We also conducted flow cytometric analysis on the expression of chemokine receptors on myeloma cells present in the peripheral blood of patient with multiple myeloma. As shown in Fig. 1*b*, PBMC from patients with multiple myeloma but not those from healthy adult donors contained a substantial fraction of cells expressing CD38 at high levels. We confirmed that these cells were also positive for another plasma cell marker CD138 (data not shown) (15, 16). These cells that represented floating myeloma cells in PBMC of myeloma patients also consistently expressed CXCR4 and CXCR6 at high levels, CCR10 at intermediate levels, and CCR3 at low levels (Fig. 1*b*).

Responses of plasma cells to the ligands of CXCR4, CXCR6, CCR10, and CCR3

We next examined chemotactic responses of bone marrow plasma cells to CXCL12 (CXCR4 ligand), CXCL13 (CXCR5 ligand), CXCL16 (CXCR6 ligand), CCL11 (CCR3 ligand), CCL27 (CCR10 ligand), and CCL28 (CCR10 and CCR3 ligand) (1). As shown in Fig. 2*a*, CXCL12, CXCL16, and CCL28 induced migration of plasma cells with similar potencies and efficiencies. CCL11 and CCL27 also induced modest migratory responses, while CXCL13 induced only marginal responses. The chemotactic responses of plasma cells to CCL28, which signals via both CCR10 and CCR3 (10, 11), were roughly about the summation of those to CCL27 and CCL11, the specific ligands of CCR10 and CCR3, respectively (1). These results were in good accordance with the expression profile of chemokine receptors on plasma cells (Fig. 1).

Previously, CXCL12 was shown to induce transient adhesion of fresh myeloma cells to fibronectin and VCAM-1 via activation of very late Ag-4 (VLA-4) (12). Therefore, we examined whether CXCL16 and CCL28 were also capable of inducing adhesion of plasma cells to fibronectin. As shown in Fig. 2*b*, not only CXCL12 but also CXCL16 and CCL28 efficiently induced adhesion of bone marrow plasma cells to fibronectin. The adhesion responses were rapid and transient, reaching the maximal levels within 5 min and returning to basal levels by 30 min (12), and also VLA-4 dependent as shown by the ability of anti-α_4 integrin mAb to block cell adhesion to fibronectin.

Direct adhesion of plasma cells to immobilized CXCL16

CXCL16, the ligand of CXCR6, is a novel transmembrane-type chemokine whose structure is very similar to that of another transmembrane chemokine fractalkine/CX_3C chemokine ligand (CX_3CL)1 (8, 9). Previously, we have shown that immobilized CX_3CL1 induces firm adhesion of CX_3CR1-expressing cells via its chemokine domain in both static and flow conditions without requiring signaling via $G_{\alpha i}$ or divalent cations (14, 17). The structural similarity of CXCL16 to CX_3CL1 prompted us to examine whether immobilized CXCL16 was also capable of inducing direct adhesion of plasma cells expressing CXCR6. As shown in Fig. 2*c*, CXCL16-SEAP immobilized to the plastic surface efficiently induced adhesion of plasma cells, which was effectively blocked by anti-CXCR6 but not by control Ab. Furthermore, pretreatment of plasma cells with pertussis toxin or presence of EGTA during the binding assay did not affect the levels of adhesion. Collectively, immobilized CXCL16 was indeed capable of inducing adhesion of plasma cells via CXCR6 without requiring signaling via $G_{\alpha i}$ or divalent cations (integrins).

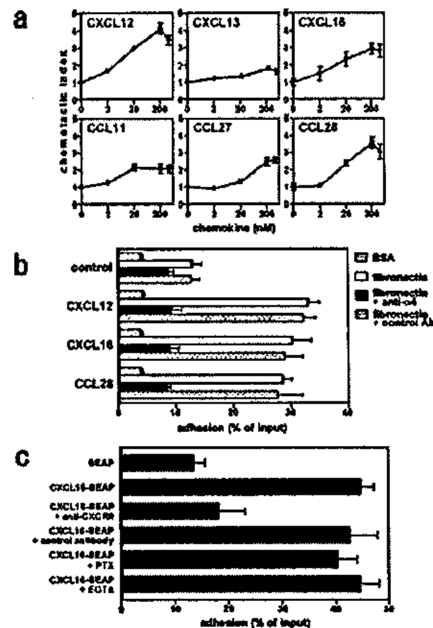

FIGURE 2. Chemotactic and adhesion responses of plasma cells. *a*, Migratory responses of human bone marrow plasma cells to indicated chemokines were determined in Transwell plates. After incubation for 2 h at 37°C, input cells and cells migrated into lower wells were double stained with PE-labeled anti-CD38 and Cy5-labeled anti-CD45, and measured by flow cytometry. All assays were done in duplicate. Chemotaxis index = migration in response to chemokine/background migration. Representative results of $CD38^{high}CD45^{low}$ mature plasma cells from five donors are shown. Each point represents mean ± SEM from three separate experiments. *b*, Bone marrow mononuclear cells were resuspended without or with chemokines at 200 nM and immediately added in triplicate to 96-well plates coated with BSA or fibronectin. After washing, bound cells were harvested and stained with PE-labeled anti-CD38. Plasma cells in original and bound populations were counted by flow cytometry. For blocking experiments, cells were pretreated for 20 min with 10 μg/ml anti-integrin α_4 (HP1/2) before treatment with chemokines. Representative results from three donors are shown. Each point represents mean ± SEM from three separate experiments. *c*, Bone marrow mononuclear cells obtained from adult donors (n = 3) were seeded in triplicate into 96-well microtest plates coated with CXCL16-SEAP or SEAP and incubated for 30 min at room temperature. In some experiments, cells were pretreated with 30 μg/ml anti-CXCR6 or 500 ng/ml pertussis toxin (PTX; Invitrogen) for 30 min, or the assay was conducted with adhesion buffer containing 5 mM EGTA. After gentle washing, original cell populations and adherent cells were stained with PE-labeled anti-CD38, and $CD38^{high}$ plasma cells were counted by flow cytometry. Representative results from three donors are shown. Each point represents mean ± SEM from three separate experiments.

Expression of CXCL12, CXCL16, and CCL28 by human bone marrow stromal cells

The selective expression of CXCR4, CXCR6, CCR10, and CCR3 by bone marrow plasma cells suggests that their respective chemokine ligands may be involved in the homing and tissue microenvironmental localization of plasma cells in the bone marrow and other target tissues. However, except for CXCL12 (CXCR4 ligand) (7), expression of these chemokines in the bone marrow has not been reported. Therefore, we conducted RT-PCR analysis for expression of these chemokines in bone marrow and other human tissues known to be enriched with plasma cells. As shown in Fig. 3*a*, all the tissues examined including bone marrow expressed CXCL12 (CXCR4 ligand),

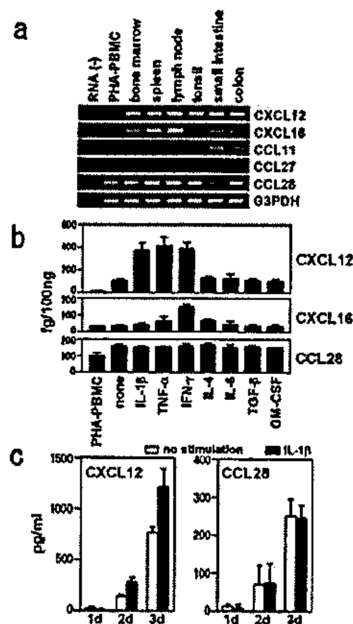

FIGURE 3. Expression of CXCL12, CXCL16, and CCL28 in human bone marrow tissue and stromal cells. *a*, cDNA samples from PHA-stimulated PBMC (positive control), human bone marrow, spleen, lymph node, tonsil, small intestine, and colon were analyzed for expression of CXCL12, CXCL16, CCL11, CCL27, CCL28, and G3PDH by PCR. Representative results from two separate experiments are shown. *b*, cDNA samples from PHA-stimulated PBMC (positive control) and human bone marrow stromal cells treated without or with 10 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ, 10 ng/ml IL-4, 10 ng/ml IL-6, 10 ng/ml TGF-β, and 10 ng/ml GM-CSF for 24 h were analyzed for expression of CXCL12, CXCL16, CCL28, and G3PDH by real-time PCR. Representative results from two separate experiments are shown. *c*, Bone marrow stromal cells were seeded in triplicate in 24-well plates and cultured without or with 10 ng/ml IL-1β. At indicated time points, CXCL12 and CCL28 in the culture supernatants were measured by using ELISA. Each point represents mean ± SEM. Representative results from two separate experiments are shown.

CXCL16 (CXCR6 ligand), and CCL28 (CCR10 and CCR3 ligand) at high levels. Thus, these chemokines can be collectively involved in the homing and localization of plasma cells in the bone marrow and other target tissues. In contrast, CCL11 (CCR3 ligand) or CCL27 (CCR10 ligand) was hardly expressed in the bone marrow.

CXCL12 was originally identified as a secretory product of a mouse bone marrow stromal cell line (7). Therefore, we next examined expression of CXCL12, CXCL16, and CCL28 by human primary bone marrow stromal cells. We confirmed that stromal cells were $CD10^+$ and $CD34^{low}$ (data not shown) (18). As shown in Fig. 3*b*, real-time RT-PCR revealed that stromal cells indeed constitutively expressed CXCL12, CXCL16, and CCL28 at high levels. The expression of CXCL12 was enhanced by treatment with IL-1β, TNF-α, or IFN-γ, while that of CXCL16 was enhanced by IFN-γ. In contrast, the expression of CCL28 was hardly affected by any cytokines. We also examined secretion of CXCL12 and CCL28 by bone marrow stromal cells. As shown in Fig. 3*c*, stromal cells indeed secreted copious amounts of CXCL12 and CCL28 in the culture supernatants. Consistent with the results from RT-PCR (Fig. 3*b*), treatment of stromal cells with IL-1β significantly enhanced secretion of CXCL12 ($p < 0.05$) but not that of CCL28.

Discussion

We have demonstrated that human bone marrow plasma cells as well as myeloma cells consistently express CXCR4, CXCR6, CCR10, and CCR3 (Fig. 1). Accordingly, CXCL12 (CXCR4 ligand) (7), CXCL16 (CXCR6 ligand) (8, 9), and CCL28 (CCR10 and CCR3 ligand) (10, 11) efficiently induced chemotactic responses and VLA-4-dependent transient adhesion to fibronectin in plasma cells (Fig. 2). Importantly, we have also shown that human bone marrow and other tissues known to be enriched with plasma cells contain transcripts of CXCL12, CXCL16, and CCL28 at high levels (Fig. 3). Furthermore, we have shown that cultured human bone marrow stromal cells constitutively express CXCL12, CXCL16, and CCL28 at high levels (Fig. 3). Collectively, our results support the roles of CXCR4, CXCR6, CCR10, and CCR3 and their respective chemokine ligands in the migration and tissue localization of human plasma cells in bone marrow and other tissues known to be enriched with plasma cells.

CXCL16 is a novel transmembrane-type chemokine (8, 9), which was also identified as a novel scavenger receptor for oxidized low density lipoprotein (13). In the present study, we have shown that immobilized CXCL16 is capable of inducing adhesion of plasma cells expressing CXCR6 without requiring $G_{\alpha i}$-mediated signaling or divalent cations (Fig. 2), an observation quite similar to that of another transmembrane-type chemokine CX_3CL1 (14, 17). Thus, like CX_3CL1, CXCL16 may function as a chemoattractant in its soluble form and a cell adhesion molecule in its membrane-anchored form. This may allow CXCL16 to contribute to plasma cell localization in the bone marrow and other target tissues not only by its chemotactic activity but also by its direct cell adhering activity.

In conclusion, we have shown for the first time that human bone marrow plasma cells and myeloma cells consistently express CXCR6, CCR10, and CCR3 besides CXCR4. The important role of CXCR4 in migration and tissue localization of plasma cells has been reported previously (2). Thus, the exact roles of CXCR6, CCR10, and CCR3 in the migration and tissue localization of plasma cells remain to be seen. It also remains to be seen whether human plasma cells producing different Ig isotypes express a different set of chemokine receptors to migrate to different anatomical sites as mouse plasma cells do (4).

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LETTER TO THE EDITOR

Effects of MAPK inhibitors on CCR4-mediated chemotaxis against thymus and activation-regulated chemokine (TARC/CCL17)

To the Editor,

Tissue recruitment of immunocompetent cells is a central event in the inflammatory response. Most of peripheral blood T cells polarized to type 2 helper T (Th2) cells express CC chemokine receptor 4 (CCR4) and also include almost all skin-seeking memory T cells positive for cutaneous lymphocyte antigen [1,2]. Thymus and activation-regulated chemokine (TARC/CCL17) is one of the ligands for CCR4 and the ligation of TARC and CCR4 plays an important role in the migration of Th2 cells into inflamed tissues [1,3]. Moreover, serum levels of TARC were shown to correlate well with disease severity in Th2-prone skin disorders, such as atopic dermatitis [4]. Keratinocytes also express TARC in vivo and in vitro under an appropriate condition [4]. Therefore, TARC- and CCR4-mediated chemotaxis may play a crucial role in the initiation and perpetuation of the allergic inflammation.

Various cellular events such as proliferation, differentiation and migration responses are associated with phosphorylation of intracellular signaling molecules such as p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases1/2 (ERK1/2) and MAPK/Erk kinase 1/2 (MEK1/2). Recently, several researchers have reported on the differential involvement of MAPKs in chemotaxis [5,6]. However, the intracellular signaling pathways linking to TARC—CCR4-mediated chemotaxis are not well characterized. We have investigated the effects of various MAPK inhibitors on CCR-4-mediated chemotaxis to TARC using mouse T-cell lymphoma cell line EL4 constitutively expressing recombinant human CCR4 (CCR4/EL4 cells) with high level [7].

Recombinant human TARC was obtained from Sigma, St. Louis, MO. SB203580 (p38 MAPK inhibitor), SD-282 (p38 MAPK inhibitor), U0126 (MEK 1/2 inhibitor) and PD098059 (ERK 1/2 inhibitor) were obtained from Calbiochem (Dermstadt, Germany). CX-659S (MEK 1/2 inhibitor) was obtained from Kowa Co., Japan [8]. CCR4/EL4 cells were obtained from Kyowa Hakko Kogyo Co., Ltd., Japan. These cells expressed large amounts of cell surface CCR4 [7] and exhibited marked chemotactic activity to TARC (Fig. 1). All the inhibitors inhibited the TARC-induced chemotaxis in a dose-dependent manner (Fig. 1 and Table 1). However, the inhibition was partial in each agent. Both p38 MAPK inhibitors (SB203580 and SD-282) showed similar level of inhibition. Among MEK/ERK inhibitors, U0126 exhibited the most potent inhibitory capability. Neither agent suppressed the viability of the CCR4/EL4 cells under the experimental concentrations examined.

The inhibitory effects on chemotaxis by MAPK inhibitors are diverse, depending on the cell types and kinds of stimuli. In granulocytes, IL-8-mediated chemotaxis was inhibited by PD98059 (about 40% inihibition) but not by SB20358 [6], whereas the inhibition of MEK1/2 by PD98059 did not block the chemotaxis in response to classical nonchemokine chemoattracts such as fMLP [5]. In monocyte chemoattractant protein-1 (MCP-1)-induced chemotaxis, p38-MAPK, but not MEK1/2, was involved in chemotaxis of CCR2-expressing THP-1 monocytic cells [9].

Ligation of TARC to CCR4 is known to activate the pertussis toxin-sensitive G proteins not only in the aggregation of platelets [10]. However, little is known on the involvement of MAPKs on TARC—CCR4 chemotaxis. In the present study we investigated the inhibitory effects of five different MAPK inhibitors on CCR4-expressing EL4 cells. All the inhibitors partially inhibited the TARC-induced chemotaxis in a dose-dependent manner. These results suggest that p38-MAPK and MEK/ERK pathways are both involved in the CCR4-mediated chemotaxis to TARC. Although these results using CCR4/EL4 do not exactly correspond to human Th2 lymphocytes, those inhibitors might be candidates that suppress the migration of T cells expressing CCR4, and could be therapeutic reagents for allergic skin diseases.

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Table 1 Effects of various MAPK inhibitors on TARC–CCR4-mediated chemotaxis

	6.25 μM	12.5 μM	25 μM	50 μM
SB203580 (p38 MAPK inhibitor) (%)	19.1 ± 0.68	24.4 ± 2	27.3 ± 0.6	47.2 ± 0.9
	5 μM	10 μM	20 μM	40 μM
SD-282 (p38 MAPK inhibitor) (%)	20.8 ± 0.6	31.5 ± 2	38.9 ± 0.8	38.4 ± 1.2
	5 μM	10 μM	20 μM	40 μM
U0126 (MEK 1/2 inhibitor) (%)	48.3 ± 0.6	52.6 ± 3	54.3 ± 0.4	62.8 ± 1.1
		10 μM		100 μM
CX-659S (MEK 1/2 inhibitor) (%)		0		16.6 ± 0.8
	5 μM	10 μM	20 μM	40 μM
PD098059 (ERK 1/2 inhibitor) (%)	7 ± 1.5	23.2 ± 0.9	30 ± 1.2	32.8 ± 2.5

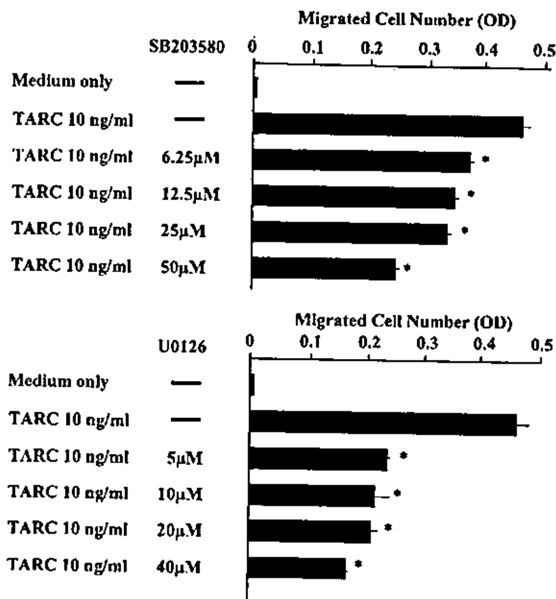

Fig. 1 Inhibitory effects of SB203580 and U0126 on the TARC-mediated chemotaxis of CCR4/EL4 cells. Number of migrated cells through 5-μm pore was determined using a cell counting kit (cell proliferation kit, Biovision Inc., Mountain View, CA). TARC induced a potent chemotaxis of CCR4/EL4 cells compared to negative control (medium only). Both agents significantly and dose-dependently inhibited the chemotaxis. *$p < 0.01$.

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Enhanced TARC production by dust-mite allergens and its modulation by immunosuppressive drugs in PBMCs from patients with atopic dermatitis

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KEYWORDS
Atopic dermatitis (AD);
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Disease activity;
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Dexamethasone (Dex);
Cyclosporine (CsA);
FK506 (Tacrolimus)

Summary *Background:* Thymus and activation regulated chemokine (TARC) is a CC chemokine that attracts CCR4$^+$ T cells. We reported previously that TARC is an important chemokine that defines Th2 imbalance in the pathogenesis of atopic dermatitis (AD). *Objectives:* This study was undertaken to clarify TARC producing cells in peripheral blood mononuclear cells (PBMCs), the regulation of dust mite-allergen clude extract (DME) and different immunosuppressive drugs (Tacrolimus (FK506), cyclosporine (CsA), dexamethasone (Dex)) on TARC production by peripheral PBMCs from AD patients in vitro. *Methods:* Monocyte derived dendritic cells (MoDCs) were generated from and TARC mRNA levels were examined and comapared with those from T cells in PBMCs from AD patients. PBMCs were cultured with or without DME and/or immunosuppressive drugs (Tacrolimus, CsA, Dex) for 7 days and TARC levels were measured. *Results:* PBMCs from AD patients which were cultured with DME stimulation for 7 days showed significantly higher levels of TARC production than those from healthy controls. RT—PCR demonstrated that TARC mRNA was expressed in CD4$^+$ T cells, CD8$^+$ T cells and MoDCs. Tacrolimus, CsA and Dex individually suppressed TARC production by PBMCs from AD patients which were co-cultured with DME for 7 days. Gel shift analysis revealed differential inhibitory effects of these immunosuppressive drugs on NFκB activity in PBMCs from AD patients. *Conclusion:* Our data demonstrate that TARC producing cells are MoDCs, T cells as well as epidermal keratinocytes in AD. We suggest that MoDCs might regulate the immune responses by attracting T cells and CD25$^+$ T cells in the pathogenesis of AD. We also showed the important role of DME on TARC production and the inhibitory effect of the immunosuppressive drugs on TARC production by PBMCs from AD patients, that can regulate ongoing immune responses in the pathogenesis of AD.
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Abbreviations: AD, atopic dermatitis; DME, dust mite allergen extract; TARC, thymus and activation-regulated chemokine; CCR4, CC chemokine receptor 4; PBMC, peripheral blood mononuclear cell; Dex, dexamethasone; CsA, cyclosporine

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1. Introduction

Chemokines are chemoattractants which bring different types of leukocytes to sites of inflammation [1]. They are produced locally in tissues and act on leukocytes through specific receptors [2]. Chemokines are divided into four subfamilies, CXC, CC, CX_3C and C, depending on the position of their first two N-terminal cysteine residues [1,2]. Thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokine superfamily, and is produced by monocyte-derived dendritic cells (DCs), endothelial cells and keratinocytes [3–6].

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is associated with an elevated serum IgE level, IgE specific to environmental allergens such as mites, and tissue and the occurrence of peripheral blood eosinophilia. It is characterized by predominant infiltration of Th2-type cells, increased secretion of Th2-type cytokines in acute phase of lesional skin [7] and high responsiveness of peripheral blood mononuclear cells (PBMCs) to IL-4 [8]. The production of Th2 cytokines, such as IL-4, IL-10 and IL-13, are significantly increased when PBMCs of AD patients are stimulated with dust mite-allergen extracts (DME) compared without stimulation [9].

We showed previously that serum TARC levels, and the expression of CC chemokine receptor 4 (CCR4) are significantly increased in patients with AD [10]. These data strongly suggest that the interaction of TARC with CCR4 may be important for recruiting Th2-type T cells in the pathogenesis of AD. However, little is known about the effects of DME and various immunosuppressive drugs (Taclorimus (FK506), cyclosporine (CsA), dexamethasone (Dex)) on TARC production by PBMCs of AD patients. In the present study, we quantified the effect of DME and the inhibitory effects of immunosuppressive drugs on TARC production by DME-stimulated PBMCs of AD patients in order to clarify the action of immunosuppressive drugs on Th2 chemokine regulation.

2. Materials and methods

2.1. Samples and reagents

Eight AD patients (mean age ± S.D., 25.4 ± 7.9) and eight healthy controls (mean age ± S.D., 29.8 ± 5.8) were examined. The levels of IgE RIST, peripheral blood eosinophil numbers, SCORAD indexes were listed in Table 1. All patients with AD were diagnosed according to the criteria of Hanifin and Rajka [11] and were treated with topical corticosteroids in combination with oral anti-histamines.

2.2. Culture of PBMCs

PBMCs were isolated from peripheral blood samples by centrifugation on a Ficoll-metrizoate density gradient (Pharmacia Biotech, Uppsala, Sweden) at 2000 rpm for 20 min at room temperature. PBMCs were washed three times with PBS, and dispensed into 10^6 cells per tube in RPMI1640 medium. Isolated PBMCs were cultured at 37 °C for 7 days in RPMI 1640 medium with dust-mite clude extract (DME) (Greer Laboratories Inc., NC) (10 µg/ml) with or without various immunosuppressive drugs (Tacrolimus, CsA or Dex) (10^{-6} M to 10^{-10} M).

2.3. Cell viability

After culture, cell viability was evaluated with trypan blue dye exclusion. The viable cells and dead cells were counted in each experiment.

2.4. ELISA

Polystyrene microplates were coated with a murine mAb against human TARC (TECHNE Corp., Minneapolis, MN). TARC levels in the supernatants of cultured PBMCs were measured [6]. Briefly, the samples were added to each well and the plates were incubated for 2 h at room temperature (RT). After washing, anti-TARC Ab conjugated to

Table 1 The laboratory data of AD patients examined in this experiment are shown

Age	Sex	IgE (IU/ml)	Dust mite specific IgE	Eosinophils (mm^{-3})	SCORAD
22	Female	420	0.86	210	28.9
21	Female	6100	65.3	610	37.6
23	Male	2100	69.9	120	41.2
37	Female	9000	76.3	730	48.4
39	Female	83	0.39	260	29.2
21	female	16000	78.0	690	44.6
20	Male	36	5.69	60	28.8
20	Female	4200	68.7	360	40.3

horseradish peroxidase (HRP) was added to each well and the plates were incubated for 1 h at RT. After washing, a substrate solution (including tetramethylbenzidine) was added and the plates were incubated for 30 min. The optical density of each well was determined using an ELISA reader (Spectra and Rainbow Readers). The minimum amount of TARC detectable was less than 7 pg/ml. ELISAs were performed at least three times in each experiment.

2.5. Purification of $CD4^+$ T cells, $CD8^+$ T cells and monocytes, and separation of monocyte derived dendritic cells in PBMCs from AD patients

PBMCs were incubated with or without DME for 24 h, and $CD4^+$ T and $CD8^+$ T cells were purified from PBMCs using RosetteSep™ antibody cocktail (Atemcell Technologies Inc., Vancouver, Canada). These cells were used for RT—PCR analysis. Monocytes were isolated from un-stimulated PBMCs and monocyte derived dendritic cells (DCs) were generated, according to published methods [12]. Briefly, to generate immature monocytes derived DCs (MoDCs), monocytes purified from PBMCs using RosetteSep™ antibody cocktail were cultured for 7 days with recombinant human GM—CSF (R&D) (50 ng/ml) and recombinant human IL-4 (R&D)(50 ng/ml). These MoDCs were then cultured with or without DME for 24 h and used for RT—PCR analysis.

2.6. Preparation of mRNA and RT-PCR

$PolyA^+$ mRNAs were extracted from cells using a Micro-FastTrack™ 2.0 kit (Invitrogen Co., Carlsbad, CA) [13]. Briefly, cells were lysed and transferred to vials containing oligo (dT) cellulose. The oligo (dT) cellulose was washed and the $polyA^+$ mRNA was retrieved. Approximately 1 μg of $polyA^+$ mRNA was reverse-transcribed using a cDNA CYCLE™ kit (Invitrogen Co.). The reaction mixture contained 1 μg of $polyA^+$ mRNA, 1 μl of random primers, 1 μl of RNase inhibitor, 4 μl of 5 × RT buffer, 1 μl of 100 mM dNTPs, 1 μl of 80 mM sodium pyrophosphate and 0.5 μl of AMV reverse transcriptase. After incubation at 42 °C for 60 min and 95 °C for 3 min, the resulting cDNA was used for PCR. Sequences were amplified from cDNAs using specific primers for TARC and β-actin. The TARC primer sequences were: sense, 5′-CAC GCA GCT CGA ACC AAT GTG-3′, antisense, 5′-TCA AGA CCT CTC AAG GCT TTG CAG G-3′. The β-actin primer sequences were: sense, 5′-GAC TAT GAC TTA GTT GCG TTA-3′, antisense, 5′-GCC TTC ATA CAT CTC AAG TTG-3′. PCR amplifications were carried out in cDNA, each primer (25 pmol), $MgCl_2$, dNTPmix and Taq polymerase (TaKaRa, Tokyo, Japan). Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for TARC or at 55 °C for β-actin for 1 min, and extension at 72 °C for 2 min. For negative controls, cDNA was omitted from the PCR reaction. After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel.

2.7. Gel shift assay

Nuclear extracts were prepared from PBMCs (3×10^6) incubated with or without DME and immunosuppressive drugs using a Cell Lytic™ Nu kit (Sigma Co., St Louis, Ms.). The cells were collected by centrifugation, washed with PBS, and resuspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol. The cells were incubated on ice for 30 min. The cells were then treated with 10% Igepal, and centrifuged at 1300 rpm for 3 min. The pellets were collected and treated with extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, and 25% glycerol. The suspension was centrifuged at 14,000 rpm for 30 min at 4 °C. The pellets were freeze-thawed in liquid N_2 three times and centrifuged to remove debris. Approximately 7 μg of protein was incubated with or without an excess of unlabeled competitor. The probe was annealed to form oligomers and labeled using T4 polynucleotide kinase (Promega), [γ-^{32}P]ATP (Amersham Pharmacia Biotech). P-labeled oligonucleotide probe (5000 cpm) was added and the incubation was continued for additional 10 min at room temperature. The free and the protein-bound DNAs were separated on 5% polyacrylamide gels (29:1—acrylamide: bisacrylamide). The gels were pre-run for 30 min in 1 times TBE buffer and then run for 2—2.5 h at 125 V. The gels were transferred onto filter paper, and exposed to x-ray film (X-Omat, Kodak Co.) at −70 °C for 24—48 h with screen ontensifiers. The band density was scanned and analyzed using NIH image scanning.

2.8. Statistical analysis

The data were analyzed using the Mann—Whitney's *U* test. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. Levels of TARC production by PBMCs from patients with atopic dermatitis (AD)

Peripheral blood mononuclear cells (PBMCs) from AD patients cultured for 7 days produced high levels of TARC (440.3 ± 72.8 pg/ml) which were significantly higher than those from healthy controls (24.4 ± 10.1 pg/ml) ($P < 0.001$) (Fig. 1a and b). PBMCs from AD patients which were cultured with DME for 7 days showed enhanced levels of TARC production up to 1652.3 ± 368.5 pg/ml, which was significantly higher than by those cultured without DME ($P < 0.001$). TARC levels of PBMCs from AD patients which were cultured with DME for 7 days were significantly higher than those of healthy controls (163.4 ± 23.3 pg/ml, $P < 0.001$).

3.2. TARC mRNA expression by CD4$^+$ T cells, CD8$^+$ T cells and monocyte-derived dendritic cells (MoDC) from AD patients analyzed by RT-PCR analysis

We examined TARC mRNA expression of purified CD4$^+$ T and CD8$^+$ T cells derived from non-stimulated or DME-stimulated PBMCs from AD

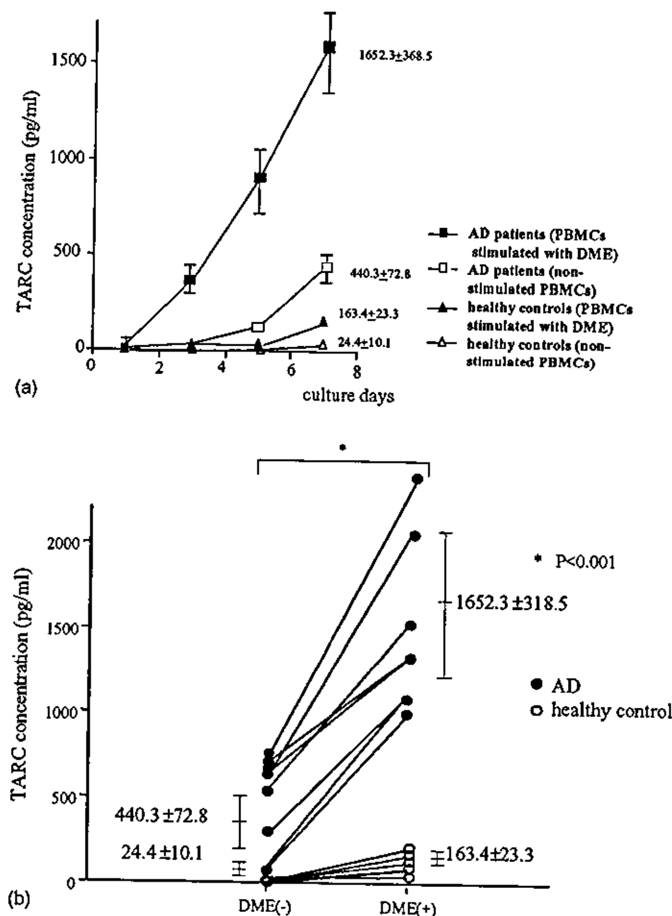

Fig. 1 TARC production from atopic dermatitis (AD) patients and healthy controls cultured with dust-mite allergen extract (DME). (a) Time course of TARC production by PBMCs is shown. PBMCs isolated from AD patients and healthy controls were cultured with DME (10 μg/ml) up to 7 days and TARC levels in the culture supernatants were measured at 3, 5 and 7 days. The levels of TARC production by PBMCs from AD patients were significantly higher than healthy controls ($P < 0.001$). The levels of TARC production by PBMCs from AD patients which were cultured with DME for 7 days were significantly higher than by those cultured without DME ($P < 0.001$). (b) TARC production by PBMCs cultured with DME (10 μg/ml) for 7 days is shown. The TARC levels by cultured PBMCs isolated from AD patients is significantly higher compared with those from healthy controls. Closed circles show TARC levels of AD patients and open circles show those of healthy controls.

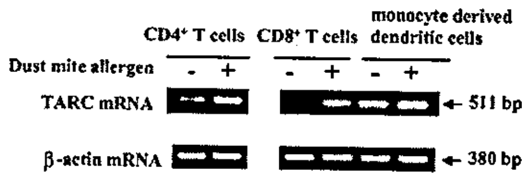

Fig. 2 TARC mRNA expression in $CD4^+$ T cells, $CD8^+$ T cells and monocyte derived dendritic cells (MoDCs) in AD patients. $CD4^+$ T cells and $CD8^+$ T cells were purified from non-stimulated or DME-stimulated PBMCs from AD patients for 24 h. Very little TARC mRNA was detected in non-stimulated T cells. After stimulation with DME for 24 h, TARC mRNA levels were enhanced in purified $CD4^+$ T cells and $CD8^+$ T cells. Monocytes were purified from non-stimulated PBMCs and monocyte derived DCs (MoDCs) were generated. MoDCs were stimulated with DME for 24 h and TARC mRNA expression was examined. Both non-stimulated and DME-stimulated MoDCs expressed high levels of TARC mRNA. Each experiment was performed at least three times and representative data are shown. Upper panel shows bands for TARC mRNA (511 bp) and lower panel shows bands for β-actin (380 bp).

patients. When the PBMCs were not stimulated, $CD4^+$ T and $CD8^+$ T cells expressed very little TARC mRNA, however TARC mRNA expression was dramatically enhanced in both $CD4^+$ T and $CD8^+$ T cells, when PBMCs from AD patients were stimulated with DME for 48 h (Fig. 2). We next examined TARC mRNA expression in MoDCs. Both DME-stimulated and non-stimulated MoDCs from AD patients strongly expressed TARC mRNA (Fig. 2).

3.3. Effects of immunosuppressive drugs on TARC production by DME-treated PBMCs from AD patients

We next investigated TARC production by PBMCs from AD patients which had been cultured with DME and immunosuppressive drugs (Tacrolimus, CsA or Dex) for 7 days and found that the drugs dose dependently inhibited TARC production by PBMCs as shown in Fig. 3. There were no significant differences of viability between non-treated and immunosuppressive drugs-treated groups (data not shown). ELISA showed that the inhibitory rates of TARC production by PBMCs cultured with Tacrolimus, CsA and Dex in AD patients were as follows; Tacrolimus: 87.4% (10^{-6} M), 79.4% (10^{-8} M), 47.1% (10^{-10} M); CsA: 79.5% (10^{-6} M), 29.2% (10^{-8} M), 13.8% (10^{-10} M); Dex: 74.2% (10^{-6} M), 29.1% (10^{-8} M), 2.1% (10^{-10} M), respectively.

3.4. Effects of immunosuppressive drugs on NFκB DNA binding by DME-treated PBMCs from AD patients

We investigated the effects of immunosuppressive drugs on NFκB activity in PBMCs from AD patients which were stimulated with DME. As shown in Fig. 4, NFκB DNA binding activity increased when

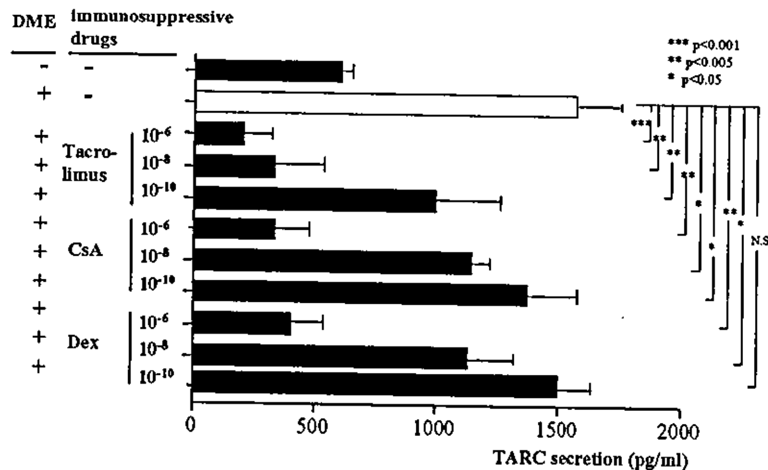

Fig. 3 Inhibitory effects of immunosuppressive drugs on TARC protein production by PBMCs from AD patients cultured with DME. PBMCs were cultured with DME (10 μg/ml); and various immunosuppressive drugs (10^{-8} M); for 7 days, and TARC levels in the supernatants were measured. The inhibitory effects of various immunosuppressive drugs on TARC protein production by PBMCs from AD patients cultured with DME for 7 days were as follows: Tacrolimus: 87.4% (10^{-6} M) 79.4% (10^{-8} M); 47.1% (10^{-10} M); CsA: 79.5% (10^{-6} M), 29.2% (10^{-8} M), 13.8% (10^{-10} M); Dex: 74.2% (10^{-6} M), 29.1% (10^{-8} M), 2.1% (10^{-10} M), respectively. The each experiment was performed at least three times and the average inhibitory rate was determined.

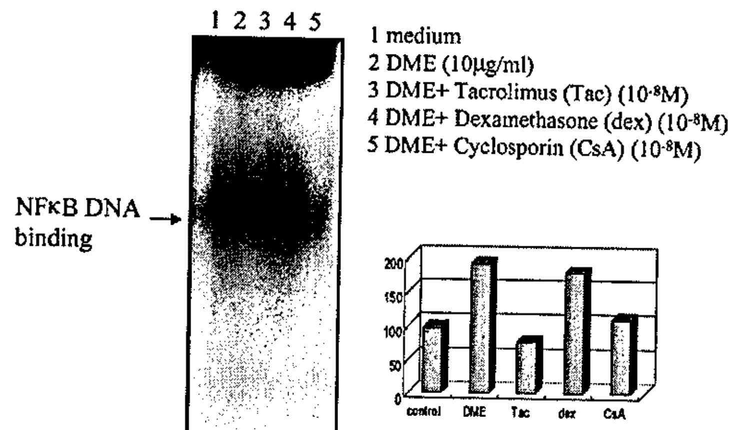

Fig. 4 Inhibitory effects of immunosuppressive drugs on NFκB activity in PBMCs from AD patients cultured with DME. The inhibitory effects of immunosuppressive drugs on NFκB activity by PBMCs cultured with DME were examined. NFκB activity was enhanced by DME- stimulation of PBMCs from AD patients. The inhibitory effects of Tacrolimus and CsA on NFκB activity were dramatic, while Dex inhibited NFκB activity only slightly. Each experiment was performed at least three times and representative data are shown. The band density was shown in the right panel.

PBMCs were cultured with DME (lane 2). Tacrolimus (10^{-8} M) and CsA (10^{-8} M) strongly suppressed NFκB binding while Dex (10^{-8} M) slightly suppressed NFκB DNA binding in nuclear protein obtained from stimulated PBMCs from AD patients.

4. Discussion

This study demonstrated the following: (1) PBMCs from AD patients which were cultured without stimulation for 7 days produced significantly higher levels of TARC protein (440.3 ± 72.8 pg/ml) compared to those from healthy controls (24.4±10.1 pg/ml, $P<0.01$). (2) When PBMCs from AD patients were stimulated with DME for 7 days, the levels of TARC production markedly increased up to 1652.3 ± 368.5 pg/ml, which was significantly higher than those without stimulation ($P < 0.001$). (3) PBMCs from AD patients which were stimulated with DME for 7 days produced significantly higher levels of TARC compared to those from healthy controls (163.4 ± 23.3 pg/ml, $P < 0.001$). (4) RT–PCR analysis demonstrated that in AD patients TARC mRNA was expressed in $CD4^+$ T cells, $CD8^+$ T cells and MoDCs. (5) ELISA showed that immunosuppressive drugs such as Tacrolimus, CsA and Dex suppressed TARC production by PBMCs from AD patients stimulated with DME for 7 days. (6) Gel shift assays revealed that immunosuppressive drugs differentially suppress NFκB activity in PBMCs from AD patients which had been stimulated with DME.

AD is an inflammatory skin disease characterized by infiltration of eosinophils, mast cells and Th2-type T cells in the lesional skin [7]. The immunological abnormality is characterized by overproduction of Th2 cytokines such as IL-4, IL5 and IL-13 and GM–CSF [7,8,14]. Among several factors which induce skin manifestations of AD, dust mite allergens is one of the major triggers that affects Th2 cytokine production in PBMCs of AD patients [9]. We showed previously that DME induces secretion of Th2 cytokines such as IL-4, IL-5 and IL-13 by PBMCs from AD patients [9]. Thus these data indicate that leukocytes in peripheral blood from AD patients produced predominantly Th2 cytokine patterns. The recent paper revealed that the lesional skin of AD showed Th2 cytokine mRNAs in acute lesional skin and the participation of Th1 cytokines such as IL-12 mRNA in chronic phase, and the mechanism of Th1/Th2 balance in the lesional skin of AD patients has not been completely elucidated [15].

TARC is a Th2 chemokine which can bind and attract $CCR4^+$ Th cells. We reported high levels of TARC in the sera of AD patients and demonstrated that TARC plays an important role in defining Th2 shift imbalance in the pathogenesis of AD [6]. In this paper we demonstrated that TARC production by PBMCs from AD patients is increased by the stimulation with DME. It has been reported that monocytes and DCs secrete significant amounts of TARC protein when stimulated, however un-stimulated T cells did not express TARC mRNA [16]. In this paper we clearly demonstrated that $CD4^+$ T and $CD8^+$ T cells from AD patients have the capacity to express TARC mRNA. Interestingly, when MoDCs were purified from PBMCs from AD patients, they strongly expressed TARC mRNA with or without the stimulation

by DME. These observation, strongly suggest that DCs, as well as $CD4^+$ T and $CD8^+$ T cells, produce TARC in PBMCs from AD patients, and we suggest that the TARC producing cells in AD are DCs, T cells in peripheral blood in addition to epidermal KCs as we previously demonstrated [6]. It has been reported that TARC/CCL17-deficient mice showed diminished contact hypersensitivity responses with decreased levels of antigen presenting capacity of TARC-deficient DCs [17]. Thus, we also suggest that TARC producing DCs attract Th2 cells as well as $CD25^+$ T cells and that these may participate in the regulation of immune responses in AD [18].

Immunosuppressive drugs are very effective and widely used for the treatment of AD. Tacrolimus and CsA bind calcineurin resulting in the inhibition of NF–AT nuclear translocation [6]. Glucocorticosteroids bind glucocorticoid receptors and inhibit IL-2 promoter activities by the interaction of glucocorticoid receptor with AP-1 to prevent it from binding to the IL-2 promoter [19]. In addition, glucocorticoids inhibit IL-5 production through RE-II region which contain NF–AT transcription region [20]. It has been reported that Tacrolimus inhibits IL-4 production through NF–AT activity in basophils [21]. We examined the effects of DME and the immunosuppressive drugs on NFκB activity in PBMCs from AD patients. Our data showed that DME strongly induced NFκB activity in PBMCs of AD patients. When PBMCs were stimulated with PMA, the enhancement of TARC production by PBMCs was detected in both AD patients and healthy controls, however, the enhanced levels was lower than those stimulated with DME stimulation (data not shown). Our data suggest that TARC production by PBMCs in AD patients may be enhanced by DME through the enhanced NFκB DNA binding activity.

In addition, we demonstrated that immunosuppressive drugs differentially inhibited NFκB activity of PBMCs. The inhibitory effect of tacrolimus and CsA on NFκB activity in PBMCs of AD patients was observed prominent. Because it is reported that the NFκB binding site may be located in TARC promoter lesion [22], we suggest that the inhibitory effect of immunosuppressive drugs such as Tacroimus and CsA on TARC production from PBMCs in AD may be partially explained by the inhibition of NFκB DNA binding activity in PBMCs. Further investigation will be required to clarify the mechanism of the regulation of TARC promoter activity in PBMCs of AD.

This paper demonstrated that overproduction of TARC levels was induced by DME in PBMCs of AD patients and that TARC mRNA was produced by $CD4^+$ T cells, $CD8^+$ T cells and MoDCs in AD patients. Immunosuppressive drugs inhibited TARC protein by PBMCs in AD patients. We conclude that Th2 chemokine, TARC, is produced by MoDCs and lymphocytes as well as epidermal KCs in AD, and that that these may be important in the regulation of T cells recruitment in peripheral blood in AD patients. Further study will be required to elucidate Th1/Th2 balance in the pathogenesis of AD.

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