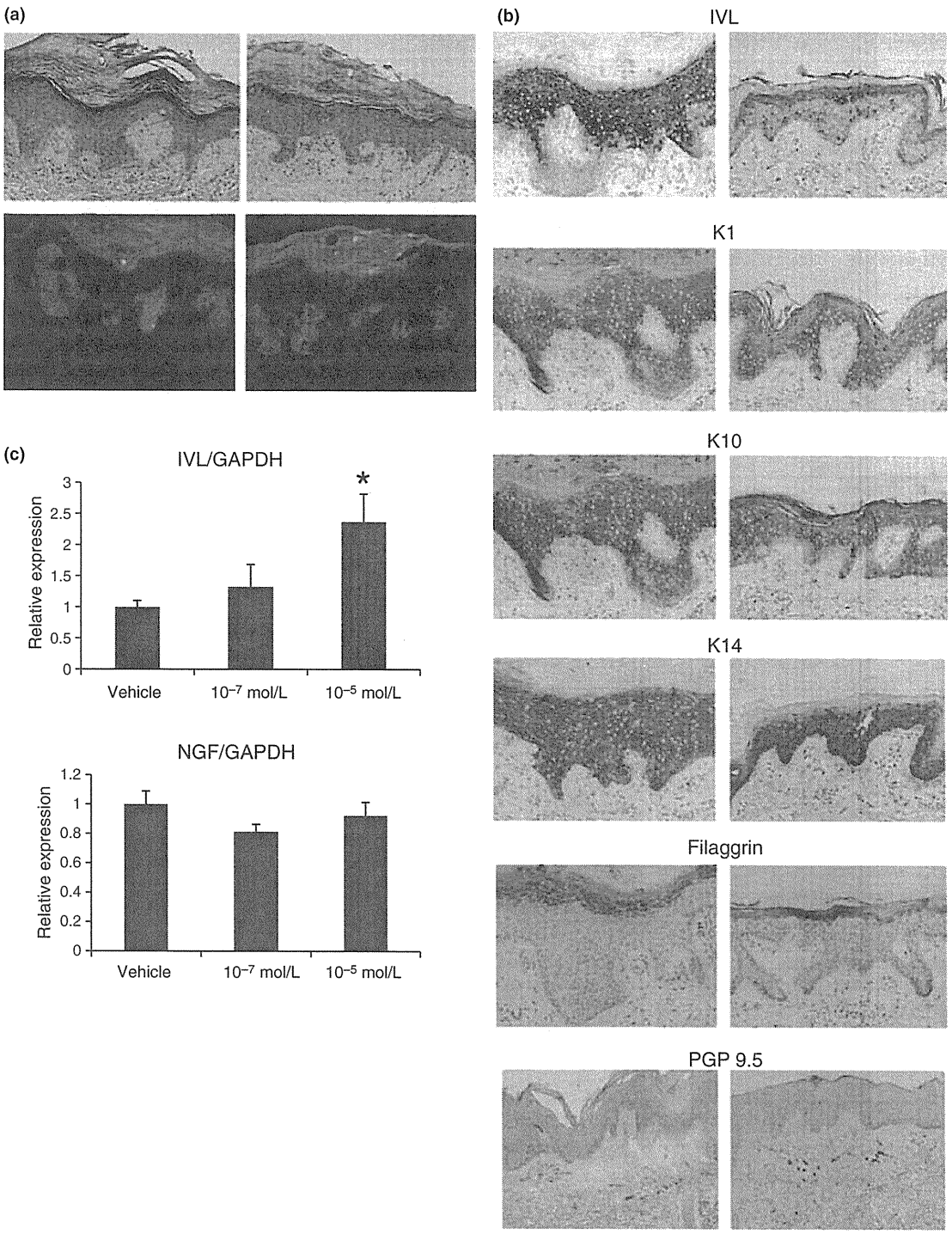

Figure 2. (a) Hematoxylin–eosin and thioflavin T staining of biopsied lichen amyloidosis lesion from patient 2 before (left) and after (right) tocoretinate treatment. (b) Immunohistochemistry of involucrin (IVL), cytokeratin 1 (K1), cytokeratin 10 (K10), cytokeratin 14 (K14), Filaggrin and protein gene product (PGP) 9.5 before (left) and after (right) tocoretinate treatment. (c) Relative expression of IVL and nerve growth factor (NGF) mRNA in normal human epidermal keratinocytes 6 h after adding tocoretinate (10^{-7} mol/L or 10^{-5} mol/L) or vehicle. Histogram shows the mean (± standard deviation). *P = 0.034 vs vehicle-treated group.

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patients (20%) did not respond after 10 weeks of treatment (Table 1). The mean response duration for the responders was 2.1 months (range 1–4). Pruritus and pigmentation were reduced and papules were flattened in LA patients (Fig. 1a–d). In MA patients, pigmentation and scratch marks on the upper back were significantly reduced (Fig. 1e,f). The lesions recurred gradually after tocoretinate treatment was discontinued in four patients. Of these four patients, two patients were treated with tocoretinate again and experienced satisfactory responses.

The downward extension of the epidermis improved and the granular layer became thickened after 3 months of topical tocoretinate treatment. Although the lesions clinically improved, amyloid deposits still existed in the upper dermis (Fig. 2a). Tocoretinate treatment also normalized the disturbed epidermal differentiation in LA lesions (Fig. 2b). The width of the broadened band of involucrin staining in LA lesions was dramatically reduced in the upper spinous and granular layers by tocoretinate application. Also, the expression of the spinous layer keratins K1 and K10 was reduced by tocoretinate treatment. Expression of the suprabasal layer keratin K14, which was extended to the spinous layer in lesions, was reduced after tocoretinate treatment although still expressed in the suprabasal layer. Filaggrin expression was observed in the entire granular layer in both pre- and post-treatment samples. The amount of PGP 9.5 stain was not significantly altered between pre- and post-treatment although cutaneous innervation was more apparent in pretreated skin. The mRNA expression of the keratinocyte differentiation marker IVL was dose-dependently upregulated by tocoretinate *in vitro* treatment (Fig. 2c), suggesting that tocoretinate causes keratinocyte differentiation, rather than keratinocyte proliferation. The mRNA expression of NGF did not change significantly after tocoretinate treatment (Fig. 2c).

DISCUSSION

Tocoretinate, the synthetic compound of tocopherol and retinoic acid, has been used for the treatment of skin ulcers because it enhances fibroblast migration and proliferation.[15] It also accelerates neovascularization (manufacturer information). In addition to its beneficial effects for the treatment of skin ulcers, tocoretinate is reported to improve skin manifestations of scleroderma, morphea and hypertrophic scars.[15] Although tocoretinate has not been reported to be useful for the treatment of LA or MA, the differentiation promoting properties of tocoretinate suggests that it may act similarly to the oral amyloidosis treatment acitretin. Acitretin is reported to be effective for the treatment of LA and biphasic amyloidosis.[2] The apoptosis-inducing and phagocytosis-stimulating effects of acitretin are thought to mediate its beneficial effects for amyloidosis. We evaluated the effect of tocoretinate, the compound of tocopherol and retinoic acid in this study as tocoretinate is a widely used ointment in Japan with rare side-effects. Tocoretinate had two differentiation-promoting effects for keratinocytes which may mediate its beneficial effects for amyloidosis treatment: (i) normalized epidermal differentiation *in vivo*; and (ii) elevation of the expression of the differentiation marker involucrin *in vitro*. Even though amyloid deposits remained detectable following tocoretinate treatment, clinical improvement was evident as a result of treatment. Tocoretinate also reduced pruritus and pigmentation, suggesting that it has other actions such as increasing macrophage phagocytosis and affecting peripheral sensory nerves. We measured NGF expression of keratinocyte, although tocoretinate did not alter its expression. It is also reported that innervations of the epidermis were unexpectedly diminished in LA recently.[16] We investigated the amount of nerve fibers by staining PGP 9.5. The amount of nerve fibers in the dermoepidermal junction was not significantly altered although cutaneous innervation was more apparently observed in pretreatment skin compared with post-treatment skin. This may be because of the reduction of pruritus. Further study is needed to understand the mechanisms of the tocoretinate effects on amyloidosis. Because of the rare side-effects and fast clinical improvement within 4 months of treatment, we propose that tocoretinate is a potential treatment for LA and MA cases that do not respond to other treatments.

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The course of pregnancy and childbirth in three mothers with recessive dystrophic epidermolysis bullosa

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Summary

Background. Recessive dystrophic epidermolysis bullosa (RDEB) is an autosomal recessive skin disease caused by mutations in the type VII collagen gene (*COL7A1*), resulting in detachment of the entire epidermis due to loss or hypoplasticity of the anchoring fibrils that normally secure the basement membrane to the underlying dermis. Trauma-induced blistering is often complicated by chronic erosions and scarring. From that perspective, pregnancy in RDEB might be considered an indication for elective caesarean section in a bid to minimize perineal blistering. To date, only four cases of pregnancy and delivery in patients with RDEB have been reported.

Cases. We report three more women, each with RDEB-generalized other (RDEB-GO), all of whom had successful vaginal deliveries without major cutaneous or mucosal complications. One woman also had a second child, by vaginal delivery, indicating a lack of vaginal stenosis after the first birth.

Conclusions. These cases show that RDEB-GO is not an absolute primary indication for elective caesarean section and that, perhaps surprisingly, genital/perineal blistering and scarring are not inevitable consequences of childbirth. Moreover, breastfeeding is also feasible in women with RDEB-GO.

Introduction

Epidermolysis bullosa (EB) comprises a heterogeneous group of inherited blistering skin diseases with widely varying degrees of severity.[1] Recessive dystrophic (RD)EB is an autosomal recessive disease producing severe blistering beneath the lamina densa of the dermoepidermal junction. It is caused by mutations on both alleles of the *COL7A1* gene, resulting in structural defects of the anchoring fibrils that normally secure the basement membrane to the underlying dermis.[2–4]

Severe generalized RDEB (RDEB-SG), which was previously known as the Hallopeau–Siemens type of RDEB, is generally caused by a combination of premature termination codon mutations resulting from nonsense, frameshift or splice-site mutations on both *COL7A1* alleles. These mutations lead to either nonsense-mediated decay of the mRNA, or to truncated polypeptides that are unable to assemble into functional anchoring fibrils. Less severe forms of RDEB, called RDEB-generalized other (RDEB-GO), are often caused by compound heterozygous mutations such as one premature termination codon mutation and one missense mutation. As a result, full-length type VII collagen

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polypeptides can be synthesized from the allele with the missense mutation, but the substituted amino acid affects the stabilization of the anchoring fibrils by disulfide bonding, or results in other structural changes.[4–6]

Because the skin fragility in RDEB is often followed by delayed wound healing and scarring, pregnant women with RDEB (or their carers) may be concerned about vaginal delivery because of the possibility of severe genital blistering and scar formation. However, caesarean section is not a straightforward alternative in patients with RDEB because there may be erosions on the back that interfere with spinal anaesthesia, or blisters/scarring in the mouth and upper airway that compromise the safety or practicality of general anaesthesia. Guidelines for best practice and indeed sensible advice for the management of pregnancy and childbirth in RDEB are currently lacking. Moreover, only three previously published reports have addressed pregnancy and delivery in RDEB.[1,2,7] We report three further women with RDEB who had safe pregnancies and vaginal deliveries.

Case reports

All three patients were Japanese, and all had been monitored since birth. All three had generalized cutaneous blisters and scars at sites of minimal trauma that had occurred since birth. Their fingers had adhered to each other, resulting in pseudosyndactyly. Their nails were deformed and some nail plates were lost.

Patient 1

This patient had cutaneous blisters present from birth, and over the years, many generalized cutaneous blisters, erosions, ulcers and scars had developed as a result of gentle mechanical trauma (Figs 1a,b). The molecular diagnosis of RDEB-GO was based on the detection of two compound heterozygous *COL7A1* mutations, c.8644G>A (p.Glu2882Lys) and c.8569G>T (p.Glu2857X), resulting in abnormal type VII collagen production. The first mutation is a missense mutation inherited from the patient's mother, and the second is a nonsense mutation inherited from her father.

The patient became pregnant at the age of 30 years. Pregnancy was confirmed by ultrasonography examination, and during gestation, there were no major complications or abnormalities related to the pregnancy. After 36 weeks and 6 days of gestation, the patient was admitted because of severe general fatigue and inability to manage her skin lesions by herself at home. She had no genital mucosal ulcers, but the number of skin ulcers around her lower abdomen had increased as it became distended in pregnancy.

On admission, the patient had a poor appetite and she had not been gaining weight during the latter part of her pregnancy. Laboratory investigations revealed the following: haemoglobin 9.0 g/dL (normal range 12.0–15.0 g/dL), albumin 2.5 g/dL (3.6–4.7 g/dL), cholinesterase 1018 U/L (2700–5600 U/L), total cholesterol 105 mg/dL (150–220 mg/dL), iron 30 mg/dL (41–127 mg/dL), prothrombin international normalized

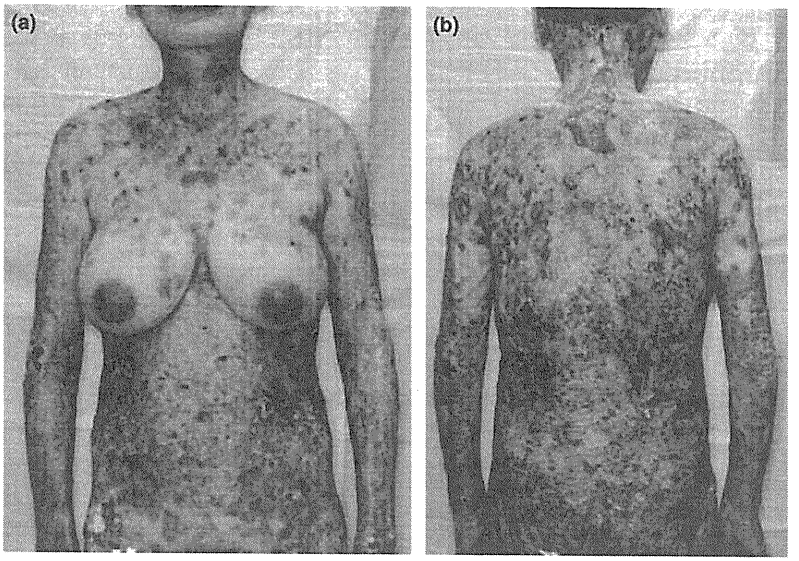

Figure 1 Patient 1. Several days after delivery: (a) anterior and (b) posterior views showing extensive cutaneous blisters.

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ratio 1.35 (0.85–1.15), white cell count 7040/mm^3 (3300–9400/mm^3), C-reactive protein 4.6 mg/dL (≤ 0.2 mg/dL), and platelets 34.5×10^4/mm^3 ($13.0–32.0 \times 10^4$/mm^3). The patient's Birmingham Epidemolysis Bullosa Severity (BEBS) score was 19.0.[8] Nevertheless, the growth of the fetus was normal.

On the estimated date of delivery (full term), the patient delivered a healthy boy by vaginal delivery with episiotomy. The baby was 2508 g (−1.6 SD) in weight, and had no signs of EB on his skin or mucous membranes.

The mother did not develop any major cutaneous or vaginal ulcers, and the total course of her delivery was similar to that of a woman without RDEB. The episiotomized perineum healed normally, and there was no stenosis of the patient's vagina. We advised her not to breastfeed the baby because his suckling might have caused new blisters on her breasts. The mother had no aftereffects of delivery, and the child developed normally.

Patient 2

The second patient had many generalized cutaneous blisters present since birth (Fig. 2), and the clinical diagnosis of RDEB-GO was confirmed by detection of compound heterozygous *COL7A1* mutations, c.7741G>A (p. Gly2581Arg) and c.8329C>T (p. Arg2777X), resulting in abnormal type VII collagen production.

The patient was 27 years old when she became pregnant, which was confirmed by ultrasonography examination. She had no genital mucosal ulcers, but she was admitted to our hospital for 2 weeks at 19 weeks gestation because of severe lower abdominal pain induced by a threatened miscarriage. However, the symptoms abated after bed rest and administration of ritodrine and cefotiam. The growth of the fetus was normal. The patient was admitted to our hospital at 38 weeks and 5 days of gestation in preparation for delivery. Her BEBS score was 11.6.

On the estimated date of delivery, labour pains started, and on the next day, a healthy boy, 2884 g in weight, was delivered vaginally after episiotomy. Significant bleeding accompanied the delivery, which almost induced hypovolaemic shock, but which settled after rapid infusion of intravenous fluids.

The mother's episiotomized perineum healed normally and there was no stenosis of her vagina. As there were no skin blisters around the breast, we encouraged her to breastfeed her baby. Breastfeeding was not associated with blistering of the nipples or areolae; she continued breastfeeding for 3 months and the baby developed normally.

Two years later, in 2002, the patient became pregnant for the second time. In week 27 of gestation, mild anaemia was detected, with haemoglobin levels of 9.9 g/dL. Intravenous administration of iron maintained the patient's haemoglobin levels. No significant genital mucosal lesions or scar-induced stenosis were noted before the birth, and the growth of the fetus was normal. On the estimated date of delivery, the patient was admitted to our hospital, and a healthy girl weighing 3108 g was delivered vaginally after episiotomy. The patient did not develop any major cutaneous or vaginal erosions or scarring during the subsequent follow-up period she breastfed her second baby for 3 months as well.

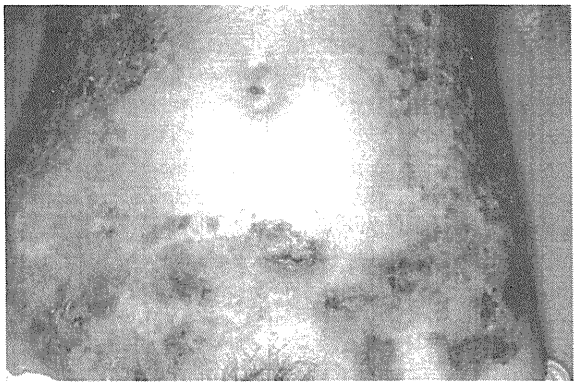

Figure 2 Patient 2. Abdomen with extensive blistering at the time the patient's first pregnancy was confirmed.

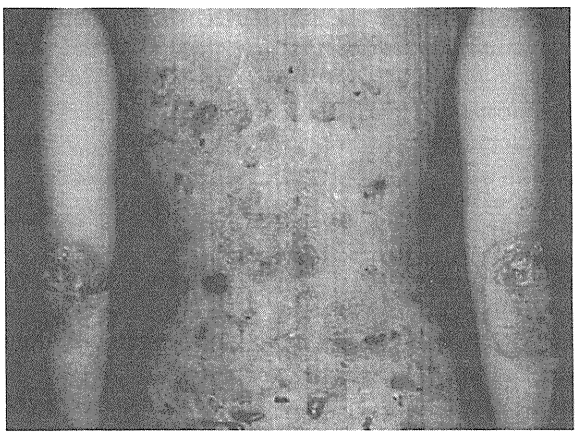

Figure 3 Patient 3. Posterior view taken 3 years after delivery; no cutaneous adverse events were seen.

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Table 1 Summary of RDEB pregnancies.

Ref.	Phenotype	Genotype	Complications	Delivery method	Adverse events
Bianca *et al.*[1]	RDEB-SG	c.8441-14del21	Genital mucous lesions, fetal growth retardation, premature rupture of membranes	Caesarean	Blistering around caesarean scar
Baloch *et al.*[2]	RDEB	Unknown	None	Vaginal	None
	RDEB	Unknown	Strong fear of vaginal delivery	Caesarean	None
Buscher *et al.*[7]	RDEB-SG	Unknown	Mild anaemia, polyhydramnios	Vaginal (twice)	None
Present study					
Patient 1	RDEB-GO	c.8644G>A and c.8569G>T	Severe general fatigue, moderate anaemia	Vaginal	None
Patient 2	RDEB-GO	c.7741G>A and c.8329C>T			
First delivery			Threatened spontaneous abortion	Vaginal	Much bleeding
Second delivery			Slight anaemia	Vaginal	None
Patient 3	RDEB-GO	Unknown	Moderate anaemia, HCV carrier	Vaginal	None

HCV, hepatitis C virus; RDEB, recessive dystrophic epidermolysis bullosa; RDEB-SG, RDEB-severe generalized; RDEB-GO, RDEB-generalized other.

Patient 3

The third patient also had many generalized cutaneous blisters since birth (Fig. 3), and had been diagnosed with RDEB-GO based on her clinical and skin biopsy findings but without genetic testing. She was a carrier of the hepatitis C virus.

At the age of 21 years, pregnancy was confirmed by ultrasonography examination. The number of skin ulcers around patient's lower abdomen increased during the pregnancy, but no genital mucosal ulcers occurred; however, she was admitted to hospital for several weeks to treat the skin ulcers. She was found to have moderate anaemia, with a haemoglobin level of 9.0 g/dL at 10 weeks of gestation, and received an iron preparation. Her BEBS score was 12.5. and the growth of the fetus was normal.

At 39 weeks and 6 days, a 2996 g healthy boy was delivered vaginally. The baby had no signs of EB on his skin or mucous membranes. The patient did not develop any major cutaneous or vaginal ulcers, and there was no scarring noted in subsequent follow-up. In this case, we recommended avoidance of breastfeeding because of skin ulceration and pain around the nipple.

Discussion

We report three women with RDEB-GO who each had successful vaginal delivery to give birth to healthy children who had no signs of skin fragility. Of note, all three women were engaged in sexually active relationships, emphasizing that this form of mechanobullous disease is not necessarily an impediment to regular vaginal intercourse. As shown in the summary of RDEB pregnancies, including our cases reported here (Table 1), caesarean section was selected only in two previously reported cases, because of presence of genital blistering or a strong fear of the potential complications that might follow vaginal delivery. Surprisingly, however, for those women who underwent vaginal delivery, there were no major adverse sequelae such as vaginal stenosis, even in an individual with RDEB-SG,[7] suggesting that the genital mucosa in RDEB can withstand vaginal delivery.

In general, caesarean section may be indicated for pregnant women with a history of previous caesarean section or dystocia, or in cases of fetal distress or breech presentation. It may also be indicated for women with placenta praevia, severe pre-eclampsia, eclampsia or active genital herpes simplex virus infection.[9] Medical considerations for caesarean section may also include heart failure, which could be relevant in RDEB, as the disease can be associated with dilated cardiomyopathy, and therefore careful assessment of cardiac function is advisable.[10] None of our three patients had any general obstetric indications for caesarean section, although all presented with slight or moderate iron-deficiency anaemia, a common complication in RDEB.[11] Heart function was assessed and deemed normal in all cases, and none presented with any local contraindication to vaginal delivery such as any pre-existing genital mucosal ulcers or blisters. Taken together, our three cases suggest that vaginal delivery can occur safely in patients with RDEB in the absence of any other obstetric or medical contraindications or concerns.

Another possible issue for mothers with RDEB is whether breastfeeding is appropriate, given the possibility of inducing significant blistering.[7] However, our

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second patient breast-fed without adverse consequences, suggesting that it may not be necessary to avoid breastfeeding if there are no pre-existing skin lesions around the breasts or nipples.

Conclusion

We report three cases of successful vaginal delivery for childbirth in pregnant mothers with RDEB-GO. Although further cases will add to the clinical experience and help refine the advice, our observations indicate that pregnancy, vaginal delivery and breastfeeding are not specifically associated with major problems or sequelae in women with RDEB-GO.

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Diminished regulatory T cells in cutaneous lesions of thymoma-associated multi-organ autoimmunity: a newly described paraneoplastic autoimmune disorder with fatal clinical course

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Summary

Thymoma-associated multi-organ autoimmunity is a rare, autoimmune disease that causes colitis, liver dysfunction and cutaneous graft-*versus*-host (GVH)-like skin damage. This paraneoplastic autoimmune disorder may be due to inadequate T cell selection in the tumour environment of the thymus. Although sporadic case reports have revealed its clinical features, little is known about its pathological mechanism. By comparing the skin-infiltrating T cell subsets with those of GVH disease (GVHD) and other inflammatory skin diseases, we sought to elucidate the pathological mechanism of thymoma-associated multi-organ autoimmunity. Histopathological and immunohistochemical analysis of skin biopsies was performed for three patients with thymoma-associated multi-organ autoimmunity. Histopathological findings of thymoma-associated multi-organ autoimmunity were indistinguishable from those of patients with acute GVHD, although the aetiologies of these diseases are completely different. The frequency of regulatory T cells (T_{regs}) is reduced in cutaneous lesions and $CD8^+$ cytotoxic T lymphocytes that massively infiltrate into the epidermis of patients with thymoma-associated multi-organ autoimmunity. Additionally, the ratio of T helper type 17 (Th17) cells to $CD4^+$ cells in patients with thymoma-associated multi-organ autoimmunity and acute GVHD was higher than that in healthy controls, but similar to that in psoriasis vulgaris patients. Similarity of the skin-infiltrating T cell subsets with those of acute GVHD suggested that skin damage in patients with thymoma-associated multi-organ autoimmunity might be induced by self-reactive cytotoxic T lymphocytes under the diminished suppressive capacity of T_{regs}.

Keywords: autoimmunity, graft-*versus*-host disease (GVHD), regulatory T cells, thymus, Th17

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Introduction

Thymoma-associated multi-organ autoimmunity, a rare autoimmune disorder, is known to cause colitis, liver dysfunction and cutaneous graft-*versus*-host (GVH)-like skin damage [1–9]. Although sporadic case reports have revealed its clinical features, little is known about its pathological mechanism.

As reported previously, thymoma patients occasionally develop paraneoplastic autoimmunity, such as myasthenia gravis (MG), pure red cell aplasia and acquired hypogammaglobulinaemia [10,11], probably because of abnormal T cell maturation within the tumour environment.

GVH disease (GVHD) is caused by the activation of donor T cells that recognize recipient antigens in normal tissues and show clonal expansion after haematopoietic cell transplantation [12]. In normal individuals, peripheral tolerance is maintained by regulatory T cells (T_{regs}), even if self-reactive T cells escape negative selection in the thymus. The development of $CD4^+CD25^+$ T_{regs} depends on the forkhead box P3 transcription factor (FoxP3), which is a specific marker for T_{regs} [13,14]. Recent studies have revealed that the number of T_{regs} is reduced in allografts, peripheral blood and the skin lesions of recipients of transplants with acute or chronic GVHD [15–18]. More recently, it was demonstrated that increased numbers of interleukin (IL)-17-producing $CD4^+$ [T helper

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type 17 (Th17)] cells in the peripheral blood correlate strongly with inflammatory processes and the clinical status of acute GVHD (aGVHD) and active, chronic GVHD [19].

Here, we demonstrate that the frequency of T_{regs} is reduced in the cutaneous lesions of patients with thymoma-associated multi-organ autoimmunity compared with healthy individuals or individuals with other inflammatory skin diseases. Similar to aGVHD, dominant $CD8^+$ cytotoxic T lymphocyte (CTL) infiltration in the epidermis suggests that skin damage in patients with thymoma-associated multi-organ autoimmunity might be induced by self-reactive CTLs under the diminished, suppressive capacity of T_{regs}.

Case reports

The clinical data of our three thymoma patients are summarized in Table 1).

Case 1

We have reported previously the case of a 40-year-old Japanese woman presenting with psoriasiform erythroderma caused by thymoma-associated multi-organ autoimmunity [7], whereas others have reported similar cases [1–6,8,9]. This patient was diagnosed with MG associated with type-B1 thymoma, as defined by the World Health Organization (WHO) classification, at 23 years of age, and extended thymectomy followed by radiation therapy (RT) was performed [20]. After several years, tacrolimus was introduced for recurrent MG. At 37 years of age, ablative surgery and RT were performed for multiple disseminated tumours in the left pleural cavity. Two years later, multiple recurrent tumours appeared in the peritoneal cavity, which were resistant to chemotherapy. Subtotal resection of the peritoneal tumours with bilateral oophorectomy was performed. For mass reduction and relief of MG, steroid pulse therapy replaced treatment with tacrolimus. A few days after steroid pulse therapy was started, generalized erythema appeared. Generalized, psoriasiform erythematous patches were fused on the trunk, developing into generalized erythroderma (Fig. 1). Although high doses of oral steroids and cyclosporin were continued, the patient developed liver dysfunction and diarrhoea. A skin biopsy specimen was taken from an erythematous skin lesion of the left dorsal foot. Erythroderma gradually improved over 2 months after the initiation of high-dose oral steroid therapy, but reappeared with discontinuation of steroid therapy. Five months after the first appearance of erythroderma, the patient died of sepsis.

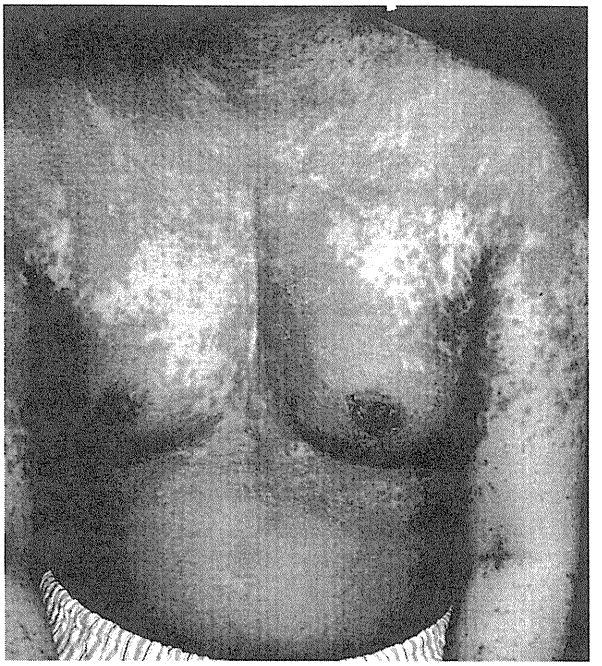

Fig. 1. Clinical appearance of thymoma-associated multi-organ autoimmunity on the trunk of case 1. Scaly erythemas were fused on the chest.

Case 2

A 36-year-old Japanese woman presented with psoriasiform erythroderma after thymoma relapse (Fig. 2). Extended

Table 1. Summary of patient characteristics.

	Case 1	Case 2	Case 3
Sex	Female	Female	Female
WHO classification of thymoma	B1	B1	B2
Age of thymoma detection (year)	23	31	39
Duration from thymoma detection to GVH-like disease onset (year)	18	5	3
Damaged organ due to GVH reaction			
Skin	+	+	+
Liver	+	+	–
Intestine	+	–	–
Complications	MG	MG	MG, SLE
Relapse of thymoma	+	+	+
Course of skin lesion	Relapse	Relapse	Better
Prognosis	Died after 5 months	Died after 3 years	Exacerbating thymoma

GVH, graft-*versus*-host; MG, myasthenia gravis; SLE, systemic lupus erythematosus.

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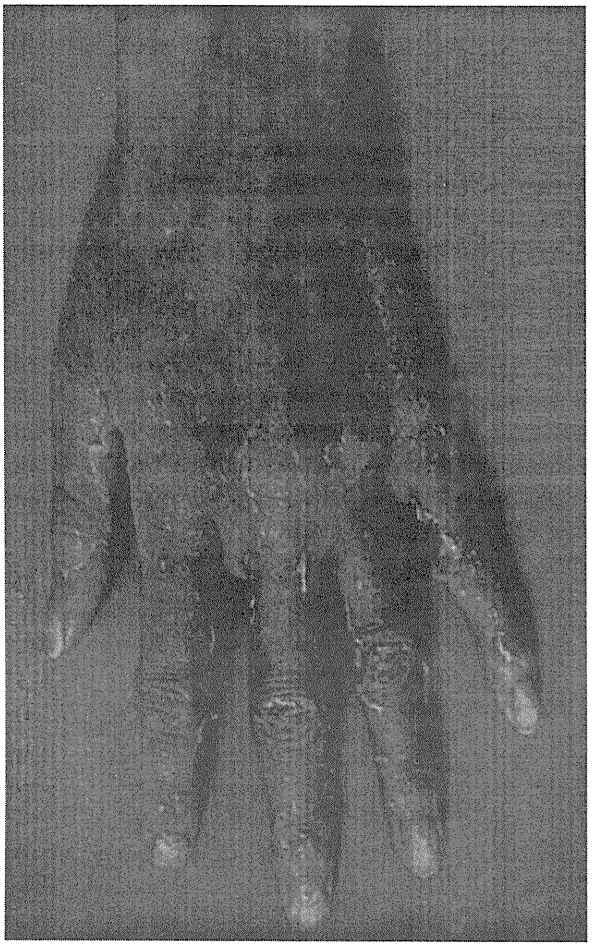

Fig. 2. Clinical appearance of thymoma-associated multi-organ autoimmunity on the hand of case 2. Erythroderma lesions were seen on the entire body.

thymectomy was performed for type-B1 thymoma associated with MG when the patient was 31 years of age. Despite treatment with predonisolone (10 mg/day), tacrolimus and ambenonium, the thymoma reappeared. Generalized erythema was improved by higher-dose prednisolone (20 mg/day) and topical steroid treatments; however, skin lesions recurred after withdrawal of prednisolone administration. Generalized erythemas were fused and developed into erythroderma after systemic steroid withdrawal, accompanied by alopecia, pneumonia and liver dysfunction. A skin biopsy was performed on an erythematous area of the abdomen. Three years after the first appearance of erythroderma, the patient died of pneumonia.

Case 3

A 42-year-old Japanese woman presented with scaling erythematous patches during the treatment of systemic lupus erythematosus with prednisolone (10 mg/day) since age 38 years. Thymoma was revealed by chest computed tomography, and needle biopsy showed type-B2 thymoma-infiltrating pleura. Subsequently, she developed eyelid ptosis and was diagnosed with MG. Chemotherapy was slightly effective in reducing the tumour size and subtotal resection was performed for thymoma removal, followed by chemotherapy and RT. Generalized psoriasiform erythroderma and oral erosions appeared during the RT course. A skin biopsy was taken from the involved area on the left upper arm (Fig. 3). Skin lesions disappeared with prednisolone (30 mg/day); however, oral aphthae recurred after withdrawal of systemic steroids. The patient developed pleural dissemination and thymoma metastasis to the lymph nodes and was started on a weekly docetaxel regimen. However, the adverse effect of glossitis was too severe to continue treatment.

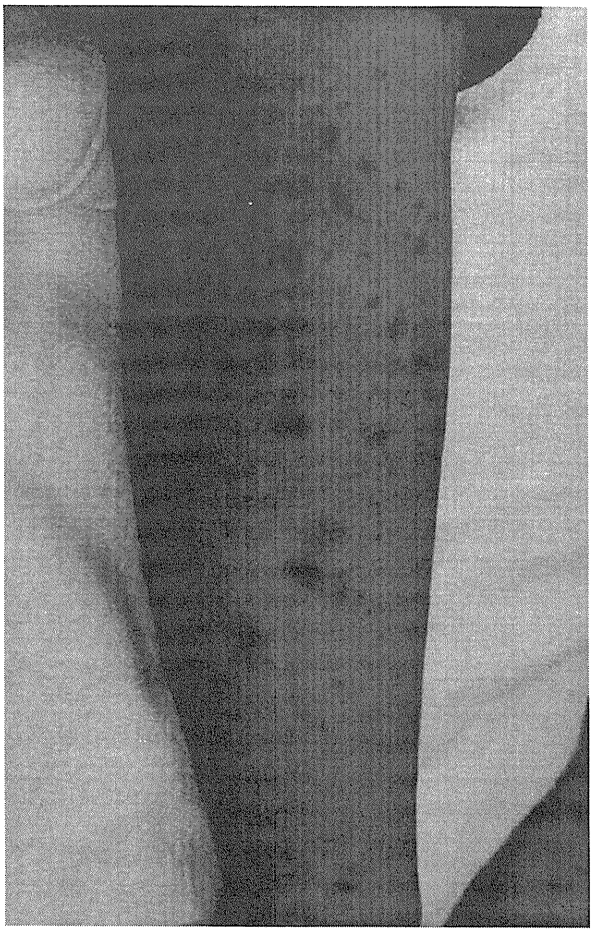

Fig. 3. Clinical appearance of thymoma-associated multi-organ autoimmunity on the left upper arm of case 3. Multiple erythemas approximately 10 mm in diameter were seen on the entire body. Aphthas in the oral cavity were also observed.

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Materials and methods

Samples and immunohistochemical analysis

Skin biopsy tissues were fixed with 10% formaldehyde, and paraffin-embedded sections were stained with haematoxylin and eosin and analysed by immunohistochemistry. Immunohistochemical staining was performed on skin sections from the three thymoma-associated multi-organ autoimmunity patients described in the case reports, three acute GVHD (aGVHD) patients, three lichen planus (LP) patients, three psoriasis vulgaris patients and three healthy controls. aGVHD, LP and psoriasis vulgaris were diagnosed on clinical appearance and histopathology. All aGVHD patients were treated with immunosuppressive therapy. All psoriasis vulgaris patients were treated only with topical steroids. No LP patients were treated. Because our thymoma-associated multi-organ autoimmunity patients and aGVHD patients were treated with immunosuppressive therapy, the effect of this medication on their immune condition cannot be excluded in this study. Three-micrometer-thick sections were stained with the following monoclonal antibodies (mAbs): anti-CD4 antibody (CD4 mAb, clone 1F6, dilution 1:25; Novocastra, Newcastle, UK); anti-CD8 mAb (CD8 mAb, clone C8/144B, dilution 1:100; DakoCytomation, Minneapolis, MN, USA); anti-CD1a mAb (CD1a mAb, clone 010, dilution 1:50; DakoCytomation); anti-FoxP3 mAb (FoxP3 mAb, clone 236A/E7, dilution 1:100; Abcam, Cambridge, UK); and anti-IL-17 antibody (polyclonal IL-17 antibody, dilution 1:150; R&D Systems, Minneapolis, MN, USA). Immunohistochemistry was performed as described previously [21,22]. For FoxP3 staining, Dako LSAB$^+$/AP was used, whereas for other immunohistochemical staining, the Dako ChemMate Envision Kit/horseradish peroxidase (HRP) was used.

Quantification of the frequency of immunostained cells in the upper dermis was performed in single-stained serial sections. The number of FoxP3$^+$ T_{regs} and IL-17$^+$ Th17 cells was quantified (mean number/high power field calculated in three non-adjacent, high-power fields) and related to the number of CD4$^+$ T lymphocytes (FoxP3$^+$/CD4$^+$ ratio and IL-17$^+$/CD4$^+$ ratio, respectively). The number of FoxP3$^+$ T_{regs} was also related to the number of CD8$^+$ T lymphocytes (i.e. FoxP3$^+$/CD8$^+$ ratio).

Results

Figure 4 shows the results from histopathological and immunohistochemical analyses of skin biopsies from three thymoma-associated multi-organ autoimmunity patients described in the case reports. As shown by haematoxylin and eosin staining, focal liquefaction degeneration of the basal epidermal layer, presence of superficial perivascular lymphocytes, infiltration with exocytosis and the presence of dyskeratotic keratinocytes (satellite cell necrosis) were found in all three cases. CD1a$^+$ Langerhans cells disappeared completely from the epidermis, and CD8$^+$ CTLs infiltrated massively into the epidermis (Fig. 4). These findings are consistent with the histopathological features of aGVHD (data not shown), as reported previously [23,24].

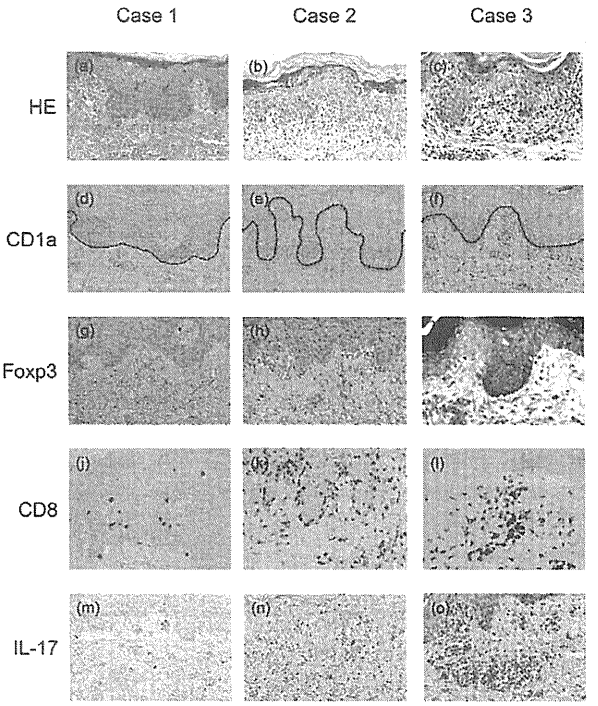

Fig. 4. Haematoxylin and eosin staining revealed graft-*versus*-host (GVH)-like reactions in cases 1–3 (a–c). Immunohistochemical staining revealed the following: CD1a$^+$ Langerhans cells disappearing from the epidermis (d–f); few forkhead box P3 (Fox P3)$^+$ regulatory T cells (T_{regs}) expressed (g–i); CD8$^+$ cytotoxic T lymphocytes infiltrating the epidermis (j–l); and presence of interkeukin (IL)-17$^+$ cells in the dermis (m–o). Dotted lines represent the basal epidermal layer (original magnifications: ×100).

Because thymoma-associated multi-organ autoimmunity has many clinical and histopathological similarities with post-haematopoietic cell transplantation aGVHD, we further examined infiltrating T cell subsets in the skin for the presence of FoxP3$^+$ T_{regs}. As reported previously, T_{regs} are found sparsely in the skin lesions of patients with aGVHD [18]. In this study, compared to healthy controls, the percentage of skin-infiltrating FoxP3$^+$ T_{regs} per number of CD4 T cells decreased in patients with thymoma-associated multi-organ autoimmunity, whereas T_{regs} increased in LP and psoriasis vulgaris patients (Fig. 5a). The percentage of skin-infiltrating FoxP3$^+$ T_{regs} per number of CD8$^+$ T cells was also profoundly decreased in thymoma-associated multi-organ autoimmunity and aGVHD compared with psoriasis vulgaris (Fig. 5b).

The scaling erythematous skin lesions seen in our three thymoma-associated multi-organ autoimmunity cases was clinically indistinguishable from patients with psoriasis

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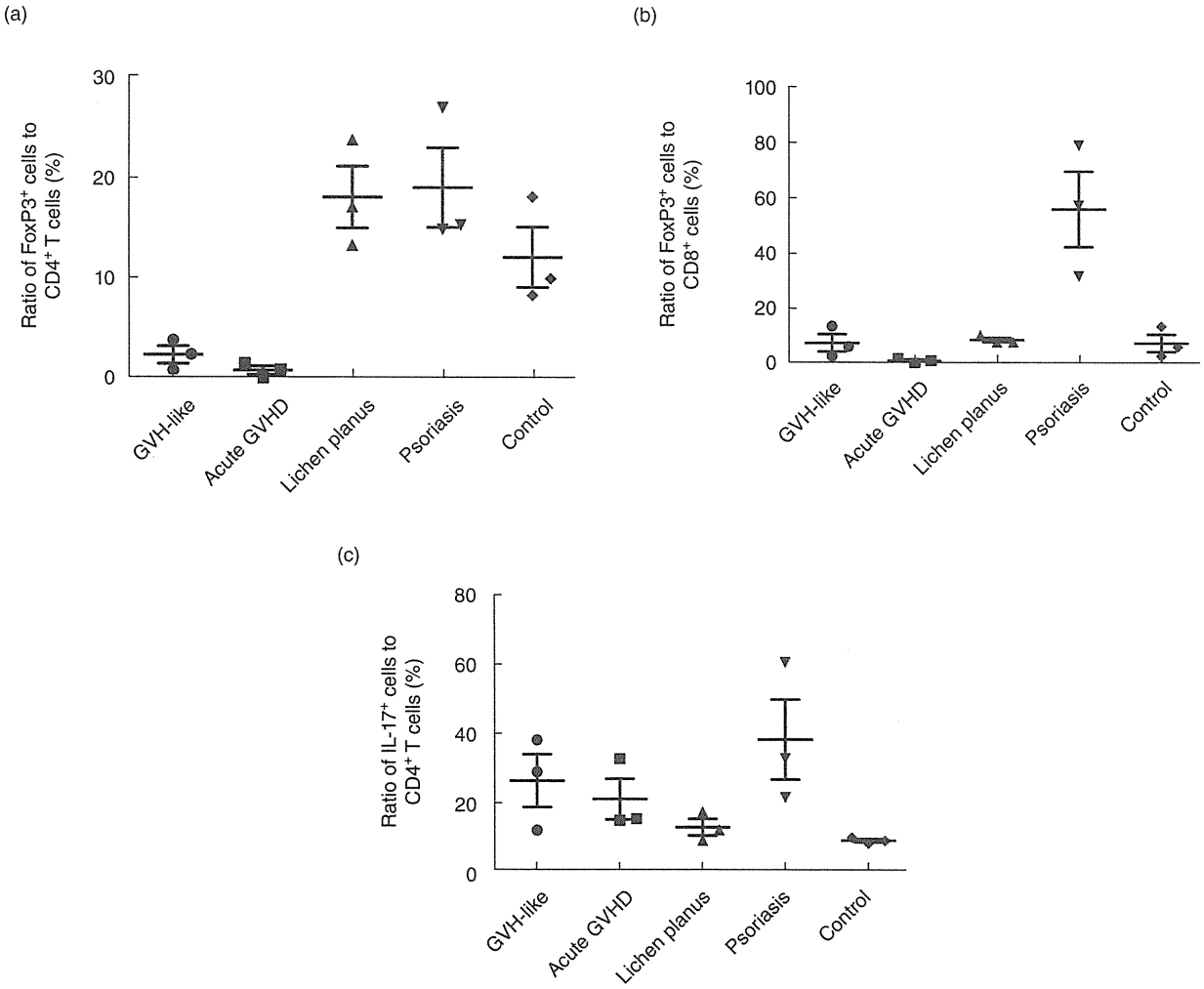

Fig. 5. (a) In cases 1–3 and patients with acute graft-*versus*-host disease (aGVHD), the ratio of regulatory T cells (T_{regs}) to $CD4^+$ T cells was reduced compared to that in healthy controls, while the ratio in lichen planus (LP) and psoriasis vulgaris patients increased. (b) In cases 1–3 and patients with aGVHD, the ratio of T_{regs} to $CD8^+$ T cells was reduced compared to that in patients with psoriasis vulgaris. (c) In cases 1–3 and patients with aGVHD, the ratio of T helper type 17 (Th17) cells to $CD4^+$ cells was increased as much as that seen in psoriasis vulgaris patients compared to that in either LP or healthy controls. Horizontal bars represent the mean value and the mean ± standard deviation of each group.

vulgaris, although the histopathology was quite different between the two sets of patient materials (data not shown). Recent reports suggest that Th17 cells are key players in the induction of psoriatic skin lesions and putative targets for therapeutic intervention [19]. Therefore, we assessed skin-infiltrating Th17 cells. In thymoma-associated multi-organ autoimmunity and aGVHD patients, IL-17-producing cells infiltrated into the upper dermis, mainly into the perivascular regions. The ratio of Th17 cells per number of $CD4^+$ cells in patients with thymoma-associated multi-organ autoimmunity and aGVHD was higher than that in LP or healthy controls, but similar to that in psoriasis vulgaris patients (Fig. 5c). Thus, skin-infiltrating T cell subsets are quite similar between patients with thymoma-associated multi-organ autoimmunity and those with aGVHD.

Discussion

In the normal thymus, immature T cells are positively selected by major histocompatibility complex peptides, depending on T cell receptor affinity [9,25,26]. Self-reactive T cells are usually depleted by medullary thymic epithelial cells. Central tolerance depends largely on the autoimmune regulator (Aire) gene, which controls the ectopic expression of a wide range of peripheral tissue-specific antigens in medullary thymic epithelial cells [27,28]. Recently, the complete lack of Aire and minimal expression of FoxP3 in intratumoural T cells were reported in patients with enterocolonopathy caused by thymoma-associated multi-organ autoimmunity [9], suggesting that self-reactive T cells, but not T_{regs}, might be preferentially differentiated in thymomas.

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In addition, self-reactive T cells might escape negative selection because professional antigen-presenting cells that 'educate' naive T cells in the normal thymic medulla are absent in thymoma [29]. This failure of central tolerance might cause autoimmune diseases in thymoma patients. In our study, sparse FoxP3$^+$ T_{regs} in the dermis and massive CD8$^+$ CTL infiltration in the epidermis were common features of both thymoma-associated multi-organ autoimmunity and aGVHD patients. T_{regs} are reduced in the skin lesions of patients with systemic sclerosis, which may be responsible for the loss of tolerance in the autoimmune skin diseases [30,31]. CD8$^+$ CTLs are the major cellular effectors of aGVHD in either the Fas–Fas ligand or perforin/granzyme pathway [32]. We speculate that insufficient generation or skin recruitment of FoxP3$^+$ T_{regs} might cause self-reactive CTL-induced cutaneous GVH-like reactions.

We found that the frequency of Th17 cells in the skin lesions of patients with thymoma-associated multi-organ autoimmunity was increased by showing an increased number of IL-17$^+$ cells among the CD4$^+$ population. Increased numbers of Th17 cells in the peripheral blood are correlated strongly with inflammatory processes in GVHD and have been shown previously [19]. The clinical appearances of our three cases were similar to those of psoriasis, another Th17-mediated dermatosis [33,34]. As seen in patients with aGVHD or psoriasis vulgaris, the ratio of IL-17$^+$ cells to CD4$^+$ T cells increased in patients with thymoma-associated multi-organ autoimmunity.

In conclusion, thymoma-associated multi-organ autoimmunity provides useful information for understanding the pathological differences and similarities between autoimmune skin diseases and GVH-like reactions, especially for the involvement of T_{regs}, CTLs and Th17 cells. To understand more about thymoma-associated autoimmunity, long-term observations of the T cell repertoire might be useful for monitoring effector and T_{regs}.

Disclosure

None.

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Case report

Psoriatic arthritis in two patients with an inadequate response to treatment with tocilizumab

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ABSTRACT

Psoriatic arthritis (PsA) is considered as one of the seronegative spondylarthropathies. Like rheumatoid arthritis (RA), the increased production of interleukin (IL)-6 suggests a pathogenic role of IL-6 in PsA. However, whether humanized anti-IL-6 receptor antibody such as tocilizumab (TCZ) might be effective for PsA as well as RA has yet to be determined. We report herein two cases of PsA treated using TCZ. Although, TCZ treatment resulted in disappearance of serum CRP in both patients, arthritis and skin lesions were not improved despite 6-month administration of TCZ. In contrast, tumor necrosis factor (TNF) inhibitor proved effective against arthritis and skin lesions in these patients. Collectively, these findings not only indicate that IL-6 has distinct pathological roles in RA and PsA, but also suggest that TNF inhibitor therapy (but not TCZ) is effective for arthritis and skin lesions of PsA.

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1. Introduction

Psoriatic arthritis (PsA) is a common inflammatory arthritis that is associated with psoriasis. Like rheumatoid arthritis (RA), increased production of interleukin (IL)-6 is well known in psoriasis and PsA [1,2].

Most reports have demonstrated correlations between serum levels of IL-6 and disease severity of PsA [3], mouse with epidermal over expression of IL-6 (K14-IL-6 transgenic mouse) exhibits phenotype of psoriasis [4]. The transcription factor signal transducer and activator of transcription 3 (STAT3) is up regulated in psoriasis and IL-6 induces STAT3 phosphorylation and are also thought to be potential therapeutic targets [5].

Characteristic features differ between PsA and RA. In PsA, peripheral arthritis evolves with a distinct joint pattern, potentially involving the distal interphalangeal joints. Dactylitis with enthesitis involving the entire digit is a characteristic feature. Furthermore, articular damage as assessed by radiographic erosion is more common than in RA and typically shows an asymmetric pattern in PsA. Despite these differences in characteristic features, therapeutic options including tumor necrosis factor (TNF) inhibitors and methods of assessing disease activity are mostly held in common. This suggests that IL-6 may play a similar role in the inflammatory arthritis in both RA and PsA.

A humanized anti-IL-6 receptor antibody, tocilizumab (TCZ), was recently approved for RA patients and efficacy of TCZ in patients with RA has been demonstrated [6]. PsA is included in the category of seronegative spondylarthritides, which are defined by the absence of rheumatoid factor and include diseases such as ankylosing spondylitis (AS) and reactive arthritis (ReA). Variable efficacy of TCZ has also been reported in patients with ReA [7] and AS [8–10], since the efficacy is variable pending of the evaluation. However, whether TCZ is effective for PsA as well as RA has not been determined. We therefore tried using TCZ in patients with PsA. Severity of inflammation was evaluated using erythrocyte sedimentation rate (ESR), and levels of C-reactive protein (CRP) and matrix metalloproteinase (MMP)-3. Severity of arthritis was evaluated by two measures of composite disease activity: Disease Activity Score including the 28-joint count (DAS28) and Clinical Disease Activity Index (CDAI). Severity of psoriatic skin was evaluated using the Psoriasis Area-and-Severity Index (PASI).

2. Case 1

A 35-year-old man was diagnosed with psoriasis in July 2002 and developed complications of arthritis in April 2004, particularly in bilateral knee and shoulder joints. Since prednisolone (5 mg/day) and cyclosporine (5 mg/kg/day) proved ineffective for improving arthritis, methotrexate was started at 6 mg/week, then the anti-TNF antibody Infliximab (IFX) was started at 3 mg/kg in November 2006. Although, arthritis and eruptions initially showed marked improvements, symptoms exacerbated in June 2008. Informed consent from the patient and approval by the ethics committee of Osaka

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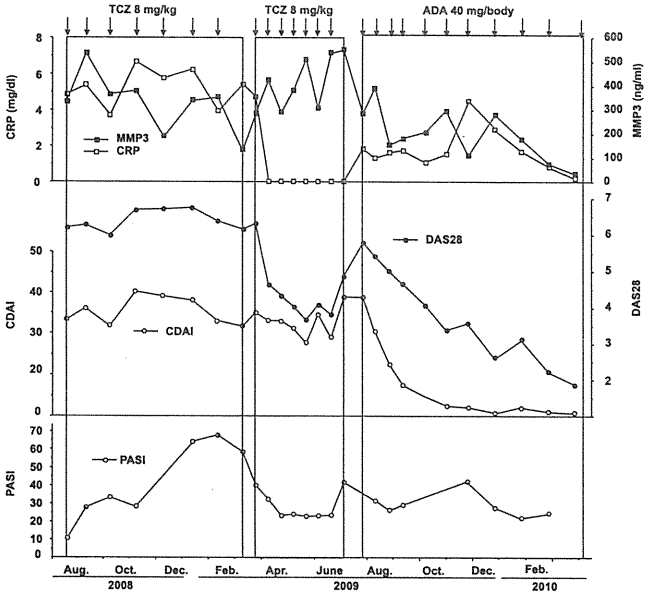

Fig. 1. Changes in C-reactive protein (CRP) level, matrix metalloproteinase (MMP)-3 level, Disease Activity Score including the 28-joint count (DAS28), Clinical Disease Activity Index (CDAI) and Psoriasis Area-and-Severity Index (PASI) score in case 1 during tocilizumab and adalimumab therapy.

University Hospital were obtained for TCZ treatment, which was then started at 8 mg/kg every 4 weeks in July 2008. IL-6 concentration was 77.6 pg/mL (normal, <4 pg/mL) at the time of starting TCZ. Clinical course is shown in Fig. 1. Before TCZ treatment, DAS-28 was 6.44, CDAI was 30.8 and PASI was 51.3. After seven infusions of TCZ, CRP was not improved (7.20 mg/dL to 5.71 mg/dL) suggesting that the 4-week interval infusion of TCZ was not sufficient to inhibit IL-6 function in this patient. Two-week interval infusion was then performed from April 2009 to June 2009. ESR and CRP then showed complete normalization. However, clinical symptoms remained unimproved and MMP-3 level tended to increase (290 mg/dL to 555 mg/dL). DAS28 was slightly improved (6.32 to 4.85), but this was attributed to ESR normalization (54.2 mm/hour to 4.0 mm/hour) by TCZ. If ESR was not included, clinical assessment by CDAI was unimproved (35.0 to 38.7). Skin disease activity as evaluated by PASI showed no improvement (40.5 to 42.1). Administration of TCZ was therefore suspended and injection of adalimumab (ADA) was started in June 2009. Although, ESR and CRP level increased (18 mm/hour and 1.31 mg/dL, respectively), clinical symptoms of arthritis, DAS28, CDAI and MMP3 levels were significantly improved (2.19, 1.6 and 73.5 mg/dL, respectively) by February 2010. Although, skin disease activity as assessed by PASI did not improve rapidly, significant improvement was achieved using ADA by April 2010 (42.1 to 16.5).

3. Case 2

A 28-year-old man was diagnosed with psoriasis in 1991 and developed complications with arthritis in 2002. Since response to DMARDs (disease-modifying antirheumatic drugs) including sulfasalazopiridine (1000 mg/day) and methotrexate (6 mg/week) was inadequate, he joined the Japanese phase III study of ADA in 2006. Arthritis and eruptions were significantly improved during ADA treatment. However, symptoms showed flare-up after the 1-year ADA therapy. In September 2008, he joined a Japanese phase III clinical study of Ustekinumab (fully human monoclonal immunoglobulin [IgG1] antibody targeting the interleukin [IL]-12/23 shared P40 subunit). CRP and MMP-3 levels and

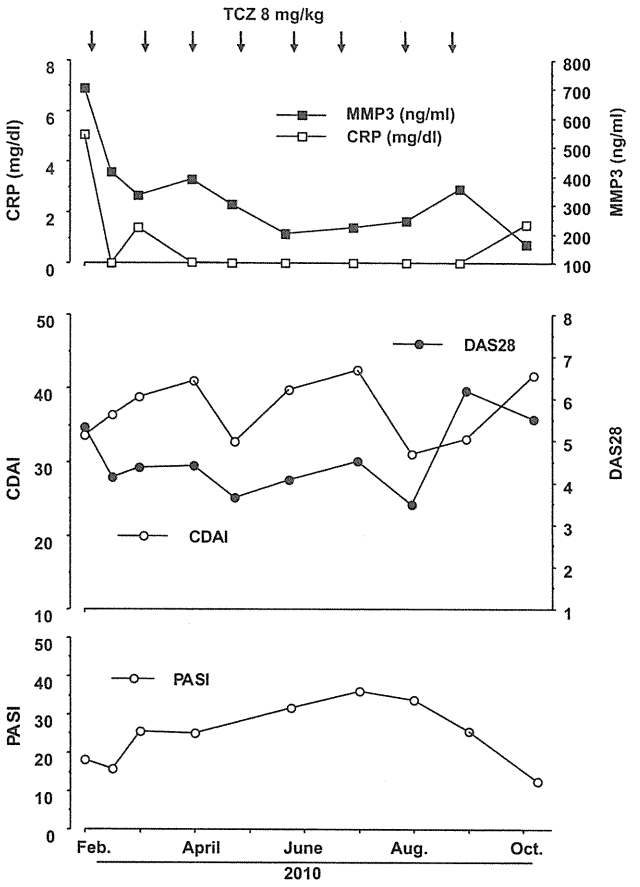

Fig. 2. Changes in C-reactive protein (CRP) level, matrix metalloproteinase (MMP)-3, Disease Activity Score including the 28-joint count (DAS28), Clinical Disease Activity Index (CDAI) and Psoriasis Area-and-Severity Index (PASI) score in case 2 during tocilizumab therapy.

symptoms improved slightly during Ustekinumab therapy. After the 1-year Ustekinumab therapy, symptoms again flared up. CRP level increased to 5.08 mg/dL and MMP-3 level increased to 704 ng/mL. Informed consent from the patient and approval by the ethics committee of Osaka University Hospital were obtained for TCZ treatment. TCZ was then started at 8 mg/kg every 4 weeks in February 2010, at 47 years old. Clinical course is shown in Fig. 2. Inflammatory markers of ESR and CRP showed complete normalization (21 mm/hour to 1.2 mm/hour and 5.08 mg/dL to 0 mg/dL, respectively). However, clinical symptoms as evaluated by CDAI and PASI remained unimproved after seven infusions of TCZ (28.5 to 33.2 and 18.1 to 25.6, respectively). DAS28 was slightly improved (5.33 to 3.48), but this was attributed to ESR normalization by TCZ. Administration of TCZ was therefore suspended in August 2010.

4. Discussion

Although, the characteristics of arthritis differ between PsA and RA, most therapeutic options for these diseases are similar. The differences in mechanism of arthritis between RA and PsA are thus unclear. We report herein the inadequate response to TCZ in PsA, suggesting a different pathogenic role of IL-6 in RA and PsA. In our patients, TCZ normalized CRP levels. Since CRP is induced by IL-6, we can monitor the efficacy of TCZ in suppressing IL-6 by monitoring CRP. IL-6 function thus appeared to be completely suppressed. However, arthritis and skin manifestations of PsA

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were unimproved, suggesting that inhibition of IL-6 function was insufficient to improve arthritis in PsA. In contrast, use of ADA as a TNF inhibitor just after TCZ therapy was effective for arthritis in case 1. Although, inflammatory markers (CRP level and ESR) increased, clinical symptoms were improved. In case 2, TNF inhibitor could not be started just after TCZ suspension as the cost proved prohibitive. However, the patient had a history of adequate response to ADA. Unfortunately, ADA could not be continued, again due to financial reasons. Disease activity flared up after suspension of ADA, suggesting that TNF is more important than IL-6 in the pathogenesis of PsA.

During TCZ treatment, the method of evaluating arthritis is important. In RA, TCZ improved CRP and ESR completely and rapidly. However, clinical improvement with TCZ was slow. Improvements of inflammatory markers and clinical symptoms thus do not occur in parallel. We sometimes observe patients in whom arthritis does not improve even if CRP levels normalize. Improvement of inflammatory markers may not match improvement of arthritic symptoms during TCZ therapy. Disease activity has recently been seen to be reduced by TCZ irrespective of the measure of composite disease activity [11]. In our case, DAS28 overestimated the efficacy of TCZ due to the effect of TCZ on ESR and the high weighting for ESR in the DS28.

The exact reason for the different efficacy of TCZ in RA and PsA is unclear. We hypothesized that this may be related to different pathogenic roles of IL-6 in RA and PsA. The traditional model of pathogenesis for psoriasis and PsA hypothesizes that chronic inflammation occurs as a result of T-cell-directed autoimmunity against a common skin and joint autoantigen. However, recent imaging, histological and genetic studies have challenged this view. Clinically unrecognized enthesitis is commonly seen in early PsA and induces frequent microdamage. Tissue repair at normal enthesis attachment sites in healthy joints has resulted in the proposal of a new model of PsA pathogenesis embracing the concept of auto-inflammation [12]. Based on observations of the cytokine dependency of arthritis models, type II collagen-induced arthritis (CIA) requires both IL-6 and TNF [13], whereas anti-type II collagen antibody-induced arthritis (CAIA) requires only TNF, not IL-6 [14]. This suggests that IL-6 is necessary for the production of antibodies specific for joint components (autoimmune phase) and TNF is necessary for the generation of arthritis (inflammation phase) [15]. TCZ may inhibit only inflammatory markers, not arthritis directly. The arthritic improvement effects of TCZ may thus depend on immune modulation rather than inflammatory suppression. TCZ might therefore be ineffective in auto-inflammatory arthritides like PsA.

Our observations suggest that the pathogenic role of IL-6 differs between RA and PsA. Inhibition of IL-6 functions is not indispensable for the treatment of PsA. The present observations are limited by the small number of patients, and larger clinical studies are necessary to confirm the efficacy of TCZ in PsA.

Disclosure of interest

AO received consultant fee from Chugai Pharmaceutical Co. Ltd. NU, IK. AK and TT have no conflict interest.

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Identification of Semaphorin 4B as a Negative Regulator of Basophil-Mediated Immune Responses

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Basophils are strong mediators of Th2 responses during helminthic infections. Recently, basophils were shown to function as APCs and promote both Th2 skewing and humoral memory responses. However, the mechanisms that regulate basophils are still unclear. In this article, we show that a class IV semaphorin, Sema4B, negatively regulates basophil functions through T cell–basophil contacts. In a screen to identify semaphorins that function in the immune system, we determined that Sema4B is expressed in T and B cells. Interestingly, Sema4B$^{-/-}$ mice had considerably increased serum IgE levels despite normal lymphocyte and dendritic cell functions. Recombinant Sema4B significantly inhibited IL-4 and IL-6 production from basophils in response to various stimuli, including IL-3, papain, and FcεRI cross-linking. In addition, T cell-derived Sema4B, which accumulated at contact sites between basophils and CD4$^+$ T cells, suppressed basophil-mediated Th2 skewing, suggesting that Sema4B regulates basophil responses through cognate cell–cell contacts. Furthermore, Sema4B$^{-/-}$ mice had enhanced basophil-mediated memory IgE production, which was abolished by treating with an anti-FcεRIα Ab. Collectively, these results indicate that Sema4B negatively regulates basophil-mediated Th2 and humoral memory responses. *The Journal of Immunology*, **2011, 186: 2881–2888.**

Basophils are rare granulocytes that are found in the circulation and are effector cells of the innate immune system that are associated with allergic inflammation and infections with helminth parasites (1–4). Recent studies indicated that basophils regulate Th1/Th2 homeostasis (5, 6) and humoral immunity (7). Basophils produce large amounts of IL-4, a key cytokine in Th2 skewing, in response to various stimuli, including IL-3, the protease allergen papain, and cross-linking of surface-bound IgE molecules (8, 9). In addition, basophils have been shown to express MHC class II and costimulatory molecules and to function as APCs (10–12), although the role of basophils as APCs in vivo is still controversial (13–16). Previous studies have shown that basophil-mediated Th2 skewing can be promoted when these cells are activated by papain (11) or internalize Ags through Ag-specific IgE on their cell surface (12). In addition, basophils have been implicated in humoral memory responses; on re-exposure to Ags, Ag and Ag-specific IgE complexes activate basophils to release IL-4 and IL-6, resulting in enhanced humoral immune responses (7). However, the mechanisms that regulate basophil-mediated Th2 responses are still unknown.

Semaphorins were originally identified as axon guidance molecules during neuronal development (17). However, cumulative findings indicate that semaphorins have diverse functions in many physiological processes (18–22). Semaphorins have been shown to be involved in various phases of immune responses, including the activation of B cells (23), T cells (24), and dendritic cells (DCs) (25); the regulation of Th differentiation (26); and the navigation of immune cell trafficking (27). In particular, membrane-bound class IV semaphorins, Sema4A and Sema4D/CD100, have been extensively investigated. Sema4D has been shown to be important for B cell and DC activation (23). In addition, Sema4A has been demonstrated to be critical for Th differentiation (26). However, the impact of other class IV semaphorins on other immune functions has not been determined.

In this study, we searched for semaphorins that function in the immune system and identified a class IV semaphorin, Sema4B, as a novel immune semaphorin. To determine the physiological roles of Sema4B, we generated Sema4B$^{-/-}$ mice and determined that Sema4B negatively regulates IL-4 and IL-6 production by basophils. We also determined that T cell-derived Sema4B suppresses basophil-mediated Th2 skewing. In addition, Sema4B$^{-/-}$ mice not only had increased serum IgE levels under steady-state conditions but also enhanced memory IgE responses caused by defects in Sema4B-mediated negative regulation of basophils.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; hIgG, human IgG; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl; NP-CGG, 4-hydroxy-3-nitrophenylacetyl-chicken-γ-globulin conjugate; PSD, postsynaptic density; rSema4B, recombinant Sema4B; Tg, transgenic; WT, wild-type.

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Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from CLEA Japan. Sema4B$^{-/-}$ mice on C57BL/6 and BALB/c backgrounds were generated as described later. Sema4B$^{-/-}$ mice were backcrossed >6× onto the C57BL/6 or BALB/c background. Transgenic (Tg) mice expressing the TCR specific for OVA (323–339; BALB/c background) were kindly provided by S. Habu (28). OVA-TCR Tg mice on a Sema4B$^{-/-}$ background were established by crossing the OVA-TCR Tg mice with Sema4B$^{-/-}$ BALB/c mice. All mice were bred in specific pathogen-free conditions. All animal experimental protocols were reviewed and approved by our institutional animal care committees.

Generation of Sema4B$^{-/-}$ mice

A targeting vector was designed to replace the fifth to eighth exons of the *Sema4B* gene with a Neo resistance cassette. In addition, the HSV thymidine kinase gene was inserted to select against random integration of this targeting vector. The linearized targeting plasmid DNA was transfected into embryonic stem cells by electroporation. After double selection with G418 and ganciclovir, we screened for homologous recombination of the Sema4B allele by PCR and FACS analysis. PCR was performed using 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The following oligonucleotide primers were used to identify the rearranged Sema4B locus: forward primer (P1): 5′-TAGTGGCATATGTGGACCTG-3′; reverse primer (P2): 5′-TCCCTGGAAGCTACTGACTGTT-3′; and reverse primer (P3) that includes the Neo cassette: 5′-TGCTCGACGTTGTCACTGAA-3′.

RT-PCR

Sema4B mRNA expression was examined by RT-PCR using a panel of multiple mouse tissue cDNAs (Clontech). RT-PCR was performed using 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with the forward primer (5′-AACAGCAACCTCAGCTTCTTGC-3′) and reverse primer (5′-GGCCTCATCTTGGGCTAAAGTA-3′).

Generation of the anti-Sema4B Ab

Rats were repeatedly immunized with purified mouse recombinant Sema4B (rSema4B) and alum, and their spleens were harvested to generate hybridomas using standard methodologies. The hybridomas were screened by ELISA for the production of Sema4B-specific Abs. To confirm the specificity of the Ab for Sema4B, we transfected COS7 cells with Sema4B, Sema4A, Sema4D, or the control vector using Lipofectamine (Invitrogen). After 48 h, the transfectants were stained with the biotinylated anti-Sema4B Ab (TK-2) followed by allophycocyanin-conjugated streptavidin.

Reagents and Abs

Anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD49b (DX5), anti-FcεRIα (MAR-1), anti-CD62L (MEL-14), anti–c-Kit (2B8), MHC class II (I-A/I-E; M5/114.15.2), and allophycocyanin-, FITC-, and Cy5-conjugated streptavidin were obtained from eBioscience. Anti-CD16/CD32 (2.4G2), anti-CD11c (HL3), anti-CD40 (HM40-3), and anti-CD3ε (145-2c11) were from BD Pharmingen. Anti-T1/ST2 was from MD Biosciences. Anti-p44/42 MAPK (SC-154) and anti-STAT5 (SC-835) were from Santa Cruz Biotechnology. Anti–phospho-p44/42 MAPK (9101) was from Cell Signaling Technology. Anti–phospho-STAT5 (47) was from BD Biosciences. Papain was purchased from Calbiochem. The monoclonal IgE anti-DNP Ab (SPE-7), OVA, DNP-human serum albumin (HSA), and LPS were from Sigma. 4-Hydroxy-3-nitrophenylacetyl-chicken-γ-globulin conjugate (NP-CGG), NP-BSA, and DNP-OVA were from Biosearch Technologies.

Cell purification

To prepare bone marrow (BM)-derived basophils, BM cells were cultured for 14 d with IL-3 (30 ng/ml, conditioned medium from a mouse IL-3–producing cell line) in RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (29). Samples were enriched for basophils by single-cell sorting on CD11c$^-$c-Kit$^-$FcεRIα$^+$ cells or by positively selecting CD11c$^-$c-Kit$^-$DX5$^+$ cells with MACS (Miltenyi Biotech). Otherwise, BM-derived basophils were enriched by depleting mast cells using anti–c-Kit–biotin and streptavidin-coupled Dynabeads (Invitrogen) (30). T cells, B cells, and DCs were isolated from the spleen by MACS. The resulting purity was >95% for each experiment. BM-derived DCs (BMDCs) were generated by stimulating with GM-CSF as previously described (31).

Flow cytometry

For the rSema4B binding assay, BM-derived basophils were incubated with either biotinylated rSema4B or biotinylated human IgG (hIgG), followed by streptavidin-allophycocyanin. For in vivo Th2 skewing, single-cell suspensions from the spleens of adoptively transferred mice were stained with allophycocyanin–anti-CD4 and FITC–anti-T1/ST2. To evaluate Sema4B expression in specific immune cell populations, we identified CD4$^+$ T cells (CD4$^+$CD8$^-$), CD8$^+$ T cells (CD4$^-$CD8$^+$), B cells (B220$^+$CD11c$^-$), DCs (CD11c$^+$B220$^-$), and basophils (FcεRIα$^+$DX5$^+$) using the indicated markers.

In vitro stimulation assays

c-Kit–depleted BM-derived basophils were stimulated with IL-3 (30 ng/ml) after being starved for 8 h, or stimulated with papain (20 μg/ml) or IgE cross-linking without starvation. Basophils were activated with IgE cross-linking by incubating with IgE anti-DNP (1 μg/ml), followed by various concentrations of DNP-HSA for 16 h. Basophils were stimulated on plates coated with rSema4B (10 μg/ml) or hIgG (10 μg/ml). The concentrations of IL-4 and IL-6 in the culture supernatants were measured by ELISA (R&D Systems). B cells were cultured with anti-CD40 (0.2 μg/ml) and various concentrations of IL-4 for 7 d (32). The IgM, IgG1, and IgE levels in the culture supernatants were measured by ELISA (Bethyl Laboratories).

In vitro and in vivo basophil-mediated Th2 skewing assays

For the in vitro basophil-mediated Th2 skewing assay, BM-derived basophils (CD11c$^-$c-Kit$^-$FcεRIα$^+$) were enriched by flow cytometry sorting. Naive CD4$^+$CD62L$^+$ T cells from OVA-TCR Tg mice (4×10^5 cells/ml) and irradiated basophils (5×10^5 cells/ml) were cultured with the OVA peptide (1 μM), OVA (100 μg/ml) and papain (20 μg/ml), or IgE anti-DNP (1 μg/ml) and DNP-OVA (100 μg/ml) (11, 12) in plates coated with Sema4B (10 μg/ml) or hIgG (10 μg/ml). In some experiments, naive CD4$^+$CD62L$^+$ T cells from wild-type (WT) or Sema4B$^{-/-}$ OVA-TCR Tg mice were used instead of rSema4B. After 5 d of coculture, CD4$^+$ T cells isolated by anti-CD4–conjugated magnetic beads (autoMACS) were restimulated with immobilized anti-CD3 for 24 h. For the in vivo basophil-mediated Th2 skewing assay, naive CD4$^+$CD62L$^+$ T cells (5×10^6 cells/mouse) from WT or Sema4B$^{-/-}$ OVA-TCR Tg mice were i.v. transferred into nude mice. The next day, basophils (CD11c$^-$c-Kit$^-$FcεRIα$^+$, 5×10^6 cells/mouse) pulsed with IgE anti-DNP (2 μg/ml) and DNP-OVA (100 μg/ml) were transferred through the tail vein into these recipient nude mice. After 4 d, CD4$^+$ T cells (1×10^5 cells/well) isolated from the spleen by flow cytometry sorting were cultured with BMDC (1×10^5 cells/well) and DNP-OVA (100 μg/ml) in 96-well plates for 36 h. Cytokine concentrations in the supernatants were measured by ELISA (R&D Systems).

In vivo T cell priming and Ab production assays

Mice were immunized in the hind footpads with keyhole limpet hemocyanin (KLH; 100 μg) in CFA or alum. Five days after priming, cells were purified from the draining lymph nodes. Lymph node cells (1×10^5 cells/well) were stimulated for 48 h with various concentrations of KLH. For proliferation assays, the cells were pulsed with 2 μCi [^{3}H]thymidine for the last 14 h (26). To induce Ab responses to T cell-dependent Ags, we immunized mice i.p. with 50 μg NP-CGG as an alum-precipitated complex on day 0 and then boosted on day 14. Serum was collected on days 0, 14, and 21. NP-specific Abs were detected with NP-BSA–coated ELISA plates and quantified using isotype-specific Abs as previously described (33).

Measurement of serum Igs

The concentrations of serum Igs were measured using a mouse IgE ELISA quantitation kit (Bethyl Laboratories).

In vivo memory response assays

WT and Sema4B$^{-/-}$ mice were immunized i.p. with 100 μg OVA without adjuvant. Four weeks after the primary immunization, the mice were administered 10 μg OVA i.v. (7). Serum was collected on days 0, 5, 7, and 9 after rechallenging with the Ag. To deplete basophils, we injected primary immunized mice twice daily for 3 d with 5 μg anti- FcεRIα. The mice were allowed to rest for 2 d and then were i.v. injected with 10 μg OVA. OVA-specific serum IgE was measured with a mouse OVA-IgE ELISA kit (Dainippon Sumitomo Pharma). OVA-specific serum IgG1 was measured with a mouse OVA-IgG1 ELISA kit (Shibayagi).

Immunohistochemistry

Sema4B$^{-/-}$ BM-derived basophils (CD11c$^-$c-Kit$^-$DX5$^+$) and OVA-TCR Tg-derived CD4$^+$ T cells were cultured with OVA peptide for 1 h. Then the

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