Differential Contribution of Adhesion Molecules to Th1 and Th2 Cell-Mediated Lung and Bowel Inflammation

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CD4+ T cells play a critical role in the development of allergic inflammation in several target organs. Various adhesion molecules are involved in the local recruitment of T cells and other inflammatory cells. We investigated the differential contribution of adhesion molecules to T helper 1 (Th1) and Th2 cell-mediated allergic lung and bowel inflammation by employing their neutralizing antibodies. BALB/c mice transferred with in vitro-differentiated antigen-specific Th1 and Th2 cells were intratracheally or intrarectally challenged with a relevant antigen. Infiltration of infused T cells occurred, along with the accumulation of neutrophils and eosinophils in the lungs of Th1 and Th2 cell-transferred recipients, respectively. Th1-mediated neutrophil and Th2-mediated eosinophil accumulation in the large intestine, which occurred after intrarectal challenge with the antigen, was indicated by the significant elevation of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activity. Blocking experiments with neutralizing antibodies indicated that intercellular cell adhesion molecule (ICAM)-1; vascular cell adhesion molecule (VCAM)-1; and αβ-2 integrins contribute to Th1-mediated neutrophilic inflammation in the bowel, though only MadCAM-1, a4, αL, and β2 were involved in Th2-mediated eosinophilic inflammation. We conclude that distinct sets of adhesion molecules are involved in Th1- and Th2-mediated allergic lung and bowel inflammation.

Key words allergy; bronchial asthma; helper T cell; inflammatory bowel disease.

CD4+ T cells are involved in the development of immunological, inflammatory, and allergic diseases.1) Research has demonstrated that antigen-induced CD4+ T cell-mediated inflammation is inducible in various target tissues, including the upper and lower airways, skin, and intestinal tract.2–6) CD4+ T cells are divided into subsets, e.g., T helper 1 (Th1) and Th2, according to their cytokine producing characteristics, with each subset inducing a distinct type of local inflammation.2,3,6) However, mechanisms by which CD4+ T cell-mediated inflammation develops in a tissue-specific fashion have not been fully clarified.

The local accumulation of inflammatory cells, including CD4+ T cells, is regulated by adhesion molecules.7) The fact that expression of adhesion molecules is enhanced by various T cell-derived cytokines,8) suggests that activated CD4+ T cells play a critical role in the upregulation of adhesion molecules and subsequent accumulation of both inflammatory and T cells. In addition, it has been suggested that distinct sets of adhesion molecules are involved in the accumulation of inflammatory cells in different target tissues. For example, it is considered that interaction of the mucosal addressin cell adhesion molecule (MadCAM)-1 with its ligand α4/β7-integrin is the preferentially used pathway for entry of inflammatory cells into intestinal sites; intercellular cell adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1-dependent pathways contribute in other tissues.9–11)

To examine the role of adhesion molecules in the pathogenesis of CD4+ T cell-mediated inflammatory diseases, we carried out a cell-transfer procedure using antigen-specific and Th1/Th2-differentiated T cells, and monitored the initial accumulation of inflammatory cells in the antigen-challenged lungs and colons of mice. Blocking experiments using neutralizing antibodies against adhesion molecules were carried out to determine differences in the underlying mechanisms of Th1 and Th2 cell-mediated responses; differences between target tissues were also examined.

METHODS

Animals and In Vitro Polarization of T Cells Mice expressing transgenes for the DO11.10 T cell receptor (TCR) αβ, which recognizes residues 323-339 of ovalbumin (OVA), are described elsewhere.12) Male BALB/c mice, 6 to 8 weeks of age, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). OVA-specific Th1 and Th2 cells were generated from DO11.10 mice, using methods described previously.3,6) OVA-specific naïve CD4+ T cells were isolated from the spleens of DO11.10/RAG-2−/− mice by positive selection using CD4 microbeads and a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Cells were cultured with X-ray-irradiated BALB/c splenocytes in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.3 μM OVA 323–339 peptide, and 10 U/mL recombinant interleukin (IL)-2 (BD Biosciences, San Jose, CA, U.S.A.). For Th1 phenotype...
development, 10 U/mL recombinant murine IL-12 (PeproTech, Rocky Hill, NJ, U.S.A.) and 1 μg/mL neutralizing anti-IL-4 monoclonal antibody (mAb) (BD Biosciences) were added, while, for Th2 phenotype development, 10 U/mL recombinant murine IL-4 (PeproTech) and 1 μg/mL anti-IL-12 mAb (BD Biosciences) were used. Seven to ten days after stimulation, cells were harvested, purified by centrifugation over Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden), and used as Th1 and Th2. Successful differentiation of polarized cells was achieved using enzyme-linked immunosorbent assay and the intracellular staining of produced cytokines.\(^6,13\) Experimental procedures in this study were approved by the Animal Use and Care Committee of Tokyo Metropolitan Institute of Medical Science.

**Cell Transfer and Challenge Procedures**

OVA-specific Th1 or Th2 DO11.10 T cells were injected into the tail vein of normal BALB/c mice (3×10^7/ head). In some experiments, cells were stained with the fluorescein-based dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, U.S.A.) before the transfer, using methods previously described.\(^6,13\) For bowel inflammation experiments, 24 h after the transfer, mice were challenged by administering an enema of 0.2 mL of 10% OVA or bovine serum albumin (BSA) dissolved in 0.9% saline; the process was repeated 7 times with 10 min intervals.

For lung inflammation experiments, mice were challenged by intratracheal administration of antigens. Accumulation of eosinophils and neutrophils was evaluated by bronchoalveolar lavage, using methods described previously.\(^6,16\) The number of leukocytes in the bronchoalveolar lavage fluid (BALF) was counted using a hemocytometer. Differential cell counts based on morphologic criteria were performed for at least 200 cells on a cytocentrifuged preparation after staining with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of antigen-specific T cells recovered in the BALF was determined by detection of CFSE+ cells, using flow cytometry, as described previously.\(^6\) Lung and colon sections (5 μm thick) were stained with hematoxylin and eosin, and observed under optical microscopy.

For blocking studies, anti-ICAM-1 (YN1/17.4), anti-VCAM-1 (M/K-2.7), anti-MadCAM-1 (MECA-367), anti-α4 (PS/2), anti-α4 (M17/4), anti-β1 (TS2/16), anti-β2 (M17/5.2), or anti-β7 (FIBS06.64) monoclonal antibodies, purified from ascites or culture supernatant of the corresponding hybridomas (ATCC, Manassas, VA, U.S.A.), were administered (4 mg/kg) intravenously 30 min before each antigen challenge. The expression profiles of these adhesion molecules were retrieved by BioGPS (http://biogps.org/) (Figs. S1–S10).

**Tissue Myeloperoxidase (MPO) and Eosinophil Peroxidase (EPO) Activity**

**EPO** Activity

MPO and EPO activity in the large intestine was measured using methods described previously,\(^14–16\) but with slight modifications. Briefly, the middle portion of the large intestine (3 cm long; 2 cm from anus) was isolated and cut into fragments with a scalpel. Tissue fragments were suspended in 0.5% hexadecyltrimethylammonium bromide (KPO_4-HTAB and Tris-HTAB) in 2 mL of 0.05 M potassium phosphate buffer (pH 6.0) and 0.05 M Tris buffer (pH 8.0) for the MPO and EPO assays, respectively. Tissue fragments were homogenized using Polytron homogenizer (Kinematica, GmbH, Switzerland) for 30 s, and homogenates were centrifuged (12000×g) for 30 min at 20°C; the supernatants were used for assays.

For the MPO assay, the substrate solution (0.02% H_2O_2, 0.4 mg/mL o-dianisidine in KPO_4-HTAB) was added to 1/20 volume of the homogenate and mixed for 5 min at room temperature. The reaction was terminated by adding the same volume of 0.2 N NaOH, and absorbance at 450 nm was measured. Similarly, for the EPO assay, the substrate solution (0.04% H_2O_2, 1.2 mg/mL α-phenylenediamine dihydrochloride in Tris-HTAB) was added to the same volume of the filtrated homogenate and mixed for 5 min at room temperature. The reaction was terminated by adding the same volume of 4 M H_2SO_4, and absorbance at 492 nm was measured. Tissue MPO and EPO activity was expressed in units using standard horse-radish peroxidase, the specific activity of which was known.

**Statistics**

Results are presented as mean±standard error. The statistical analysis was performed by one-way ANOVA with Dunnett’s multiple comparison tests, p<0.05 was considered to indicate statistical significance.

**RESULTS AND DISCUSSION**

The role of CD4+ T cells in the pathogenesis of inflammatory respiratory disorders, especially bronchial asthma, is well established.\(^12,18\) The first experiment was carried out to clarify the contribution of adhesion molecules to antigen-induced and CD4+ T cell-dependent lung inflammation. BALB/c mice were transferred with OVA-reactive Th1 or Th2 cells, and challenged with intratracheal administration of the relevant antigen. As reported in our previous study,\(^6\) infiltration of neutrophils and eosinophils in the lungs of mice transferred with Th1 and Th2 cells, respectively, occurred when challenged with OVA but not BSA (Figs. 1A, S11). Along with these features, hyperplasia of bronchial epithelial cells was especially observed in antigen-challenged lungs of Th2-transferred mice at histopathological examination (Fig. S11). Antigen-specific Th1 and Th2 cells accumulated to the same degree in the lungs and in an antigen-specific manner (Fig. 1B).

We demonstrated that the accumulation of Th2 cells in the lungs was mediated by IL-5, chemokine (CC motif) ligand (CCL)11, CCL17, CCL22, and their receptors, chemokine (CC motif) receptor (CCR)3 and CCR4, while the contribution of only CCL11 in Th1 cell migration was confirmed.\(^6,19,20\) In addition, antigen-induced upregulation of VCAM-1 and ICAM-1 expression on the vascular endothelial cells was demonstrated in an antigen-specific T cell clone-transfer model.\(^15\) Therefore, the contributions of ICAM-1, VCAM-1, and MadCAM-1 and their counter ligands, to T cell-induced lung inflammation were next determined. Administration of blocking antibodies indicated that ICAM-1; VCAM-1; and αL, β2, and β7 integrins participate in accumulation of Th2 cells and eosinophils in the lungs (Figs. 1A, B).

The fact that expression of α4/β1 integrin is restricted on eosinophils and lymphocytes, combined with the fact that its receptor VCAM-1 on vascular endothelial cells is upregulated by Th2 cytokines, suggests these molecules are associated with allergic diseases.\(^21–23\) The interaction of αL/β2 integrin and ICAM-1 is also a target for the treatment of allergic diseases.\(^23,24\) Eosinophil and T cell infiltration recorded in the lungs of Th2 cell-transferred mice in this study generally supports previous research, but the contribution of α4/β1 was
not observed. As the adhesion molecule network is highly redundant (Figs. S1–S8), and VCAM-1 ligands other than α4β1 such as moesin and ezrin are potentially expressed on T cells and granulocytes (Figs. S9, S10), these molecules could be involved in Th2 cell-mediated airway inflammation.

Th1 cells induce allergic airway inflammation that differs from Th2 cell-mediated and eosinophil-dependent inflammation, and is characterized by neutrophil infiltration. ICAM-1 contributed to the migration of neutrophils (Fig. 1A) but not T cells (Fig. 1B) in the lungs of Th1 cell-transferred mice, although its expression is regulated by Th1 cytokines. Interestingly, blocking of the ICAM-1 ligand, αLβ2, suppressed the migration of T cells but not neutrophils (Figs. 1A, B). These contradictory findings might be caused by the participation of remaining receptors/ligands, such as ICAM-2, ICAM-3, and αM. Furthermore, we previously showed that a fraction of infused T cells spontaneously distributed in the lungs before antigen provocation rather than those migrated in response to the antigen plays a primary role in the development of lung inflammation. This might explain the lack of suppression of neutrophil accumulation regardless of the significant inhibition of Th1 cells by anti-αL and -β2.

The development of inflammatory bowel diseases (IBD) is also associated with CD4+ T cell-mediated pathology. In addition, the development of IBD is associated with the enhanced expression of cell adhesion molecules in the colonic mucosa of experimental animals and humans. In order to elucidate the tissue specific contribution of adhesion molecules to T cell-mediated inflammation, parallel experiments were performed in the large intestine of Th1 and Th2 cell-transferred mice by intrarectal administration of the relevant antigen. The development of neutrophilic and eosinophilic inflammation, represented by elevation of MPO and EPO activity in homogenates of the large intestine, was induced in Th1- and Th2-transferred mice, respectively, upon challenge with OVA but not BSA (Fig. 2) In the histological sections,
weak inflammatory features such as slight villous branching and crypt elongation was seen in antigen-challenged Th1- and Th2-transferred mice (Fig. S12).

Th1 cell-mediated and antigen-induced migration of neutrophils (upregulation of MPO activity) was suppressed by the treatment of antibodies against ICAM-1 and its ligands, αL and β2. Interaction of MadCAM-1 with its ligand, α4/β7, was also involved. Antibodies against VCAM-1 and β1 suppressed the MPO activity with less potency (Fig. 2A). In contrast, while MadCAM-1, α4, αL, and β2 were involved in the development of Th2-mediated bowel eosinophilia, ICAM-1, VCAM-1, β1, and β7 were not (Fig. 2B).

It is considered that interaction of MadCAM-1 is preferentially used for entry of inflammatory cells into intestinal sites, though ICAM-1- and VCAM-1-dependent pathways contribute in other tissues.9–11) Consistently, treatment with the anti-MadCAM-1 antibody suppressed both Th1- and Th2-dependent neutrophilic and eosinophilic bowel inflammation (Fig. 2). Moreover, it was a little unexpected that antigen-induced Th2 cell migration in the lungs was also suppressed by the anti-MadCAM-1 (Fig. 1B). Recently, the involvement of MadCAM-1 in T cell migration in the lungs was demonstrated in patients with lung cancer.32) Therefore, the contribution of MadCAM-1 may not be restricted to the gastrointestinal tract in some inflammatory conditions.

Distinct types of IBD include Crohn’s disease, which develops anywhere along the gastrointestinal tract, and ulcerative colitis, which develops in the large bowel. Crohn’s disease is characterized by specific expression of Th1 cytokines, whereas Th2 cytokine production is observed in most patients with ulcerative colitis.33,34) Contribution of each phenotype to the pathogenesis of IBD seems to differ with types of clinical features and experimental systems.33,34) Our present study showing the differential effects of antibodies against adhesion molecules of Th1 and Th2 cell-transferred mice suggests the different mechanisms involved in the development of these bowel diseases. Given that neither the blocking of a part of MadCAM-1 ligand, β7, nor the blocking of a receptor of αL/β2 integrin, ICAM-1, showed efficacy in suppressing Th2-mediated bowel inflammation, the contribution of other adhesion molecule sets is suggested.

In conclusion, various adhesion molecule sets are involved in antigen-induced local inflammation in different tissues, and distinct subsets of CD4+ T cells play important roles (Table 1). Some of the adhesion molecules tested should be targeted for treatment of CD4+ T cell-mediated inflammatory diseases.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

### Table 1. Summary for the Contribution of Adhesion Molecules to Th1 and Th2-Mediated Lung and Bowel Inflammation

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**Fig. 2. Effect of Antibodies against Adhesion Molecules on Th1 and Th2 Cell-Mediated Bowel Inflammation**

Th1 and Th2 cells (3×10⁷) were transferred to normal mice by intravenous injection. After 24 h, mice were challenged with 7 administrations of 0.2 mL of 10% OVA or BSA. Each antibody (0.5 mg/head) was injected intravenously 30 min before the challenge. MPO activity in the large intestine of Th1 cell-transferred mice (A) and EPO activity of Th2 cell-transferred mice (B) were measured 24 and 48 h after antigen challenge, respectively (n=5–6). *p<0.05, **p<0.01, ***p<0.001, compared with OVA-challenged control (Dunnett’s test).
REFERENCES