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Novel Approach to Inhibit Asthma-Mediated Lung Inflammation Using Anti-CD147 Intervention

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Extracted cyclophilins have well been described as chemotactic factors for various leukocyte subsets. This chemotactic capacity is dependent upon interaction of cyclophilins with the cell surface signaling receptor CD147. Elevated levels of extracellular cyclophilins have been documented in several inflammatory diseases. We propose that extracellular cyclophilins, via interaction with CD147, may contribute to the recruitment of leukocytes from the periphery into tissues during inflammatory responses. In this study, we examined whether extracellular cyclophilin-CD147 interactions might influence leukocyte recruitment in the inflammatory disease allergic asthma. Using a mouse model of asthmatic inflammation, we show that 1) extracellular cyclophilins are elevated in the airways of asthmatic mice; 2) mouse eosinophils and CD4+ T cells express CD147, which is up-regulated on CD4+ T cells upon activation; 3) cyclophilins induce CD147-dependent chemotaxis of activated CD4+ T cells in vitro; 4) in vivo treatment with anti-CD147 mAb significantly reduces (by up to 50%) the accumulation of eosinophils and effector/memory CD4+ T lymphocytes, as well as Ag-specific Th2 cytokine secretion, in lung tissues; and 5) anti-CD147 treatment significantly reduces airway epithelial mucin production and bronchial hyperreactivity to methacholine challenge. These findings provide a novel mechanism whereby asthmatic lung inflammation may be reduced by targeting cyclophilin-CD147 interactions. The Journal of Immunology, 2006, 177: 4870 – 4879.

The pathogenesis of allergic asthma is characterized by an influx of eosinophils and Th2 lymphocytes into lung tissues and airways. Cytotoxic proteins, bronchoactive mediators, and cytokines such as IL-4, -5, and -13 released by eosinophils and Th2 effector cells are thought to contribute to the eosinophilia as well as mucus production and airway hyperresponsiveness (AHR) associated with disease pathology (1, 2). Chemokines have been well described as critical factors involved in the recruitment of these inflammatory cells from the circulation into lung tissues and airways via interaction with chemokine receptors present on leukocytes (3–5).

Cyclophilins are ubiquitously distributed intracellular proteins possessing peptidyl-prolyl cis-trans isomerase activity, which are believed to play a critical role in protein folding. The two most-characterized cyclophilins are cyclophilin A (CypA) (18 kDa), which is cytosolic and cyclophilin B (CypB) (21 kDa), which is directed to the endoplasmic reticulum by an N-terminal signal sequence (6, 7). CypA is the most abundant cyclophilin, accounting for ~0.1–0.4% of total cellular protein (8) and is best known as the high-affinity intracellular receptor for the immunosuppressive drug cyclosporin A (9). However, cyclophilins are also released by necrotic cells or are secreted (10–14) and thus can function extracellularly. Specifically, extracellular cyclophilins have been shown to induce the chemotaxis of various human leukocyte subsets in vitro, including monocytes, eosinophils, neutrophils, and T lymphocytes (10, 15–17). In vivo injection of CypA into mouse footpads has also been shown to induce a local influx of neutrophils (10). Cyclophilins thus represent a novel class of extracellular proteins possessing chemokine-like activity.

CD147 (50–60 kDa), also known as extracellular matrix metalloproteinase inducer (EMMPRIN) in humans, is a type I transmembrane glycoprotein that is expressed by a wide array of cell types, including all human peripheral blood leukocytes (18). Furthermore, CD147 is up-regulated on activated human T cells (19). CD147 is the principal cell surface signaling receptor for extracellular CypA and CypB (17, 20), and most importantly, cyclophilin-mediated chemotactic activity has been shown to be CD147 dependent in that anti-CD147 mAb blocks cyclophilin-mediated chemotaxis of human leukocytes in vitro (17).

Due to the relevance of cyclophilins and CD147 to leukocyte chemotaxis, we propose that cyclophilin-CD147 interactions may be important in the regulation of inflammatory processes by directly promoting leukocyte migration into inflamed tissues (6, 21). In fact, indirect evidence exists suggesting that cyclophilin-CD147 interactions might contribute to several inflammatory diseases. For example, elevated levels of extracellular cyclophilins have been reported during ongoing tissue inflammation in rheumatoid arthritis (22) and vascular smooth muscle cell disease (11). Elevated serum CypA and CypB have also been detected in patients with severe inflammatory sepsis (23). In the case of rheumatoid arthritis, CD147 expression was shown to be up-regulated on inflammatory granulocytes, fibroblast-like cells, and monocytes/macrophages in the synovial fluid of patients with ongoing inflammation...
flammation may be reduced, by targeting cyclophilin-CD147 interactions in a mouse model of acute lung inflammation (27).

In the current study, we address whether extracellular cyclophilin-CD147 interactions might play a role in asthmatic lung inflammation. Using a mouse model of allergic asthma that induces a characteristic Th2-driven inflammatory response, we propose that extracellular cyclophilins, via interaction with cell surface CD147 on leukocytes, directly contribute to the influx of eosinophils and/or CD4+ Th2 effector lymphocytes into inflamed lung tissues and airways. As will be shown, our findings demonstrate that extracellular cyclophilins are highly elevated in the airways of asthmatic mice and that in vivo blocking of cyclophilin-CD147 interactions using anti-CD147 mAb significantly reduces the accumulation of both eosinophils and effector/memory CD4+ T cells, as well as Ag-specific Th2 cytokine secretion, in lung tissues during asthma-mediated inflammation. Moreover, this treatment led to a significant reduction in airway epithelial mucin production and bronchial hyperreactivity to methacholine challenge. Such findings provide a novel mechanism whereby asthmatic lung inflammation may be reduced, thereby targeting cyclophilin-CD147 interactions.

Materials and Methods

Animals

All in vivo studies and most in vitro studies were conducted using female BALB/c mice at 6 wk or older purchased from the National Cancer Institute (Bethesda, MD). IL-5 transgenic (Tg) mice, either on a BALB/c (28) or on a C57BL/6 (29) genetic background, were provided by L. A. Dent (University of Adelaide, Adelaide, Australia) and J. J. Lee (Mayo Clinic, Scottsdale, AZ), respectively. Tg mice were used at 6 wk or older where specified. All studies were reviewed and approved by the institutional animal care and use committee at The George Washington University Medical Center.

Abs and reagents

Rat anti-mouse CD147 mAb was purified from the RL73.2 hybridoma (30), originally provided to us by H. R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). The rat IgG2a hybridoma (HB-189) was obtained from American Type Culture Collection and was used as a source of isotype control mAb. Both mAbs were purified by the National Cell Culture Center (Minneapolis, MN). FITC-conjugated F(ab′)2 anti-rat IgG (secondary) Ab was purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor 647-conjugated anti-mouse CCR3 mAb, Cy-Chrome-conjugated anti-mouse CD4 mAb, and FITC-conjugated anti-mouse CD62L mAb were purchased from Bio-Rad Systems. Rat anti-mouse CypA Ab, which specifically recognizes only CypA, was obtained from U.S. Biological. Rabbit anti-CypB Ab was obtained from ABR and specifically recognizes only CypB. HRP-conjugated anti-rabbit secondary Ab was purchased from Amershams Biosciences or, for ERK blots, from Jackson ImmunoResearch Laboratories. Phospho-p44/42 (Thr202/Tyr204) and p44/42 MAPK (Thr202/Tyr204) MAPK Abs were purchased from Cell Signaling Technology. Human recombinant CypA (CDCA site-mutant) mAb was purchased from Bio-Rad Systems. Mouse recombinant CypB was by only one amino acid residue, was purchased from Calbiochem. Human recombinant CypB, which has a chomotoxins-signalining amino acid sequence motif highly homologous to mouse CypB, was expressed and purified in house. The plasmid encoding the gene for human recombinant CypB was provided by F. Allain (Universite des Sciences et Technolgies de Lille, Lille, France). Mouse recombinant CYP1A1 and IL-5 were purchased from R&D Systems. Mouse RANTES was obtained from PeproTech. OVA was purchased from ICN Biomedicals. Injact Alum was obtained from Pierce. BSA fraction V and Con A were purchased from Sigma-Aldrich.

Regimen for OVA-induced airway inflammation and its inhibition

We used a modified model of airway inflammation, previously described by Umetsu and coworkers (31). Female BALB/c mice were primed by i.p. injection of 50 μg of OVA in PBS plus 100 μl of alum (200-μl total volume per mouse) on day 0. Control mice were injected i.p. with PBS plus 100 μl of alum (200-μl total volume per mouse). OVA/alum-primed (OVA) and alum-primed (control) mice were then challenged under light anesthesia (isoflurane) by intranasal (i.n.) delivery of 100 μg of OVA in PBS (50-μl total volume) on days 7–10. Naive (untreated) mice were also used in some experiments as a negative control. Mice were sacrificed by exposure to CO2 on day 12. For in vivo blocking studies, a previously optimized dose (10 μg) of anti-CD147 or isotype control mAb was administered i.p. on days 6–7–10 (at least 1 h before i.n. challenge), and 11. All animals were sacrificed on day 12.

Bronchoalveolar lavage (BAL) and lung tissue processing

Following sacrifice, cells were collected from the airways of individual mice by BAL, in which a cannula was inserted into the trachea, and three 1-ml washes of cold PBS were infused in and out of the airways. BAL cells were then washed, treated with ammonium chloride lysing buffer to remove RBC, counted and centrifuged onto glass slides, and stained with Wright-Giemsa (Camco) to determine leukocyte distribution. After BAL was performed, lungs were perfused via the right ventricle with 20 ml of ice-cold PBS, finely chopped, and then pushed through a metal strainer, followed by a nylon mesh screen, to generate single-cell suspensions. Lung cells were treated with ammonium chloride lysing buffer, counted, and analyzed by FACS after staining with Alexa Fluor 647-conjugated anti-mouse CCR3 to determine numbers of CCR3+ granulocytes (eosinophils) or Cy-Chrome-conjugated anti-mouse CD4 in combination with FITC-conjugated anti-mouse CD62L to determine numbers of CD4+Cy62L+ lymphocytes (effector/memory CD4+ T cells).

In vitro OVA restimulation

For OVA restimulation, perfused lungs were pooled within each group, finely chopped, and incubated at 37°C for 20 min in 5 ml of a digestion mixture consisting of Click’s medium (BioSource International), 5% FBS, 150 U/ml collagenase type IV (Worthington Biochemical), and 20 μg/ml DNase I (ICN Biomedicals). Digested lung tissue was pushed through a metal strainer followed by a nylon mesh screen to generate single-cell suspensions. Lymphocytes were isolated using lymphocyte separation medium (Mediatech), counted, and then cultured (1 × 106 cells per well) in 48-well plates in Click’s medium supplemented with 5% FBS at 37°C in the presence of T cell-depleted, mitomycin C-treated splenocytes (1 × 105 per well) prepared from naive BALB/c mice and increasing doses of OVA protein Ag (4, 20, or 100 μg/ml) to induce cytokine secretion by OVA-specific T cells, or medium alone. Culture supernatants were collected after 4 days of OVA restimulation for cytokine analysis. IFN-γ and IL-5 were measured using ELISA mini-kits purchased from Endogen. IL-13 was measured using an ELISA kit from R&D Systems. Baseline cytokine values detected in medium alone (unstimulated) supernatants were subtracted from values for each dose of OVA.

Histology

After lung perfusion, 1 ml of 10% formalin was infused into the trachea to inflate and fix the lung tissue on day 12 of the OVA regimen. The trachea was tied off using suture thread, and the fixed lungs were isolated and stored in 10% formalin until further processing. Fixed lungs were embedded in paraffin, and 5-μm sections were cut and stained with periodic acid-Schiff (PAS) reagent to identify changes in airway epithelial mucin production (Histoserv). The frequency of PAS+ vs PAS− airways present in each stained section was counted blindly by two independent investigators.

 Bronchial hyperreactivity

Bariometric whole-body plethysmography was used to measure bronchial hyperreactivity on day 12 of the OVA regimen. This method measures changes in box flow waveform from both inspiration and expiration of the test animal in the chamber and is expressed as enhanced pause (Penh) following challenge with an increasing dose of inhaled methacholine (Sigma-Aldrich) in PBS (3.125 up to 50 mg/ml) using a Buxco pressure transducer. The animal was placed in the chamber and a baseline reading for Penh was taken for 3 min. The mouse was then exposed to aerosolized PBS for 3 min and PenhPBS was recorded. The mouse was then exposed to aerosolized methacholine for 3 min and PenhMch was recorded at each dose. The average Penh value over the 3-min period was calculated.
Eotinophil isolation

Peripheral blood was obtained from IL-5 Tg mice by cardiac puncture. Leukocytes were isolated from whole blood as previously described (29) using a Percoll-E gradient (60% Percoll, 9.5% 10X HBSS, 1.4% 1 M HEPES, and 3% 0.1 N HCl in water), of which the majority of cells are eosinophils (60%) and lymphocytes (40%). Eosinophils were then purified by negative selection using anti-mouse Thy1.2 and B220-labeled MACS beads purchased from Miltenyi Biotec.

In vitro activation and purification of CD44+ T cells

Splenocytes were prepared from spleens harvested from naive BALB/c mice. The cells (3 x 10^6 cells per well) were activated overnight at 37°C in 24-well plates with Con A at a final concentration of 1 µg/ml. Con A-activated and naive CD44+ T cells were then purified by negative selection using the CD44+ T cell isolation kit from Miltenyi Biotec.

Chemotaxis assays

Chemotaxis of peripheral blood-derived, purified eosinophils from IL-5 Tg mice to CypA and CypB was assessed using 24-well Transwell plates (Costar) in a method previously described (32) in which IL-5 Tg-derived eosinophils were shown to be chemotactic to a number of chemokines in vitro. Medium only (5% BSA fraction V in RPMI 1640) or different doses of recombinant CypA or CypB (400, 200, or 100 ng/ml) in medium were placed in the lower chambers (100-µl volume) in triplicate. Recombinant eotaxin-1 (270 ng/ml) was used as a positive control. Eosinophils, in medium containing rIL-5 (30 ng/ml), were placed into the Transwell polycarbonate inserts (5 x 10^6 cells per insert in a 200-µl volume). The Transwells were then incubated at 37°C, and the number of eosinophils that had migrated into each well was counted after 2 h. The chemotactic index was determined by dividing the number of migrated cells for each well by the mean number of migrated cells from the wells containing medium only.

Leukocytes were isolated from whole blood as previously described (29) and purified. Eosinophils, in medium containing rIL-5 (30 ng/ml), were placed into the Transwell polycarbonate inserts (5 x 10^6 cells per insert in a 200-µl volume). The Transwells were then incubated at 37°C, and the number of eosinophils that had migrated into each well was counted after 2 h. The chemotactic index was determined by dividing the number of migrated cells for each well by the mean number of migrated cells from the wells containing medium only.

Western blot analysis

Day 12 BAL fluids were cleared of leukocytes by centrifugation, and 1 ml of this fluid was used for Western blot analysis. Equivalent volumes (20 µl) of fluid from individual mice (naive (untreated) or OVA) were separated by SDS-PAGE under reducing conditions. Recombinant protein standards were used as positive controls for the detection of CypA and CypB. Separated proteins were then transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). After blocking in 10% milk, the membranes were stained with Wright-Giemsa to discriminate lymphocytes. The chemotactic index was determined by dividing the number of migrated cells for each well by the mean number of migrated cells from the wells containing medium only.

Analysis of CD44 expression on leukocytes

CD44 expression levels were assayed by FACS analysis on peripheral blood and lung tissue-derived CCR3+ granulocytes (eosinophils) and CD44+ T cells from OVA mice on day 12 of the OVA regimen, as well as on naive and Con A-activated CD44+ T cells purified using MACS beads. In some experiments, CD44 expression was examined on CCR3+ granulocytes (eosinophils) from IL-5 Tg mice. Cells were stained with 1.25 µg/ml anti-mouse CD44 or isotype control mAb, followed by FITC-conjugated anti-rat IgG secondary for detection. Alexa Fluor 647-conjugated anti-mouse CCR3 or CyChrome-conjugated anti-mouse CD44 was added after the second step. Based on the two-parameter distribution of FITC expression on CD44+ and CCR3+ leukocytes, we confirmed that cross-reactivity between anti-CD44/CCR3 and FITC-conjugated anti-rat IgG was not an issue.

Statistical analysis

Statistical significance was established by Student’s t test using Prism software.

Results

Establishing the model of asthma-mediated lung inflammation

Our initial studies were designed to confirm that our mouse model of asthma-mediated lung inflammation demonstrated the characteristic pathology associated with allergic asthma. As shown in Fig. 1A, differential analysis of cytospins generated from day 12 BAL fluid obtained from each group of mice demonstrated a >300-fold increase in the number of eosinophils and a >10-fold increase in the number of lymphocytes in the OVA/alum-primed (OVA) group, compared with that of the alum-primed (control) group and naive mice. We observed very few to no neutrophils in any of the groups. FACS analysis of cells from disaggregated lungs harvested on day 12 from each group of animals revealed a 10- to 13-fold increase in the number of CCR3+ granulocytes (eosinophils) in OVA mice compared with control or naive mice (Fig. 1B). Whereas CCR3 is known to be expressed on mast cells, Th2 cells, and basophils in humans, CCR3 expression is restricted to eosinophils in mice (33). There was also a 9- to 12-fold increase in the number of CD4+CD62L- lymphocytes in OVA mice (Fig. 1B), which are defined as effector/memory CD4+ T cell populations. To establish the profile of cytokines generated in this model, pooled lung cells collected on day 12 were restimulated in culture for 4 days with OVA protein to induce cytokine secretion by OVA-specific T cells. Fig. 1D shows that the Th2 cytokines IL-5 and IL-13 were highly elevated in supernatants from OVA mice compared with control mice. IL-4 was low and the Th1 cytokine IFN-γ was undetectable in both groups (data not shown). Our asthma protocol also induced airway dysfunction, as measured by bronchial hyperreactivity to methacholine challenge (Fig. 1C). Although changes in Penh were not a direct measure of changes in classical AHR, Penh measurements in BALB/c mice have been demonstrated to correlate closely with changes in lung resistance, a parameter used to characterize lung mechanical function/dysfunction as well as classical AHR (34). Thus, our current findings of an increase in Penh ratios in OVA mice provide evidence of airway dysfunction that is correlative with the induction of AHR. Taken together, these initial data indicate that our OVA regimen generates a potent asthma-like response, as indicated by an influx of eosinophils and effector/memory CD44+ T lymphocytes (including Ag-specific, cytokine (IL-5 and IL-13)-secreting CD44+ Th2 cells) into lung tissues and airways, and bronchial hyperreactivity to methacholine challenge.

ECL chemiluminescence detection system (Amersham Biosciences), and images were taken on film.

Enzyme-linked immunosorbent assay (ELISA)
Extracellular cyclophilins are elevated in the airways of OVA mice

In preparation for our studies investigating the contribution of cyclophilin-CD147 interactions during asthmatic inflammation, we next established whether extracellular CypA and CypB were elevated in the airways of OVA mice. Thus, Western blot analysis was conducted on BAL fluid from OVA vs naive (untreated) mice. As shown in Fig. 2A, high levels of CypA and CypB were readily detected in the fluid of individual OVA mice compared with untreated animals. By densitometric analysis, both increases were statistically significant (Fig. 2B). A comparison of cyclophilin levels in BAL fluid from alum-primed (control) mice vs untreated mice showed that levels were low in both types of control groups (Fig. 2C).

Mouse eosinophils and CD4\(^+\) T cells express cell surface CD147

We next examined whether CD147, the principal signaling receptor for extracellular CypA and CypB, was expressed on mouse effector leukocytes associated with asthmatic inflammation, specifically eosinophils and CD4\(^+\) T cells. We initially focused on peripheral leukocytes, because these are the cells most likely to be recruited out of the circulation and into inflamed lung tissues and airways by extracellular cyclophilins. Fig. 3A indicates that both circulating eosinophils and CD4\(^+\) T cells express CD147. Interestingly, we observed that this expression of CD147 was decreased on cell subsets isolated from lung tissues of the same mice (Fig. 3B). This decrease was small on eosinophils, but pronounced on CD4\(^+\) T cells.

Anti-CD147 mAb treatment reduces numbers of eosinophils and effector/memory CD4\(^+\) T cells, as well as Ag-specific Th2 cytokine secretion, in lung tissues

Having established the presence of elevated extracellular cyclophilins during asthma-mediated lung inflammation and the presence of CD147 signaling receptor on circulating eosinophils and CD4\(^+\) T cells, we next addressed the possibility that these cyclophilins play a role in leukocyte recruitment, via interaction with CD147, during in vivo tissue inflammation. Thus, we examined whether targeting CD147 in vivo, using anti-CD147 mAb, might block the chemotraction of eosinophils and/or CD4\(^+\) T cells into inflamed lung tissues. Fig. 4 shows that anti-CD147 treatment resulted in a significant decrease (46%) in the number of eosinophils in lung tissues of OVA mice relative to the isotype control group. Anti-CD147 treatment also resulted in a significant decrease (53%) in the number of effector/memory CD4\(^+\) T cells in lung tissues of OVA mice.

Our finding that anti-CD147 treatment led to a major decrease in the number of effector/memory CD4\(^+\) T cells accumulating in lung tissues led us to examine how this might impact on Th2 cytokine secretion by Ag (OVA)-specific T cells in lung tissues. For these studies, pooled lung cells were restimulated in vitro with OVA Ag to induce OVA-specific T cells to secrete cytokines. Fig. 5 shows levels of Th2 cytokines (IL-5 and IL-13) detected in cultures from OVA-treated mice. As shown in Fig. 5, the secretion of IL-5 and IL-13 was significantly reduced in cultures from anti-CD147-treated mice compared with isotype control-treated mice. These results suggest that anti-CD147 treatment may be a potential therapeutic strategy for the treatment of asthma.
OVA mice treated with anti-CD147 or isotype control mAb. Both IL-5 and IL-13 were significantly decreased (by 29 and 40%, respectively) in mice treated with anti-CD147. Such findings suggest that anti-CD147 treatment is reducing the frequency of OVA-specific CD4^+ Th2 effector cells being recruited into lung tissues. Although an alternative explanation is that anti-CD147 could be impacting on the activation status of CD4^+ T cells, as reported by others (35) for anti-human CD147 (specifically mAb MEM-M6/6), we have established in separate studies that our anti-mouse CD147 clone (RL73.2) has no direct inhibitory activity on mouse CD4^+ T cell activation (our unpublished observations). Taken together, these in vivo data suggest that treatment of OVA mice with anti-CD147 mAb results in decreased accumulation of eosinophils and effector/memory CD4^+ T cells, as well as decreased Ag-specific Th2 cytokine secretion, in inflamed lung tissues.

Anti-CD147 mAb treatment reduces airway epithelial mucin production and bronchial hyperreactivity

We next investigated whether the reduction in eosinophils, effector/memory CD4^+ T cells, and Th2 cytokines in lung tissues might be impacting on functional parameters of allergic asthma, specifically airway epithelial mucin production and bronchial hyperreactivity. Fig. 6A shows that there was a significant reduction (39%) in the percentage of airways staining positive with PAS, which is a histochemical marker for goblet cells that identifies mucin (the major glycoprotein constituent of mucus), in lung sections from OVA mice treated with anti-CD147 compared with the isotype control group. In addition, we observed that bronchial hyperreactivity was significantly decreased in mice treated with anti-CD147 mAb, relative to isotype control, upon aerosolized challenge with methacholine (Fig. 6B).

Cyclophilins induce CD147-dependent chemotaxis of activated mouse CD4^+ T cells

Based on previous studies demonstrating the capacity of extracellular cyclophilins to induce leukocyte chemotaxis (10, 15–17), we postulated as a potential mechanism that anti-CD147 treatment...
was likely inhibiting the cyclophilin-mediated recruitment of eosinophils and/or CD4+ T cells into inflamed lung tissues. To provide support for this possibility, we investigated whether cyclophilins could induce the chemotaxis of mouse eosinophils and CD4+ T cells in vitro and, if so, whether the response was dependent on interaction with CD147. Surprisingly, we observed that eosinophils (purified from IL-5 Tg mice on a BALB/c genetic background) were not chemotactic either to CypA or CypB (Fig. 7A). Tg mice were used as a source of eosinophils for these experiments, because the frequency of circulating eosinophils in naïve wild-type mice is very low (29). However, we obtained similar results using either circulating (nontransgenic) eosinophils from OVA mice or eosinophils from IL-5 Tg mice on a C57BL/6 background or using modified Boyden chamber instead of Transwell chemotaxis assays. In previous studies, we had shown that binding of CypA or CypB to CD147 induces signaling that culminates in ERK activation and leukocyte chemotaxis (17, 20). In support of the observed lack of migration by eosinophils, stimulation with CypA failed to induce ERK phosphorylation in eosinophils (Fig. 7B) despite the presence of CD147 on their cell surface (Fig. 7C). However, potent ERK phosphorylation was induced in these same eosinophils using eotaxin-1, demonstrating their capacity for ERK-dependent signaling with other chemokines.

In contrast, CypA and CypB were both able to induce the chemotaxis of mouse CD4+ T cells (Fig. 8, A and B). This chemotactic capacity was most potent with activated, compared with naïve, CD4+ T cells and was comparable to the migration observed in response to RANTES (Fig. 8A). We also confirmed that the observed cyclophilin-mediated chemotaxis of CD4+ T cells was dependent upon interaction with CD147, because anti-CD147, but not isotype control mAb, significantly blocked the observed migration (Fig. 8B). Furthermore, activated CD4+ T cells showed marked ERK phosphorylation in response to CypA (Fig. 8C), supporting our observations of potent cyclophilin-mediated chemotaxis in this subset of cells (Fig. 8B). To establish a potential mechanism for the observed enhanced migratory capacity of activated vs naïve CD4+ T cells, CD147 expression was compared on the two subsets of cells. As shown in Fig. 8D, the expression of CD147 was strongly up-regulated on the activated subset, suggesting a likely increased capacity to respond to cyclophilin-mediated signaling. Taken together, these findings suggest that mouse CD4+ T cells (notably activated CD4+ T cells), but not eosinophils, are induced to migrate directly in response to extracellular cyclophilins and that this response is dependent upon interaction with cell surface CD147.

**Discussion**

Chemokines have been well documented in the recruitment of leukocytes into lung tissues and airways during asthma-mediated inflammation (3–5). More recently, leukocytes have also been shown to undergo chemotaxis in vitro in response to another class of chemokine-like proteins, extracellular cyclophilins, via a mechanism that is dependent on interaction with cell surface CD147 (21). Elevated levels of cyclophilins have been reported in inflamed tissues in several inflammatory diseases and thus may recruit CD147-bearing cells into these sites from the periphery (6, 21). In the current study, we are the first to report of a role for extracellular cyclophilins, via interaction with CD147, in asthma-mediated lung inflammation.

Using a mouse (OVA) model of asthmatic lung inflammation, characterized by an influx of effector cells (eosinophils and Th2 lymphocytes), we initially showed that CypA and CypB are both elevated in BAL fluid from the airways of these mice. Elevated...
levels of chemokines, such as eotaxin-1 and RANTES, have similarly been reported in BAL fluid of mice with induced asthma (36, 37). We also found that circulating eosinophils and CD4 T cells express CD147, the ligand for cyclophilins, on their surface. Interestingly, we observed a marked decrease in CD147 expression on lung tissue-derived CD4 T cells, compared with circulating cells, supporting the idea that CD147 may be most critical for the recruitment phase of these cells into tissues during inflammatory processes. Similar findings of a decrease in expression on tissue vs circulating leukocytes have been reported for other chemokine receptors (38). Although it might be argued that a decrease in chemokine receptor expression could be due to interference by bound chemokine, in vitro studies in which leukocytes were incubated with or without CypA before anti-CD147 staining demonstrated equivalent CD147 expression (our unpublished observations). Such findings suggest that the decreases in CD147 expression observed on tissue-derived leukocytes are most likely not due to interference of anti-CD147 binding by prebound cyclophilins. Intriguingly, several studies have reported increased expression of CD147 on inflammatory cells within tissues, notably synovial tissue of rheumatoid arthritis patients (24 –26). Such variable findings suggest that CD147 expression might be regulated differentially on distinct leukocyte subsets and/or within different tissue sites. We are investigating this possibility as part of ongoing studies.

Our major interest for the current studies was to establish the contribution of cyclophilin-CD147 interactions to eosinophil and CD4 T cell recruitment during in vivo asthma-mediated lung inflammation. Specifically, anti-CD147 mAb intervention was...
used to disrupt these interactions. We had previously published the feasibility of targeting cyclophilin-CD147 interactions using anti-CD147 intervention to reduce the accumulation of neutrophils in a mouse model of acute lung inflammation (27). Other groups have described similar strategies to reduce lung tissue and airway eosinophilic inflammation in OVA mice by targeting chemokine receptors, such as CCR3, using anti-CCR3 mAb (39). Our current in vivo blocking studies showed that systemic administration of anti-CD147 mAb resulted in a significant (up to 50%) reduction in the total numbers of eosinophils and effector/memory CD4+ T cells accumulating in lung tissues of asthmatic mice. In preliminary studies working with anti-CD147 mAb, we had previously established that the cross-linking of bound Ab had no impact either on leukocyte survival or on leukocyte activation (our unpublished observations), suggesting the most likely effect mediated by anti-CD147 mAb would be the structural interference of CD147 with exogenous ligands, including extracellular cyclophilins. Based on our previous findings with mouse neutrophils (27), our initial interpretation for the current findings was that anti-CD147 treatment was likely impacting on the cyclophilin-mediated chemotactic recruitment of eosinophils and effector/memory CD4+ T cells into asthmatic lung tissues. Therefore, to establish the capacity of extracellular cyclophilins to induce migration of these two leukocyte subsets, we conducted in vitro chemotaxis assays. To our surprise, mouse eosinophils failed to chemotax either to CypA or to CypB. Indeed, similar results have recently been observed in a study by Lee and coworkers (40) comparing the capacity of small molecules to induce mouse eosinophil migration in vitro. Although one group has reported cyclophilin-mediated chemotaxis of eosinophils in vitro (15), it should be noted that the study was conducted using eosinophils differentiated from a human leukemia cell line rather than using primary circulating eosinophils. The different outcome for their study may reflect the unusual migratory properties of many cultured cell lines, relative to primary eosinophils. It is intriguing that, despite the presence of CD147 on their cell surface, eosinophils failed to demonstrate cyclophilin-induced ERK activation, correlating with their inability to migrate in response to cyclophilins. The same finding was observed whether the eosinophils were obtained from peripheral blood of IL-5 Tg mice (Fig. 7A) or from peripheral blood or lung tissue of OVA-primed mice (data not shown), suggesting this lack of response might be a general property of primary eosinophils. It should be noted that the presence/absence of exogenous IL-5 had no impact on the outcome of these assays, ruling out the possibility that the priming status of the eosinophils might be responsible for the observed failure to respond to cyclophilins. Although we do not have an explanation for why some CD147-expressing leukocytes, and not others, can respond to cyclophilin-induced signaling events, it is possible that some leukocyte subsets lack the necessary intracellular adapter molecules or cell surface coreceptors to efficiently signal and/or bind via CD147. Other than ERK-associated events, little is known of the CD147 signaling pathway, making it difficult to suggest which adapter proteins might differ between cell subsets. In the case of cell surface coreceptors, heparan sulfates are known to be necessary for optimal binding of extracellular cyclophilins to CD147 (16, 20, 41). It is possible that primary eosinophils lack the necessary abundance and/or profile of cell surface heparans for interaction with cyclophilins, thereby preventing their interaction with CD147. This possibility is currently under investigation in our laboratory. Another possibility that we are examining is whether cyclophilins might be failing to induce clustering/multimerization of CD147 molecules on eosinophils. For example, studies using human CD4+ T cells have shown that PHA activation not only induces the up-regulation of CD147 molecules (19), but also their clustering (35). Signaling via clustered CD147 may be necessary to provide an optimal threshold of ERK activation for chemotactic function. Such a model fits well with our current observations that activated CD4+ T cells (unlike naive T cells) expressed elevated CD147, demonstrated potent cyclophilin-mediated ERK phosphorylation, and migrated well in response to extracellular cyclophilins.

Our observations that cyclophilins do not induce eosinophil migration directly suggest that the reduction in eosinophilia observed in mice treated with anti-CD147 could be due to an indirect effect. As part of our studies, we examined changes in Th2-derived cytokines from Ag-specific effector T cells isolated from lung tissues. Both IL-5 and IL-13 production were decreased in anti-CD147-treated mice, suggesting that the frequency of OVA-specific Th2 cells might be reduced in lung tissues. Interestingly, several studies suggest that IL-5 and IL-13 are critical regulators of pulmonary eosinophilia in allergen-challenged mice (42–44). Furthermore, IL-13 has been shown to directly induce the migration of human eosinophils in vitro (45). Thus, the reduction in eosinophil numbers observed in our mice treated with anti-CD147 could be explained by a decrease in Th2-derived cytokines. We also observed a significant reduction in airway epithelial mucin production and bronchial hyperreactivity to methacholine challenge in anti-CD147-treated mice. Although the regulation of airway mucus hypersecretion and hyperresponsiveness (AHR) has been extensively studied, the relative importance of different regulatory factors to each of these pathological parameters is still in debate (1). For example, whereas some studies have reported a decrease in AHR when eosinophilia is reduced or abolished, others have reported no decrease (46). Similarly, the relative contribution of IL-5 and IL-13 cytokines to these parameters remains to be definitively established (31, 47–54), although there is a strong consensus that IL-13 plays a major role in regulating both mucus hypersecretion and AHR (55). Thus, the observed decrease in IL-13, as well as in IL-5 and/or eosinophilia, might be contributing factors for the reduction in airway epithelial mucin and bronchial hyperreactivity to methacholine challenge observed in anti-CD147-treated mice. Based on our findings, we propose the following model. In vivo treatment with anti-CD147 mAb blocks cyclophilin-induced recruitment of activated Th2 effector cells into inflamed lung tissues of asthmatic mice. Due to a decrease in Th2 effector cells, there is a concurrent decrease in the local production of IL-5 and IL-13, which in turn affects the recruitment of eosinophils (which are not chemotactic to cyclophilins) into these tissues and/or their maturation. Decreases in Th2 cytokines, as well as in eosinophilia, contribute to the observed reduction in the frequency of mucin-positive airways as well as bronchial hyperreactivity. Thus, the net effect after anti-CD147 mAb intervention is an overall decrease in several different parameters of asthma-mediated lung inflammation and associated pathology.

Because anti-CD147 mAb intervention resulted in only a partial inhibition of leukocyte accumulation in lung tissues, future studies will address potential synergisms between cyclophilins and other chemokines. It should be noted that, although the results from our current studies fit with a model whereby targeting CD147 impacts on the chemotactic properties of leukocytes, we cannot definitively conclude that this is the mechanism of action for the observed reduction in leukocyte numbers during in vivo anti-CD147 intervention. Indeed, CD147 has been shown to participate in several different physiological interactions (21), some of which might be relevant to inflammatory responses. For example, CD147 has been described to function as an integrin (56) and also as a regulator of lactate transporters (57). Whether either of these CD147-mediated functions contributes to our model of lung inflammation has not
been established, although indirect evidence from several studies supports the conclusion that any interference by anti-CD147 mAb on either of these alternative functions is unlikely to have a significant impact on leukocyte recruitment into tissues. For example, in vitro studies in which CD147 was blocked using anti-CD147 mAb demonstrated no inhibition of integrin function by leukocytes migrating in response to different chemokines (16, 20, 27), suggesting little impact on general leukocyte adhesion by anti-CD147 intervention. This is supported by an in vivo study showing that, although anti-CD147 treatment led to impaired erythrocyte recirculation, the same treatment did not affect the circulation and extravasation of leukocytes (56), suggesting normal integrin function on leukocytes. In the case of CD147 as a regulator of lactate transporters, its major function was shown to be as intracellular chaperone for cell surface expression of the metabolic transporters, MCT1 and MCT4 (57). Although both the transmembrane and cytoplasmic domains of CD147 were found to be necessary for interaction with these transporter proteins, no disruption of cell metabolism was observed when the extracellular domain of CD147 was targeted (57, 58). Based on these findings, it is unlikely that extracellular anti-CD147 mAb will have a significant impact on the regulation of lactate transporters.

In conclusion, our current findings demonstrate that anti-CD147 intervention significantly reduces several parameters of asthma-mediated lung inflammation. Based on our in vitro chemotaxis assays, one potential explanation is that anti-CD147 mAb inhibits cyclophilin-CD147 interactions on specific leukocyte subsets, notably activated CD4⁺ T cells. Although such an explanation remains to be definitely proven in vivo, it provides the most likely model for our results at this time. Regardless of the exact mechanism, it is clear that anti-CD147 mAb intervention reduces leukocyte accumulation in inflamed lungs, thus, we propose that targeting of cyclophilin-CD147 interactions using anti-CD147 mAb represents a potentially novel therapy for the treatment of allergic asthma.

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Disclosures

The authors have no financial conflict of interest.

References


