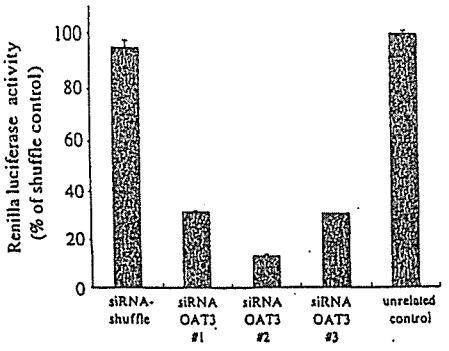

Fig. 1. Effect of siRNAs directed against the OAT3 in vitro. HEK293 cells were transfected with *Renilla* luciferase-fused OAT3 expression vector, *firefly* luciferase expression vector, and 25 nM siRNA. Reduction effect of *Renilla* luciferase activity relative to *firefly* luciferase activity was analyzed. Negative controls were the siRNA with randomized sequence of siRNA OAT3 #2 (siRNA-shuffle) and the siRNA against unrelated gene. Data were averaged from three experiments with SEM indicated.

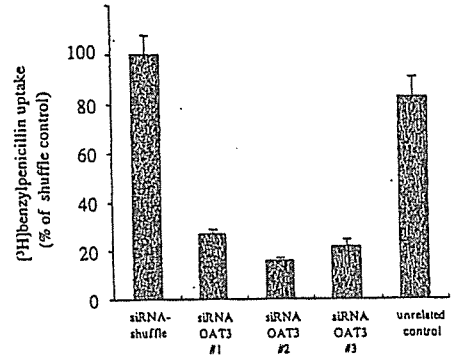

Fig. 2. Effect of siRNAs on uptake of OAT3 substrate in culture cells. Effect of siRNAs OAT3 on the OAT-3-mediated [^{3}H]benzylpenicillin uptake in HEK293 cells. After expression of OAT3 to the cells, [^{3}H]benzylpenicillin uptake was performed at 2 min, reflecting the initial uptake phase. All siRNAs were used at a concentration of 100 nM. Each value represents the mean ± SEM ($n = 4$). The increased uptake by expression of OAT3 was significantly reduced by siRNA OAT3 compared to siRNA-shuffle and siRNA-unrelated control. ($p < 0.0001$).

to HEK293 cells, the uptake mediated OAT3 was increased, and siRNA OAT3 #2 significantly inhibited the increased uptake of the substrate in HEK293 cells, compared with siRNA-shuffle and siRNA-unrelated control (Fig. 2).

In vivo delivery of siRNA to brain endothelial cells

We biochemically investigated an inhibitory effect of siRNA on expression of endogenous protein in BCECs using brain vascular fraction of small vessels from mouse brain.

For detection of endogenous protein in BCECs, we used SOD1 and siRNA to SOD1, because we have confirmed the efficient in vivo effect of this siRNA to endogenous mouse SOD1 in the siRNA-overexpressed transgenic mouse [14].

Western blot of the mouse brain small vascular fraction showed a reduction of endogenous mouse SOD1 level after hydrodynamic injection of siRNA SOD1 (Fig. 3A, left), whereas SOD1 level in the total homogenate of brain did not change (data not shown). There was a potentially more significant level of reduction on a per-BCEC basis, because the brain small vascular fraction contained proteins from cells other than BCECs such as pericytes and astrocytes [15]. We roughly estimated the content of BCECs in the brain small vascular fraction by performing a Western blot analysis with antibody to glucose-transporter-1 (GLUT-1) which specifically expressed in BCECs (Fig. 3B). The band intensity of GLUT-1 in the brain small vascular fraction was 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines which we previously established [12] (Fig. 3B). Since the cell line contains more than 1/8 of GLUT-1 [12], around 50% protein of the brain small vascular fraction that we made was supposed to come from brain endothelial cells.

In contrast, there was not obvious reduction of SOD1 level in the small vascular fraction after a regular intrave-

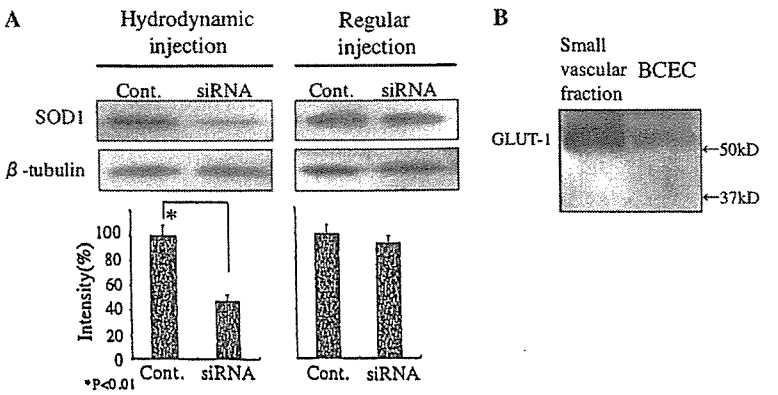

Fig. 3. Western blot analysis of mouse brain capillary-rich fraction. (A) The mouse brain small vascular fraction was examined on Western blot analysis after hydrodynamic (left) and regular (right) injection of 50 μg siRNA SOD1. The lower panels indicate percentages of signal intensities of SOD1 normalized with that of tubulin. (B) Western blot analysis with 2.5 μg protein of anti-GLUT-1 antibody of the mouse brain small vascular fraction (left) and mouse brain capillary endothelial cell lines (right). Signal intensity of GLUT-1 in the mouse brain small vascular fraction is 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines. BCEC, brain capillary endothelial cell line cells.

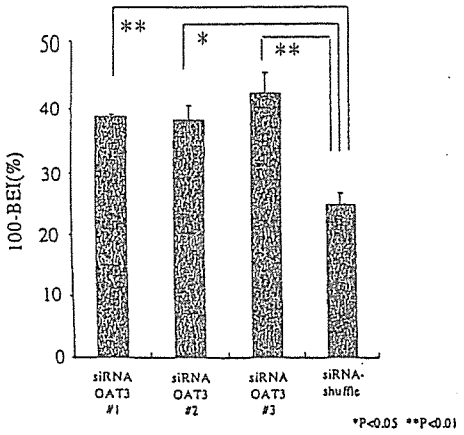

Fig. 4. Effect of siRNA on transport function of OAT3 by BEI. The 50 μg siRNA dissolved in the 5–10% volume PBS of mouse body weight was rapidly injected into the tail vein 36 h before the BEI assay. The residual radioactivity of OAT3 substrate, [^{3}H]benzylpenicillin in the brain, was measured at 40 min after intracerebral injection.

nous injection (Fig. 3A, right). These results indicate that hydrodynamic injection method is effective for delivery of siRNA to brain capillary endothelial cells.

In vivo effect of siRNA on transporter function of OAT3 in vivo

The in vivo inhibitory effect of siRNA OAT3 on the brain-to-blood efflux transport was examined with BEI method with intracerebral injection of OAT3 substrate, [^{3}H]benzylpenicillin. We intravenously injected siRNA OAT3 #2 to 11 mice, siRNA OAT3 #1 and #3 to 3 mice each, and siRNA-shuffle (control) to 7 mice with hydrodynamic method. Transport function of OAT3 was evaluated by BEI method at 36 h after the injection of siRNA. The results of 100-BEI, percentage of OAT3 substrate remaining in the brain after injection, are shown in Fig. 4. The value of 100-BEI of siRNA OAT3 #2 is significantly higher than that of siRNA-shuffle by 26.4%. Those of siRNA OAT3 #1 and #3 were also similarly higher than that of control. The results that plural different siRNAs to the OAT3 gene similarly increased 100-BEI value indicated that these siRNA influences were not "off-target effect" on molecules other than OAT3 in the brain. Taken together, these results suggested that in vivo applied-siRNA to OAT3 could suppress the brain-to-blood efflux function of OAT3.

Discussion

This is the first report of successful in vivo inhibition of endogenous gene in BCECs by systemic intravenous injection of siRNA. Furthermore, we demonstrated that our gene silencing effect was enough to suppress the transport function of OAT3 endogenously expressed in BCECs at BBB. We could deliver siRNA to BCECs by hydrodynamic injection method, but not by regular intravenous injection from the mouse tail vein. It has been thought that a rapid injection of a large bolus of solution develops a high pressure in the inferior vena cava, causing retrograde movement of the solution to the abdominal organs including liver and kidneys. Such a sharp increase in venous pressure enlarges the liver fenestrae and promotes membrane permeability of the hepatocytes, making siRNA enter the cells [16]. Since BCECs are circulated from the tail vein via lung capillary, the phasic hydrodynamic pressure in the inferior vena cava should decrease in the lung. However, rapid loading of extremely large volume of solution, 40–80% of circulating plasma volume should considerably increase hydrostatic pressure in the carotid artery due to volume overload. In addition, the rapid injection of large volume solution prevents the solution from being mixed with the serum containing RNase and keeps the concentration of siRNA extremely high when it is delivered to BCECs.

This in vivo knockdown method with siRNA to BCECs is expected to be a powerful tool for investigating function of BBB. The BBB is formed by the tight intercellular junctions of BCECs and regulates CNS homeostasis and drug delivery by restricting the transfer of substances between the circulating blood and the brain [17]. We have developed Brain Efflux Index as a reliable in vivo method of analyzing efflux transport at the BBB [18]. The efflux function of a transporter protein expressed in BCECs, such as OAT3, can be well evaluated by combining in vivo knockdown method with siRNA and BEI method.

Since synthetic siRNA does not work in the cells for no more than six days [19], long-term silencing of the target gene is necessary for investigating other functions of BCECs in the pathophysiology of atherosclerosis and Alzheimer's disease. Long-standing gene suppression can be achieved in vivo with adenovirus and adeno-associated virus (AAV) vectors expressing short hairpin RNA (shRNA) [20,21]. Actually, with the adenovirus expressing shRNA to SOD1 gene (2.0×10^9 pfu), we could efficiently suppress the endogenous SOD1 level of brain capillary-rich fraction by regular intravenous injection into mouse tail vein (unpublished data). For the evaluation of BCEC function, however, the AAV may be better than adenovirus, because BBB function should be less affected due to limited local immune response to the AAV capsid [22].

The hydrodynamic injection does not cause marked injury to organs in the animals [23], but it is hard to be clinically applied to patients because of its extremely high hydrostatic pressure and volume overload. Possible alternate is a regional delivery of large dose siRNA into carotid artery, but development of less invasive systemic delivery system in vivo is necessary for a therapeutic application of siRNA. Novel cationic liposomes have been reported to transduce efficiently siRNA into the liver [24] as well as tumor tissue [25]. These siRNAs formulated with cationic liposomes also induce interferons and cytokines in vivo through toll-like receptors [26,27] which should change the BBB function. Recently, the lipid-conjugated siRNA

at the 5′-end of the sense strand enhanced cellular uptake and gene silencing [28]. Combined with chemical modification of 2′-O-methylation and phosphorothioate to stabilize siRNA, substantial gene silencing in the liver and jejunum was achieved by a regular intravenous injection into the mouse tail vein [29]. Now, we are trying to use these new siRNA delivery methods to achieve more effective, stable, and safe gene suppression in BCECs for a clinical application.

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Type I Interferons Attenuate T Cell Activating Functions of Human Mast Cells by Decreasing TNF-α Production and OX40 Ligand Expression While Increasing IL-10 Production

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Recent studies have demonstrated that mast cells not only mediate inflammatory reactions in type I allergy but also play an important role in adaptive immunity. In the present study, we investigated the effects of interferon-α, which shares the same receptor as IFN-β, on human cord blood-derived mast cells. Mast cells produced TNF-α, and IL-10, and expressed OX40 ligand upon activation by crosslinking of FcεRI. When treated with interferon-α, TNF-α production was decreased while IL-10 and TGF-β productions were increased. Furthermore, flow cytometric analysis revealed that interferon-α downregulated expression OX40 ligand on mast cells which is crucial for mast cell-T cell interaction. We confirmed that the viability of mast cells was not affected by interferon-α treatment. Accordingly, interferon-α-treated mast cells induced lower levels of $CD4^+$ T cell proliferation compared with those without interferon-α treatment. These results suggest that type I interferons suppress T cell immune responses through their regulatory effects on mast cells.

KEY WORDS: Mast cells; $CD4^+$ T cells; Type I interferons; OX40 ligand; .

INTRODUCTION

Mast cells (MCs) are well known for their ability to mediate inflammatory reactions in pathological processes of allergic diseases (1). Upon recognition of specific antigens by IgE bound to FcεRI, MCs secrete various kinds of cytokines, chemokines, and chemical mediators, which trigger allergic reactions (2). This IgE-dependent activation of MCs has been emphasized as a major causative event of harmful type I hypersensitivity. However, MCs are originally engaged in the first line defense against bacterial as well as parasite infections. Recent evidence has indicated that in mice, MCs recruit and activate T cells in the draining lymph nodes during bacterial infection and thus positively regulate adaptive immunity (3). TNF-α has been shown to play a pivotal role in this enforcement of T cell responses (3, 4). In addition to TNF-α secretion, MCs can directly interact with T cells and even present antigens to T cells in the context of MHC class I and class II (5–7), resulting in enhanced T cell proliferation. It has been reported that human MCs and murine BMMCs express OX40 ligand (OX40L) upon activation and the OX40/OX40L system plays a crucial role in MCs-mediated T cell proliferation (8, 9).

In clinical settings, MCs are implicated in the pathogenesis of most allergic diseases in which Th2-type immune responses appear to be predominant such as bronchial asthma and atopic dermatitis (10). On the other hand, recent reports have revealed that MCs also play important roles in some Th1 dominant diseases such as inflammatory bowel diseases (11), multiple sclerosis (MS) (12), and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. MS is a demyelinating disorder of the central nervous system that preferentially affects young adults. Although myelin-specific $CD4^+$ T cells are principal effector cells, other cell types are also likely to be involved in the pathogenesis of the disease. It is noted that MCs are required for the development and aggravation of EAE because MC-deficient W/W^v mice are less sensitive to EAE than wild-type mice and reconstitution of MCs by injection of bone marrow-derived MCs from wild-type mice restore the severity of EAE(13). In humans, MCs are found in the CNS plaques of MS patients (14) and

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the concentrations of histamine and tryptase were higher in MS patients compared with normal healthy individuals (15, 16), suggesting that MCs are also involved in MS.

Treatment of MS remains to be a challenging problem. Apart from several immunosuppressive drugs, type I IFNs have been shown to be effective in a proportion of MS patients (17), although the precise mechanism of their effects has not been fully elucidated. In a study using specimens from MS patients, IFN-β has been reported to correct Th1/Th2 imbalance by increasing IL-4 production and lowering IFN-γ production (18). Since type I IFNs do not have such a direct effect on $CD4^+$ T cells, these reports suggest the presence of other target cells of type I IFNs that mediate alleviation of pathological T cell responses. Based on the roles of MCs in EAE and MS as discussed above, it is to be determined whether type I IFNs could modify MC-mediated T cell activation processes. As far as we know, effects of IFNs on MCs have not been studied in detail. In the present study, we addressed this question by using human cord blood-derived MCs and IFN-α, a type I IFN. Herewith we report that IFN-α did not affect the viability of MCs but decreased TNF-α production and OX40 ligand expression while increased TGF-β and IL-10 production. Thus, type I IFNs indirectly attenuate T cell responses through the regulatory effects on MCs. The possible involvement of MCs in the effectiveness of IFN-β therapy for MS and other Th1 diseases is discussed.

MATERIALS AND METHODS

Antibodies and Cytokines

SCF and IL-6 were purchased from Wako (Tokyo, Japan) and IL-3 and IL-4 were from Peprotech (Rock Hill, NJ). Human myeloma IgE was from Chemicon International (Temecula, CA) and goat anti-human IgE from Biosource (Camarillo, CA). Phycoerythrin (PE)-conjugated anti-human OX40 ligand monoclonal antibody (mAb) (clone ik-1) and PE-conjugated mouse IgG1 was obtained from Becton-Dickinson (Franklin Lakes, NJ). Anti-OX40L blocking mAb (mouse IgG2a, clone ik-5) was generated in our laboratory (19). Control IgG2a (clone UPC10) was purchased from Sigma (St. Louis, MO).

Preparation of MCs

Human cord blood-derived MCs (CBMCs) were generated as described (20). In brief, mononuclear cells were isolated from cord blood of healthy neonates with the informed consent of the parents and subsequently cultured in AIM-V (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (penicillin–streptomycin–glutamine; Life Technologies), 100 ng/ml SCF and 50 ng/ml IL-6. Half medium change was performed once a week. Cells cultured for more than 8 weeks were used which consisted of >95% pure MCs measured as c-kit-positive cells by flow cytometry.

Activation of MCs

Naïve MCs were cultured with 5 ng/ml IL-3 and 10 ng/ml IL-4 for 5 days. Human IgE was added at 1 μg/ml for the last 24 h to be presensitized. For the aggregation of FcεRI, presensitized MCs were collected, washed three times, and cultured with 2 μg/ml goat anti-human IgE Ab in SCF-containing medium (without IL-6) in the presence or absence of IFN-α for the indicated periods of time.

RT-PCR

Presensitized MCs were cultured with anti-IgE Ab and with or without IFN-α (Biosource, Camarillo, CA) for 6 h. Cells were then collected and washed three times. Total RNA was isolated with RNeasy mini kit (QIAGEN, Hilden, Germany). 1 μg of RNA was used in reverse transcription using ImProm-II™ reverse transcription system (Promega, Madison, WI). The same amount of complementary DNA was used in the subsequent PCR. The following primers were used: β-actin (sense 5′-TCAGCCTCTTCTCCTTCCTGATCG-3′; antisense 5′-TGAAGAGGACCTGGGAGTAGATGA-3′) (21), TNF-α (5′-GTTCCTCAGCCTCTTCTCCT-3′; antisense 5′-ATCTATCTGGGAGGGGTCT T-3′) (22), IL-10 (sense 5′-ATGCCCCAAGCTGAGAAC CAAGACC CA-3′; antisense 5′-AAGGGGCTGGGTCAGCTATCCCA-3′) (23), and TGF-β (sense 5′-CTACTAGGCCAAGGA GGTCAC-3′; antisense 5′-TTGCTGAGGTATGGCCAG GAA-3′) (24). PCR products were separated through 1.5% agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator.

Measurement of Cytokine Production

Cytokine production was measured by ELISA according to the manufacture's protocol. Presensitzed MCs were treated with goat anti-human IgE Ab and IFN-α for 6 h at 1.0×10^6/ml. Culture supernatants were then collected and stored at −80°C until they were subjected to analysis. Concentrations of TNF-α, IL-10, and TGF-β in the supernatants were measured by using the respective ELISA kits (Biosource).

MTT Assay

2×10^4 presensitized CBMCs were activated with goat anti-IgE Ab in the presence or absence of IFN-α in a total volume of 100 μl/well in a 96 well flat-bottomed plate (IWAKI, Tokyo, Japan). 10 μl cell count reagent SF (Nakalai, Kyoto, Japan) per well was added after 24 h and MCs were further incubated for 1 h. Optical density values at 450 nm (OD 450) were measured with a microplate reader (BioRad, Hercules, CA).

Flow Cytometric Analysis of OX40L Expression

MCs were presensitized and activated in the presence or absence of 100 ng/ml IFN-α. After 24 h, cells were collected and stained with either PE-conjugated anti-human OX40L mAb or PE-conjugated mouse IgG_1 (Becton-Dickinson). After immunofluorescence staining, cells were analyzed with a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

$CD4^+$ T Cell Proliferation Assay

To avoid the influence of IFN-α on $CD4^+$ T cells, MCs that had been presensitized and treated with anti-IgE Ab and IFN-α were washed thoroughly and suspended in RPMI1640 supplemented with 10% FBS. Since MCs are unable to proliferate in the absence of SCF, we used MCs without any anti-proliferative treatment such as irradiation and exposure to mitomycin C that might affect the functions of MCs. $CD4^+$ T cells were purified with CD4 isolation kit (Miltenyi Biotech) from PBMC of normal healthy donors. The purity of $CD4^+$ T cells was >95% in all assays performed. In a 96-well round-bottomed plate (IWAKI, Tokyo, Japan), 1×10^5 $CD4^+$ T cells were cultured with 1×10^5 anti-CD3-coated beads (Dynabeads CD3, Dynal Biotech, Oslo, Norway), and activated MCs (at MC/T ratios of 1/5, 1/10, and 1/20) in a total volume of 200 μl. To evaluate the involvement of the OX40/OX40L system, some cultures were set up in the presence of either 50 μg/ml ik-5 or control IgG2a. Cells were cultured in triplicate for 5 days and pulsed with [^{3}H]thymidine ([^{3}H]TdR) (0.5 μCi/well; MEN Life Science, Boston, MA) for the last 6 h of culture. After harvesting cells, the incorporated radioactivity was measured in a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL).

Statistical Analysis

Statistical analyses were performed by Student's t-test or paired t-test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

TNF-α Production was Decreased While IL-10 Production was Increased in IFN-α-Treated MCs

MCs are known to release various kinds of cytokines and chemokines upon activation. Among these, TNF-α is crucial in MC-$CD4^+$ T interaction (4). IL-10 is an immunoregulatory cytokines which inhibit T cell proliferation. Therefore, we focused on these cytokines and investigated their production in IFN-α-treated MCs. Expression of TNF-α mRNA was decreased while IL-10 and TGF-β mRNA were increased in IFN-α-treated MCs (Fig. 1a). To confirm these results, we measured the concentrations of TNF-α, IL-10 and TGF-β in the supernatants of activated MCs preactivated with or without IFN-α by ELISA. As shown in Fig. 1b, pretreatment with IFN-α decreased the production of TNF-α in a dose-dependent manner. Three independent experiments were done with different CBMC batches to give similar results. The decrease in TNF-α production by IFN-α (at 100 ng/ml) was statistically significant. Conversely, pretreatment with IFN-α increased IL-10 production reproducibly (Fig. 1c). This increase was also statistically significant. TGF-β production was slightly increased by IFN-α pretreatment, but the difference was not statistically significant (data not shown).

IFN-α Treatment did not Affect the Survival of MCs

It is known that the proliferation of human bone marrow-derived MCs was not affected by IFN-α treatment (25). However, the effect of IFN-α on the survival of CBMC has not been reported. To exclude the possibility that the decrease in cytokine production was due to impaired survival of CBMCs, we performed MTT assay of CBMCs cultured with various concentrations of IFN-α. As shown in Fig. 2, IFN-α pretreatment did not affect the survival of CBMCs at any concentrations tested.

Pretreatment with IFN-α Suppresses OX40L Expression on Activated MCs

OX40 is a costimulatory molecule that potently promotes $CD4^+$ T cell proliferation. Its ligand, OX40L, has been reported to be expressed on human tonsillar and PBMC-derived MCs upon crosslinking of FcεRI and play a vital role in MC-$CD4^+$ T cell interaction although the expression of OX40L on CBMCs has not been reported. In $OX40L^{-/-}$ mice, The severity of EAE was markedly attenuated, whereas it was significantly enhanced in OX40L

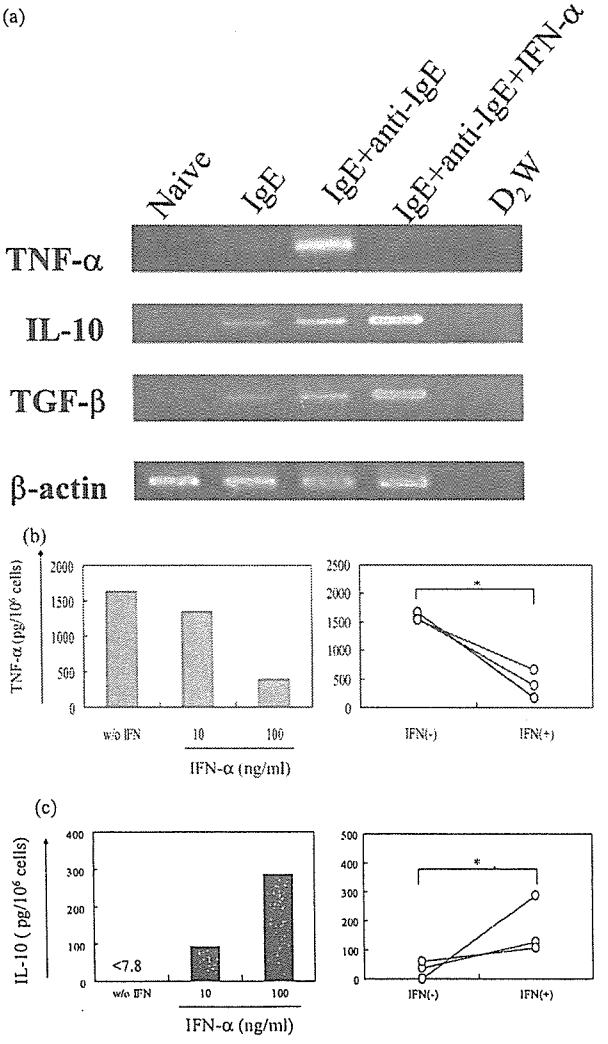

Fig. 1. (a) RT-PCR for TNF-α, IL-10, and TGF-β. PCR products were electrophoresed on 1.5% agarose gel. Data shows a representative of three independent experiments. (b) and (c) ELISA assay of TNF-α and IL-10. TNF-α and IL-10 productions of presensitized CBMCs activated in the presence or absence of IFN-α were measured by ELISA. A representative of three independent experiments for each cytokine is shown (left). The difference in each cytokine production (pg/ml) between the absence and presence of 100 ng/ml IFN-α was analyzed statistically. $^*p < 0.05$.

transgenic mice (26). If MCs participate in the pathogenesis of EAE, it is likely that OX40L expressed on MCs are involved in such process. We first confirmed that OX40L was also expressed on CBMCs after FcεRI crosslinking as reported with tonsillar and PBMC-derived MCs and found that pretreatment with IFN-α considerably suppressed its expression (Fig. 3).

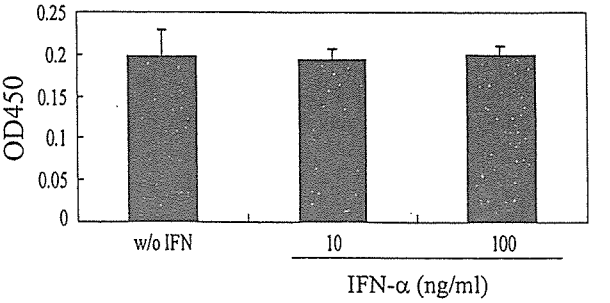

Fig. 2. Survival of IFN-α-treated MCs was measured by MTT assay. MTT assay was performed 24 h after activation with anti-IgE Ab and the indicated concentrations of IFN-α. Data indicate mean ± SD of triplicate wells of a representative of three independent experiments.

Pretreatment of MCs with IFN-α Significantly Inhibited Costimulation of CD4⁺ T Cells by CBMCsn

Finally we examined the effects of IFN-α on T cell activating functions of MCs, because TNF-α and OX40L which we showed were suppressed by IFN-α are both important factors for T cell costimulation by MCs. We cocultured $CD4^+$ T cells from normal healthy donors and preactivated MCs with or without IFN-α treatment, and analyzed T cell proliferation on day 5. In this assay, IFN-α and anti-IgE Ab were washed out before coculture in order to exclude the effect of IFN-α on $CD4^+$ T cells. As shown in Fig. 4a, IFN-α-treated MCs induced lower levels of T cell proliferation compared with non-treated MCs, indicating that IFN-α suppressed T cell activating functions of MCs. The OX40/OX40L system is reported to play a role in MC-T cell interaction in humans (8). In accordance with the previous report, an addition of anti-OX40L mAb significantly suppressed the costimulatory functions of activated CBMCs (Table I).

DISCUSSION

Accumulating evidence has indicated that MCs are not simple effector cells in allergic reactions but are multifunctional accessory cells for T cell responses that influence the magnitude and direction of adaptive immunity. Consistent with this new concept, MCs have been shown to play important roles in the pathogenesis of not only Th2 diseases but also some Th1 diseases such as inflammatory bowel disease and MS (11, 12). Thus, it is essential to understand the regulation of MC functions in order to elucidate the MC-mediated pathogenesis and control of the diseases.

In the present study, we showed that pretreatment of MCs with IFN-α decreased TNF-α production and

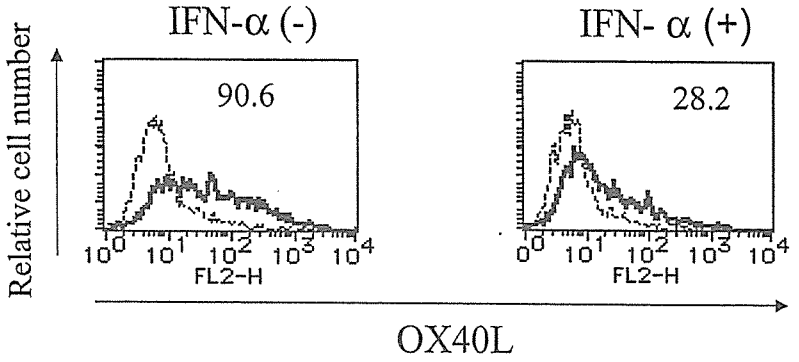

Fig. 3. OX40L expression on activated CBMCs. Presensitized MCs activated in the absence or presence of 100 ng/ml IFN-α were subjected to flow cytometric analysis. Data of a representative of three independent experiments are shown. Numbers in the histograms indicate ΔMFI of OX40L expression.

OX40L expression, while IL-10 production was increased. In accordance with these results, IFN-α significantly suppressed the costimulatory functions of MCs in coculture with $CD4^+$ T cells. In the literature, we found two primitive reports that IFN-α/β plus IFN-γ suppressed TNF-α mRNA levels in rat MC lines and rat peritoneal MCs (27), and that in vivo topical administration of IFN-α to nasal mucosa of allergic patients resulted in an decrease in TNF-α^+ mucosal MC number in the biopsy specimens (28). In these reports, however, the effects of type I IFNs on actual TNF-α secretion, on other cytokine productions, on OX40L expression, or on T cell costimulatory functions were not investigated. Thus, the effects of IFN-α on MC functions have never been studied in detail. As far as we know, the present study is the first to delineate the entire picture of the effects of IFN-α on the MC functions.

IFN-α-induced increase in IL-10 production by MCs is particularly important because IL-10 negatively affects immune responses by inhibiting the production of inflammatory cytokines. IL-10 is reported to suppress the release of inflammatory cytokine by MCs in an autocrine manner (20). Thus, it is possible that inhibition of TNF-α production presented in this study was mediated by increased IL-10 that was induced by IFN-α treatment. In allergic reactions, the role of IL-10-secreting T(R)1 cells is reported to induce tolerance (29). However, the role of IL-10 secreted by MCs in the context of adaptive immunity has never been reported. Our results suggest the possibility that immunoregulatory cytokines derived from IFN-α-treated MCs may contribute to the suppression of costimulatory function of MCs themselves and other surrounding immune cells leading to regulation of the overall inflammatory reactions.

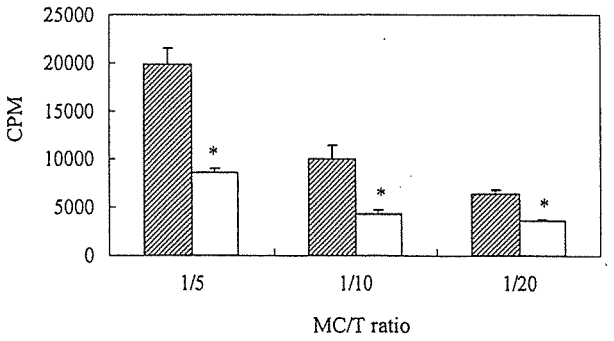

Fig. 4. Coculture of CBMCs and $CD4^+$ T from normal healthy donors. Presensitized CBMCs were activated with goat anti-IgE in the presence or absence of 100 ng/ml IFN-α for 24 h and subsequently washed. 1×10^5 T cells were cocultured with MCs at indicated MC/T ratios in the presence of anti-CD3-coated beads for 5 days. Cells were pulsed with [^{3}H]TdR for the last 6 h of culture and the incorporated radioactivity was measured by a liquid scintillation counter. Data indicate mean $\pm$ SD of triplicate wells. Striped bars indicate CBMCs activated without IFN-α and white bars indicate CBMCs activated with IFN-α. $^*p < 0.05$ compared with "without IFN-α."

Table I. The Effect of Anti-OX40L mAb on the Costimulatory Activities of CBMCs

	CPM $\pm$ SD
w/o mAb	10118 $\pm$ 1303
Control	13047 $\pm$ 490
ik-5	7132 $\pm$ 350

Notes. Presensitized CBMCs were activated with IgE and anti-IgE Ab, washed, and then cocultured with $CD4^+$ T cells and anti-CD3-coated beads in the absence or presence of either 50 μg/ml ik-5 or control IgG2a for 5 days. T cell proliferation was measured by [^{3}H]TdR incorporation for the last 6 h. Three independent experiments were done and gave similar results. Data indicate the mean $\pm$ SD of triplicate cultures of a representative experiment.

Downregulation of OX40L expression on MCs by IFN-α is also impressive. OX40L can be induced on human peripheral blood stem cells-derived MCs in vitro and human MCs isolated from tonsil and lung (8). These MCs have been reported to interact directly with $CD4^+$ T cells and augment their proliferation mainly via the OX40/OX40L system. It is now recognized that expression of OX40L on antigen presenting cells that stand for mostly dendritic cells (DCs) is the key event for promotion of inflammatory responses. In fact, OX40/OX40L system is implicated in the exacerbation of EAE (26, 30). Considering that presence of MCs is required for the development of severe EAE, it is possible that type I IFNs ameliorate the manifestations of MS by suppressing the expression of OX40L on MCs.

In conclusion, we showed here that type I IFNs attenuate T cell activating functions of human MCs by decreasing TNF-α production and OX40 ligand expression while increasing IL-10 production. Effectiveness of type I IFNs in the treatment of MS is still an enigma, since IFNs are considered to be a proinflammatory cytokine enhancing Th1 immune responses. The present study provides a novel insight into the inhibitory aspects of IFNs action and presents the possibility that IFNs might influence T cell response via their regulatory effects on MCs in inflammatory Th1 diseases including MS.

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IgE-activated mast cells in combination with pro-inflammatory factors induce T_h2-promoting dendritic cells

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Keywords: allergy, antigen-presenting cells, human, T cells

Abstract

Dendritic cells (DCs) and mast cells (MCs) co-localize in peripheral tissues of antigen entry, i.e. skin and mucosa. Due to the proximity of these two cell types, activation of MCs may affect DC functions. Here, we co-cultured human monocyte-derived DCs with cord blood-derived MCs activated by cross-linking of FcεRI to elucidate the net effect of the whole MC products on DCs. Activated MCs induced maturation of DCs, and potently suppressed IL-12p70 production by the DCs. Whereas co-culture of DCs with activated MCs alone did not significantly influence the type of $CD4^+$ T cell responses induced by the DCs, DCs co-cultured with activated MCs in the presence of pro-inflammatory or T_h1-inducing factors caused T_h2 polarization. Although histamine was involved in the induction of DC maturation and T_h2 polarization by activated MCs, a combinatorial effect of various MC-derived factors, including those acting in a cell contact-dependent manner, was required for the optimal induction of T_h2-promoting DCs. Furthermore, we demonstrated that clusters of DCs are located closely with MCs in lesions of atopic dermatitis. Collectively, this study suggests that the interaction between DCs and IgE-activated MCs in a pro-inflammatory or even T_h1-prone environment is instrumental in maintaining and augmenting T_h2 responses in allergy, and that disruption of the DC–MC interaction may constitute an effective strategy to treat ongoing allergic diseases.

Introduction

Immature dendritic cells (DCs) are located mainly in peripheral tissues through which antigens invade, particularly in skin and mucosa (1). At the initial stage of an immune response, immature DCs are activated directly by pathogens and indirectly by various inflammation-associated factors produced by tissue resident cells in the microenvironment (2). Activation of DCs induces their maturation and migration to secondary lymphoid organs, where the mature DCs prime antigen-specific naive T cells. During the process of maturation, DCs integrate signals from both pathogens and tissue-derived factors and acquire the capacity of inducing different types of $CD4^+$ T cell responses, prototypes of which are T_h1 and T_h2 types. Thereby, DCs induce appropriate types of adaptive immune responses for efficient elimination of the given pathogens.

Another abundant cell type present in skin and mucosa is mast cells (MCs) (3–6). MCs are well recognized as key effector cells in IgE-associated, T_h2-type immune responses. Upon activation by cross-linking of a high-affinity IgER, FcεRI, MCs immediately undergo degranulation and secrete a vast array of humoral mediators (reviewed in 5, 6). These include preformed granule-associated molecules [e.g. neutral proteases, tumor necrosis factor (TNF)-α and histamine], *de novo* synthesized lipid mediators [e.g. prostaglandin D_2 (PGD_2), leukotriene C_4 and B_4], cytokines and chemokines. In addition to FcεRI, MCs express a diverse spectrum of receptors for 'danger' signals, such as pathogens and endogenous inflammatory mediators (5, 6). By virtue of their ability to directly sense 'dangers' and to immediately secrete a vast array of humoral mediators, MCs play an important role in the first line of defense against microbial invasions as well as in immediate allergic reactions.

Recent studies have been revealing critical roles of MCs in inducing optimal adaptive T cell responses (6). First, MCs, by immediately secreting preformed TNF-α upon bacterial invasion, induce hypertrophy of draining lymph nodes and recruitment of circulating T cells (7). Second, MCs appear to be critical for the full manifestation of experimental autoimmune

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encephalomyelitis (8, 9). Third, several studies suggest that MCs migrate to secondary lymphoid organs and influence T cell responses (9–11). Thus, other than an established role as immediate effector cells in allergic responses, MCs are likely to be critically involved in determining the strength and quality of adaptive immune responses.

MCs and immature DCs co-localize at antigen entry sites, i.e. skin and mucosa. Both human and mouse MCs activated by cross-linking of FcεRI have been shown to express markedly high levels of chemokines that attract immature DCs: CCL2, CCL3 and CCL4 (12, 13). Reciprocally, DCs have been shown to produce CCL5 and CCL8 (14) that can interact with CCR3 on MCs (15). Due to such apparent interaction between the two cell types, a vast array of humoral and possibly membrane-associated molecules derived from MCs may influence DC functions in peripheral inflamed tissues, which leads to modulation of adaptive T cell responses in draining lymphoid organs (6). Indeed, several molecules secreted by MCs have been shown to affect DC functions. First, histamine, which is stored in MC granules and is immediately released upon activation, induce human monocyte-derived dendritic cells (MoDCs) to transiently express CD86 expression (16), to produce more IL-10 and less IL-12 and to differentiate into T_h2-promoting DCs (17–20). Second, PGD_2, a major eicosanoid from MCs, reduces IL-12 production by MoDCs and favors T_h2 development (21, 22). Third, thymic stromal lymphopoietin (TSLP), whose mRNA is expressed in MCs, promote maturation of $CD11c^+$ blood DCs and their differentiation into T_h2-promoting DCs (23–25). Lastly, MC-derived exosomes have been shown to induce DC maturation (26). These studies suggest that MCs influence DC functions via different mechanisms. However, MCs express many other secretory and membrane-associated molecules that potentially affect DCs, and the net effect of the whole MC-derived factors on DC function, which will occur in a physiological situation, remains to be determined.

Here, to investigate the effects of the whole MC products on DC functions, we co-cultured human MoDCs with cord blood-derived MCs activated by cross-linking of FcεRI, and examined DC maturation, cytokine production and naive $CD4^+$ T cell differentiation primed by the DCs. Significantly, whereas co-culture of DCs with activated MCs alone did not have any effect on polarization of T cell differentiation, DCs co-cultured with activated MCs in the presence of other DC maturation-inducing factors polarized T cell responses toward a T_h2 type. Although histamine was involved in inducing T_h2-promoting DCs, combinatorial effects of other MC-derived factors, including those acting in a cell contact-dependent manner, were required for the optimal induction of T_h2-promoting DCs.

Methods

Media and reagents

RPMI 1640 (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated FCS (ThermoTrace, Victoria, Australia), 2 mM L-glutamine, penicillin G, streptomycin (GIBCO BRL, Carlsbad, CA, USA) and 10 mM HEPES (Nacalai Tesque, Japan) was used (referred to as complete medium). Recombinant human cytokines, IL-3, IL-4, IL-6, IFN-γ, TNF-α and IL-1β were purchased from PeproTech (London, UK), and stem cell factor (SCF) was obtained from Amgen (Thousand Oaks, CA, USA). Granulocyte–macrophage colony-stimulating factor (GM-CSF) was obtained from Schering–Plough.

Generation of human umbilical cord blood-derived MCs

Umbilical cord blood was obtained from healthy volunteers in local obstetrics hospitals. Written informed consent was obtained from mothers from whom the cord blood was got, and the procedures were approved by the Human Studies Internal Review Board of Kyoto University. Cord blood-derived MCs were obtained as previously described (27). Briefly, mononuclear cells were isolated from cord blood by centrifugation on Ficoll–Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), and the cells were cultured in AIM-V medium containing 5% FCS in the presence of 100 ng ml^{-1} SCF and 50 ng ml^{-1} IL-6 for >10 weeks. Half of the medium was exchanged weekly for fresh medium supplemented with the cytokines. Then, the cells were further incubated with 1 μg ml^{-1} IgE (Biosource International, Camarillo, CA, USA), 5 ng ml^{-1} IL-3, 10 ng ml^{-1} IL-4 for 5 days in the presence of SCF and IL-6. These factors have been shown to act synergistically on cord blood-derived MCs, and prolong survival, induce maturation, enhance FcεRI expression and optimize secretion of histamine, PGD_2 and leukotriene C_4 when MCs are activated by cross-linking of FcεRI (15, 28–32). For the last 3 h of incubation, IgE was added again to assure that IgE binds to FcεRI, and then IgE-sensitized mature MCs were harvested. MCs obtained by this method were positively stained with toluidine blue and expressed FcεRI (stained with anti-FcεRI mAb: clone CRA-1). The purity of MCs was >98% as assessed by the expressions of CD117 (eBioscience, San Diego, CA, USA) and CD203c (Beckman Coulter Immunotech, Marseille, France) by flow cytometry.

Generation of human MoDCs

Buffy coats were obtained from healthy donors in the local blood bank (Red Cross Blood Center, Kyoto, Japan). PBMCs were isolated by centrifugation on Ficoll–Paque. Monocytes were purified from PBMCs by positive selection using anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured for 6–7 days in complete medium in the presence of 40 ng ml^{-1} IL-4, and 50 ng ml^{-1} GM-CSF to induce immature MoDCs. Every 3 days, half of the medium was exchanged for fresh medium supplemented with the cytokines.

Co-culture of DCs and MCs

Immature MoDCs and IgE-sensitized mature MCs were co-cultured in complete medium at a density of 5×10^5 DCs ml^{-1} per well in a 24-well microplate in the presence of 50 ng ml^{-1} GM-CSF, 40 ng ml^{-1} IL-4 and 100 ng ml^{-1} SCF at a DC:MC ratio of 2:1, unless otherwise indicated. For MC activation by cross-linking of FcεRI, goat anti-human IgE antibody (Biosource International) was added at a concentration of 3 μg ml^{-1}, and the co-culture was performed for 24 h. Where indicated, 10 μg ml^{-1} mouse anti-human TNF-α mAb (clone: MAb1, BD PharMingen, San Diego, CA, USA), a mixture of histamine receptor antagonists or 10 μM indomethacin

(Sigma–Aldrich) (33), was added. The following histamine receptor antagonists were combined: 10^{-7} M pyrilamine (Sigma–Aldrich), 10^{-4} M cimetidine (a gift from Sumitomo Pharmaceuticals, Osaka, Japan) and 10^{-6} M thioperamide (Sigma–Aldrich) for H1, H2 and H3 plus H4 receptor blocking, respectively (18, 34). Separation of DCs and MCs by a porous membrane in the co-culture was performed by using transwell culture plates with polycarbonate membrane insert with 0.4-μm pore size (Costar, Corning, NY, USA). In some experiments, DCs were stimulated with 100 ng ml^{-1} LPS (from *Escherichia coli* O111:B4, Sigma–Aldrich), 1000 IU ml^{-1} IFN-γ, 10 ng ml^{-1} TNF-α, 10 ng ml^{-1} IL-1β and/or 10^{-5} M histamine (Sigma–Aldrich), with or without MCs. Concentrations of IL-12p70 in 24-h supernatants were measured by the ELISA kits (BD PharMingen). Stimulation of DCs with CD40 ligand (CD40L) was done as described (35), using irradiated (55 Gy) CD40L-transduced L cells.

Measurement of concentrations of MC-derived humoral mediators

IgE-sensitized mature MCs were plated alone at the same density and under the same cytokine condition as the DC–MC co-culture, and were activated by cross-linking of FcεRI. Concentrations of TNF-α in 24-h supernatants were measured by an ELISA kit (Biosource International), and those of histamine and PGD_2 by enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

Phenotypic analysis of DCs and MCs

The expression of surface markers was analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA, USA) using the following mAbs: FITC-labeled anti-CD80, CD83, CD40, CD54, HLA-ABC and HLA-DR (Beckman Coulter Immunotech); FITC-labeled anti-CD86 (BD PharMingen) and FITC-labeled anti-CCR7 mAbs (R&D Systems, Minneapolis, MN, USA). For cells in DC–MC co-culture, the cells were stained with PE-labeled anti-CD11c mAb (Becton Dickinson), and $CD11c^{bright}$ cells and $CD11c^{dim}$ cells were gated as DCs and MCs, respectively. For OX40 ligand (OX40L), cells were stained with ik-5 mAb (mouse IgG2a) (36) and FITC-labeled $F(ab')_2$ goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA, USA).

Analysis of cytokine production by primed T cells

Naive $CD4^+$ T cells were isolated from human cord blood mononuclear cells with the CD4 isolation kit II or from adult PBMCs with CD4 Multisort kit and CD45RA microbeads (Miltenyi Biotec). This method yielded highly purified (>92%) $CD4^+$ $CD45RA^+$ naive T cells as assessed by flow cytometry (data not shown). Naive T cells (5×10^4 cells) were co-cultured with allogeneic DCs (5×10^3 cells) in complete medium in 96-well round-bottom microplates. DCs co-cultured with MCs were purified by FACSAria cell sorter (Becton Dickinson) by gating $CD11c^{bright}$ population as DCs before they were used to stimulate T cells. On day 3, 10 ng ml^{-1} IL-2 (teceleukin, Takeda Pharmaceuticals, Japan) was added. T cells were further expanded and subjected to analysis of cytokine production on days 12–14. For intracellular cytokine staining, primed T cells were re-stimulated with 50 ng ml^{-1} phorbol myristate acetate (PMA) (Sigma–Aldrich) and 500 ng ml^{-1} A23187 (Calbiochem) for 6 h. Brefeldin A (10 μg ml^{-1}) (Sigma–Aldrich) was added during the last 3 h. The cells were fixed, permeabilized and stained with FITC-labeled anti-IFN-γ mAb (BD PharMingen) plus PE-labeled anti-IL-4 mAb (BD PharMingen). For ELISA, T cells were re-stimulated with PMA/A23187 at 1×10^6 cells ml^{-1} for 24 h, and the supernatants were harvested. For IFN-γ, a matched antibody pair (clone 2G1 and B133.5; Pierce Biotechnology, Rockford, IL, USA) was used. For IL-4, IL-5, IL-10 and IL-13, commercially available ELISA kits (Biosource International) were used.

Real-time reverse transcription–PCR analysis of Notch ligands

Total RNA was isolated from FACS-sorted DCs after DC–MC co-culture using RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA (0.5 μg) was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR was performed using qPCR Mastermix Plus (Eurogentec, Belgium) and TaqMan Gene Expression Assays for JAG1, JAG2 and DLL4 (Applied Biosystems) on the ABI PRISM 7700 Sequence Detection System. Relative quantitations of mRNA expressions were performed by the relative standard curve method and mRNA expression levels of each gene were normalized to those of β-glucronidase.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded sections were prepared from biopsied specimens of lesional skins from patients with atopic dermatitis. After deparaffinization, endogeneous peroxidase activity was blocked by 0.3% H_2O_2 in methyl alcohol. The slides were pre-incubated with 1% normal horse serum and incubated with anti-human MC tryptase mAb (clone: G3, Chemicon International, Temecula, CA, USA). Subsequently, they were incubated with biotinylated horse anti-mouse serum, and the development of staining was performed using avidin–biotin–peroxidase complex (ABC-Elite, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine. After incubation with 0.1 M glycine–HCl (pH 2.2) and antigen retrieval by autoclave methods (37), fascin was stained with mouse anti-human fascin mAb (clone: 55K-2, DakoCytomation, Carpinteria, CA, USA) by the same procedure as the above, using avidin–biotin–alkaline phosphatase and New fuchsin in the development step. Using isotype-matched control mAbs instead of the anti-tryptase or anti-fascin mAb did not show non-specifically stained cells.

Results

Activated MC-derived factors induce DC maturation

First, we examined whether the *in vitro*-generated MCs secrete humoral factors upon activation that have been reported to affect DC functions. IgE-sensitized mature MCs were plated at the same cell density and under the same cytokine condition as the DC–MC co-culture, and activated by cross-linking of FcεRI. They secreted considerable amounts of TNF-α (256 ± 0.6 pg ml^{-1}), histamine (6.7 ± 0.6 μM) and PGD_2

(21.3 ± 0.86 ng ml^{-1}) for 24 h. As expected, addition of a cyclooxygenase inhibitor, indomethacin, inhibited the production of PGD_2 by activated MCs, whereas a mixture of histamine receptor antagonists did not affect the secretion of the three factors (data not shown). Thus, the *in vitro*-generated MCs are physiologically relevant in that they secrete major humoral factors produced by MCs, including the ones which can affect DC functions.

To examine the net effect of the whole activated MC-derived factors on DC functions, we co-cultured IgE-sensitized mature MCs with immature MoDCs, and then activated MCs by cross-linking of FcεRI by adding goat anti-human IgE antibody. GM-CSF, IL-4 and SCF were added to the co-culture to maintain the viability of DCs and MCs and to optimize mediator release from MCs (32, 38, 39). SCF alone did not induce DC maturation or affect DC maturation induced by LPS (data not shown). First, we analyzed phenotypes of DCs after 24 h of co-culture with activated MCs by flow cytometry, gating CD11c^{bright} population as DCs (Fig. 1). Without activation, MCs had no effect on the phenotypes of the DCs. In contrast, MCs activated by cross-linking of FcεRI induced up-regulation of CD80, CD86, CD83, CCR7, HLA-ABC and HLA-DR on the co-cultured DCs. Activated MCs did not affect maturation of DCs induced by LPS (Fig. 1) or LPS/IFN-γ (data not shown) added at the same time as the MC activation. These data indicate that activated MC-derived factors induce DC maturation, and that they do not have antagonistic effects on Toll-like receptor 4-mediated maturation of DCs.

To investigate relative contributions of each MC-derived factor to DC maturation, we added histamine receptor antagonists, neutralizing anti-TNF-α mAb or indomethacin (33) to the DC–MC co-culture to block the actions of histamine or TNF-α, or to inhibit the synthesis of PGD_2 in activated MCs, respectively. Because immature MoDCs express histamine H1, H2, H3 and H4 receptors (18, 20, 40), a mixture of antagonists against all the receptors (pyrilamine, cimetidine and thioperamide) was used. In addition, to evaluate the effect of cell contact on DC maturation, we separated DCs from MCs by a porous membrane using transwell plates. Although MCs can also produce IL-4 (4–6), which affects DC function, the addition of exogenous IL-4 to the co-culture precludes us from evaluating the influence of MC-derived IL-4 on DCs. As shown in Fig. 2, up-regulation of co-stimulatory molecules, CD80 and CD86, on DCs was largely inhibited by histamine receptor antagonists, whereas anti-TNF-α mAb or indomethacin did not show a considerable effect. Separation of DCs and MCs diminished the levels of the up-regulation. These data indicate that histamine is responsible for the up-regulation of co-stimulatory molecules on DCs, whereas TNF-α and PGD_2 are not involved. Cell contact has an augmenting effect on the up-regulation. It remains to be determined whether this augmentation is mediated by membrane-associated molecules on MCs that act in combination with histamine or by possible increases in local concentrations of MC-derived soluble factors around DCs.

IL-12p70 production by DCs is potently suppressed by activated MCs

IL-12p70 is a key DC-derived cytokine that plays a crucial role in induction of T_h1 responses (41). Thus, we next examined the effects of activated MC-derived factors on IL-12p70 production by DCs. We co-cultured DCs and MCs, and stimulated DCs with either LPS or CD40L in the presence or absence of IFN-γ to induce IL-12p70 production by DCs (42, 43). At the same time as the DC stimulation, MCs were activated by cross-linking of FcεRI. After 24 h of culture, concentrations of IL-12p70 in the supernatants were measured by ELISA (Table 1). Stimulation with either LPS or CD40L in the presence or absence of IFN-γ induced variable amounts of IL-12p70

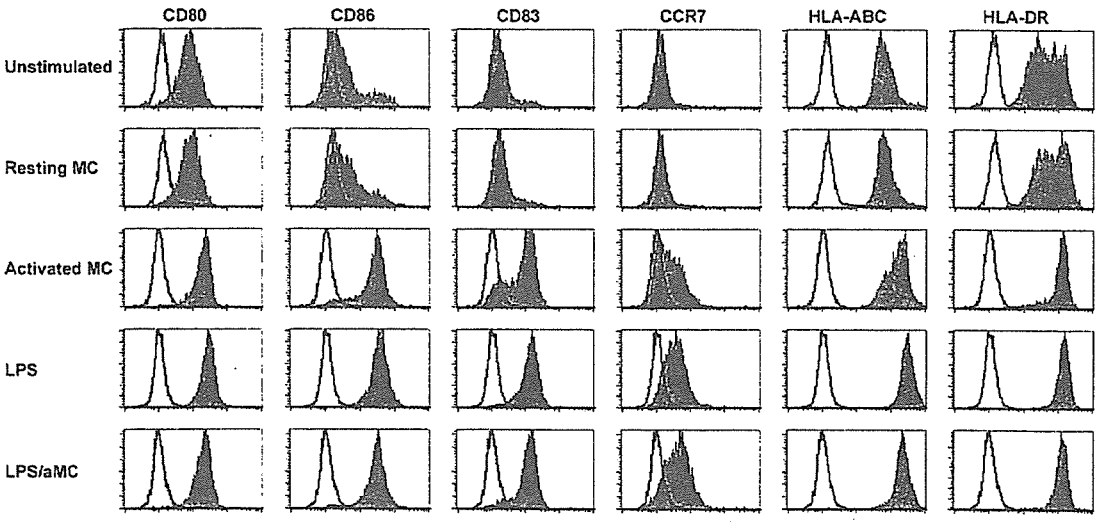

Fig. 1. Activated MC-derived factors induce DC maturation. Immature MoDCs were either cultured alone or co-cultured with IgE-sensitized mature MCs at a DC:MC ratio of 2:1. To activate IgE-sensitized MCs, FcεRI was cross-linked with goat anti-human IgE antibody. Where indicated, 100 ng ml^{-1} LPS was added to immature DCs either alone or with activated MCs (at the same time as FcεRI cross-linking). After 24 h of co-culture, surface phenotypes of DCs were analyzed by flow cytometry. CD11c^{bright} cells were gated and analyzed as DCs. Open histograms indicate background staining with an isotype-matched control mAb. These results are representative of three independent experiments.

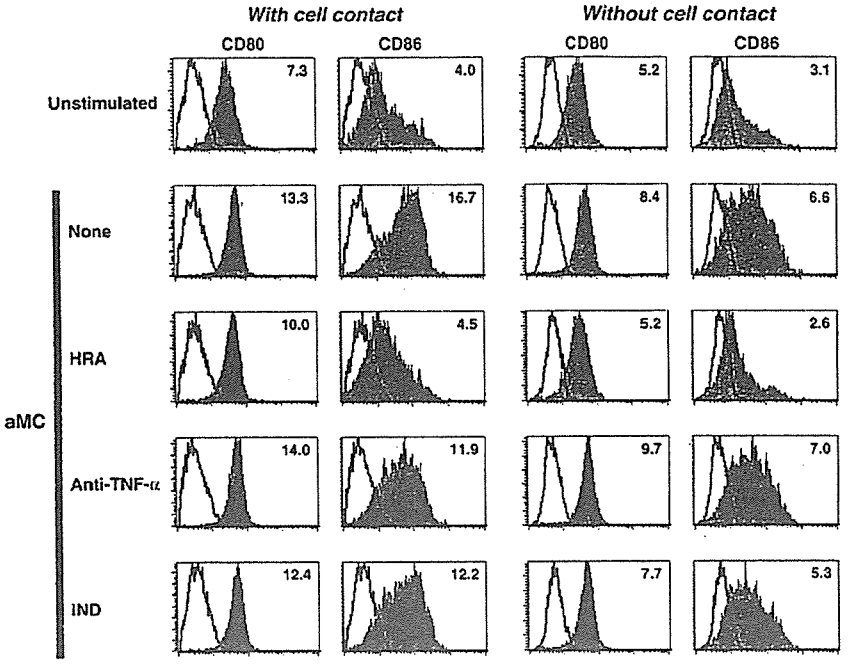

Fig. 2. Contribution of each activated MC-derived factor to the induction of DC maturation. Immature MoDCs and activated MCs were co-cultured, in the same chamber (with cell contact) or separated by a porous membrane (without cell contact) in transwell culture plates. A mixture of histamine receptor antagonists (10^{-7} M pyrilamine, 10^{-4} M cimetidine and 10^{-6} M thioperamide), 10 μg ml^{-1} neutralizing anti-TNF-α mAb or 10 μM indomethacin was added to the co-culture as indicated. After 24 h of co-culture, cells were analyzed by flow cytometry as above. Open histograms indicate background staining with an isotype-matched control mAb. Numbers indicate the relative fluorescent intensity of each marker, obtained by dividing the mean fluorescent intensity of each marker by that of the isotype control. aMC, activated mast cells; HRA, histamine receptor antagonists; IND, indomethacin. These results are representative of three independent experiments.

production by DCs, depending on donors. Remarkably, IL-12p70 production by DCs was potently suppressed by activated MCs irrespective of the type of DC stimulation, indicating that activated MCs suppress IL-12p70 production whether DCs are activated by a pathogen-derived signal in the peripheral tissues or by a T cell-derived signal during interaction with T cells. Separation of DCs and MCs only slightly diminished the suppression, indicating that soluble factors play a major role. Histamine receptor antagonists only partially reversed the suppression, and addition of histamine at 10^{-5} M, the concentration comparable to that produced by activated MCs in this system, was not sufficient to reproduce the suppressive effect of activated MCs, indicating that although histamine plays an important role, combinatorial effects of other MC-derived soluble factors are also present. Although PGD_2 has been reported to suppress IL-12p70 production by MoDCs (21, 22), indomethacin did not have a considerable effect on the suppression, even when combined with histamine receptor antagonists, indicating that PGD_2 is not a critical factor for the suppression in this culture system. Thus, activated MCs potently suppress IL-12p70 production by DCs mainly through histamine and other synergistic soluble factors except PGD_2. Cell contact appears to play only a minor role in this suppression.

DCs co-cultured with activated MCs in combination with other maturation-inducing factors induce T_h2-promoting DCs

The cytokine profile of $CD4^+$ T cells primed by mature DCs is profoundly affected by signals given to DCs during maturation (2). Thus, we primed allogeneic naive $CD4^+$ T cells with DCs that had been co-cultured with activated MCs in the presence or absence of other maturation-inducing factors: LPS or TNF-α/IL-1β. Some of MC-derived factors such as IL-4, histamine and OX40L have been shown to have direct effects on T cells (11, 44). To eliminate direct effects of activated MCs on T cell priming, we purified DCs from the DC–MC co-culture by a cell sorter before they were used to prime T cells. The purity of DCs was always >98% as assessed by the CD11c expression by flow cytometry. After 12–14 days of expansion, the T cells were re-stimulated with PMA/A23187 and their cytokine profiles were analyzed by intracellular cytokine staining for IFN-γ and IL-4 (Fig. 3A).

Immature DCs or DCs stimulated with LPS or TNF-α/IL-1β mainly induced IFN-γ single-producing T_h1 cells with a minor population of T cells exhibiting an IFN-γ/IL-4 double-positive pattern of uncommitted 'T_h0-like' T cells. Co-culturing DCs with activated MCs in the absence of other maturation-inducing factors did not affect the cytokine profile of T cells. Remarkably, however, when DCs were co-cultured with activated MCs in the presence of LPS or TNF-α/IL-1β, the frequency of IL-4 single-producing T_h2 cells considerably increased, while the frequency of both T_h1 and T_h0-like cells decreased. The degree of the T_h2-polarizing effect was correlated with the DC:MC ratio, while this effect was still observed at the DC:MC ratio of 32:1 (Fig. 3B). Moreover, the T_h2-polarizing effect was observed even when DCs were co-cultured with activated MCs in the presence of LPS/IFN-γ, a combination that strongly

Table 1. Effects of MC-derived factors on IL-12p70 production by DCs[a]

		Experiment 1	Experiment 2	Experiment 3
Unstimulated		<7.8	<15.6	<15.6
LPS/IFN-γ	None	12067 ± 34	393 ± 5	3403 ± 27
	Histamine	2810 ± 66	138 ± 5	1103 ± 16
	aMC (contact)	816 ± 14	<15.6	187 ± 3
	aMC (Transwell)	1320 ± 53	<15.6	542 ± 15
	aMC (contact) + HRA	4320 ± 29	301 ± 2	375 ± 1
	aMC (contact) + IND	1210 ± 41	<15.6	131 ± 3
	aMC (contact) + HRA/IND	4490 ± 226	348 ± 28	ND
LPS	None	313 ± 1	ND	ND
	Histamine	53 ± 1	ND	ND
	aMC (contact)	<7.8	ND	ND
	aMC (Transwell)	36 ± 1	ND	ND
	aMC (contact) + HRA	96 ± 1	ND	ND
	aMC (contact) + IND	<7.8	ND	ND
	aMC (contact) + HRA/IND	84 ± 1	ND	ND
		Experiment 4	Experiment 5	Experiment 6
Unstimulated		<7.8	<7.8	<7.8
CD40L/IFN-γ	None	55914 ± 3150	61335 ± 1438	63327 ± 2879
	aMC (contact)	18793 ± 833	24169 ± 250	15420 ± 411
CD40L	None	21537 ± 627	10047 ± 120	16863 ± 725
	aMC (contact)	6415 ± 260	5172 ± 116	5157 ± 42

[a]Immature DCs (5×10^5 cells ml^{-1}) were cultured without stimulation, or stimulated with LPS or CD40L in the presence or absence of IFN-γ for 24 h. Histamine, activated MCs (aMCs), histamine receptor antagonists (HRA) and/or indomethacin (IND) were also added as indicated. The concentrations of IL-12p70 (pg ml^{-1}) in culture supernatants were measured by ELISA. Results shown are means ± SD of duplicate ELISA values. ND: not done.

induces T_h1-promoting DCs (42; Fig. 3C). Thus, IgE-activated MCs, when combined with additional DC maturation factors, induce DCs that diminish T_h1 and promote T_h2 differentiation.

Activated MCs induce T_h2-promoting DCs by combinatorial effects of different factors

We then investigated relative contributions of each MC-derived factor to the induction of T_h2-promoting DCs (Fig. 3C). DCs stimulated with LPS/IFN-γ induced IFN-γ single-producing T_h1 cells with a minor population of IFN-γ/IL-4 double-producing T cells, as observed with DCs stimulated with LPS or TNF-α/IL-1β. DCs co-cultured with activated MCs in the presence of LPS/IFN-γ increased IL-4 single-producing T_h2 cells and decreased T_h1 and T_h0-like cells. Both addition of histamine receptor antagonists and separation of DCs and MCs considerably reduced the T_h2 polarization of DCs, indicating that both histamine and cell contact contribute to the induction of T_h2-promoting DCs. Importantly, addition of histamine at 10^{-5} M only slightly antagonized the T_h1 induction by DCs stimulated with LPS/IFN-γ. Indomethacin did not have any considerable effect on T cell polarization in accordance with the absence of its effect on activated MC-induced suppression of IL-12p70 production by DCs (data not shown).

We also examined cytokine production by T cells by ELISA (Fig. 3D). $CD4^+$ T cells primed by LPS/IFN-γ-stimulated DCs produced a high level of IFN-γ and low or undetectable levels of T_h2 cytokines (IL-4, IL-5 and IL-13) as well as IL-10. Addition of histamine alone slightly suppressed the T_h1 induction, as shown by a decrease in IFN-γ production and slight increases in IL-5, IL-10 and IL-13 production. Activated MCs decreased IFN-γ production more potently than histamine, and remarkably increased the production of T_h2 cytokines and IL-10 far more than histamine did. Histamine receptor antagonists or separation of DCs and MCs considerably suppressed the production of T_h2 cytokines and IL-10, whereas did not significantly increase the IFN-γ production.

The intracellular staining data (Fig. 3A and C) indicate that a considerable number of naive T cells differentiated toward IFN-γ/IL-4 double-producing T_h0-like cells in the absence of MCs. Consequently, the overall frequency of IL-4-producing T cells (i.e. T_h2 cells plus T_h0-like cells) does not change much irrespective of the presence or absence of MCs. However, the ELISA data (Fig. 3D) suggest that IL-4 single-producing T_h2 cells induced by MC-stimulated DCs are qualitatively distinguished from IFN-γ/IL-4 double-producing T_h0-like cells induced without MCs, because the former T cells appear to produce large amounts of other T_h2 cytokines (IL-5 and IL-13) and IL-10, whereas the latter T cells produce little amounts of these cytokines (Fig. 3D). These data indicate that the $CD4^+$ T cells induced by MC-stimulated DCs appear to be truly T_h2-polarized cells, and thus such DCs have T_h2-promoting activity as well as T_h1-suppressing activity.

Collectively, activated MCs, even in the presence of T_h1-promoting stimuli (LPS/IFN-γ), induce DCs that suppress T_h1 and promote T_h2 differentiation by combinatorial effects of different factors, including histamine, other soluble factors except PGD_2 and cell contact-dependent factors.

Close anatomical associations between DCs and MCs in atopic dermatitis

Finally, to obtain insights into the DC–MC interactions *in vivo*, we examined the anatomical relationship between DCs and MCs in inflammatory skin lesions. We visualized DCs and MCs in lesional skins of chronic atopic dermatitis by

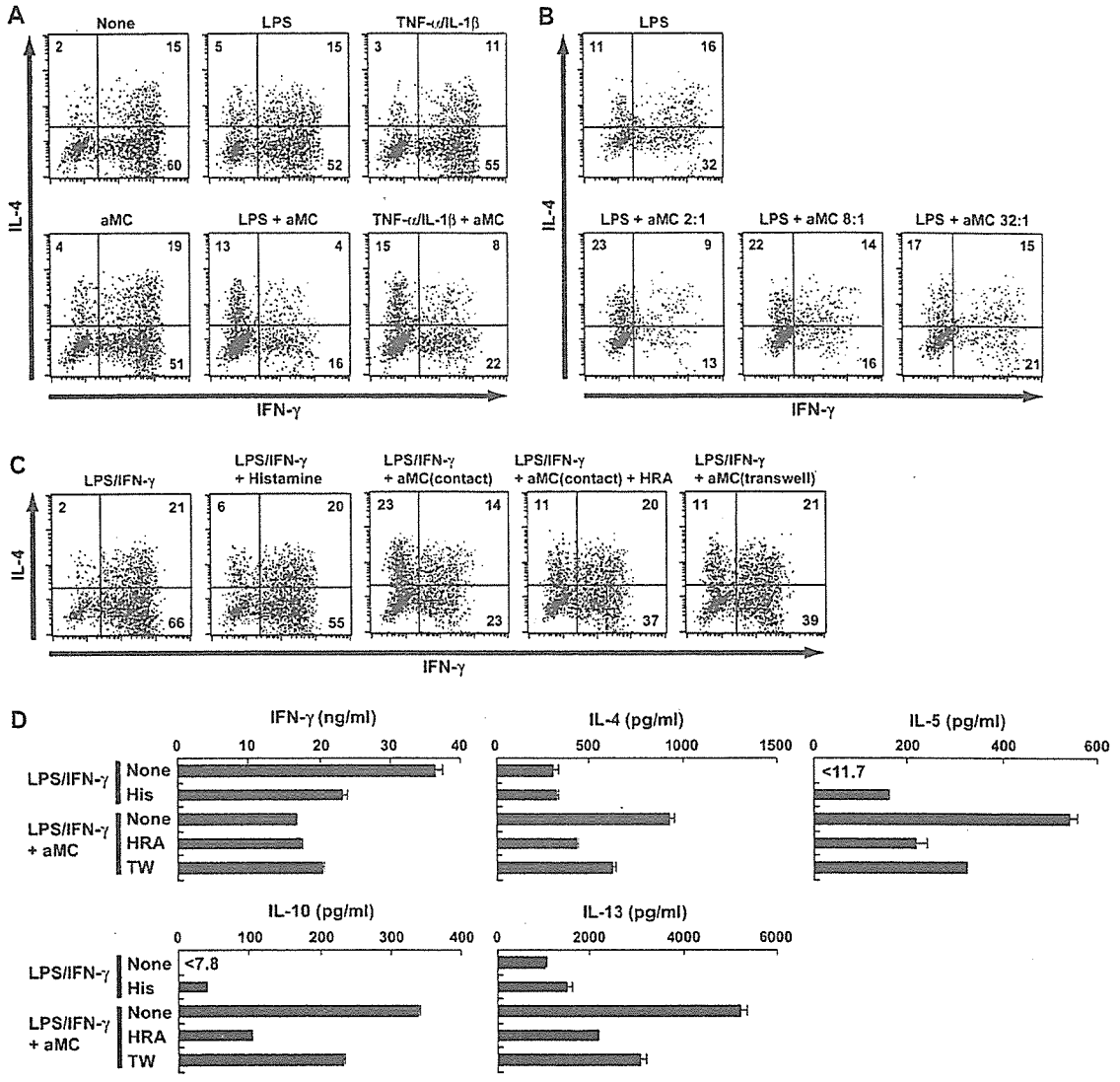

Fig. 3. DCs were polarized toward T_h2 by co-culture with activated MCs. (A) Immature DCs were cultured with or without activated MCs in the presence or absence of LPS or TNF-α/IL-1β. After 24 h, $CD11c^{high}$ cells were sorted as DCs by a cell sorter, and were used to prime cord blood allogeneic naive $CD4^+$ T cells. After 12- to 14-day expansion, T cells were re-stimulated with PMA/A23187, and were analyzed by intracelluiar cytokine staining for IFN-γ and IL-4. Percentages of cytokine-producing cells are indicated in each quadrant. (B) Immature DCs were cultured with activated MCs at different DC:MC ratios in the presence of LPS for 24 h. The cytokine profile of adult allogeneic naive $CD4^+$ T cells primed by the DCs was analyzed as in (A). (C) Immature DCs were stimulated with LPS/IFN-γ in the presence or absence of either histamine or activated MCs for 24 h. In some co-culture, a mixture of histamine receptor antagonists was added, or DCs and MCs were separated by a porous membrane in transwell plates. The cytokine profiles of cord blood allogeneic naive $CD4^+$ T cells primed by the DCs were analyzed as in (A). (D) Cord blood allogeneic naive $CD4^+$ T cells were primed with the DCs as in (C), and were re-stimulated with PMA/A23187 at 1×10^6 cells ml^{-1} for 24 h. The concentrations of cytokines in supernatants were measured by ELISA. Error bars indicate standard deviation of duplicate measurements. aMC, activated MCs; His, Histamine; HRA, histamine receptor antagonists; TW, transwell. The results in (A–D) are representatives of three independent experiments.

immunohistochemical staining using anti-fascin mAb for DCs and anti-tryptase mAb for MCs (Fig. 4). Anti-fascin mAb has been reported to react with DCs, endothelial cells and some neuronal cells in central nervous system (45–47). In all of four patients examined, both fascin-positive cells with DC morphology and tryptase-positive cells were detected. Fascin-positive cells were present in the superficial layer of dermis, forming aggregates (Fig. 4A, arrows). Tryptase-positive cells were scattered throughout the dermis, and some of them surrounded the aggregates of fascin-positive cells with a few intermingled with fascin-positive cells (Fig. 4B and C). The anatomical proximity of the two cell types was observed in all patients, suggesting functional associations between DCs and MCs in inflammatory skin lesions.

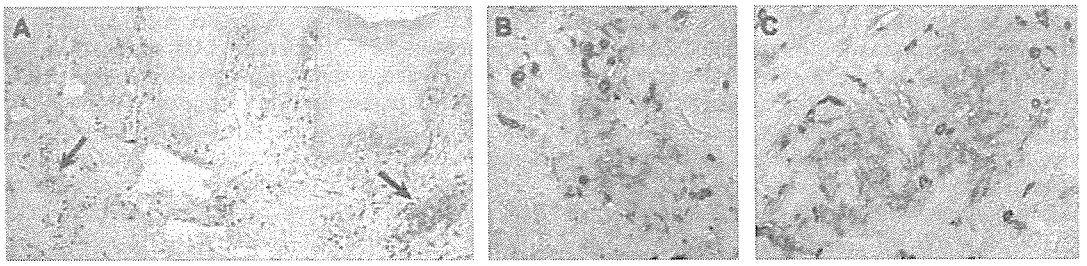

Fig. 4. Close anatomical associations between DCs and MCs in atopic dermatitis. A biopsied specimen of lesional skin of atopic dermatitis was immunohistochemically stained by anti-fascin mAb for DCs (red) and anti-tryptase mAb for MCs (brown). (A) Arrows indicate aggregates of fascin-positive cells in the superficial layer of the dermis. Original magnification: ×100. (B and C) Magnified views of the aggregates of fascin-positive cells shown in (A). Note that tryptase-positive cells surround the aggregates, and some of them are intermingled with fascin-positive cells. Original magnification: ×400. These results are representative of four different patients.

Discussion

Co-localization of DCs and MCs in sub-epithelial areas as sentinels for invading antigens, and immediate production of a variety of inflammatory mediators by activated MCs, suggest that MCs may influence the type of adaptive T cell immune responses through modulating the function of maturing DCs in inflamed tissues. However, no studies have directly addressed this question by co-culturing the two cell types *in vitro*. In this study, we utilized *in vitro* cultured human MCs, and examined the net effect of activated MCs as a whole on DCs. We found that (i) IgE-activated MCs induce DC maturation, as shown by the up-regulation of several surface molecules (Fig. 1), and potently suppress IL-12p70 production by DCs (Table 1). (ii) activated MCs alone do not have the ability to polarize DCs, but when combined with other DC maturation-inducing factors, such as LPS, TNF-α/IL-1β (pro-inflammatory cytokines) or even LPS/IFN-γ (potent IL-12-inducing factors), activated MCs induce DCs that suppress T_h1 differentiation and promote T_h2 differentiation (Fig. 3A–D) and (iii) histamine is an important mediator of these effects as reported (16–20), but a combinatorial effect of different MC-derived factors, including other soluble and cell contact-dependent factors, is required for the optimal induction of T_h2-polarizing DCs (Fig. 3C and D).

There have been several MC-derived molecules reported to affect DC functions. First, histamine has been shown to up-regulate CD86 on DCs, suppress IL-12p70 production by DCs and polarize DCs toward a T_h2-inducing type (16–20). In line with these reports, up-regulation of CD86 appeared to be almost totally dependent on histamine (Fig. 2). However, the addition of histamine together with LPS/IFN-γ or LPS only partially suppressed IL-12p70 production, compared with the stronger suppressive effect of activated MCs (Table 1). In addition, histamine receptor antagonists only partially reversed the suppression of IL-12p70 production by activated MCs. Accordingly, the T_h2-promoting effect of histamine and the T_h2-attenuating effect of histamine receptor antagonists in the DC–MC co-culture were also partial (Fig. 3C and D). These data indicate that histamine alone is not sufficient to reproduce the IL-12-suppressing and T_h2-promoting effects of activated MCs.

PGD_2 is another mediator synthesized by activated MCs (4–6). Stimulation of MoDCs with PGD_2 has been shown to diminish IL-12p70 production and favors a T_h2 response (21, 22). In our study, however, inhibition of PGD_2 synthesis by indomethacin (a cyclo-oxygenase inhibitor) did not influence the MC-induced suppression of IL-12p70 production (Table 1) and T_h2 polarization of DCs (data not shown), even when it was combined with histamine receptor antagonists. Thus, PGD_2 is not likely to be involved in T_h2 induction by MCs in our system.

As other potential MC-derived soluble factors inducing T_h2-promoting DCs, we examined two recently reported cytokines, IL-25 and TSLP. It has been reported that IL-25 is produced by a murine *in vitro* cultured MCs (48), and is implicated in induction of T_h2 responses (49, 50). However, we could not detect IL-25 mRNA expressions in the DC–MC co-culture at several time points within 24 h (data not shown). Human *in vitro* cultured MCs have been shown to express TSLP mRNA, and TSLP induces maturation and T_h2 polarization of human myeloid $CD11c^+$ DCs (23, 25). However, stimulation of MoDCs with TSLP did not affect their phenotypes, indicating that they do not express a receptor for TSLP, and moreover, addition of anti-TSLP-blocking antibody to the DC–MC co-culture did not diminish the induction of T_h2 responses by the DCs (data not shown). Thus, it is unlikely that IL-25 or TSLP is involved in the induction of T_h2-promoting DCs in our co-culture system.

Due to localization of DCs and MCs in anatomical proximity and high-level expressions of DC-attracting chemokines by IgE-activated MCs (12, 13), MCs may well have chances to directly contact with DCs in inflamed tissues. When DCs and MCs were co-cultured in direct contact, suppression of IL-12p70 production and T_h2 polarization of DCs were maximal, whereas separation of DCs and MCs by a porous membrane diminished these effects (Table 1 and Fig. 3C and D). These data suggest the presence of membrane-associated molecules on the surface of activated MCs that exert these effects in cooperation with MC-derived soluble factors.

Interestingly, DCs exhibited T_h1-suppressing as well as T_h2-promoting capacities only when DCs were co-cultured with activated MCs in the presence of other exogenous pro-inflammatory factors (Fig. 3A), including strong T_h1-inducing factors (LPS/IFN-γ) (Fig. 3C). It has been shown that helminth antigens, which induce T_h2 responses, more profoundly altered gene expressions in DCs when mixed with LPS than used alone (51). This and our observations suggest that cooperation of T_h2-inducing factors with pro-inflammatory, or even

T_h1-inducing, factors results in a full-blown T_h2 response. This is consistent with the observations in mice where LPS can promote T_h2 responses (52, 53), and may explain exacerbation of allergic symptoms by superimposed bacterial infections (54). These findings may also give a warning to an anti-allergy vaccine strategy that attempts to treat allergies by deviating the immune response toward T_h1 (55); simple application of T_h1-inducing factors may not alleviate a T_h2 response, but may rather exacerbate it.

Whereas IL-12 plays a dominant role in T_h1 development, the absence of IL-12 does not appear to be sufficient for T_h2 development (25, 56). Using different experimental systems, several molecules inducing naive $CD4^+$ T cells to differentiate into a T_h2 type have been reported to be expressed by DCs (57). OX40L (25, 58–62) is one of the candidates of such T_h2-inducing molecules. Moreover, Amsen *et al.* (63) demonstrated in a murine system that different Notch ligands on antigen-presenting cells instruct naive T cells to differentiate into different effector T cells; Delta promotes T_h1 responses, while Jagged promotes T_h2 responses, suggesting that DCs polarized by activated MCs may express high levels of Jagged. However, OX40L (Supplementary Fig. 1, available at *International Immunology* Online) was not detected on the T_h2-inducing, LPS/IFN-γ-stimulated DCs co-cultured with activated MCs by flow cytometry. In addition, there was no correlation between expression levels of Notch ligands (Jagged-1 and Jagged-2) measured by real-time reverse transcription–PCR and T_h2-inducing capacity of DCs stimulated with different stimuli (Supplementary Fig. 2, available at *International Immunology* Online). These data indicate that neither OX40L nor Notch ligands are responsible for the T_h2 polarization in our system.

de Jong *et al.* (64) demonstrated that T_h1- or T_h2-promoting DCs express diverse T_h-polarizing signals according to types of microbial stimuli, and some T_h2-promoting DCs exert its function via an OX40L-dependent mechanism, while others via an OX40L-independent, unknown mechanisms. Thus, T_h2-inducing molecules on DCs may be diverse, depending on types of stimuli and DCs. It is possible that an unspecified T_h2-inducing molecule, other than OX40L and Notch ligands, is expressed on T_h2-promoting DCs co-cultured with activated MCs.

Finally, we examined anatomical relationships between DCs and MCs in inflammatory skin lesions. We chose biopsied specimens from patients with chronic atopic dermatitis, because we thought that DC–MC interactions would be most prominently visualized in T_h2-type inflammatory lesions. In all specimens, both DCs and MCs were located in proximity in the dermis (Fig. 4). Intriguingly, MCs surrounded and entered the aggregates of DCs, suggesting production of MC-attracting chemokine by the DC aggregates, such as CCL5 and CCL8 (14) that can interact with CCR3 on MCs (15). These histological findings, together with the *in vitro* data that a relatively few MCs can influence T cell polarization induced by DCs (Fig. 3B), suggest that interactions of MCs with DCs are physiologically relevant *in vivo*, and that MCs may affect DC function through soluble and also possibly membrane-associated factors in the dermis of atopic lesions.

An IgE antibody is an end product of a T_h2 response, and thus, IgE-mediated activation of MCs occurs as a consequence of a previous T_h2 response. Our observation that IgE-activated MCs polarizes DCs toward T_h2 implies that a DC–MC interaction may constitute a positive feedback loop to maintain or augment T_h2 responses. Recently, Kalinski and Moser (65) proposed a 'success-driven consensual immunity' model, wherein outcomes of a 'successful' adaptive immune response induce polarization of DCs toward the same type of responses, constituting a positive feedback loop that stabilizes the type of adaptive immune responses. Our study is consistent with this model, demonstrating a successful T_h2 response where IgE-activated MCs deliver signals to DCs results in the stabilization of the T_h2 response. This mechanism may favor a defense against extracellular parasites by augmenting a T_h2 response, but may also lead to persistence of unwanted T_h2 responses, such as allergies. Thus, disruption of the DC–MC interaction may constitute an effective strategy to treat ongoing allergic reactions.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

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Abbreviations

CD40L	CD40 ligand
COE	Center of Excellence
DC	dendritic cell
GM-CSF	granulocyte–macrophage colony-stimulating factor
MC	mast cell
MoDC	monocyte-derived dendritic cell
OX40L	OX40 ligand
PGD2	prostaglandin D_2
PMA	phorbol myristate acetate
SCF	stem cell factor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin

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