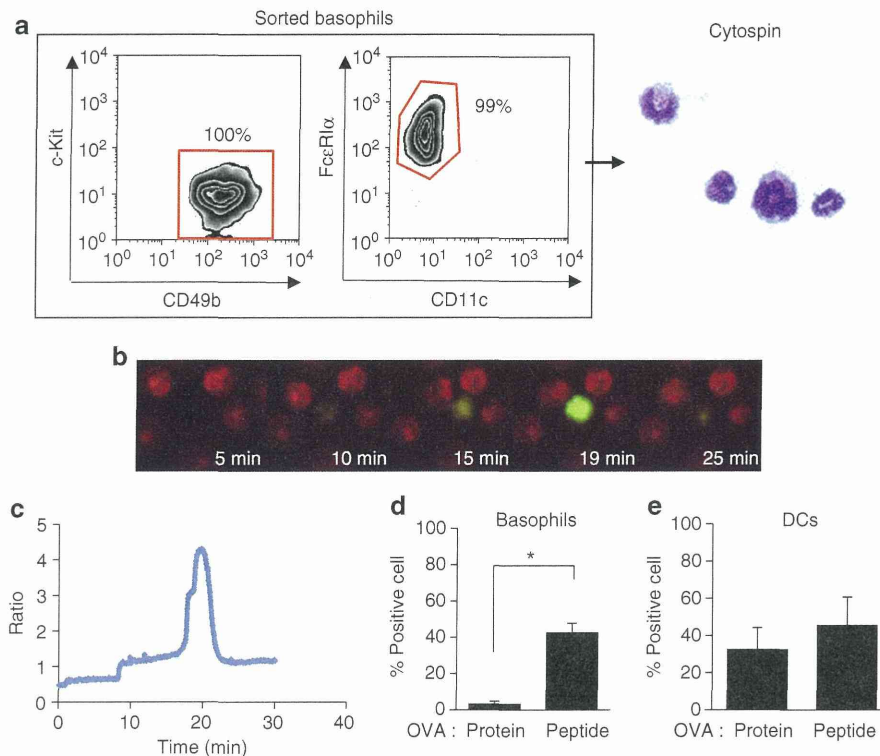

Figure 4 | Stimulation of T cells by BMBs with OVA peptide. (**a**) Sorted basophils exhibited their characteristic multilobed nuclei by cytology. (**b**,**c**) Incubation of Fluo-8-labelled naive CD4$^+$ T cells from DO11.10 and CellTracker Red-labelled BMBs with OVA protein or OVA peptide. Mixing with OVA peptide induced a prominent Ca^{2+} increase in T cells as shown in green fluorescence (**b**). The fluorescence intensity was expressed as a ratio to the initial value after subtracting background fluorescence (**c**). (**d**,**e**) The percentage of T cells demonstrating high Ca^{2+} concentration after mixing with OVA protein or OVA peptide in the presence of basophils (**d**) or DCs (**e**). All data are presented as the mean ± s.d. and are representative of three experiments.

of proteins preferentially promotes TSLP responses and impairs IL-3 response.

We next evaluated the nature of basophil populations using a repeated hapten application model in wild-type and TSLP receptor-deficient mice. The numbers of basophils in the skin and draining LNs in TSLP receptor-deficient mice was significantly decreased compared with those in wild-type mice (Fig. 6c,d). These results suggest that the nature of the basophil populations recruited to the skin and the draining LNs to be determined as TSLP-dependent at least in part in this model.

Discussion

In this study, we have demonstrated that basophils are responsible for Th2 skewing to haptens and peptide antigens but not protein antigens *in vivo*. We also found that basophils expressed MHC class II, CD40, CD80, CD86 and IL-4 in the hapten-induced Th2 model, but that basophils cannot take up or process OVA protein sufficiently using the DQ-OVA system. Basophils directly stimulated T cells via MHC class II/peptide complex and promoted IL-4 production in CD4$^+$ T cells upon exposure to peptide antigens but not to protein antigens *in vitro*. Intriguingly, basophils sufficiently promoted Th2 skewing upon OVA protein exposure in the presence of DCs. These results suggest that basophils alone are able to induce Th2 skewing with haptens and peptide antigens but require DCs for the induction of Th2 for protein antigens.

Despite recent reports that basophils act as APCs for helminth antigens or skin-injected papain[7,9,10], another group reported that basophils do not function as APCs for inhaled house dust mite allergens[11]. Therefore, there remained a discrepancy in the ability of basophils to act as APCs. Although the experimental models differ (for example, routes of antigen administrations), we assume that this discrepancy stems from the different type of antigens, such as protein, peptide and hapten. Hapten antigens may bind to MHC class II on the surface of basophils directly, and peptide can be acquired and presented by basophils, while protein antigens are not presented efficiently by basophils because they are hardly digested by these cells. In fact, previous reports demonstrated that basophils promote Th2 induction using OVA peptide but not OVA protein *in vitro*[7,9]. The protease allergen papain reaches the LNs after its cutaneous immunization and induces MHC class II expression on basophils in accord with preparing OVA peptide antigens from OVA protein *in vivo*[9]. Another group has reported that basophils pulsed with anti-2, 4-dinitrophenyl (DNP IgE) enhance Th2 skewing upon exposure to DNP-conjugated OVA by taking up DNP-OVA–IgE anti-DNP immune complexes[7]. Although house dust mites also contain cysteine protease activity, they are not sufficient for Th2 induction as they do not upregulate MHC class II on basophils in this model, even though cysteine proteases may function to generate peptide antigens from protein antigens *in vivo*[11].

Herein, we have shown that DCs support basophils to increase the frequency of IL-4–eGFP-positive CD4$^+$ T cells. As basophils cannot take up or process protein antigens efficiently, DCs may prepare peptide to be presented by basophils or may promote basophils to produce IL-4 to skew Th2. In line with this, our recent study has demonstrated that Langerhans cells, an epidermal DC subset, mediate epicutaneous sensitization with OVA protein antigens to induce Th2-type immune responses[23]. In addition, Th2 reactions are reduced upon sensitization with protein antigens or Schistosome infections using a CD11c-depletion model[11,12]; therefore, DCs seem to be necessary for Th2

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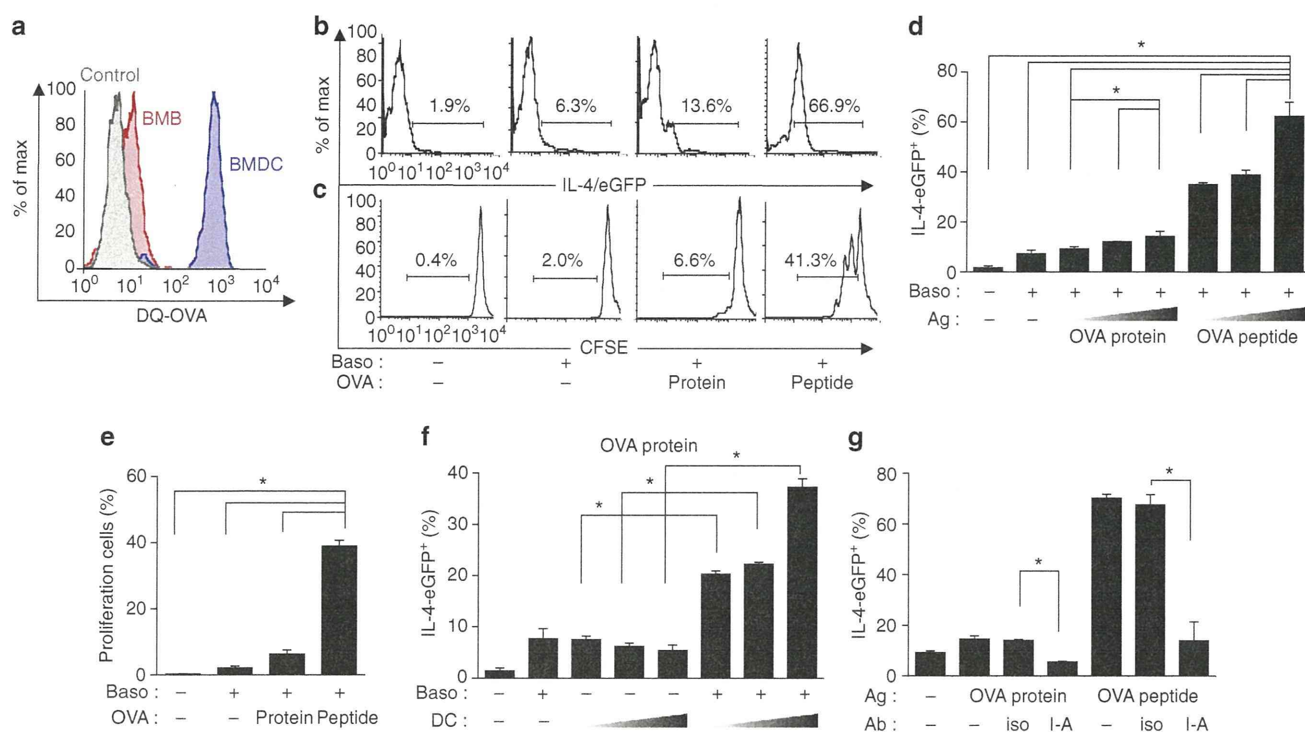

Figure 5 | Basophils were unable to take up and process protein antigens but induce Th2 differentiation with peptide antigens *in vitro*. (**a**) Using DQ-OVA that becomes fluorescent upon hydrolysis by phagocyte, we tested the ability to take up and process OVA by BMBs and DCs. (**b–d**) *In vitro* system of Th2 differentiation and T-cell stimulatory capacity. OVA-specific CD4$^+$ T cells from 4get × DO11.10 double transgenic mice (**b**,**d**) or from DO11.10 mice (**c**,**e**) were cocultured with basophils (Baso) in the presence of OVA protein or peptide. The frequency of IL-4/eGFP-positive cells, an indicator for Th2 differentiation, is shown (**b**,**d**). The populations of CFSE-diluted OVA-specific CD4$^+$ T cells after incubation for 1 day, as an indication for T-cell proliferation, were shown (**c**,**e**). (**f**) OVA-specific CD4$^+$ T cells were co-cultured with basophils (Baso) and/or DCs in the presence of OVA protein, and the frequency of IL-4-eGFP-positive CD4$^+$ T cells was shown. (**g**) The frequency of IL-4-eGFP$^+$ cells in the presence or absence of neutralizing anti-MHC class II antibody (iso: isotype-matched control, I-A: anti-MHC class II antibody). All data are presented as the mean ± s.d. and are representative of three experiments.

induction both *in vivo* and *in vitro* upon protein antigen exposure. As basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs, basophils, T cells and DCs may act towards Th2 induction in a coordinated way. In future, it will be intriguing to further evaluate whether DCs present peptide to basophils directly or by trogocytosis.

Taken together, basophils seem to be important for Th2 induction when stimulated with OVA peptide or hapten alone. Consistently, basophils hardly take up and process protein antigen compared with DCs (Fig. 5a). In line with this, DCs seem to be sufficient for Th2 induction when stimulated with OVA protein or both OVA protein and OVA peptide *in vivo*. Intriguingly, however, our *in vitro* data suggest that basophils support induction of Th2 responses upon OVA protein exposure in the presence of DCs (Fig. 5f). Therefore, DCs can to be sufficient for Th2 induction in the context of complex inflammatory environments like post-*S. mansoni* infection, where presumably various different ratios of small soluble antigens and larger proteins are present.

A fraction of basophils have the capacity to produce IL-4 in draining LNs in our model (Fig. 3c). Previously, Sullivan *et al.*[24] demonstrated that basophils produce IL-4 only in the lung tissues but not in the draining LNs when they used an *Nippostrongylus brasiliensis* infection model. In this study, we used a repeated hapten application model in which mice were immunized epicutaneously. We previously demonstrated that epidermal keratinocytes express a Th2 inducer TSLP and that TSLP-TSLP signalling is essential for IgE induction[23]. In addition, the number of basophils in the draining LNs in TSLP receptor-deficient mice was decreased compared with that in wild-type mice (Fig. 6d). At present, we still do not know how IL-4 is produced by basophils in the draining LNs in our model, but we assume that the TSLP-dependent basophils might be involved in our context.

Our results suggest that delivery of haptens and proteins differentially regulate TSLP and IL-3 response of basophils (Fig. 6b), which would provide critical insight into the reasons why some infections (which may be IL-3 or TSLP inducing) may be more DC- or basophil- dependent. It has been reported that serum IgE levels are one of the major factors that influence basophil populations[25]. Antigen-specific IgE was not detected in OVA protein or peptide sensitization models. On the other hand, TSLP is known to be elevated in the skin lesions of atopic dermatitis, upon barrier disruption, or by protease-containing antigen-exposure[26–28]. These conditions may affect heterogeneity in the basophil lineage (TSLP/IL-3), which has a role in each of these systems. It will be interesting to investigate the amounts of IL-3 and TSLP *in situ* upon stimulation with a variety of foreign antigens, including hapten, protein and bacteria, in a context-dependent manner.

Methods

Mice. Mice expressing human diphtheria toxin receptor under the control of 3′-UTR element in the *Il4* locus were generated for Bas TRECK by a transgenic strategy as reported previously[14,16]. C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). We treated C57BL/6 (B6) mice with DT as control and Bas TRECK Tg mice with DT as basophil-depleted mice. DO11.10 transgenic mice and 4get mice were purchased from Jackson Laboratory (Bar Harbor, ME). TSLP receptor-deficient mice (BALB/c) were kindly provided by Dr Steven Ziegler (University of Washington).

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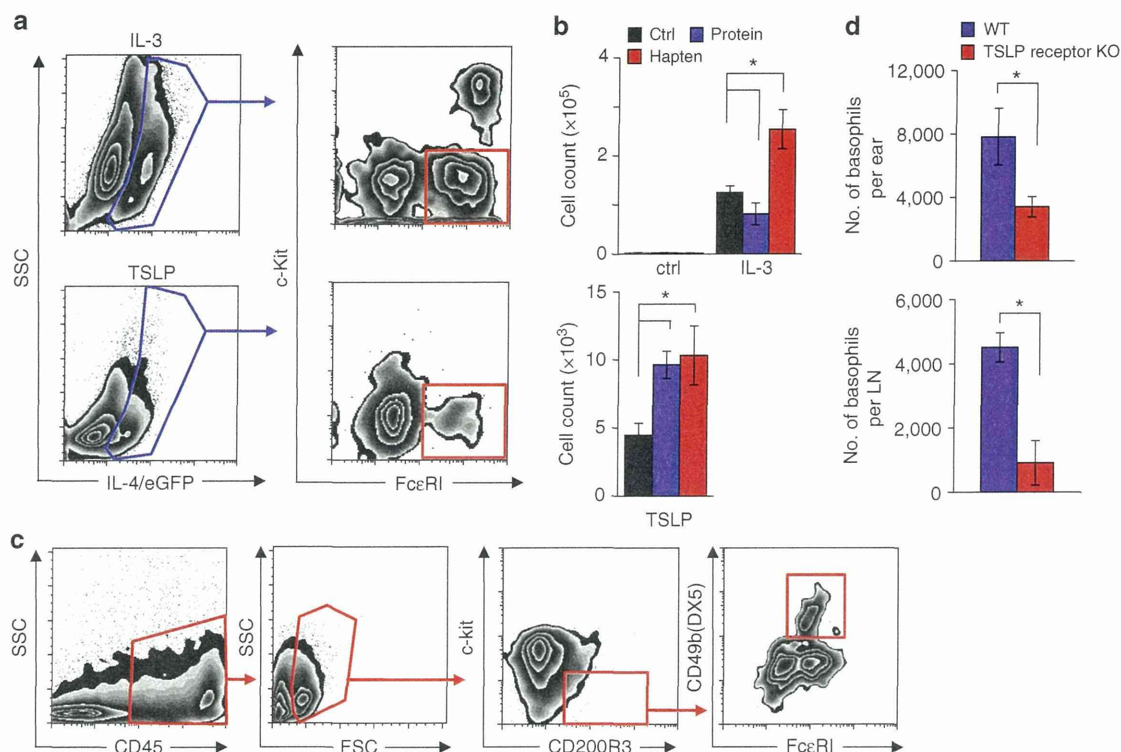

Figure 6 | Basophil development depends on the type of antigens. (**a**) BM cells were taken from 4get mice and cultured in the presence of IL-3 or TSLP. In IL-4/eGFP subsets, the FcεRI$^+$ c-Kit$^-$ basophil population is induced. (**b**) BM cells of mice with non-treatment, repeated hapten application (hapten) or OVA cutaneous patch (protein) were cultured in the absence (ctrl) or presence of IL-3 or TSLP, and basophil numbers were determined. (**c**) Basophils in the skin were detected as CD45$^+$ CD49b$^+$ FcεRI$^+$ CD200R$^+$ c-Kit$^-$ by flow cytometry. (**d**) The numbers of basophils in the skin and draining LNs of wild-type and TSLP receptor-deficient (KO) mice after repeated hapten application. All data are presented as the mean ± s.d. and are representative of two or three independent experiments.

For DT treatment, mice were injected intraperitoneally with 500 ng of DT per mouse on the first day. Then Bas-TRECK mice were treated at 3-day intervals[16]. Eight- to ten-week-old female mice were used for all of the experiments and were bred in specific pathogen-free facilities at Kyoto University. All experimental procedures were approved by the institutional animal care and use committee of the Kyoto University Graduate School of Medicine.

Reagents and antibodies. We purchased oxazolone from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), OVA from Sigma-Aldrich (St Louis, MO), OVA peptide from MBL (Nagoya, Japan), and DQ-OVA from Invitrogen (Carlsbad, CA). FITC-, PE-, PE-Cy7-, APC-, APC-7- and Pacific Blue-conjugated 145-2C11 (anti-CD3), N418 (anti-CD11c), 1C10 (anti-CD40), DX5 (anti-CD49b), 16-10A1 (anti-CD80), GL1 (anti-CD86), 2B8 (anti-CD117), M5/114.15.2 (anti-MHC class II), MEL-14 (anti-CD62L), eBioL31 (anti-CD207), 2B11 (anti-CXCR4), 4B12 (anti-CCR7), IgE (23G3), blocking antibody to IL-4 (11B11) (eBioscience, San Diego, CA), Ba103 (anti-CD200R3) (Hycult Biotech, Plymouth Meeting, PA), IL-4 (BVD4-1D11) (BD, Franklin Lakes, NJ), TUG8 (anti-MCP8) and 2G12 (anti-CCR4) (BioLegend, San Diego, CA) were purchased. Intracellular staining was performed with Cytofix/Cytoperm reagents (BD) according to the manufacturer's instructions and analysed with FACS Fortessa (BD). For the pretreatment of IL-4 staining in CD4$^+$ T cells, splenocytes were collected and incubated with PMA (20 ng ml^{-1}) and ionomycin (1 μM) for four hours. For the *in vivo* intracellular staining assay[29], mice were pretreated with intravenous injection with 500 μl of PBS solution containing 100 μg monensin (Sigma-Aldrich) for 6 h.

Epicutaneous sensitization and clinical severity. Epicutaneous sensitization with protein antigens, the clinical severity scoring of skin lesions, and the histological scoring were performed as reported previously[23,30]. In brief, mice were anesthetized with diethylether (Nacalai Tesque, Kyoto, Japan) and shaved with an electric shaver (THRIVE Co. Ltd., Osaka, Japan). A single skin site on each mouse was tape-stripped five times with adhesive cellophane tape (Nichiban, Tokyo, Japan). One-hundred milligram of OVA in 100 μl of normal saline was placed on the patch-test tape (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). Each mouse had a total of three 2-day exposures to the patch, separated by 1-day intervals. The total clinical score for skin lesions was designated as the sum of individual scores (graded none as 0, mild as 1, moderate as 2 and severe as 3) for the symptoms of pruritus, erythema, oedema, erosion and scaling. Immunohistochemical analysis was performed as reported previously[14].

Th2 induction model and ELISA. B6 and basophil-depleted mice were sensitized with intraperitoneal injections of alum (20 mg ml^{-1}) with OVA protein (100 μg ml^{-1}) or OVA peptide (10 μg ml^{-1}) on day 0, day 14, day 21 and day 28. Total serum IgE/IgG1/IgG2a levels were measured using a Bio-Rad (Hercules, CA) Luminex kit according to the manufacturer's instructions. To measure OVA/oxazolone-specific IgE/IgG1/IgG2a levels, the appropriate mouse IgE/IgG1/IgG2a ELISA kit (Bethyl Laboratories, Montgomery, TX) was used with slight modifications. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

Repeated oxazolone application. Repeated oxazolone treatment was performed essentially as described[31]. Mice treated with vehicle without oxazolone were used as control. Ear thickness was measured using a micrometre before, 1, 6, 24 and 48 h after challenge to assess inflammation. Ears were then collected for histology.

Quantitative PCR analysis. Quantitative PCR analysis was performed as reported previously[30]. The primer sequences for *TSLP*: 5′-ACGGATGGG GCTAACTTA CAA-3′ (forward) and 5′-AGTCCTCGATTTGCTCGAACT-3′ (reverse).

Generation of BMDC and BMB. Complete RPMI (cRPMI), RPMI 1640 medium (Sigma, St Louis, MO) containing 10% fetal calf serum (FCS) (Invitrogen), was used as the culture medium. For BMDC induction, 5×10^6 BM cells from BALB/c mice were cultured supplemented with 10 ng ml^{-1} recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 5 days (>90% expressed CD11c).

For BMB induction by IL-3, 5×10^6 BM cells from BALB/c mice were cultured supplemented with 10 ng ml^{-1} recombinant murine IL-3 (PeproTech) containing 20% FCS for 10 days. In some experiments, for BMB induction by TSLP or IL-3, BM cells from 4get mice were cultured in the presence of 1 μg ml^{-1} of TSLP for 5 days as reported previously[15].

Cytoplasmic Ca^{2+} *imaging*. T cells were incubated with 5 μM Quest Fluo-8 AM (ABD Bioquest, CA), and BMBs and BMDCs were stained with 2 μM CellTracker

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