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

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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 cyclo-oxygenase 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

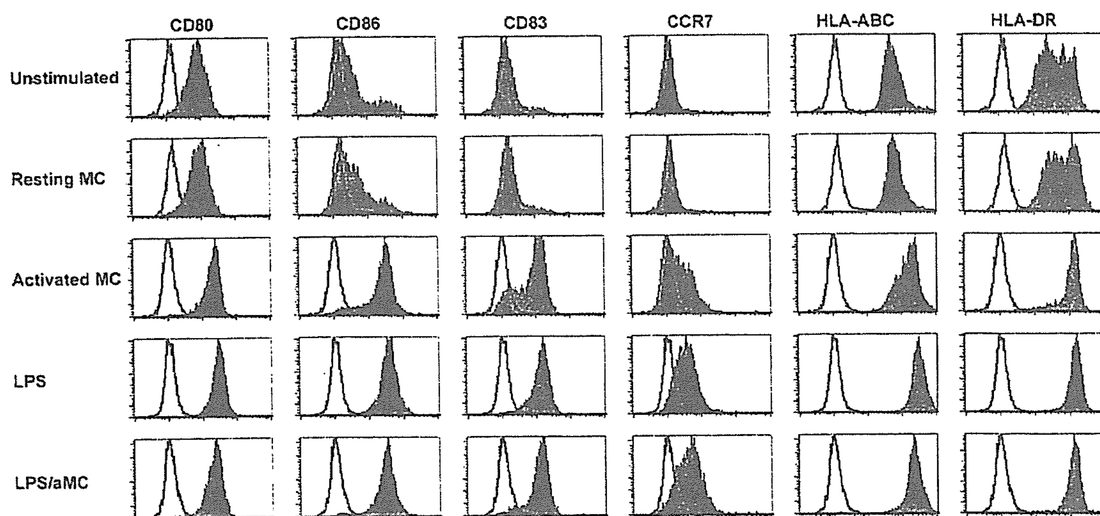

Fig. 1. Activated MC-derived factors induce DC maturation. Immature MoDCs were either cultured alone or co-cultured with IgE-sensitized mature MCs at a DC:MC ratio of 2:1. To activate IgE-sensitized MCs, FcεRI was cross-linked with goat anti-human IgE antibody. Where indicated, 100 ng ml^{-1} LPS was 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.

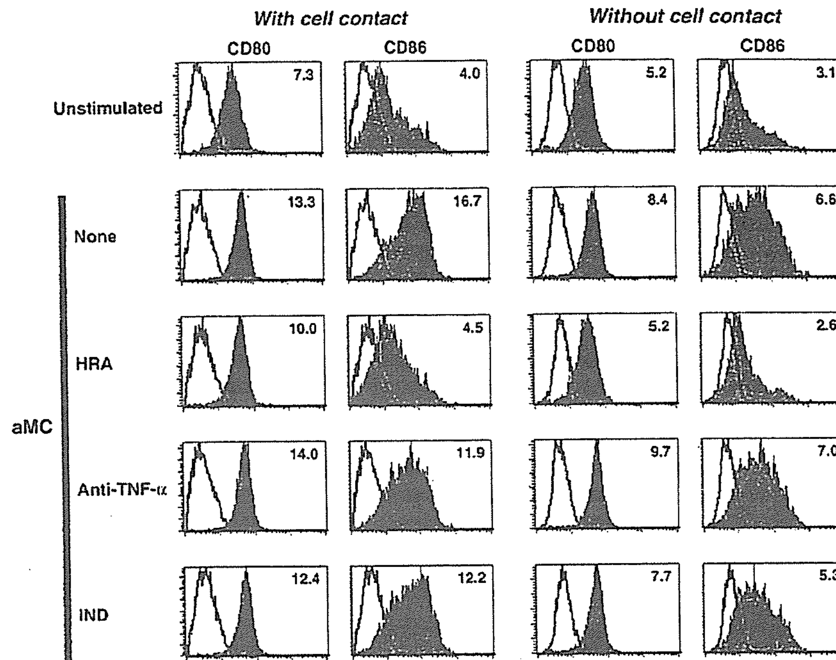

Fig. 2. Contribution of each activated MC-derived factor to the induction of DC maturation. Immature MoDCs and activated MCs were co-cultured, in the same chamber (with cell contact) or separated by a porous membrane (without cell contact) in transwell culture plates. A mixture of histamine receptor 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

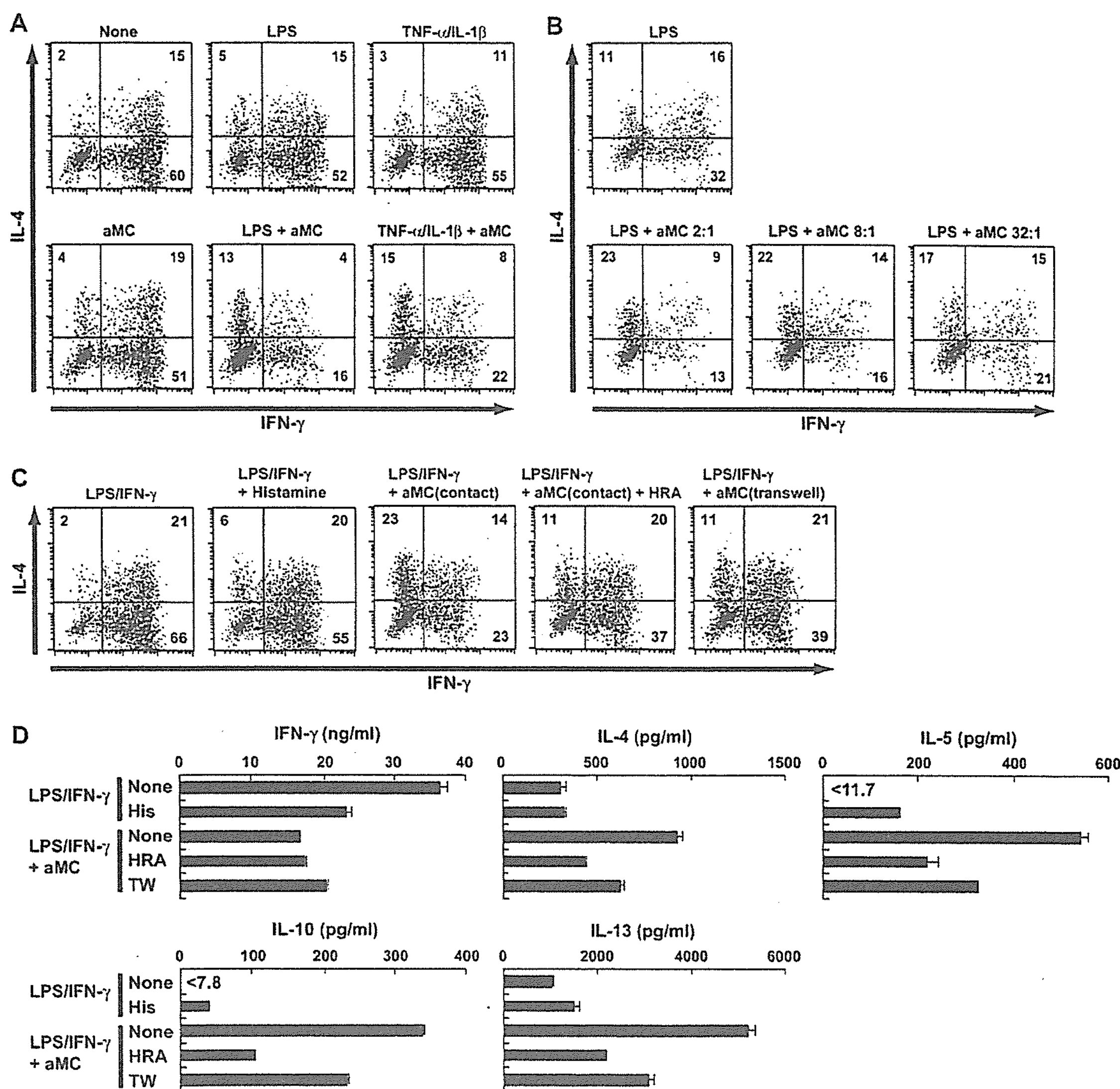

Fig. 3. DCs were polarized toward T_h2 by co-culture with activated MCs. (A) Immature DCs were cultured with or without activated MCs in the presence or absence of LPS or TNF-α/IL-1β. After 24 h, CD11c^{bright} cells were sorted as DCs by a cell sorter, and were used to prime cord blood allogeneic naive CD4$^+$ T cells. After 12- to 14-day expansion, T cells were re-stimulated with PMA/A23187, and were analyzed by intracellular 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.

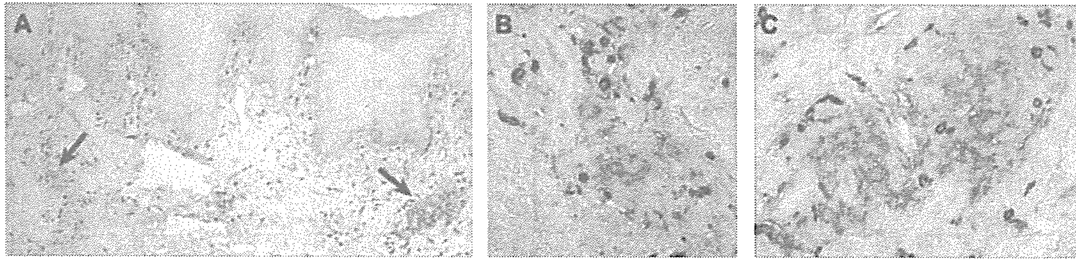

Fig. 4. Close anatomical associations between DCs and MCs in atopic dermatitis. A biopsied specimen of lesional skin of atopic dermatitis was immunohistochemically stained by anti-fascin mAb for DCs (red) and anti-tryptase mAb for MCs (brown). (A) Arrows indicate aggregates of fascin-positive cells in 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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TROY and LINGO-1 expression in astrocytes and macrophages/microglia in multiple sclerosis lesions

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Nogo constitutes a family of neurite outgrowth inhibitors contributing to a failure of axonal regeneration in the adult central nervous system (CNS). Nogo-A is expressed exclusively on oligodendrocytes where Nogo-66 segment binds to Nogo receptor (NgR) expressed on neuronal axons. NgR signalling requires a coreceptor $p75^{NTR}$ or TROY in combination with an adaptor LINGO-1. To characterize the cell types expressing the NgR complex in the human CNS, we studied demyelinating lesions of multiple sclerosis (MS) brains by immunohistochemistry. TROY and LINGO-1 were identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes. TROY was up-regulated, whereas LINGO-1 was reduced in MS brains by Western blot. These results suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A on oligodendrocytes, might modulate glial–neuronal interactions in demyelinating lesions of MS.

Keywords: LINGO-1, macrophages/ microglia, multiple sclerosis, reactive astrocytes, TROY

Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability [1]. Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in inducing axonal degeneration [2]. However, the alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an accumulation of glial scar and neurite growth inhibitors provide a non-permissive environment for regrowth of damaged axons [3].

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration, composed of three isoforms named A, B and C, all of which share a C-terminal 66-amino-acid segment named Nogo-66 [4]. Nogo-A, the longest isoform, is expressed exclusively on oligodendrocytes and myelin sheath and a subpopulation of neurones [5]. Nogo-66 as well as oligodendrocyte-myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) binds to the Nogo receptor (NgR), a glycosylphosphatidylinositol (GPI)-anchored membrane protein expressed chiefly on a subpopulation of neurones and their axons, including cerebral cortical pyramidal neurones and cerebellar Purkinje cells [6]. NgR transduces inhibitory signals via a coreceptor $p75^{NTR}$ that acts as a displacement factor releasing a small GTP-binding protein RhoA, an

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intracellular regulator of the cytoskeleton, from the Rho GDP dissociation inhibitor [6,7]. Neurones lacking $p75^{NTR}$ neither show RhoA activation nor exhibit neurite growth inhibition in the presence of myelin components, suggesting a key role of $p75^{NTR}$ in the NgR signal transduction *in vitro* [6]. However, the role of $p75^{NTR}$ remains unclear in the adult central nervous system (CNS) *in vivo* where NgR is identified in many neurones that exhibit little or no $p75^{NTR}$ expression [8].

Recently, two novel components of the NgR signalling complex, named TROY and LINGO-1, have been identified. TROY is a type I membrane protein belonging to the tumour necrosis factor receptor superfamily that substitutes for $p75^{NTR}$ in the NgR signalling complex to activate RhoA following exposure to myelin-associated neurite growth inhibitors [9,10]. TROY is strongly expressed during development in neural stem cells located in the ventricular and subventricular zones, and is more broadly expressed in various neurones in the adult rodent CNS than $p75^{NTR}$ [9–12]. Overexpression of a truncated form of TROY lacking its intracellular domain blocks neuronal response to myelin-associated inhibitors in a dominant-negative manner [9]. Neurones from TROY-deficient mice are resistant to the suppressive activity of myelin-associated inhibitors [10]. LINGO-1, also a type I membrane protein, consisting of 12 leucine-rich repeats, an immunoglobulin (Ig)-like domain, a transmembrane domain, and a short cytoplasmic tail containing a canonical epidermal growth factor receptor-like tyrosine phosphorylation site, acts as an adaptor that connects NgR (the ligand-binding component) with $p75^{NTR}$ or TROY (the signal transducing component) by forming the trimolecular complex [13,14]. The expression of LINGO-1 is enriched in the limbic system and the neocortex, identified exclusively in subpopulations of neurones in the rodent CNS [13,15]. Coexpression of NgR, TROY and LINGO-1 confers responsiveness to myelin-associated inhibitors in transfected COS-7 cells [13]. Exposure of cultured rat cerebellar granular (CG) neurones to LINGO-1-Fc fusion protein attenuates Nogo-66, OMgp or MAG-mediated neurite outgrowth inhibition [13]. These observations suggest that the NgR/TROY/LINGO-1 complex plays a key role in inhibiting axonal regeneration in the rodent CNS. However, at present, the precise cellular distribution of TROY and LINGO-1 remains to be characterized in the adult human CNS.

Recently, we demonstrated that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting a pathological role of Nogo-A/NgR interaction in persistent demyelination and axonal degeneration in MS lesions [16]. In contrast, we found that $p75^{NTR}$ expression is restricted in small regions such as substantia gelatinosa in the spinal cord [16]. The present study by using immunohistochemistry was designed to investigate TROY and LINGO-1 expression in demyelinating lesions of MS.

Materials and methods

Human brain tissues

For immunohistochemistry, 10 micron-thick serial sections were prepared from autopsied brains of five MS patients and 10 non-MS cases. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. MS cases included a 29-year-old woman with secondary progressive MS (SPMS) (MS#1), a 40-year-old woman with SPMS (MS#2), a 43-year-old woman with primary progressive MS (PPMS) (MS#3), a 70-year-old woman with SPMS (MS#4) and a 33-year-old man with SPMS (MS#5). Non-MS neurological and psychiatric disease cases included a 47-year-old man with acute cerebral infarction, an 84-year-old man with acute cerebral infarction, a 62-year-old man with old cerebral infarction, a 56-year-old man with old cerebral infarction, a 36-year-old woman with schizophrenia (SCH) and a 61-year-old man with SCH. Neurologically normal control cases included a 79-year-old woman who died of hepatic cancer, a 75-year-old woman who died of breast cancer, a 60-year-old woman who died of external auditory canal cancer and a 74-year-old woman who died of gastric and hepatic cancers.

For Western blot analysis, MS cases included MS#1, #2, #3 and #4. Non-MS cases included a 76-year-old woman with Parkinson's disease (PD#1), a 61-year-old woman with amyotrophic lateral sclerosis (ALS#1), a 74-year-old woman with ALS (ALS#2), a 61-year-old man with ALS (ALS#3), a 66-year-old man with ALS (ALS#4), a 73-year-old man with SCH (SCH#1) and a 77-year-old woman with depression (DEP#1). The *post mortem* interval of the cases ranges from 1.5 h to 10 h prior to freezing the brain tissues. Autopsies were performed either at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan or at the Nishitaga National

Hospital, Sendai, Japan. Written informed consent was obtained form all the cases examined. The present study was approved by the Ethics Committee of NCNP.

Human neural cell lines and cultures

Human astrocytes in culture were established from neuronal progenitor cells isolated from the brain of a human foetus at 18.5-week gestation obtained from BioWhittaker, Walkersville, MD, USA [16]. Human neural cell lines were maintained as described previously [17]. Human microglial cell line HMO6 is provided by Dr Seung U. Kim, Division of Neurology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada [18].

Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 125°C for 30 s in a temperature-controlled pressure chamber (Dako, Tokyo, Japan). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit serum or 10% normal goat serum at RT for 15 min to block non-specific staining. The serial sections were incubated in a moist chamber at 4°C overnight with goat anti-TROY antibody (E-19, sc-13711; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-LINGO-1 antibody (#07-678; Upstate, Lake Placid, NY, USA), mouse monoclonal anti-p75^{NTR} antibody (clone ME20.4; Sigma, St Louis, MO, USA) or the antibodies against cell type-specific markers described previously [16]. After washing with PBS, the tissue sections were labelled at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with a colourizing solution containing diaminobenzidine tetrahydrochloride (DAB) and a counterstain with haematoxylin. For negative controls, the step of incubation with primary antibodies was omitted, or the tissue sections were incubated with a negative control reagent (Dako) instead of primary antibodies.

In some experiments, tissue sections were initially stained with mouse anti-GFAP monoclonal antibody (GA5; Nichirei), then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colourized with New Fuchsin substrate. After inactivation of the antibody by autoclaving the sections in 10 mM citrate sodium buffer, pH 6.0, they were relabelled with anti-TROY antibody (sc-13711) or anti-LINGO-1 antibody (#07-678), followed by incubation with peroxidase-conjugated secondary antibody (Nichirei) and colourized with DAB substrate. Immunoreactivity was graded as intense, intermediate, weak or undetectable.

Western blot analysis

To prepare total protein extract, frozen brain tissues prepared from the frontal cerebral cortex or the cerebellar cortex were homogenized in RIPA lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Roche Diagnositics, Tokyo, Japan), followed by centrifugation at 13 400 g for 20 min at RT. The supernatant was collected for separation on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (Bio-Rad, Hercules, CA, USA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, and immunolabelled at RT overnight with anti-TROY antibody (sc-13711), anti-LINGO-1 antibody (#07-678), mouse monoclonal anti-NgR antibody (clone 188428; R&D Systems, Minneapolis, MN, USA), anti-p75^{NTR} antibody (ME20.4) or goat anti-heat shock protein HSP60 antibody (N-20, sc-1052; Santa Cruz Biotechnology) for the internal control. Then, the membranes were incubated at RT for 30 min with HRP-conjugated anti-mouse, rabbit or goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by using a chemiluminescent substrate (Pierce, Rockford, IL, USA). After the antibodies were stripped by incubating the membranes at 50°C for 30 min in stripping buffer composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, the membranes were processed for relabelling several times with different antibodies.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then, cDNA was amplified by polymerase chain reaction (PCR) using HotStar Taq DNA polymerase (Qiagen,

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Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5′-tcccatgggtggtgacaacatctc-3′ and 5′-gaatgagactggactggaacagcc-3′ for a 159-bp product of TROY: 5′-cctccctacccttctacacacgtt-3′ and 5′-gtgtcggttcg tcggctttcaact-3′ for an 175-bp product of LINGO-1: 5′-agcagccaggtgtgtgtacatacg-3′ and 5′-cgccgaaccctgtaaaca tgatgg-3′ for a 154-bp product of NgR: 5′-gaccacacttcct gtccagagaga-3′ and 5′-atatgacacctgctgtggtgggag-3′ for a 142-bp product of p75^{NTR}: and 5′-ccatgttcgtcatgggtgtga acca-3′ and 5′-gccagtagaggcagggatgatgttc-3′ for a 251-bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as an internal control. The amplification program consisted of an initial denaturing step at 95°C for 15 min. followed by a denaturing step at 94°C for 1 min. an annealing step at 60°C for 40 s and an extension step at 72.9°C for 50 s for 38 cycles, except for G3PDH amplified for 32 cycles. For the positive control, total RNA of the human frontal cerebral cortex (Clontech, Mountain View, CA, USA) was processed in parallel for RT-PCR.

Results

The constitutive expression of TROY and LINGO-1 mRNA in various human neural cell lines and primary cultures

First, the expression of TROY and LINGO-1 mRNA was studied in human neural cell lines and cultures by RT-PCR analysis. High levels of NgR and TROY transcripts were identified in all cell types, including cultured astrocytes, neuronal progenitor cells, NTera2 teratocarcinoma-derived neurones (NTera2N), Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HeLa cervical carcinoma, HepG2 hepatocellular carcinoma and HMO6 microglial cell line, along with in the human cerebral cortex (Figure 1a,b, lanes 1, 3–12). LINGO-1 and p75^{NTR} transcripts were also detected in various cell lines, although LINGO-1 mRNA levels were fairly low in U-373MG and HeLa, and p75^{NTR} mRNA levels were extremely low in HMO6 cells (Figure 1c,d, lanes 1, 3–12). The levels of G3PDH mRNA, a housekeeping gene, were almost constant among the cells examined (Figure 1c, lanes 1, 3–12), while no products were amplified when total RNA was processed for PCR without inclusion of the reverse transcription step, confirming that a contamination of genomic DNA was excluded (Figure 1a–e, lane 2).

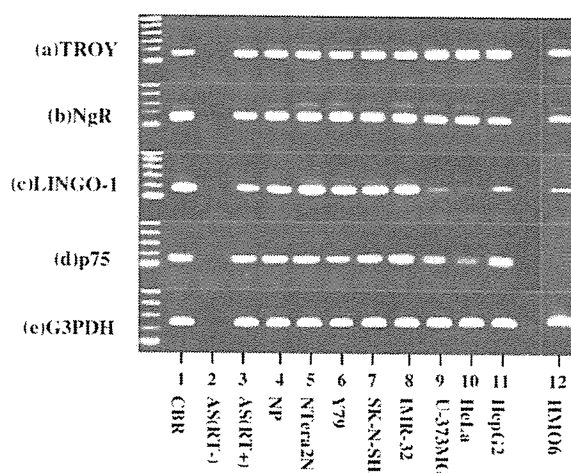

Figure 1. TROY and LINGO-1 mRNA expression in human neural cells. The expression of (a) TROY, (b) NgR, (c) LINGO-1, (d) p75^{NTR} and (e) G3PDH (an internal control) mRNA was studied in human neural cells by RT-PCR analysis. The lanes (1–12) represent: (1) the frontal cerebral cortex (CBR), (2) cultured astrocytes (AS) without inclusion of the reverse transcription step (RT–), (3) cultured astrocytes (AS) with inclusion of the reverse transcription step (RT+), (4) cultured neuronal progenitor (NP) cells, (5) NTera2 teratocarcinoma-derived neurones (NTera2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, (10) HeLa cervical carcinoma, (11) HepG2 hepatocellular carcinoma and (12) HMO6 microglial cell line. The DNA size marker (100-bp ladder) is shown on the left.

These results indicate that a panel of human neural cells in culture express both NgR/TROY/LINGO-1 and NgR/p75^{NTR}/LINGO-1 complexes.

Neurones, reactive astrocytes and macrophages/microglia but not oligodendrocytes expressed TROY and LINGO-1 in MS and control brains

In the next step, the expression of TROY and LINGO-1 proteins was studied in MS and non-MS brains by immunohistochemistry. A subpopulation of neurones in the cerebral cortex, brainstem and spinal cord expressed constitutively variable intensities of immunoreactivity for TROY or LINGO-1 in both MS and non-MS brains (Figure 2a,b). An intermediate or weak immunolabelling of TROY or LINGO-1 was identified in a subset of reactive hypertrophic astrocytes in chronic active demyelinating lesions of MS (Figure 2c,d) and ischaemic lesions of acute cerebral infarction (not shown), although the percentage and intensity of immunopositive cells/total hypertrophic reactive astrocytes was much greater and stronger in

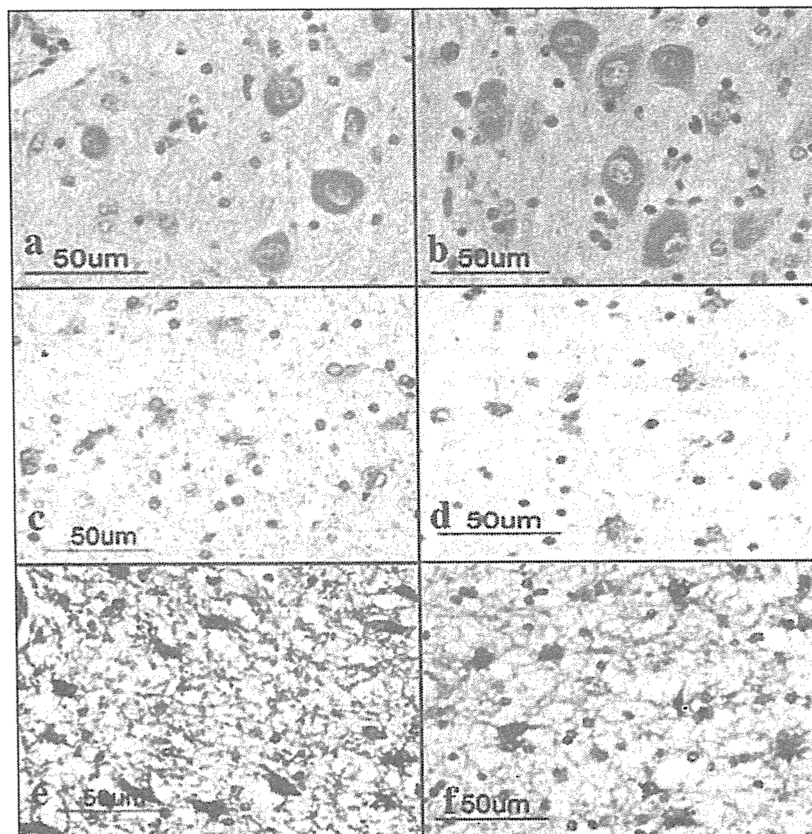

Figure 2. TROY and LINGO-1 immunoreactivities are identified on neurones and reactive astrocytes in multiple sclerosis (MS) brains. The expression of (**a,c,e**) TROY and (**b,d,f**) LINGO-1 was studied in MS brains by immunohistochemistry. The panels represent (**a**) TROY immunoreactivity in the pontine base of MS#1. (**b**) LINGO-1 immunoreactivity in the pontine base of MS#1. (**c**) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#2. (**d**) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3. (**e**) TROY (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#2, and (**f**) LINGO-1 (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#3. Neurones (**a,b**) and reactive hypertrophic astrocytes (**c–f**) express TROY and LINGO-1.

TROY (> 80%) than in LINGO-1 (< 10%), suggesting that not all TROY-expressing astrocytes coexpress substantial levels of LINGO-1. Double immunolabelling verified that a population of the cells expressing TROY or LINGO-1 immunoreactivity was comprised of GFAP⁺ astrocytes (Figure 2e,f). The great majority of macrophages and microglia expressed an intense/intermediate immunoreactivity for TROY and LINGO-1 in MS and non-MS brain lesions (Figure 3a,b). In contrast, both TROY and LINGO-1 was neither detectable in surviving oligodendrocytes remaining in demyelinating lesions nor in oligodendrocytes in the normal appearing white matter of MS (Figure 3c,d) and non-MS brains (not shown). $p75^{NTR}$ expression was restricted in small regions such as substantia gelatinosa in the spinal cord (Figure 3e), as described previously [16].

Up-regulated expression of TROY in MS brains

Finally, the expression of TROY and LINGO-1 proteins was studied in brain tissues of four MS and seven non-MS cases by Western blot analysis. TROY protein levels were much higher in four MS cases MS#1, #2, #3 and #4 (Figure 4a, lanes 1–7), when compared with five non-MS cases PD#1, ALS#1, #2 and #3, and SCH#1 (Figure 4a, lanes 8–14 and 17), or comparable to two non-MS cases ALS#4 and DEP#1 (Figure 4a, lanes 16 and 19), when standardized against the levels of HSP60, an internal standard detected

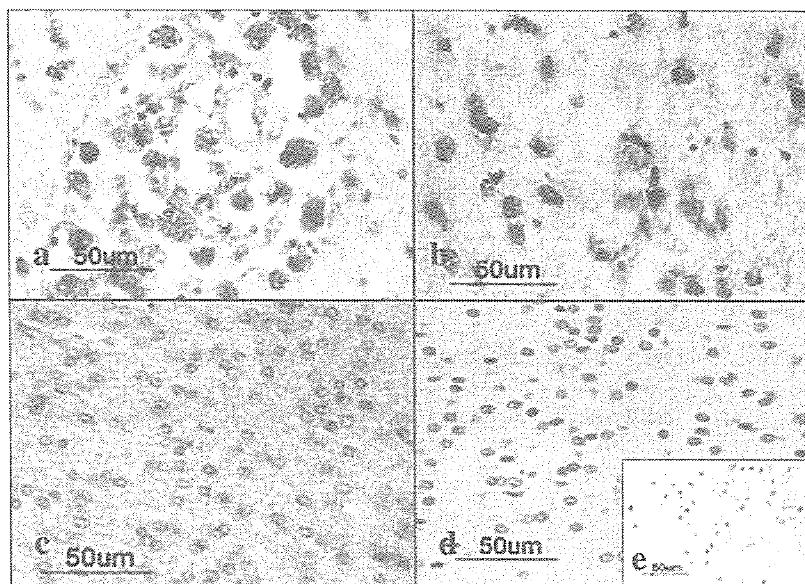

Figure 3. TROY and LINGO-1 immunoreactivities are identified on macrophages/microglia but not on oligodendrocytes in multiple sclerosis (MS) brains. The expression of (a,c) TROY, (b,d) LINGO-1 and (e) p75^{NTR} was studied in MS brains by immunohistochemistry. The panels represent (a) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3, (b) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the parietal cerebral cortex of MS#3, (c) TROY immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, (d) LINGO-1 immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, and an inset, (e) p75^{NTR} immunoreactivity in the substantia gelatinosa of the spinal cord of MS#2. Macrophages/microglia (a,b) but not oligodendrocytes (c,d) express TROY and LINGO-1.

in corresponding blots, which appeared to be almost constant among the samples (Figure 4e, lanes 1–19). In contrast, LINGO-1 protein levels were reduced in all MS cases and the cerebellum of the case of DEP#1, compared with other cases (Figure 4c, lanes 1–7 and 19). The pattern of p75^{NTR} protein expression (Figure 4d, lanes 1–19) was generally similar to that of TROY (Figure 4a, lanes 1–19), except for one case of MS MS#2 that expressed a high level of TROY but a trace of p75^{NTR} (Figure 4a,d, lane 2). In contrast, NgR protein levels varied among the cases and the regions examined, although there existed a trend for higher levels of NgR in the cerebellum *vs.* lower levels in the cerebrum (Figure 4b, lanes 1–19).

Discussion

By immunohistochemistry, the present study for the first time demonstrated that the expression of TROY and LINGO-1 was more widespread than p75^{NTR} in the brains of MS and non-MS cases, identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinating lesions of MS and ischaemic lesions of cerebral infarction. The expression of TROY and LINGO-1 in astrocytes and microglia, the findings previously unreported [9–15], was verified by immunohistochemistry of brain tissue sections and RT-PCR analysis of human astrocytes in culture and the microglia cell line HMO6. Non-neuronal distribution of TROY and LINGO-1, both of which constitute pivotal components of the NgR signalling complex [9,10,13], is not so surprising. Previous studies showed that TROY mRNA was detected in glioma and embryonal carcinoma cells [11], and recently, we reported that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting an active role of NgR signalling in non-neural cell types [16]. Our observations put forth the hypothesis that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, plays some role in regulating glial–neuronal and glial–glial interactions in active demyelinating lesions of MS.

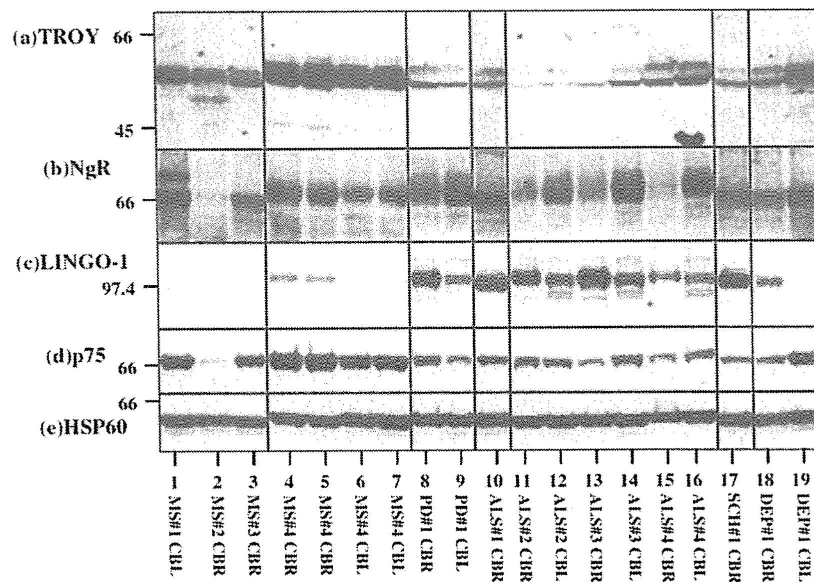

Figure 4. TROY and LINGO-1 protein expression in MS and non-MS brains. The expression of (a) TROY (55 kDa), (b) NgR (67 kDa), (c) LINGO-1 (97 kDa), (d) $p75^{NTR}$ (68 kDa) and (e) HSP60 (60 kDa, an internal control) proteins was studied by Western blot analysis in brain homogenates prepared from either the frontal cerebral cortex (CBR) or the cerebellar cortex (CBL) of four MS and seven non-MS cases. Sixty micrograms of total protein separated on a 12% SDS-PAGE gel was transferred onto nitrocellulose membranes, and processed for relabelling several times with different antibodies. The lanes (1–19) represent (1) MS#1 CBL, (2) MS#2 CBR, (3) MS#3 CBR, (4) MS#4 CBR, (5) a different part of MS#4 CBR, (6) MS#4 CBL, (7) a different part of MS#4 CBL, (8) PD#1 CBR, (9) PD#1 CBL, (10) ALS#1 CBR (11) ALS#2 CBR, (12) ALS#2 CBL, (13) ALS#3 CBR, (14) ALS#3 CBL, (15) ALS#4 CBR, (16) ALS#4 CBL, (17) SCH#1 CBR, (18) DEP#1 CBR and (19) DEP#1 CBL. PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; SCH, schizophrenia; DEP, depression. The position of molecular weight marker is indicated on the left.

By Western blot analysis, we found that TROY levels were elevated, whereas LINGO-1 levels were reduced in MS brains, although the sample size of the present study was too small, i.e. seven samples of four MS cases *vs.* 12 samples of seven non-MS cases, to obtain definitive conclusions. In contrast, immunohistochemical studies showed that both TROY and LINGO-1 immunoreactivities were enhanced in subpopulations of astrocytes and macrophages/microglia in MS brains. Because a subset of neurones expressed variable levels of TROY and LINGO-1, the discrepancy in the results between Western blot and immunohistochemistry is in part attributable to the varying degree of axonal loss in the lesions examined. Recent studies indicate that LINGO-1 expression is regulated by neuronal activity [19]. The expression of LINGO-1 mRNA is strongly up-regulated, while NgR mRNA levels are decreased in the dentate gyrus of rat brain following treatment with brain-derived neurotrophic factor or kainic acid, suggesting a role of LINGO-1 in activity-dependent neuronal plasticity responses [19]. Reduced neuronal activity causes irreversible axonal damage in demyelinating lesions of MS [20]. Recent studies indicate that LINGO-1 is expressed on rat oligodendrocytes where it negatively regulates differentiation and myelination competence of oligodendrocytes [21]. Expression of dominant-negative LINGO-1, LINGO-1 RNA-mediated interference, or an exposure to soluble LINGO-1-Fc enhances differentiation of rat oligodendrocytes in culture, accompanied by down-regulation of RhoA activity [21]. Furthermore, LINGO-1 knockout mice have greater numbers of myelinated axons [21]. These observations do not apparently agree with our present and previous studies showing that LINGO-1, along with NgR and TROY, was undetectable on oligodendrocytes in the brains of any cases examined [16]. This discrepancy is attributable in part to the differences between the previous study [21] and our own in the species and age, developing rat *vs.* adult human, or to the methods, cell cultures *vs.* immunohistochemistry, or both.

In conclusion, the expression of TROY and LINGO-1 was identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinating lesions of MS

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and ischaemic lesions of cerebral infarction. These observations suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, might play a regulatory role in glial–neuronal and glial–glial interactions under demyelinating conditions of MS and other pathological conditions.

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