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Myosin light chains 9 and 12 are functional ligands for CD69 that regulate airway inflammation

Koji Hayashizaki,¹* Motoko Y. Kimura,¹* Koji Tokoyoda,¹,²* Hiroyuki Hosokawa,¹ Kenta Shinoda,¹ Kiyoshi Hirahara,¹ Tomomi Ichikawa,¹ Atsushi Onodera,¹ Asami Hanazawa,¹,² Chiaki Iwamura,¹ Jungo Kakuta,³ Kenzo Muramoto,³ Shinichiro Motohashi,⁴ Damon J. Tumes,¹,⁵ Tomohisa linuma,⁶ Heizaburo Yamamoto,⁶ Yuzuru Ikehara,⁷ Yoshitaka Okamoto,⁶ Toshinori Nakayama¹,⁸†

Recent decades have witnessed a rapid worldwide increase in chronic inflammatory disorders such as asthma. CD4⁺ T helper 2 cells play critical roles in the pathogenesis of allergic airway inflammation, and CD69 expression on activated CD4 T cells is required to induce allergic inflammation in tissues. However, how CD69 mechanismically controls allergic inflammation remains poorly defined. In lymphoid tissues, CD69 regulates cellular retention through inhibition of S1P1 expression and requires no specific ligands to function. In contrast, we show herein that myosin light chain (Myl) 9 and Myl12 are new functional ligands for CD69. Blockade of CD69-Myl9/12 interaction ameliorates allergic airway inflammation in ovalbumin-induced and house dust mite–induced mouse models of asthma. Within the inflamed mouse airways, we found that the expression of Myl9/12 was increased and that platelet-derived Myl9/12 localized to the luminal surface of blood vessels and formed intravascular net-like structures. Analysis of nasal polyps of eosinophilic chronic rhinosinusitis patients revealed that Myl9/12 expression was increased in inflammatory lesions and was distributed within net-like structures in the intravascular space. In addition, we detected Myl9/12 in perivascular spaces where many CD69⁺ cells were positioned within Myl9/12 structures. Thus, CD69-Myl9/12 interaction is a key event in the recruitment of activated CD69⁺ T cells to inflamed tissues and could be a therapeutic target for intractable airway inflammatory diseases.

INTRODUCTION

CD69 is a type II transmembrane glycoprotein with C-type lectin domains that is expressed on activated leukocytes, especially on T cells (1, 2). CD69 expression is rapidly induced upon T cell receptor (TCR) stimulation (3) and is therefore used as a marker for early activation of T cells. CD69 is also expressed on developing T cells undergoing selection in the thymus (2). Furthermore, several recent studies have shown that CD69 plays important roles in establishing tissue inflammation by promoting cell recruitment and retention in various sites (4–8). CD69 regulates cellular retention within lymphoid tissues by inhibiting S1P1 expression through direct interaction (4), whereas it remains unknown whether there are any specific ligands for CD69 in peripheral tissues.

We have previously reported that CD69 expression on effector CD4 T cells (5, 8) and neutrophils (6) is crucial for the establishment of tissue inflammatory responses. For example, CD69-deficient antigen-specific CD4 T cells failed to migrate into and be retained in the lung, resulting in reduced airway inflammatory responses after antigenic airway challenge (5). CD69-deficient mice are resistant to dextran sulfate sodium (DSS)–induced colitis, and this is due to the inability of CD4 T cells to infiltrate the colon mucosa after DSS treatment (8).

Furthermore, CD69-deficient CD4 T cells failed to generate memory CD4 T cells and to induce secondary antibody responses because of their inability to relocate into and persist in the bone marrow (BM), where antigen-specific resting memory CD4 T cells are maintained (7, 9). These inflammatory responses were efficiently prevented by in vivo treatment with blocking anti-CD69 antibodies (5, 7, 8), suggesting that CD69 may function through specific interactions with one or more ligands expressed in inflamed sites. However, such ligands have not yet been determined.

Accumulation of CD69-expressing leukocytes was observed in a number of human chronic inflammatory disorders, such as asthma (10) and eosinophilic pneumonia (11). CD69 expression on eosinophils is induced by cytokine stimulation, including interleukin-3 (IL-3), IL-5, granulocyte-macrophage colony-stimulating factor, and interferon-γ (10, 11), suggesting that cytokines in the inflammatory environment may induce CD69 expression on effector leukocytes and promote their retention within inflammatory tissues. Accumulation of effector leukocytes in tissues promotes efficient immune response but also may result in exacerbation of inflammatory disorders. The mechanism by which CD69-expressing effector leukocytes are retained at sites of inflammation remains unknown.

An example of chronic inflammatory disorders in humans is chronic rhinosinusitis (CRS), which is categorized as an upper respiratory inflammatory disorder with tissue remodeling of the sinuses and distinct cytokine production profiles by infiltrating inflammatory cells (12–14). CRS with nasal polyps often shows T helper 2 (Th2)–type inflammation, and the nasal polyps include infiltration of large numbers of eosinophils [eosinophilic CRS (ECRS)], lymphocytes, and plasma cells (12, 13, 15–17). ECRS patients usually undergo surgical resection and topical or systemic administration of steroids, which improve their symptoms; however, the recurrence rate remains very high (16, 18). In addition, approximately one-third of ECRS patients suffer from asthma, indicating...
some similarities in the pathogenesis of inflammation between ECRS and asthma (16). Our recent study showed that infiltrating cells in nasal polyps from ECRS patients highly express CD69 (19), implying that CD69 expression on effector leukocytes may play roles in the pathogenesis of ECRS.

We herein identified myosin light chain (Myl) 9, Myl12a, and Myl12b (Myl9/12) as new functional ligands for CD69 in inflamed lungs. Upon airway inflammation, Myl9/12 proteins were predominantly detected on the luminal surface of blood vessels and also as net-like structures inside the vessels. Myl9/12 proteins appeared to be derived from activated platelets in the clots. Blockade of the interaction between CD69 and Myl9/12 by specific antibodies resulted in reduced leukocyte infiltration and ameliorated airway inflammation, indicating that Myl9/12 protein detected on the luminal surface of blood vessels and net-like structures in the clots may play an important role as a platform for CD69-expressing effector leukocytes to be recruited into and maintained in inflamed tissues. A similar localization of Myl9/12 was observed in the polyps of ECRS, a human chronic upper airway inflammatory disease. This study identifies a new mechanism by which effector lymphocytes are recruited and retained in inflamed tissues through CD69-Myl9/12 interaction.

RESULTS
Identification of Myl9/12 as functional ligands for CD69
To identify potential CD69 ligands, we decided to use BM cells because previous studies within our laboratory found that BM cells constitutively express CD69-interacting molecules (7). Affinity purification, using an anti–glutathione S-transferase (GST) antibody with a mixture of BM extracts and recombinant GST–His–CD69 extracellular domain (GST-His-CD69EC) protein, identified an associated 19-kDa protein (Fig. 1A, black arrowhead), which was identified as Myl9 by mass spectrometry (fig. S1A). To determine whether the Myl9 molecule interacts with CD69, we performed co-immunoprecipitation (IP) assays (fig. S1B, lanes 1 and 2) and enzyme-linked immunosorbent assays (ELISAs) (Fig. 1B) and observed a direct interaction between Myl9 and CD69EC molecules. Because the CD69 molecule is known to be highly glycosylated (1), we next examined the impacts of the glycosylation of CD69 on interaction with Myl9. A stronger interaction of Myl9 with CD69EC was detected when N-linked glycans were removed by N-glycosidase F (PNGase F) treatment (fig. S1B, lanes 3 and 4, and Fig. 1B, right).

Fig. 1. Identification of Myl9/12 as interacting proteins for CD69. (A) BM extracts from C57BL/6 mice mixed with recombinant GST-His-CD69EC proteins were subjected to affinity purification using anti-GST antibody, followed by SDS-PAGE and Coomassie brilliant blue staining. Black arrowhead indicates CD69EC-interacting proteins (Myl9/12), and white arrowheads indicate recombinant CD69EC protein. Input was subjected to SDS-PAGE in parallel. (B) ELISAs were used to detect interactions between GST–His–CD69 protein and 3×Flag CD69EC protein with or without PNGase F treatment. (Left) **P = 0.00039, ***P = 0.0000015, ****P = 0.000003, and *****P = 0.000018 (t test; n = 3). (Right) **P = 0.000001, ****P = 0.00000001, and *****P = 0.00000006, and ****P = 0.000004 (t test; n = 3). (C) PNGase F–treated 3×Flag-CD69EC proteins were mixed with GST–His–Myl9 WT or mutant proteins. The mixture was subjected to co-IP with anti-Flag antibody, followed by IB with anti-His antibody. Input was also subjected to IB in parallel. Schematic representations of the GST–His–Myl9 WT and mutants are shown (bottom). (D) ELISA was used to detect interaction between PNGase F–treated 3×Flag-CD69EC protein and GST–His–Myl9 in the presence of anti-Flag antibody, followed by IB with anti-His antibody. Input was also subjected to IB in parallel. Schematic representations of the GST–His–Myl9 WT and mutants are shown (bottom). (D) ELISA was used to detect interaction between PNGase F–treated 3×Flag-CD69EC protein and GST–His–Myl9 in the presence of anti-Flag antibody, followed by IB with anti-His antibody. Input was also subjected to IB in parallel. Schematic representations of the GST–His–Myl9 WT and mutants are shown (bottom). Data are representative of at least three (A, B, D) or two (C) independent experiments.
These data demonstrate that Myl9 specifically interacts with CD69 and that this interaction is partially dependent on the glycosylation status of CD69.

We next examined which regions of Myl9 protein were interacting with CD69 by co-IP assays with the recombinant Myl9 mutant proteins, and we found that positive charges at the N-terminal regions (positions 7 to 13) of Myl9 were required for sufficient interaction with CD69EC (Fig. 1C). Therefore, we generated polyclonal and monoclonal anti-Myl9 antibodies specific for the N-terminal region of Myl9 (positions 1 to 27), with the aim of specifically inhibiting the interaction between Myl9 and CD69. The Myl9 amino acid sequence has high homology to Myl12a (94%) and Myl12b (94%) (fig. S1C), and CD69EC proteins were able to interact with Myl9, Myl12a, and Myl12b proteins in vitro (fig. S1D). As we expected, both monoclonal and polyclonal antibodies bound to Myl9, Myl12a, and Myl12b (fig. S1E); therefore, we refer to these antibodies as anti-Myl9/12 antibody. Both monoclonal and polyclonal anti-Myl9/12 antibodies efficiently inhibited the interaction between Myl9 and CD69 (Fig. 1D), as did the anti-CD69 antibody (fig. S1F). Next, we performed in vitro adhesion assays with in vitro activated CD69-expressing wild-type (WT) CD4 T cells and CD69-deficient CD4 T cells (fig. S1G). WT activated CD4 T cells adhered to plate-coated Myl9 at significantly higher levels compared with CD69-deficient activated CD4 T cells, indicating that CD69 on activated CD4 T cells may functionally interact with Myl9 in vitro. These results indicate that CD69 interacts with Myl9/12 through their N-terminal regions and that the generated antibodies efficiently inhibit the interaction of CD69 with Myl9/12.

Allergic airway inflammation ameliorated by administration of anti-Myl9/12 antibody

To examine the impact of inhibiting CD69-Myl9/12 interaction on the induction and exacerbation of in vivo inflammatory responses, we injected anti-Myl9/12 antibody into mice with ovalbumin (OVA)–induced allergic airway inflammation (Fig. 2A). Injection of either monoclonal (Fig. 2) or polyclonal (fig. S2) anti-Myl9/12 antibodies 1 day before and after the first OVA inhalation significantly attenuated airway inflammation. We detected reduced lung tissue mononuclear cell infiltration and mucin secretion (Fig. 2B), reduced Muc5ac mRNA expression (fig. S2B), and reduced accumulation of leukocytes in bronchoalveolar lavage (BAL) fluid (Fig. 2C). In addition, T\textsubscript{i}12-associated cytokines were undetectable in BAL fluid (Fig. 2D),
and methacholine-induced airway hyperresponsiveness (AHR) was significantly attenuated (Fig. 2E) after anti-Myl9/12 antibody treatment. The inhibition of OVA-induced allergic airway inflammation by anti-Myl9/12 antibody treatment was observed to the same extent as that by anti-CD69 antibody treatment (Fig. S2C). To further examine the impact of anti-Myl9/12 antibody treatment on airway inflammation induced by a naturally occurring allergen, we next used house dust mite (HDM) for sensitization and challenge at the mucosal surface of the airway (Fig. 2F). Anti-Myl9/12 antibody treatment significantly ameliorated HDM-induced airway inflammation (Fig. 2, G and H). These results indicate that blockade of the interaction between Myl9/12 and CD69 by anti-Myl9/12 antibody inhibits not only OVA-induced allergic airway inflammation but also HDM-induced airway inflammation.

**Increased Myl9/12 expression on the luminal surface of blood vessels in the lungs during airway inflammation.**

To elucidate the cellular mechanisms by which Myl9/12 regulates airway inflammation, we next examined whether Myl9/12 protein expression was induced during OVA-induced allergic airway inflammation and where the protein was located in the inflamed lung. Total amounts of Myl9/12 protein in the whole lung were increased upon airway inflammation (Fig. 3A), and immunohistological analysis revealed that Myl9/12 protein was predominantly located around the luminal surface of blood vessels but not on bronchi in the inflamed lung (Fig. 3B). Blood vessels that had Myl9/12 present on more than 10% of their surface were classified as Myl9/12-positive vessels. More than 60% of blood vessels in inflamed lung tissue were Myl9/12-positive, whereas the lung from control mice (no OVA inhalation) had a limited number of Myl9/12-positive vessels (Fig. 3C). Because anti-Myl9/12 antibody treatment efficiently prevented OVA-induced airway inflammation (Fig. 2), we next used goat antirabbit immunoglobulin G (IgG) secondary antibody to detect the in vivo localization of the anti-Myl9/12 antibody that we administered to mice with allergic airway inflammation. The anti-Myl9/12 antibodies were mainly detected on the luminal surface of blood vessels (Fig. S3A). These results indicate that Myl9/12 protein becomes detectable on the luminal surface of blood vessels in the lungs upon OVA-induced allergic airway inflammation.

**Platelet-dependent formation of Myl9/12 net-like structures during airway inflammation.**

Myl9/12 are proteins typically considered to be restricted to the cytosol under normal conditions. Therefore, it was essential to determine how Myl9/12 protein was relocated to the luminal surface of blood vessels during airway inflammation. We first examined Myl9/12 expression in vascular endothelial cells. Although expression of Myl9/12 on lung endothelial cells was detected by flow cytometry, no obvious increase was detected upon inflammation (Fig. S3B), and Myl9/12 mRNA expression in sorted CD45−TER119−PECAM-1− lung endothelial cells was not changed (fig. S3C). My9 is known to form a complex with nonmuscle myosin Ila (Myh9) (20), which is highly expressed in megakaryocytes and platelets (21, 22), and My9 can be detected in granules released from platelets upon activation (23). Platelets are readily activated in the intravascular space in inflamed lungs; therefore, we hypothesized that activated platelets in inflamed lungs release platelet activation mediators including My9, resulting in My9 protein attachment to the luminal surface of blood vessels. We first confirmed that Myl9/12 protein was highly expressed in megakaryocytes (Fig. 4A) and platelets (Fig. 4B). In vitro thrombin stimulation resulted in the relocation of Myl9/12 to the edge of platelets within 10 min and its disappearance from platelets in 2 hours (fig. S4A), followed by abundant Myl9/12 protein detected in the culture supernatant 2 hours after stimulation (Fig. 4C). We next examined mRNA expression levels of My9, Myl12a, and Myl12b in platelets and endothelial cells with specific primers. mRNA expression levels of My9, Myl12a, and Myl12b were high in platelets but very low in lung endothelial cells (Fig. 4D). My9 mRNA expression in platelets was extremely high, suggesting that the major molecule detected by anti-Myl9/12 antibody in the lung is My9.

We further examined at which location on the luminal surface of blood vessels in inflamed lungs was Myl9/12 protein detected by immunohistological analysis. Myl9/12 protein was detected on the luminal surface of blood vessels but was not merged with platelet endothelial cell adhesion molecule–1 (PECAM-1) staining of vascular endothelial cells (Fig. 4E), indicating that Myl9/12 protein attaches to the luminal surface of blood vessels but is not expressed in vascular endothelial cells. Furthermore, with immunohistological analysis using thicker (20 μm) lung sections, we found that some of the blood vessels in the inflamed lungs contained clots (thrombus) consisting of TER119+ red blood cells, CD41+ platelets, and Myl9/12, forming net-like structures (Fig. 4F and G, and movie S1). We further addressed the impact of platelet depletion on inflammation-induced Myl9/12 expression by injecting anti–glycoprotein 1b, alpha polypeptide (GP1bα) antibody (Fig. 4H), which efficiently depleted platelets in the peripheral blood (24) and lungs (fig. S4B). The levels of Myl9/12 protein attached to the luminal surface of blood vessels in the lungs of mice treated with anti-GP1bα antibody were reduced (Fig. 4I). The total amounts of Myl9/12 protein in the lung were also reduced (Fig. 4I). Together, we conclude that
Myl9/12 molecules are predominantly detected on the luminal surface of blood vessels, form net-like structures in the thrombus in the inflamed lungs, and are likely to be derived from platelets.

Detection of CD69+ antigen-specific CD4 T cells in the lung vasculature during airway inflammation

Considering that Myl9/12 protein expression was detected mainly on the luminal surface of blood vessels (Fig. 3) and that anti-Myl9/12 and anti-CD69 antibodies significantly ameliorated airway inflammation (Fig. 2), it is likely that antigen-specific CD69-expressing T cells in the lung are recruited into tissues by interacting with Myl9/12. Consequently, using an intravascular staining method, we examined whether there were any CD69+CD4 T cells in the lung vasculature during airway inflammation (25). We injected OVA-specific (KJ1+)CD4 T cells into host mice, induced airway inflammation, and examined whether there were any CD69+KJ1+CD4 T cells in the lung vasculature (fig. S5A). No detectable CD69-expressing KJ1+ cells were observed in the peripheral blood, whereas about 1% of injected KJ1+ cells expressed CD69 in the lung vasculature before OVA inhalation (fig. S5, B and C). The frequency of CD69-expressing KJ1+ cells in the peripheral blood was not increased, whereas that in the lung vasculature increased substantially and significantly 12 and 24 hours after OVA inhalation (fig. S5, B and C). The absolute number of CD69+KJ1+ cells in the lung vasculature was also increased upon OVA inhalation (fig. S5C, right). Increased numbers of CD69+KJ1+ cells were detected in the lung parenchyma even at 12 hours after OVA inhalation (fig. S5D). This result could indicate that CD69+KJ1+CD4+ T cells in the lung vasculature rapidly migrate into the lung parenchyma. In addition, CD69+TCRb+ T cells were detected, by immunohistochemistry, inside the blood vessels 24 hours after OVA inhalation (fig. S5E). These results indicate that antigen-specific T cells in the lung vasculature express CD69 upon airway antigen challenge and that the expression of CD69 may be important for these T cells to be recruited into the lung parenchyma.

Effects of anti-Myl9/12 and antihuman CD69 antibodies on human CD69-mediated allergic airway inflammation

To further address the feasibility of using anti-CD69 and anti-Myl9/12 antibodies for the treatment of patients, we established a mouse model of human CD69-mediated airway inflammation (fig. S6A). We generated mouse CD69-deficient OVA-specific effector T1/2 cells with or without...
human CD69 expression (fig. S6B) and confirmed that human CD69-expressing T_{H2} cells efficiently induced airway inflammation in BALB/c host mice. The amino acid sequences of the N-terminal regions of human Myl9/12 and murine Myl9/12 are almost identical, and human CD69 was confirmed to interact with mouse Myl9 almost as efficiently as mouse CD69 (fig. S6C). Both antihuman CD69 and anti-Myl9/12 antibodies significantly attenuated inflammatory responses (fig. S6, D and E), indicating that blockade of the interaction between human CD69 and Myl9/12 inhibits human CD69-mediated airway inflammation. CD69 expression is known to be induced on many activated leukocytes (3, 26); therefore, any CD69-expressing leukocytes could interact with Myl9/12 and be targets of anti-Myl9/12 and anti-CD69 antibody treatments. However, experiments using the OVA-induced asthma model described herein show that the blockade of the interaction between Myl9/12 and CD69 on T_{H2} cells was sufficient to attenuate airway inflammation. Thus, CD69 on T_{H2} cells appeared to be the major target of the antibody treatment that prevents airway inflammation in this model.

**Myl9/12 expression in the polyps of ECRS patients**

CRS is a common allergic inflammatory disease of the upper respiratory tract (27, 28), and recent reports have suggested the relationship between the pathogenesis of CRS and abnormal coagulation cascade (29). Therefore, we examined the expression of Myl9/12 in human chronic airway inflammatory lesions such as nasal polyps of ECRS patients (30, 31). Nasal mucosa obtained from nonatopic healthy donors was included as a steady-state control tissue. Myl9/12 expression was significantly increased in nasal polyps from ECRS patients, compared to control nasal mucosa, and was primarily localized in the blood vessels (Fig. 5A). With a more detailed immunohistological analysis using thicker (20 μm) sections of the polyps, we found that the luminal surface of most blood vessels contained Myl9/12 and that some of them formed net-like structures together with von Willebrand factor (vWF) (Fig. 5B). In addition, a substantial proportion of the CD69-expressing cells appeared to directly interact with the Myl9/12 protein attached on the surface of blood vessels in ECRS polyps (Fig. 5C). About 65% (64.3 ± 5.4%) of the CD3-expressing cells that were in contact with Myl9/12-positive blood vessels were CD69-expressing T cells (fig. S7, A and B). Furthermore, Myl9/12 was also distributed on the vascular walls and in perivascular spaces where many CD69-expressing cells appeared to be entangled in Myl9/12 molecules (Fig. 5D).

These results demonstrate that Myl9/12 expression is significantly increased and is distributed not only in the intravascular space but also on the vascular walls and in the perivascular stromal tissue of the nasal polyps of ECRS patients. Colocalization of CD69-expressing cells and Myl9/12 protein may suggest that CD69-Myl9/12 interaction contributes to the long-term feature of inflammation in ECRS polyps.

**DISCUSSION**

We herein identify a previously unknown mechanism that regulates airway inflammation. We show that Myl9/12 proteins are previously unknown functional ligands for CD69 and define CD69-Myl9/12 interaction as a key step in the pathogenesis of airway inflammation.
Within inflamed lungs, Myl9/12 attach to the luminal surface of blood vessels and form net-like structures inside the vessels. Blockade of the interaction between Myl9/12 and CD69 by specific antibodies ameliorates lung inflammation, suggesting that the Myl9/12 protein accumulated in inflammatory vessels may play an important role as a platform for the recruitment of CD69-expressing leukocytes into inflammatory tissues.

Leukocyte recruitment into inflammatory tissues is the first step of immune response (32, 33). Innate immune cells, such as monocytes, neutrophils, and eosinophils, are recruited into inflammatory tissues through inflammatory vessels (where they are activated), whereas antigen-specific T cells are thought to be activated in draining lymph nodes and then recruited into inflammatory tissues (34). Previous studies have reported that migration of activated leukocytes into inflammatory sites is mediated by inflammatory chemokines and that recruitment of the cells into inflammatory tissues is mediated by the interaction of PSGL-1 (P-selectin glycoprotein ligand−1) with P-selectin (35, 36). Here, we propose a new mechanism, named the “CD69-Myl9/12 system,” that directs the recruitment of activated T cells into inflammatory tissues. In the CD69-Myl9/12 system, Myl9/12-containing net-like structures are created in inflammatory vessels, which play an important role as a platform for the recruitment of CD69-expressing leukocytes into inflammatory tissues. T cells that are activated in the lymph nodes proliferate, down-regulate CD69 expression, and then leave the lymph nodes to migrate into inflammatory sites in an S1pr1-dependent manner (37). However, our study revealed that about 10% of antigen-specific CD4 T cells in the lung vasculature express CD69 upon airway antigen stimulation (OVA inhalation). This supports the idea that the CD69-Myl9/12 system is important for the migration and recruitment of CD69− T cells into the inflamed lung. However, it is not known how CD69 expression is induced on antigen-specific CD4 T cells in the lung vasculature after antigen challenge via the airway. One possibility is that the Myl9/12 net-like structures may include antigen-presenting cells that directly instruct antigen-specific CD4 T cells to express CD69 on their surface. Another possibility is that the cytokine-rich inflammatory environment, induced after antigen challenge via the airway, activates CD4 T cells to express CD69. Various cytokines, including those that can stimulate T cells [such as IL-1β, IL-2, IL-4, IL-6, IL-9, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)], were detected in the lungs of allergic asthma models and in human patients (38–41). Further detailed analyses are required to answer this question.

Recent studies have reported a crucial relationship between abnormal coagulation and allergic airway inflammatory diseases such as asthma (42). Platelet abnormalities in patients with allergy have been particularly noticed (43, 44). Although there is accumulating evidence of the importance of platelets in airway inflammation (42), how platelets are involved in the process of airway inflammation appears to be complex and not well defined. The CD69-Myl9/12 system that we herein identified is a possible candidate for antibody-based immunotherapy in asthma (5) and colitis (8). In the current study, we found that anti-Myl9/12 antibodies inhibited allergic airway inflammation as efficiently as anti-human and antinouse CD69 antibodies did, and the expression of Myl9/12 was selectively detected in inflamed vessels. Therefore, Myl9/12 could be a new therapeutic target for chronic airway inflammatory disorders for which effective treatments have not been established.

In conclusion, the present study identifies Myl9/12 as previously unknown functional ligands for CD69 that regulate airway inflammation and proposes a mechanism called the CD69-Myl9/12 system, which is necessary for the recruitment of antigen-specific CD69-expressing CD4+ T cells into inflammatory tissues because it interacts with Myl9/12 protein in net-like structures within the blood vessels. Because Myl9/12 are derived from activated platelets that play an essential role in the blood coagulation system (49), the CD69-Myl9/12 system may also play a critical role in a range of other inflammatory diseases where tissue damage and blood coagulation occur. Thus, blocking the interaction between Myl9/12 and CD69 could be a new therapeutic target not only for airway inflammatory disorders but also for other inflammatory diseases.

MATERIALS AND METHODS

Study design
To elucidate the mechanism that CD69 expression on activated leukocytes contributes to inflammation, we first identified CD69 ligands by IP with recombinant CD69 protein, followed by mass spectrometry analysis, and then made specific functional antibodies against the identified CD69 ligands (anti-Myl9/12 antibodies). Using the specific antibodies, we (i) assessed the impacts of the administration of anti-Myl9/12 antibodies on airway inflammation in a mouse model and (ii) identified where and how Myl9/12 are located on blood vessels in inflamed tissues, including human polyps from ECRS patients. Differential counts of inflammatory cells in BAL fluid (Fig. 2, C and H) and Myl9/12-positive–expressing vessels (Fig. 3C) were performed double-blinded by at least two independent researchers. Statistical significance was determined as P < 0.05 by two-tailed t test for all experiments.

Mice
C57BL/6 and BALB/c mice were purchased from CLEA Co. (Tokyo, Japan). CD69-deficient mice were created in our laboratory (6) and were backcrossed onto both C57BL/6 and BALB/c genetic backgrounds more than 15 times. All mice, including OVA-specific OTI/TCR transgenic (DO11.10) mice (50), were maintained under specific pathogen-free conditions and were used at 6 to 16 weeks of age. All animal experiments were approved by the Chiba University Review Board for Animal Care.

Purification of recombinant proteins
Recombinant GST-His-CD69EC, GST-His-Myl9, Myl9 mutants, Myl12a, Myl12b, and Myl3 proteins were expressed in the BL21 (DE3) or Rosetta (DE3) strains (Novagen) transformed with pET42b-based constructs and induced by isopropyl-β-thiogalactopyranoside (1 mM) for 4 hours. GST-His-CD69EC protein was further processed for purification and refolding as previously described (51). GST-His-Myl9, Myl9 mutants, Myl12a, Myl12b, and Myl3 proteins were solubilized in a suspension buffer [50 mM tris–HCl (pH 8.0), 300 mM NaCl, and 10 mM imidazole] and further lysed by sonication with a Misonix sonicator (Microson). The recombinant proteins were purified with Ni-NTA Agarose (Qiagen) according to the manufacturer’s protocol. Recombinant 5×His-CD69EC proteins were expressed in FreeStyle 293-F cells transfected with p3×Flag-CMV9-mCD69EC plasmid, and the proteins were purified using an anti-Flag M2 affinity column (A2220; Sigma). After elution with 0.1 M

glycine-HCl (pH 3.5), purified proteins were concentrated and the buffer was exchanged for phosphate-buffered saline (PBS) with Amicon Ultra-4 (Merck Millipore). Protein purity was defined by SDS–polyacrylamide gel electrophoresis (PAGE), followed by staining with SimplyBlue SafeStain (Invitrogen).

**Identification of CD69-interacting proteins**

Adult femurs and tibias were lysed with the following protease inhibitor–containing lysis buffer [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.05% NaN₃, 0.1% Triton X-100, 1 mM phenylmethysulfonyl fluoride, and Protease Inhibitor Cocktail (Roche)]. The cell lysates were subjected to a preclearing process with Protein G Sepharose (GE Healthcare) at 4°C for 1 hour and then mixed with GST-His-CD69EC or GST-His (10 μg each) overnight at 4°C. The mixtures were subjected to IP with an anti-GST antibody (013-21851; Wako) at 4°C for 3 hours, and then the immunocomplexes were precipitated with Protein G Sepharose beads at 4°C for 1 hour. Immunoprecipitates were washed with lysis buffer and eluted from the beads by SDS-containing gel loading buffer. The eluted samples were separated in 15% polyacrylamide gels (ATTO), and the specific band was identified by liquid chromatography–tandem mass spectrometry analysis.

**Generation of antibodies against Myl9/12**

To generate rabbit polyclonal antibody against Myl9/12, we used as an immunogen a fusion protein of N-terminal peptides of Myl9 (residues 1 to 27) and keyhole limpet hemocyanin. Serum was further purified on an affinity column with N-terminal peptides of Myl9 that was originally used as an immunogen (MBL). Murine monoclonal antibody (mAb) against Myl9/12 (114-2G9, KAN Research Institute Inc.) and rabbit mAb against Myl9/12 (F-6, Abwiz bio Inc.) were made by immunization with N-terminal peptides of Myl9 (residues 1 to 27).

**IP and immunoblotting**

GST-fusion recombinant proteins (Myl9, Myl12a, Myl12b, and Myl3) were mixed with 3×Flag-CD69EC overnight and then subjected to IP with an anti-Flag antibody (M2; Sigma-Aldrich) coupled with Dynabeads (Life Technologies) at 4°C for 3 hours. Binding proteins were eluted from the beads by 3×Flag peptides (Sigma-Aldrich) and then subjected to SDS-PAGE and immunoblotting (IB), as described previously (52). As indicated, recombinant affinity-purified 3×Flag-CD69EC proteins were treated with PNGase F (NEB P0704) according to the manufacturer’s protocol and used for further assays. For detection of Myl9/12 protein expression, cell or tissue lysates were prepared using radiolabeled precipitation assay buffer and then used for IB. The antibodies used for IB were anti-GST biotin (Rockland), anti–Penta-His Biotin (Qiagen), anti-Flag (M2; Sigma-Aldrich), anti-Myl9/12, anti–tubulin-α (NeoMarkers), streptavidin–horseradish peroxidase (HRP; Jackson Laboratory), antirabbit IgG–HRP, and antimouse IgG–HRP (GE Healthcare).

**Enzyme-linked immunosorbent assay**

Purified GST-His-Myl9 or GST-His-Myl3 was directly added to a plate for coating or was added to a glutathione-coated plate (Thermo Pierce). After blocking of the coated plates with Block Ace (DS Pharma), PNGase F–treated or nontreated affinity-purified 3×Flag-CD69EC proteins were added and incubated at room temperature for 1 hour. The binding of CD69 to Myl9 was detected by HRP-conjugated anti-Flag antibody (Sigma) and tetramethylbenzidine (TMB) solution (Bio-Rad Laboratories). For detection of soluble My9 protein, anti-Myl9 mAb (Abcam) was directly coated on a plate. After blocking, culture supernatants were added and then reacted with biotinylated anti-Myl9/12 (F-6) mAb. Binding of Myl9 was detected by HRP-conjugated streptavidin (Jackson Laboratory).

**Measurement of airway hyperreactivity and airway inflammation**

OVA-induced allergic airway inflammation was induced as previously described (5, 53). In brief, BALB/c mice were intraperitoneally injected with OVA in aluminum hydroxide gel (alum) on days 0 and 7, followed by aerosolized OVA inhalation on days 14 and 16. As indicated, polyclonal and monoclonal (114-2G9) anti-Myl9/12 or isotype control antibodies (100 μg) were intraperitoneally injected on days 13 and 15. For platelet depletion, anti-GPIbα antibody (emfret ANALYTICS) was intravenously injected (4 μg/g) on days 13 and 15. BAL, AHR assessment, and immunohistological analysis were performed at 24 hours after the last OVA challenge. The absolute number of infiltrating cells in BAL fluid was obtained using percentages of cells and total cell number of BAL fluid recovered. To measure cytokine production in BAL fluid, we first removed mononuclear cells by centrifugation and measured specific cytokine production by BD Cytometric Bead Array (BD Pharmingen). AHR was assessed by methacholine (Sigma-Aldrich)–induced airflow obstruction at 24 hours after the last antigen challenge using a computer-controlled small animal ventilator (SCIREQ). For histological analysis of asthmatic lungs, the mice were killed at 48 hours after the last OVA challenge, and hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) staining was performed (5).

For HDM-induced allergic airway inflammation, 3-day consecutive intranasal administrations with HDM (50 μg) were performed in BALB/c mice for 3 weeks. Monoclonal anti-Myl9/12 (F-6) or isotype control antibodies (100 μg) were intraperitoneally injected 1 day before HDM administration and on the second day of HDM administration in each session. BAL and immunohistological analysis were performed at 24 hours after the last HDM administration. HDM were provided by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan).

**Immunofluorescent staining and confocal microscopy**

For immunofluorescent staining, cryostat sections of lungs were fixed in 4% paraformaldehyde and then stained and mounted with fluorescent mounting medium (DakoCytomation). For BM analysis, adult femurs and tibias were fixed in 4% paraformaldehyde and equilibrated in 30% glycerol, 0.05% NaN₃, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and Protease Inhibitor Cocktail (Roche). The cell lysates were subjected to a preclearing process with Protein G Sepharose (GE Healthcare) at 4°C for 1 hour and then subjected to SDS-PAGE and immunoblotting (IB), as described previously (52). As indicated, recombinant affinity-purified 3×Flag-CD69EC proteins were treated with PNGase F (NEB P0704) according to the manufacturer’s protocol and used for further assays. For detection of Myl9/12 protein expression, cell or tissue lysates were prepared using radiolabeled precipitation assay buffer and then used for IB. The antibodies used for IB were anti–Penta-His Biotin (Qiagen), anti–Penta-His Biotin (Qiagen), anti-Flag (M2; Sigma-Aldrich), anti-Myl9/12, anti–tubulin-α (NeoMarkers), streptavidin–horseradish peroxidase (HRP; Jackson Laboratory), antirabbit IgG–HRP, and antimouse IgG–HRP (GE Healthcare).

**Quantitative reverse transcription polymerase chain reaction**

Total RNA was isolated with TRIzol reagent (Invitrogen), and cDNA synthesis was performed using Superscript II (Invitrogen) with oligo(dT) primers. Quantitative reverse transcription polymerase chain reaction


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Myosin light chains 9 and 12 are functional ligands for CD69 that regulate airway inflammation


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Breathing down asthma’s neck

Worldwide increases in the prevalence of chronic inflammatory diseases such as asthma highlight the need for new targeted therapies. CD4+ T cells that express the activation marker CD69 contribute to the pathogenesis of some types of asthma, but the exact role of CD69 remains unclear. Hayashizaki et al. reported that myosin light chain (Myl) 9 and Myl12 are functional ligands for CD69. Myl9/12 expression was increased in inflamed mouse airways and in patients with eosinophilic chronic rhinosinusitis. These data suggest that CD69-Myl9/12 interaction is involved in recruiting activated cells to inflamed tissues. Indeed, blocking CD69-Myl9/12 interaction in two different mouse models decreased allergic airway inflammation, suggesting that targeting CD69-Myl9/12 interaction could treat chronic inflammatory diseases.