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A CCL24-dependent Pathway Augments Eosinophilic Airway Inflammation in House Dust Mite-challenged *Cd163*$^{-/-}$ Mice

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Abstract

CD163 is a macrophage scavenger receptor with anti-inflammatory and pro-inflammatory functions. Here, we report that alveolar macrophages (AMΦs) from asthmatic subjects had reduced cell surface expression of CD163, which suggested that CD163 might modulate the pathogenesis of asthma. Consistent with this, house dust mite (HDM)-challenged *Cd163*$^{-/-}$ mice displayed increases in airway eosinophils and mucous cell metaplasia (MCM). The increased airway eosinophils and MCM in HDM-challenged *Cd163*$^{-/-}$ mice were mediated by augmented CCL24 production and could be reversed by administration of a neutralizing anti-CCL24 antibody. A proteomic analysis identified the calcium-dependent binding of CD163 to *Dermatophagoides ptyeronyssinus* peptidase 1 (Der p1). Der p1-challenged *Cd163*$^{-/-}$ mice had the same phenotype as HDM-challenged *Cd163*$^{-/-}$ mice with increases in airway eosinophils, MCM and CCL24 production, while Der p1 induced CCL24 secretion by bone marrow-derived macrophages (BMMΦs) from *Cd163*$^{-/-}$ mice, but not BMMΦs from WT mice. Lastly, airway eosinophils and bronchoalveolar lavage fluid CCL24 levels were increased in Der p1-challenged WT mice that received adoptively transferred AMΦ's from *Cd163*$^{-/-}$ mice. Thus, we have identified CD163 as a macrophage receptor that binds Der p1. Furthermore, we have shown that HDM-challenged *Cd163*$^{-/-}$ mice have increased eosinophilic airway inflammation and MCM that are mediated by a CCL24-dependent mechanism.

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Keywords

CD163; house dust mite; asthma; eosinophils; CCL24; Der p1

Introduction

Macrophages, which play key roles in microbial defense, neoplasia, autoimmunity, fibrosis and wound healing, have increasingly been recognized to participate in the pathogenesis of asthma[1, 2]. For example, the M(IL-4) subset of alternatively activated macrophages (AAMs) produce C–C chemokines, such as CCL17 (TARC) and CCL24 (eotaxin-2), which mediate the recruitment of Th2 cells and eosinophils, respectively[3]. CD163 is a scavenger receptor superfamily member that is exclusively expressed by cells of the monocyte/macrophage lineage[1, 4–6]. CD163 functions as a high-affinity receptor that mediates the calcium-dependent endocytosis of haptoglobin-hemoglobin (Hp-Hb) complexes, which clears free hemoglobin during intravascular hemolysis[7]. CD163 also serves as a receptor for erythroblasts, TNFSF12, bacteria and viruses[8–11].

CD163 has been associated with both anti-inflammatory and pro-inflammatory functions of macrophages and monocytes[6]. Expression of CD163 on human monocytes is increased by glucocortiocoids and IL-10, which suggested a role for CD163 in modulating macrophage function[6, 12, 13]. For example, binding of Hp-Hb complexes to human monocytes expressing CD163 increased IL-10 secretion that induced heme oxygenase-1 protein synthesis via an autocrine mechanism, thereby providing anti-inflammatory and anti-oxidant effects[6, 14]. In contrast, binding of bacteria by CD163 promoted TNF production, while activation of CD163 by crosslinking with CD163-specific monoclonal antibodies induced the secretion of TNF, IL- 1β, IL-6 and nitric oxide[6, 9, 15]. In addition, $CD163^+$ macrophages that are induced by iron overloading have an unrestrained pro-inflammatory M1 activation profile and mediated persistent inflammation, tissue breakdown and impaired wound healing[16]. However, it is not known whether macrophages that express CD163 regulate the pathogenesis of asthma. Here, we show that CD163 serves as a receptor that binds the house dust mite (HDM) peptidase, Der p1, in a calcium-dependent fashion and that HDM-challenged *Cd163*$^{-/-}$ mice have CCL24-dependent increases in eosinophilic airway inflammation and mucous cell metaplasia (MCM).

Results

Alveolar Macrophages (AMΦs) from Asthmatic Patients Have Reduced Cell Surface Expression of CD163

First, we assessed whether CD163 expression by AMΦs is modified in human subjects with asthma (Table 1). Confocal microscopy demonstrated that human $CD68^+$ AMΦs in BALF express CD163 (Figure 1A), while cell surface expression of CD163 by BALF AMΦs from asthmatic subjects was significantly decreased as compared to normal individuals (Figures 1B – D). This suggested that the reduced cell surface expression of CD163 by BALF AMΦs might modify the pathogenesis of asthma.

Eosinophilic Airway Inflammation and Mucous Cell Metaplasia are Augmented in House Dust Mite-challenged *Cd163*$^{-/-}$ Mice

Cd163$^{-/-}$ mice were created from embryos that contained a targeted partial deletion of exon 1 of the *Cd163* gene, which encodes the 5′ untranslated region and the initial 15 amino acids of the signal peptide, and a total deletion of exons 2 through 16. Exon 17, which encodes the 3′ UTR, was not located within the targeted deletion. *Cd163*$^{-/-}$ mice appeared normal with no obvious developmental abnormalities. PCR genotyping confirmed the deletion of intron 15 – 16 of the *Cd163* gene in DNA isolated from *Cd163*$^{-/-}$ mice, whereas mRNA corresponding to exons 6 and 7 of *CD163* was not detected in the lungs of *Cd163*$^{-/-}$ mice by qRT-PCR (Figure 2A & 2B).

We then investigated whether airway inflammatory responses were modified in *Cd163*$^{-/-}$ mice that had been sensitized and challenged by intranasal HDM administration (Figure 2C). There was a significant increase in total bronchoalveolar lavage fluid (BALF) inflammatory cells in HDM-challenged *Cd163*$^{-/-}$ mice, which reflected a selective increase in eosinophils, whereas the number of macrophages, lymphocytes and neutrophils were not modified (Figure 2D & 2E). Lung histology similarly revealed an increase in peri-bronchial inflammatory cell infiltrates in HDM-challenged *Cd163*$^{-/-}$ mice as compared to wild type (WT) mice (Figure 2F). Serum levels of HDM-specific IgE were not significantly different in HDM-challenged *Cd163*$^{-/-}$ mice as compared to WT mice, which showed that CD163 does not modify allergic sensitization to HDM (Figure 2G). Airway remodeling was significantly increased in HDM-challenged *Cd163*$^{-/-}$ mice based upon an increase in the percentage of airways with MCM (Figure 2F and 2H) and by an increase in lung Muc5AC protein levels (Figure 2I & 2J). Lastly, neither HDM-challenged *Cd163*$^{-/-}$ mice nor WT mice developed methacholine-induced increases in airway hyperreactivity (AHR) in this model, which likely reflects the C57BL/6 genetic background[17]. Collectively, these results demonstrate that *Cd163*$^{-/-}$ mice have a phenotype of increased eosinophilic airway inflammation and MCM in experimental murine HDM-induced asthma.

We next assessed whether the generation of AAMs were modified in HDM-challenged *Cd163*$^{-/-}$ mice. As shown in Figure 2K, BALF levels of the AAM product, Chi3L3/4, were increased in HDM-challenged *Cd163*$^{-/-}$ mice[3]. We next used an intraperitoneal sensitization and intranasal challenge model of HDM-induced airway disease (Figures 3A), which again demonstrated that HDM-challenged *Cd163*$^{-/-}$ mice had an increase in BALF eosinophils as compared to WT mice (Figure 3B). Although the percentage of CD206^{+} AAMs was not significantly different between HDM-challenged *Cd163*$^{-/-}$ mice and WT mice (Figure 3C), the mean fluorescence intensity of CD206 expression on BALF AAMs was significantly increased (Figure 3D).

Eosinophilic Airway Inflammation is Augmented in HDM-challenged *Cd163*$^{-/-}$ mice by a CCL24-dependent Mechanism

Experiments were next performed using the intranasal sensitization and challenge model (Figure 2C) to identify the mechanism by which the phenotype of HDM-induced eosinophilic airway inflammation and MCM are augmented in *Cd163*$^{-/-}$ mice. There was no difference between HDM-challenged WT and *Cd163*$^{-/-}$ mice in the quantity of Th2 (IL-5

and IL-13), Th1 (IFN-γ) or Th17 (IL-17A) cytokines present in lung homogenates (Figure 4A) or secreted by mediastinal lymph node (MLN) cell cultures following *ex vivo* re-stimulation with HDM (Figure 4B). We next assessed whether CD163 modulated the production of chemokines, such as CCL11 (eotaxin- 1) and CCL24 (eotaxin-2), that mediate the chemotaxis of eosinophils to the lung[18]. As shown in Figure 4C, CCL24 protein levels were significantly increased in BALF and lungs of HDM-challenged *Cd163*$^{-/-}$ mice, whereas the levels of CCL11, CCL17 and CCL22 were either unchanged or decreased. These experiments suggest that the increase in eosinophilic lung inflammation in HDM-challenged *Cd163*$^{-/-}$ mice might be mediated by an increase in CCL24 production.

To address whether the increases in eosinophilic lung inflammation in HDM-challenged *Cd163*$^{-/-}$ mice were mediated via a CCL24-dependent mechanism, mice were treated with a neutralizing anti-CCL24 antibody (Figure 5A). HDM-challenged *Cd163*$^{-/-}$ mice that received site-directed delivery of the neutralizing anti-CCL24 antibody to the lung had significant reductions in the number of BALF eosinophils, MCM and peri-bronchial inflammatory cell infiltrates as compared to *Cd163*$^{-/-}$ mice that received a control antibody (Figures 5B – 5D). Since CD163 is expressed by AMΦs in the lung, we assessed whether CD163 attenuates HDM-mediated CCL24 release from AMΦs. AMΦs were isolated from HDM-challenged WT and *Cd163*$^{-/-}$ mice and re-stimulated with HDM for 24 hours. As shown in Figure 5E, *ex vivo* restimulation with HDM significantly increased the amount of CCL24 secreted by *Cd163*$^{-/-}$ AMΦs as compared to WT AMΦs.

We next utilized two adoptive transfer models to assess whether dendritic cells (DCs) could possibly represent the relevant cell type mediating the increases in BALF eosinophils in HDM-challenged *Cd163*$^{-/-}$ mice. As shown in Figure 6A – D, there was no difference in BALF total cells, eosinophils or CCL24 levels in HDM-challenged WT recipient mice that had received the adoptive transfer of HDM-pulsed bone marrow-derived DCs (BMDCs) from donor WT or *Cd163*$^{-/-}$ mice. Similarly, there was no difference in BALF total cells, eosinophils or CCL24 levels in HDM-challenged WT recipient mice that received the adoptive transfer of CD11c^{+} DCs isolated from MLNs from WT or *Cd163*$^{-/-}$ donor mice that had been pulsed *in vivo* with HDM (Figure 6E – 6H). Thus, CD163 expression by DCs did not appear to mediate the increases in BALF eosinophils or CCL24 levels in HDM-challenged *Cd163*$^{-/-}$ mice.

We also assessed whether the increased eosinophilic airway inflammation and MCM in the HDM-challenged *Cd163*$^{-/-}$ mice might be mediated at the level of effector CD4^{+} T cells. To address this question, splenic CD4^{+} T cells from donor WT mice that had been sensitized to HDM by intraperitoneal injection were isolated and adoptively transferred to recipient WT and *Cd163*$^{-/-}$ mice (Figure 7A). As shown in Figure 7B and 7C, *Cd163*$^{-/-}$ recipients of WT effector CD4^{+} splenic T cells showed increases in BAL eosinophils and MCM in response to multiple HDM challenges as compared to WT recipients of CD4^{+} T cells from WT mice. Therefore, the adoptive transfer of WT effector CD4^{+} T cells did not modify the phenotype of increased eosinophilic airway inflammation and MCM in HDM-challenged *Cd163*$^{-/-}$ mice.

Calcium-dependent binding of *Dermatophagoides pteronyssinus* peptidase 1 (Der p1) to CD163

Next, we hypothesized that CD163 might serve as a receptor for a HDM protein, which would be consistent with its function as a scavenger receptor[6–9, 11]. Immobilized recombinant human CD163 (rhCD163) was used to pull-down interacting proteins in *D. pteronyssinus* extracts, which were resolved by one-dimensional gel electrophoresis, visualized by silver staining and identified by mass spectroscopy. As shown in Figure 8A, 8 distinct bands were pulled-down when *D. pteronyssinus* extract proteins were incubated with immobilized rhCD163. In two independent experiments, band #6 contained a 17 amino acid peptide (GIEYIQHNGVVQESYYR) that corresponded to amino acids 177 – 193 of Der p1, a cysteine protease that is one of the major allergens in *D. pteronyssinus* [19]. Der p1 was the only *D. pteronyssinus* protein that was identified. Furthermore, band #6 displayed a molecular weight of approximately 23 kDa, which is consistent with the molecular weight of Der p1.

Additional experiments were performed to confirm and characterize the ability of CD163 to function as a Der p1-binding protein. First, immobilized rhCD163 protein pulled-down purified Der p1 protein (Figure 8B), while immobilized Der p1 pulled down rhCD163 (Figure 8C). Nominal binding occurred when the resin that had been blocked with ovalbumin alone was used for the pull-down experiments. Second, Der p1 protein that had been immobilized to plastic bound rhCD163 in a concentration-dependent and calcium-dependent fashion (Figure 8D), which was significantly attenuated by EGTA (Figure 8E). Third, immobilized Der p1 protein pulled down CD163 from a lysate of human monocyte proteins (Figure 8F) that had the same molecular weight as native CD163. Furthermore, the amount of CD163 in human monocyte lysates that was pulled-down by immobilized Der p1 protein was increased by the addition of supplemental calcium and attenuated by EGTA (Figure 8G). This is consistent with the requirement of calcium for binding of hemoglobin-haptoglobin complexes to CD163[20]. Collectively, these results demonstrate that CD163 binds Der p1 in a calcium-dependent fashion.

Experiments were next conducted to assess whether Der p1 modifies CCL24 secretion by bone marrow-derived macrophages (BMMΦs) from WT and *Cd163*$^{-/-}$ mice [21]. As shown in Figure 8H, purified Der p1 induced CCL24 secretion from BMMΦs from *Cd163*$^{-/-}$ mice, but not those from WT mice. This finding shows that CD163-deficient BMMΦs secrete increased amounts of CCL24 in response to Der p1 stimulation as compared to WT BMMΦs.

Alveolar Macrophages from *Cd163*$^{-/-}$ Mice Enhance Der p1-induced Eosinophilic Airway Inflammation and Mucous Cell Metaplasia

Having shown that CD163 binds Der p1 to suppress CCL24 secretion, we next assessed whether *Cd163*$^{-/-}$ mice that were sensitized and challenged with Der p1 had the same phenotype of increased airway eosinophils, MCM and BALF CCL24 levels as HDM-challenged *Cd163*$^{-/-}$ mice (Figure 9A). As shown in Figure 9B & 9C, Der p1 induced significant increases in total BALF cell numbers, which primarily reflected an increase in eosinophils in *Cd163*$^{-/-}$ mice as compared to WT mice. Similarly, lung histology showed

increased peri-bronchial inflammatory infiltrates and MCM in Der p1-challenged *Cd163*$^{-/-}$ mice (Figure 9C & 9D). BALF levels of CCL24 were also significantly increased in Der p1-challenged *Cd163*$^{-/-}$ mice (Figure 9E). In contrast, there was no difference in the amount of IL-5 and IL-13 produced by MLN cells from *Cd163*$^{-/-}$ and WT mice when re-stimulated with HDM (Figure 9F). Collectively, these results demonstrate that sensitization and challenge with Der p1 induces a similar phenotype of increased eosinophilic airway inflammation, MCM and CCL24 production in *Cd163*$^{-/-}$ mice as was seen with the HDM-challenge model.

Lastly, adoptive transfer experiments were performed to assess whether CD163 expression by AMΦ's modulates Der p1-induced CCL24 production and eosinophilic airway inflammation (Figure 10A). BALF CD45^{+}/CD11c^{+}/Siglec-F^{+}/CD64^{+} AMΦ's from naive donor WT and *Cd163*$^{-/-}$ mice (Figure 10B) were adoptively transferred to recipient WT mice that had been sensitized with Der p1 or saline, as a control. As shown in Figure 10C – 10D, both BALF eosinophils and CCL24 levels were significantly increased in Der p1-challenged recipient WT mice that received the adoptive transfer of *Cd163*$^{-/-}$ AMΦ's as compared to Der p1-challenged recipient WT mice that received the adoptive transfer of WT AMΦ's. Collectively, this provides additional evidence supporting the conclusion that Der p1-induced eosinophilic airway inflammation and BALF CCL24 levels are augmented by *Cd163*$^{-/-}$ AMΦ's.

Discussion

Eosinophils are key effector inflammatory cells in allergen-mediated asthma that are recruited to the airways by cooperative effects between the Th2 cytokine, IL-5, and C–C chemokines[22–24]. IL-5 is a hematopoietic growth factor for eosinophils that both mobilizes eosinophil progenitor cells from the bone marrow to the circulation and increases CCR3 expression[25, 26]. Eotaxins are C–C chemokines that mediate the chemotaxis of circulating eosinophils to the lung via binding to CCR3[18]. The eotaxin gene family is comprised of 3 genes in humans (CCL11, CCL24 and CCL26) and 2 in the mouse (CCL11 and CCL24)[22]. CCL11 (eotaxin-1) was initially identified as the major eosinophil chemotactic factor in allergen-challenged guinea pigs, while CCL24 (eotaxin-2) was cloned based upon sequence homology with known β-chemokines[27, 28]. Expression of both CCL11 and CCL24 are IL-13-dependent with CCL11 expression being restricted to lung tissue cells in the mouse, while CCL24 is primarily expressed by alveolar macrophages[29]. Consistent with this, an ovalbumin (OVA)-induced model of murine airway inflammation has shown that CCL24 has a predominant role over CCL11 in mediating eosinophil recruitment to BALF, whereas CCL11 and CCL24 act in a synergistic fashion to recruit eosinophils to lung tissue. Furthermore, CCL24 plays an important role in mediating IL-13-induced airway eosinophilia.

Here, we identify a new mechanism by which CD163 expression by alveolar macrophages may attenuate the severity of eosinophilic inflammation in HDM-induced airway disease. First, we show that cell surface expression of CD163 by alveolar macrophages is reduced in human subjects with asthma, which suggested that CD163 might participate in the regulation of airway inflammatory responses in the lung. The reduction in cell surface expression of

CD163 by alveolar macrophages occurred despite 71% of asthmatic subjects having received treatment with inhaled steroids, which are known to increase CD163 expression[12]. This is consistent with a recent report that found sputum macrophages from asthmatic subjects expressed less CD163 than macrophages from healthy subjects[30]. Next, we identified and characterized the function of CD163 in HDM-challenged *Cd163*$^{-/-}$ mice. House dust mites are a common environmental trigger and a major risk factor for the development of allergic asthma[31]. In particular, we used *D. pteronyssinus*, which is one of the two predominant species of house dust mite[32], and found that *Cd163*$^{-/-}$ mice have a phenotype of augmented HDM-induced eosinophilic pulmonary inflammation and MCM.

We then assessed the mechanisms mediating the augmented airway eosinophilia in HDM-challenged *Cd163*$^{-/-}$ mice. We did not find evidence that the augmented airway eosinophilia in HDM-challenged *Cd163*$^{-/-}$ mice occurred as a consequence of enhanced allergic sensitization or production of Th2 cytokines. Instead, the augmented eosinophilic airway inflammation appeared to be mediated by the increased production of CCL24, which is a C–C chemokine that induces the chemotaxis of eosinophils to the lung. In particular, HDM-challenged *Cd163*$^{-/-}$ mice had significantly increased CCL24 in both BALF and the lung, whereas neither CCL11, CCL17 nor CCL22 were increased. Furthermore, *Cd163*$^{-/-}$ AMΦs that were re-stimulated *ex vivo* with HDM secreted increased amounts of CCL24 as compared to WT AMΦs. This finding suggested that the augmented HDM-induced airway eosinophilia and MCM in *Cd163*$^{-/-}$ mice occurred by a CCL24-dependent mechanism. This was confirmed by administration of a neutralizing anti-CCL24 antibody to HDM-challenged *Cd163*$^{-/-}$ mice, which significantly reduced eosinophilic airway inflammation and MCM to levels similar to those found in HDM-challenged WT mice. Administration of the neutralizing anti-CCL24 antibody to WT mice, however, did not reduce either HDM-induced BAL eosinophils or MCM, which suggested that maximal responses in CD163-sufficient WT mice were not CCL24-dependent.

Our results also showed that the generation of AAMs in the setting of HDM-induced airway disease is not modified in the absence of CD163. There was, however, an increase in cell surface expression of the AAM marker, CD206 (Mrc1, mannose receptor), on cells from HDM-challenged *Cd163*$^{-/-}$ mice, as well as an increase in BALF levels of Chi3L3/4 (Ym1/2), which is a product of AAMs[3]. The importance of AAMs in the pathogenesis of HDM-induced airway inflammation, however, is unclear as HDM-challenged conditional knockout mice that lack the IL-4 receptor α chain on LysM^{+} macrophages do not have reductions in BALF eosinophil numbers, which suggests that AAMs may only be a marker of Th2 cytokine levels[33].

Next, we investigated the mechanism by which CD163 attenuates CCL24 production by HDM-stimulated alveolar macrophages. We hypothesized that CD163 might serve as a receptor for a HDM protein, which would be consistent with its function as a scavenger receptor[6–9, 11]. HDM is a complex aeroallergen that is comprised of mite-derived proteins and fecal pellets, as well as lipopolysaccharide and fungal spores[34, 35]. At least nineteen *D. pteronyssinus* proteins have been characterized as allergens that are bound by IgE. We utilized immobilized recombinant human CD163 to identify Der p1 as a binding partner by mass spectroscopy. The ability of CD163 to bind Der p1 was confirmed by *in vitro* pull-

down experiments using recombinant and purified proteins, which showed that the interaction was calcium-dependent. This is consistent with the requirement of calcium for binding of hemoglobin-haptoglobin complexes to CD163[20]. Similarly, purified Der p1 was able to pull-down CD163 from human monocyte lysates.

Der p1 is a 25-kDa glycoprotein expressed in the mite gut, which suggests that its endogenous function is related to digestion[36, 37]. Der p1 is a member of the group 1 allergens which are typical cysteine proteases that share structural similarity to papain[32]. Group 1 allergens are released into fecal pellets, where Der p1 is found in high concentrations that range from 100 to 10,000 ng/g of dust[32, 35]. Der p1 is one of the major allergens in *D. ptyeronyssinus* that is targeted by as much as 70% of the circulating IgE in atopic subjects[19, 35]. Furthermore, Der p1 has been shown to promote Th2 responses by selectively reducing IFN-γ production, as well as by mediating the cleavage of multiple cell surface molecules, including the low-affinity IgE Fc receptor (CD23), the IL-2 receptor (CD25) α subunit, DC-SIGN (CD209) and DC-SIGNR (CD299)[38–42]. Der p1 also disrupts intercellular tight junctions in the airway by cleaving the adhesion proteins occludin and claudin-1, which facilitates transepithelial movement of Der p1 to antigen-presenting dendritic cells[43]. In airway epithelial cells, Der p1 induces IL-6 and IL-8 secretion, as well as activates NF-κB, ERK1/2, p38 MAPK and JNK signaling[44, 45]. Lastly, Der p 1 binds mannose receptors on human monocyte-derived dendritic cells, which may facilitate allergen internalization[37]. Here, we show that Der p1 induced CCL24 production by *Cd163*$^{-/-}$ BMMΦs, but not from WT BMMΦs. Furthermore, *Cd163*$^{-/-}$ mice that had been sensitized and challenged with Der p1 had a similar phenotype of increased airway eosinophilia, MCM and CCL24 production as was seen when *Cd163*$^{-/-}$ mice were sensitized and challenged with HDM. In addition, Der p1-challenged WT mice that received the adoptive transfer of *Cd163*$^{-/-}$ AMΦs had significantly increased eosinophilic airway inflammation and BALF CCL24 levels as compared to recipients of WT AMΦs. It is important to point out, however, that we cannot exclude a role for other cells in the lung that have also been reported to produce CCL24, such as neutrophils and airway epithelial cells, in mediating the phenotype of increased eosinophilic airway inflammation in Der p1-challenged *Cd163*$^{-/-}$ mice[46, 47]. Lastly, since CD163 functions as a scavenger receptor with multiple known ligands, we also hypothesized that the phenotype of allergen-challenged *Cd163*$^{-/-}$ mice might not be specific for Der p1 and could possibly be seen when *Cd163*$^{-/-}$ mice are sensitized and challenged with other allergens. Consistent with this, we found that ovalbumin-challenged *Cd163*$^{-/-}$ mice have a similar inflammatory phenotype as HDM-challenged *Cd163*$^{-/-}$ mice with increases in both BALF eosinophils and CCL24 (Supplemental Figure 2).

In summary, we have shown that CD163 binds the major HDM allergen, Der p1, in a calcium-dependent fashion and that macrophages from *Cd163*$^{-/-}$ mice produce increased quantities of CCL24 when stimulated with HDM or Der p1. Furthermore, HDM-challenged *Cd163*$^{-/-}$ mice display a phenotype of increased eosinophilic airway inflammation and MCM that occurs by a CCL24-dependent mechanism. Thus, we propose that the reduced expression of CD163 by AMΦs from asthmatic patients may be associated with enhanced eosinophilic airway inflammation in response to inhalation of the house dust mite antigen, Der p1.

Materials and Methods

Human Samples

Informed consent was obtained as per NHLBI protocol 99-H- 0076. Normal individuals did not have AHR to inhaled methacholine challenge, while asthmatic subjects had either a positive bronchodilator response to an aerosolized short-acting β^2-agonist or AHR to inhaled methacholine. Allergy was defined by at least one positive skin test to short ragweed, grass mixture, cat dander, cockroach (German and American), *Dermatophagoides farinae*, or *Aspergillus fumigatus* or a prior history of positive allergy skin testing. Skin testing was performed using a Multi-Test II® applicator (provided as a generous gift from Lincoln Diagnostics, Decatur, IL). All subjects were clinically stable at the time of the bronchoscopy. Exclusion criteria included an active respiratory tract infection and current cigarette smoking. Total BALF cells were counted using a hemocytometer and differential cell counts were performed using Diff-Quik-stained cytospin slides (Siemens, Deerfield, Illinois).

Confocal Immunofluorescence Microscopy

Human BALF cell cytospin slides were fixed in 4% paraformaldehyde, incubated in blocking buffer (Aurion Blocking Solution, Electron Microscopy Sciences, Hatfield, PA) and reacted overnight with mouse anti-human CD68 (1:50 dilution, R & D Systems, Minneapolis, MN) and rabbit anti-human CD163 antibodies (1:50 dilution, Enzo Life Sciences, Inc., NY) diluted in 0.1% Aurion BSA-c (Electron Microscopy Sciences). Slides were washed 5 times in PBS, incubated with species-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568 (Life Technologies, NY) at a 1:200 dilution, washed 5 times, mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and visualized utilizing a Zeiss LSM 510 UV confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Flow Cytometry

Human BALF cells were washed and suspended in flow cytometry staining buffer (calcium- and magnesium-free PBS with 1% bovine serum albumin and 0.25 mM EDTA). Cells were incubated with mouse anti-human CD45 PECy5 (eBioscience Inc., San Diego, CA), mouse anti-human CD14 BV605 (BD Biosciences, Franklin Lakes, NJ), mouse anti-human CD68 PerCPCy5.5 (Biolegend, San Diego, CA), mouse anti-human CD163 APC (Biolegend) and fixable viability dye eFluor® 450 (eBioscience, San Diego, CA) for 45 minutes, washed with flow cytometry staining buffer and re-suspended in buffer containing 1% paraformaldehyde. Cells were acquired on a LSRII (BD Biosciences) equipped with 407, 488, 532, and 633 LASER lines using DIVA 6.1.2 software. Data were analyzed using FlowJo software version 9.1.5 (Treestar, San Carlos, CA).

Murine BALF cells were suspended in flow cytometry staining buffer (eBioscience) containing 1% mouse and rat serum and incubated with rat anti-mouse CD45 BV605 (BD Biosciences), hamster anti-mouse CD11c PerCp Cy5.5 (BD Biosciences), rat anti-mouse Siglec-F BB515 (BD Biosciences), anti-mouse CD64 (FcγRI) PE-Cy7 (Biolegend), rat anti-mouse CD206 BV 650 (Biolegend) for 45 min on ice. Cells were re-suspended in PBS containing 1% paraformaldehyde and events were acquired on a LSRII (BD Biosciences).

Cellular debris was excluded using a forward light scatter/side scatter plot. A macrophage gate was determined based upon light-scatter properties and $CD45^+/CD11c^+/Siglec\text{-}F^+/CD64^+$ AMΦs were analyzed for $CD206^+$ expression (Supplemental Figure 1).

Cd163$^{-/-}$ mice

Cd163$^{-/-}$ mice on a C57BL/6 genetic background were generated from $Cd163^{tm1(KOMP)Vlcg}$ embryos obtained from the Knockout Mouse Project at the University of California, Davis, California. $Cd163^{tm1(KOMP)Vlcg}$ embryos have a 24,636 base pair deletion from nucleotides 124,254,756 to 124,279,391 of chromosome 6. The $Cd163^{tm1(KOMP)Vlcg}$ embryos were created from ES cell clone Cd163_BA3, generated by Regeneron Pharmaceuticals, Inc. and made into live mice at the Jackson Laboratory (Bar Harbor, Maine)[48]. Wild type C57BL/6 mice were from Taconic Labs (Hudson, NY). Both female and male mice that were matched by gender were used for experiments.

Genotyping of *Cd163*$^{-/-}$ mice

The targeted deletion of the *Cd163* gene was confirmed by PCR of genomic DNA and quantitative real-time PCR (qRT-PCR) of lung mRNA. SU (5′-GTGTTCCAAAGTGGGAGGAG-3′) and LacZRev (5′-GTCTGTCCTAGCTTCCTCACTG-3′) primers were used to confirm the presence of the targeting vector in *Cd163*$^{-/-}$ mice, whereas TDF (5′-TCATTCCAGGAGAAGTGCCC -3′) and TDR (5′-CCAAGGCAAATCCCTCTCAG-3′) primers were used to confirm the absence of intron 15 – 16 of the *Cd163* gene in *Cd163*$^{-/-}$ mice. A Taqman probe directed against the boundary of exons 6 and 7 (Mm00474091_m1, Applied Biosystems/Life Technologies, Grand Island, NY) was used for qRT-PCR to confirm the absence of *Cd163* mRNA expression in the lungs of *Cd163*$^{-/-}$ mice.

Murine Models of House Dust Mite- and Der p1-induced Airway Disease

Experimental protocols were approved by the NHLBI Animal Care and Use Committee. *Dermatophagoides pteronyssinus* extract, referred to as house dust mite (HDM), was purchased from Greer Laboratories (Lenoir, NC) and contained ≤ 50 units of LPS per mg of HDM protein, which equated to administration of ≤ 5 pg of LPS with each μg of HDM[49]. **(1) Intranasal HDM sensitization and challenge model.** Six-to-eight week old WT C57BL/6 and *Cd163*$^{-/-}$ mice received daily intranasal challenges of HDM (25 μg) or saline, in a volume of 10 μl, five days a week for five weeks. End-points were analyzed 24 hrs after the last challenge. **(2) Intraperitoneal HDM sensitization and intranasal challenge model for AAM experiments.** 8–to-10 week old WT and *Cd163*$^{-/-}$ mice were sensitized on days 1 and 4 by intraperitoneal injection of HDM (50 μg/ml) emulsified in 60 μl of 2% Alhydrogel adjuvant (Invivogen, San Diego, California) plus 140 μl of sterile PBS. On day 8, all mice received a single intranasal challenge of HDM (100 μg). BALF cells were collected on day 10. **(3) CCL24 inhibition experiments.** 5 μg of a neutralizing, monoclonal rat anti-mouse CCL24 antibody (MAB528) or a monoclonal rat IgG^{2A} isotype control antibody (R&D Systems, Minneapolis, MN) were administered to the nares daily, 3 days per week, concurrent with HDM challenges for 5 weeks. End-points were analyzed 24 hours after the last challenge. **(4) Adoptive transfer of HDM-pulsed BMMΦs.** Bone marrow cells from

donor WT and *Cd163*$^{-/-}$ mice were harvested on day 1 and cultured in GM-CSF (20 ng/ml) until day 9, when cells were pulsed with HDM (100 μg/ml) or saline as a control. On day 10, CD11c^{+}/MHCII^{+}/Siglec-F^{-} BMDCs were isolated by flow sorting and 1 × 10^{5} BMDCs were adoptively transferred by intranasal administration to recipient WT mice, which received daily intranasal instillations of HDM (50 μg) on days 23 through 26. End-points were analyzed on Day 27. **(5) Adoptive transfer of HDM-pulsed MLN dendritic cells.** WT and *Cd163*$^{-/-}$ mice were pulsed with HDM (100 μg) or saline on day 1 and MLNs were harvested on day 4. CD11c^{+}/ MHCII^{+}/Siglec- F^{-}DCs were isolated by flow sorting and 2.5 × 10^{4} cells were administered to the nares of recipient WT mice, which then received daily intranasal HDM challenges (25 μg) on days 9 through 14 and endpoints were analyzed on day 15. **(6) Adoptive transfer of splenic CD4^{+} T cells.** 8 – 10 week old WT C57BL/6 mice were sensitized on days 1 and 7 by intraperitoneal injection of saline or HDM (100 μg/ml) emulsified in 60 μl of 2% Alhydrogel adjuvant (Invivogen, San Diego, California) plus 140 μl of sterile PBS. On day 14, CD4^{+} T cells were purified from the spleens of sensitized mice using the EasySep Mouse CD4^{+} T Cell Isolation kit (StemCell Technologies, Vancouver, Canada) to a purity of 95%, which was verified by flow cytometry, and 2 million CD4^{+} T cells were adoptively transferred in 100 ul of PBS via intraperitoneal administration to recipient WT and *Cd163*$^{-/-}$ mice. Recipient mice received intranasal HDM (50 μg) challenges on days 19, 21, 23 and 25 and endpoints were analyzed on day 27. **(7) Der p1 model.** Der p1 experiments were performed using a modification of a previously published protocol[17]. Mice were sensitized by intraperitoneal injection of saline (100 ul) with or without 2 μg of LoTox Der p1 (Indoor Biotechnologies, VA) on days 1 and 8. On day 15, 10 μg of LoTox Der p1 was administered via intratracheal instillation in 40 ul of saline. End-points were analyzed 72 hours after the last challenge. **(8) AMΦ adoptive transfer experiments.** Recipient mice were sensitized by intraperitoneal injection of LoTox Der p1 (2 μg), or saline as a control, on Days 1 and 7. On day 14, bronchoalveolar lavage was performed on naive donor mice using ice cold PBS without magnesium chloride and calcium chloride plus 2 mM EDTA. Viable CD45^{+}/CD11c^{+}/ Siglec-F^{+}/CD64^{+} AMΦs in BALF were isolated by flow sorting and 1 × 10^{5} cells were adoptively transferred to recipient mice by intranasal instillation. Recipient mice were challenged by intratracheal LoTox Der p1 (10 μg) administration on Day 17 and endpoints were analyzed on Day 20. Analyses of BALF cells and lung histology were performed as previously described[49].

HDM-specific IgE

Serum HDM-specific IgE was measured by incubating plates overnight with 0.01% HDM in PBS and blocked with 1% BSA prior to addition of serum for 1 h. Serum was depleted of IgG by incubating with protein G agarose (ThermoFisher Scientific, Waltham, MA) overnight at 4° C prior to analysis. Following 6 washes with PBS plus 0.05% Tween-20, biotinylated anti-mouse IgE (Pharmingen, San Jose, CA) was added for 1 h. Plates were washed six times prior to addition of streptavidin-HRP (R & D Systems, Minneapolis, MN) for 30 min. TMB substrate was added for 30 min to determine the quantity of HDM-specific antibody.

Enzyme-Linked Immunosorbent Assays

Quantification of CCL11, CCL24 and Chi3L3/4 were performed using Duoset ELISA kits from R & D Systems, Minneapolis, MN.

Western Blotting

Lung proteins (12.5 μg) were separated by SDS-PAGE using 10% Bis-Tris Nupage gels (Life Technologies, Grand Island, NY). The antibodies against Muc5AC (1:500 dilution) and β-actin (1:3000 dilution) were from Abcam (Cambridge, MA). Membranes were stripped using the Re-Blot recycling kit (Chemicon International, Temecula, CA). Densitometry was performed using NIH ImageJ software (NIH, Bethesda, MD).

Airway Hyperreactivity

Airway resistance to increasing doses of nebulized methacholine was directly measured in anesthetized mice that were mechanically ventilated using an Elan RC Fine Pointe system (Buxco, North Carolina), as previously described[49].

HDM-specific Re-stimulation of Mediastinal Lymph Node Cultures and Measurement of Th2 Cytokine Production

Mediastinal lymph nodes (MLN) were excised from HDM-challenged WT and *Cd163*$^{-/-}$ mice and single cell suspensions were generated by passage through a 100 μm strainer, followed by lysis of red blood cells using ACK buffer. Cells were suspended in RPMI 1640 medium containing 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM) and 4×10^6 cells per well were cultured in 96-well plates with "U"-shaped bottoms. Cells were stimulated with HDM (100 μg/ml), Der p1 (10 μg/ml), or saline (as a control) for 96 hours and medium was collected and analyzed for cytokines using ELISAs with a limit of sensitivity of 9.4 pg/ml for IFN-γ, 15.6 pg/ml for IL-17A, 31.25 pg/ml for IL-5 and 62.5 pg/ml for IL-13 (R & D Systems).

Alveolar Macrophage Isolation and HDM Re-stimulation

1×10^5 BALF cells from HDM-challenged WT and *Cd163*$^{-/-}$ mice were seeded in 96 well tissue culture plates or in Lab-Tek chamber slides and alveolar macrophages were allowed to adhere for 2 h in RPMI-1640 containing 10% FBS. Non-adherent cells were removed by washing with PBS. Manual counting of Diff-Quik stained slides revealed that 92% ± 0.3% of isolated cells were alveolar macrophages (n = 3). Alveolar macrophages were re-stimulated with 500 μg/ml of HDM or medium for 24 h and CCL24 secretion was quantified by ELISA.

Proteomic Identification of Der p1 Binding to CD163

10 μg of recombinant human CD163 protein (R&D Systems), *Dermatophagoides pteronyssinus* extract or purified Der p1 (Indoor Biotechnologies, Charlottesville, VA) were covalently immobilized to an aldehyde-activated 4% beaded agarose resin (Pierce Direct IP Kit, Thermo Fisher Scientific) for 2 h and blocked with 1% chicken egg white albumin (Sigma-Aldrich, St. Louis, MO). Resin-immobilized CD163 or purified Der p1 were incubated with 10 μg of pre-cleared *Dermatophagoides pteronyssinus* protein extract,

purified Der p1 or recombinant human CD163 overnight, washed 6 times with 0.1% Triton X-100, 50 mM Tris, pH 7.5, and 120 mM NaCl (Sigma-Aldrich, St. Louis, MO) and interacting proteins were eluted, resolved by SDS-PAGE using 10% Bis-Tris Nupage gels and visualized with the SilverQuest™ Silver Staining Kit (Life Technologies, Grand Island, NY). Resins to which 10 μg of chicken egg white albumin had been immobilized served as controls for background non-specific binding. Excised bands were destained and subjected to trypsin digestion, as previously described, prior to analysis by mass spectrometry using the LTQ Orbitrap Velos mass spectrometer [50]. The raw file generated by the Velos was analyzed using Proteome Discoverer v1.3 software (Thermo Fisher Scientific) using the Mascot search engine (v2.3). The Mascot search criteria were set to: database, Swiss-Prot (Swiss Institute of Bioinformatics); taxonomy, Eukaryota (eukaryotes); enzyme, trypsin; miscleavages, 3; variable modifications, oxidation (M), deamidation (NQ); fixed modifications, carbamidomethyl (C), MS peptide tolerance 10 ppm; MS/MS tolerance as 0.8 Da. The peptide confidence false discovery rate (FDR) was set to less than 1%.

CD163 binding to Immobilized Der p1

Plastic 96 well plates were coated with purified Der p1 and blocked with 1% bovine serum albumin dissolved in PBS. Recombinant human CD163 (R&D Systems, Inc., Minneapolis, MN) (120 ng) was added to the Der p1-coated plates overnight, washed 3 times with 0.05% Tween 20 in PBS, and the quantity of CD163 that bound to the immobilized Der p1 was quantified using a CD163 immunoassay kit (R&D Systems, Inc., Minneapolis, MN) with a horseradish peroxidase conjugated anti-CD163 antibody. Non-specific binding of the anti-CD163 antibody to Der p1-coated plates in the absence of CD163, as well as background related to the immobilized Der p1 protein in the absence of the anti-CD163 antibody were subtracted from the reported values.

Immunoprecipitation of Human Monocyte CD163

Elutriated human monocytes were provided under an IRB-approved protocol (01-CC-0168) by the NIH Department of Transfusion Medicine, Clinical Center. Cells were lysed in buffer containing 0.1% Triton X-100, 50 mM Tris, pH 7.5, and 120 mM NaCl supplemented with Complete™ protease inhibitor (Thermo Fisher Scientific). 300 μg of protein lysates, with or without 5 mM calcium or 5 mM EGTA, were incubated overnight with 10 μg of purified Der p1 that had been immobilized to an aldehyde-activated beaded agarose resin (Pierce Direct IP Kit, Thermo Fisher Scientific) and washed 6 times with cold lysis buffer. Eluted proteins were resolved by Western blotting and CD163 was detected using a mouse monoclonal anti-CD163 antibody (MAB1607) from R & D Systems.

Bone Marrow-derived Macrophage Cultures

Bone marrow cells from *Cd163*$^{-/-}$ mice and WT mice were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 M 2-ME, and recombinant mouse M-CSF (20 ng/ml, Biolegend, CA) for 6 days[21]. Cells were treated with IL-4 (10 ng/ml) (Biolegend) for 48 h to induce CCL24 expression prior to stimulation with purified Der p1 (20 ug/ml) for 48 h and CCL24 release into medium was quantified by ELISA.

Statistics

Data are presented as mean ± SEM. Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism version 5.0a), except where indicated that a paired or unpaired *t* test or a Mann-Whitney test were utilized. A *P* value < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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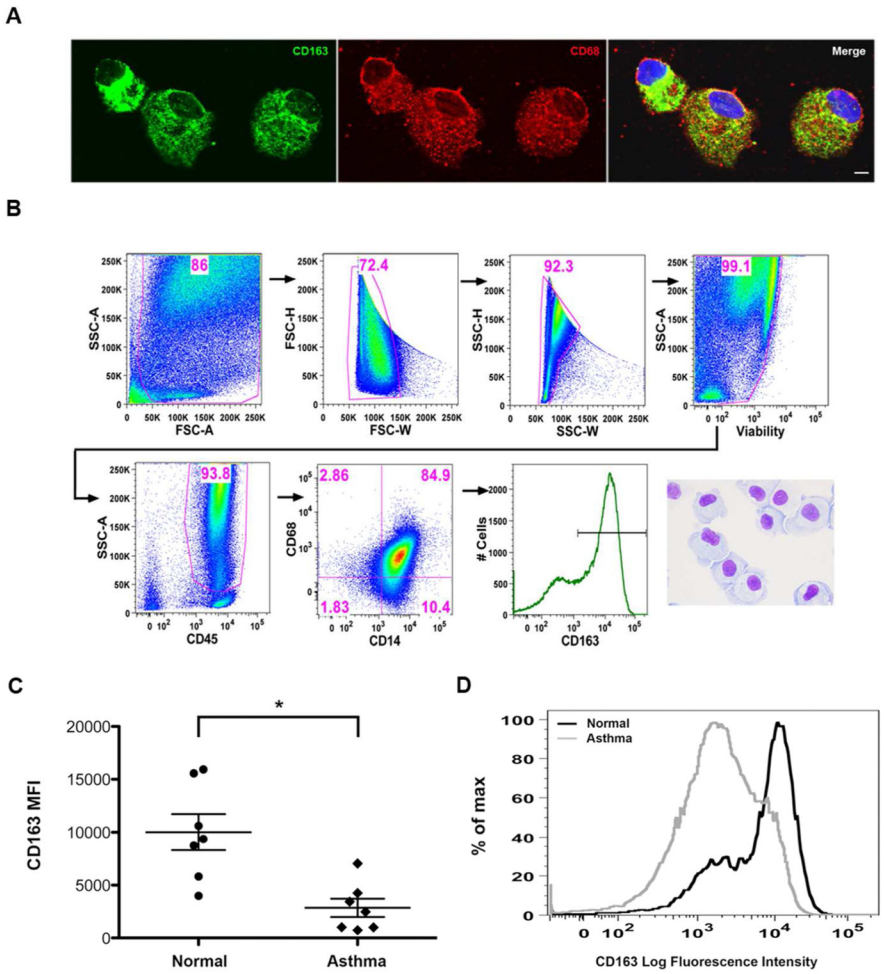

Figure 1. Alveolar macrophages (AMΦs) from human asthmatics have reduced cell surface expression of CD163

(A) Confocal immunofluorescence microscopy images of BALF cells from an asthmatic subject showing CD163 expression by $CD68^+$ AMΦs. The scale bar indicates 5 μm. **(B)** Gating strategy for identification of human $CD163^+$ alveolar macrophages in BALF. Cellular debris was excluded using a forward light scatter/side scatter plot and doublets were excluded using width parameter on FSC and SSC properties. $CD45^+$ cells, that co-expressed CD14 and CD68, were identified as alveolar macrophages using side scatter and CD45 bivariate plots from which lymphocytes had been excluded. A microscopic image of sorted $CD45^+/CD14^+/CD68^+/CD163^+$ cells shows a cellular population possessing typical cellular characteristics of alveolar macrophages. **(C)** MFI of cell surface CD163 expression by $CD45^+/CD14^+/CD68^+$ AMΦs in BALF from normal individuals and asthmatic subjects (n = 7, $P < 0.008$, paired *t* test). **(D)** A representative histogram overlay comparing cell surface CD163 expression by AMΦs from a normal individual and an asthmatic subject.

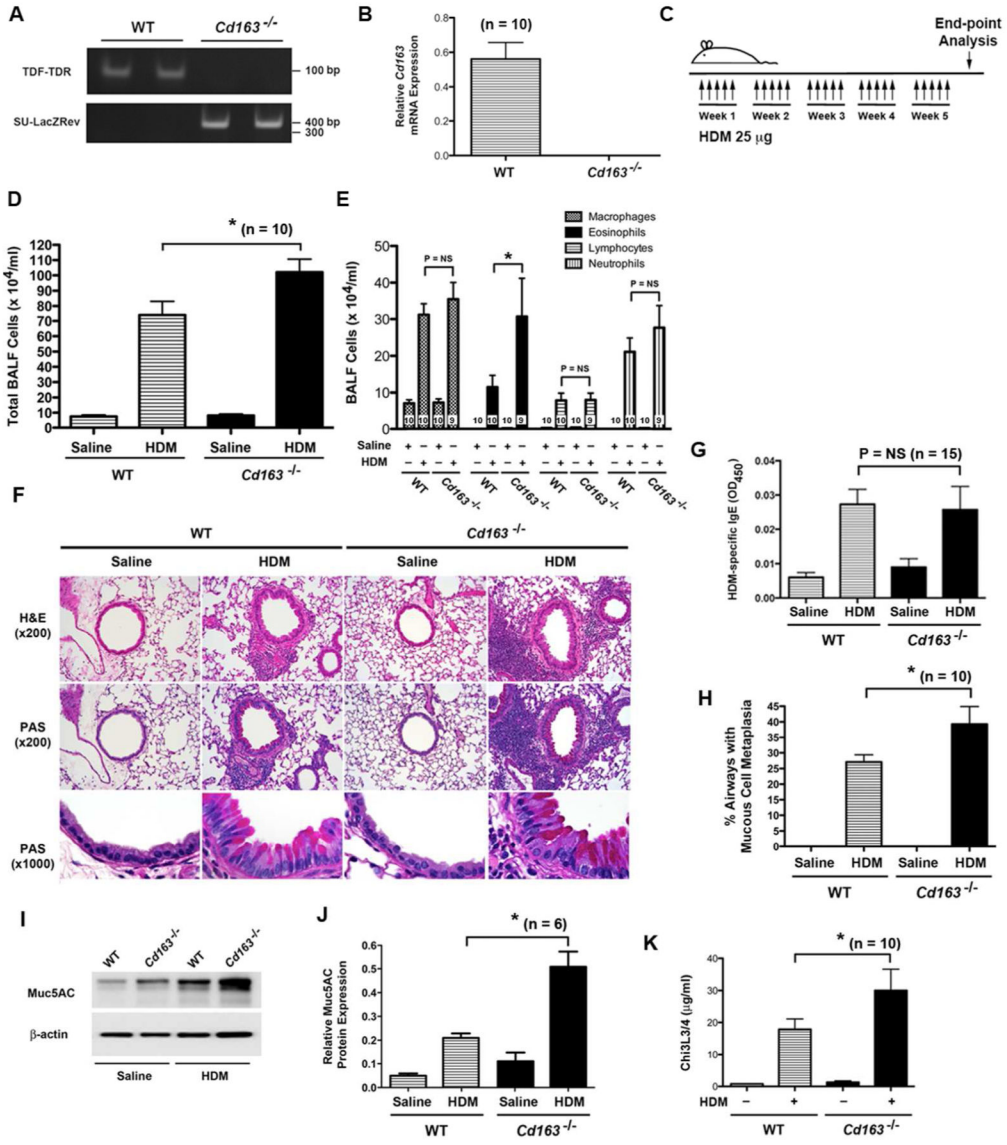

Figure 2. Eosinophilic airway inflammation and mucous cell metaplasia (MCM) are augmented in HDM-challenged *Cd163*$^{-/-}$ mice

(A) Intron 15–16 of the *Cd163* gene was not detected by PCR of genomic DNA from *Cd163*$^{-/-}$ mice, whereas the *E. coli lacZ* coding sequence from the targeting vector could only be detected in *Cd163*$^{-/-}$ mice. Results from 2 mice are shown. **(B)** *Cd163* mRNA was only detected by qRT-PCR in the lungs of wild type (WT), but not *Cd163*$^{-/-}$ mice (n = 10 mice). **(C)** WT and *Cd163*$^{-/-}$ mice were sensitized and challenged by daily nasal administration of HDM (25 µg) or saline, 5 days a week, for 5 weeks. End-point analysis was performed 24 h later. **(D and E)** Total BALF inflammatory cells (Panel D) and inflammatory cell types (Panel E) (n = 9 – 10 mice, *P < 0.05). **(F)** Representative lung histology from WT and *Cd163*$^{-/-}$ mice that had been sensitized and challenged with saline or HDM were stained with hematoxylin and eosin (H & E) (x 200) or periodic acid Schiff (PAS) (x200 and x1000). **(G)** Serum HDM-specific IgE (n = 15 mice). **(H)** Quantification of

MCM (n = 10 mice, *P < 0.01). (**I**) Western blots of lung proteins were reacted with antibodies against Muc5AC or β-actin. A representative blot from 6 replicate experiments is shown. **(J)** Relative Muc5AC expression as compared to β-actin was quantified by densitometry (n = 6, * P < 0.001). **(K)** Chi3L3/4 protein in BALF (n = 10 mice, *P < 0.05). Panels D, E, H and K are representative of two or three independent experiments. Panel G is pooled data from two independent experiments. Numbers of mice that were included in each experimental condition are shown.

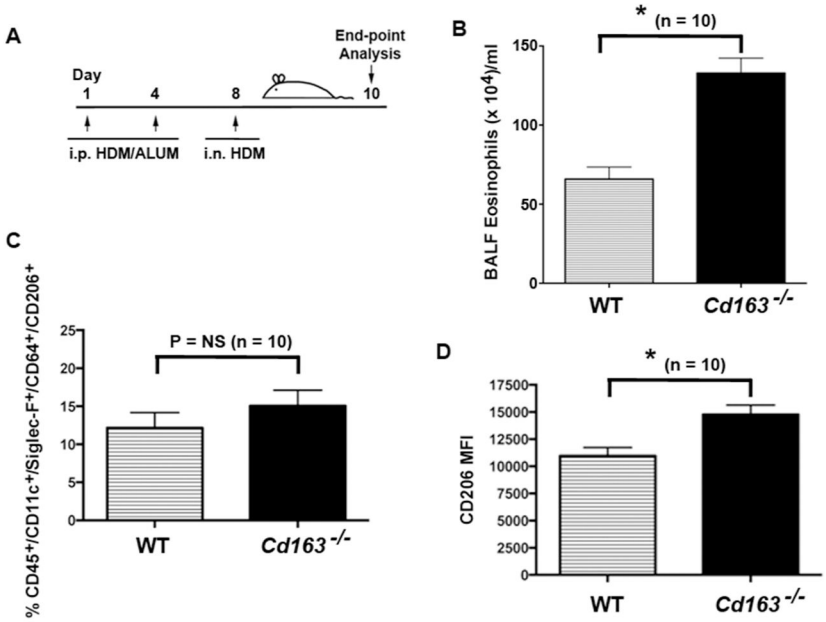

Figure 3. The percentage of M(IL-4) alternatively activated alveolar macrophages (AAMs) is not modified in HDM-challenged *Cd163*$^{-/-}$ mice

(A) WT and *Cd163*$^{-/-}$ mice were sensitized by intraperitoneal (i.p.) administration of HDM (50 μg/ml) emulsified in 2% aluminum hydroxide (ALUM) on days 1 and 4, challenged with intranasal administration of HDM (100 μg) on day 8, and end-points were analyzed on day 10. **(B)** BALF eosinophils (n = 10 mice, *P < 0.0001, Mann Whitney test). **(C)** The percentage of CD45$^+$/CD11c$^+$/Siglec-F$^+$/CD64$^+$/CD206$^+$ BALF AAMs were quantified by flow cytometry (n = 10 mice, P = NS, Mann Whitney test). **(D)** The MFI of CD206 expression by CD45$^+$/CD11c$^+$/Siglec-F$^+$/CD64$^+$/CD206$^+$ BALF AAMs was quantified by flow cytometry (n = 10 mice, P = 0.0029, Mann Whitney test). The gating strategy used to identify CD45$^+$/CD11c$^+$/Siglec-F$^+$/CD64$^+$ BALF alveolar macrophages is presented in Supplemental Figure 1. Data are pooled from two independent experiments.

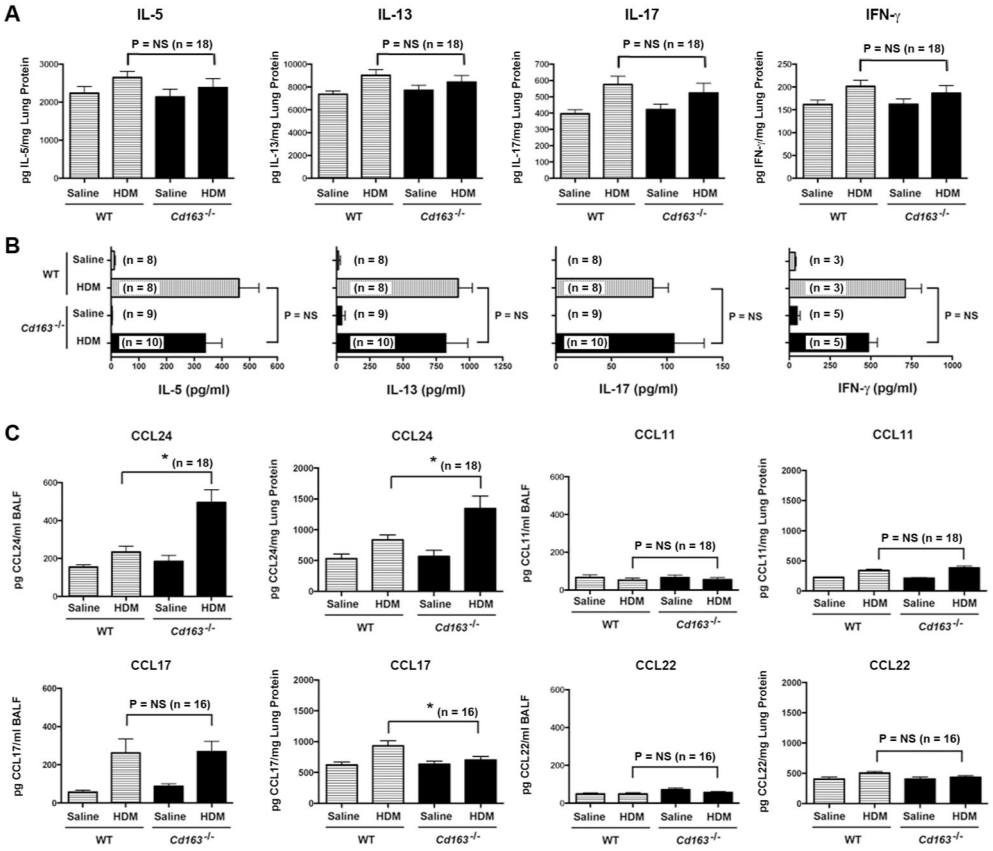

Figure 4. CCL24 is increased in the lungs of HDM-challenged *Cd163*$^{-/-}$ mice

WT and *Cd163*$^{-/-}$ mice were sensitized and challenged by daily nasal administration of HDM (25 μg) or saline, 5 days a week, for 5 weeks and end-points were analyzed 24 h later. **(A)** Cytokines in lung homogenates from HDM-challenged WT and *Cd163*$^{-/-}$ mice (n = 18 mice). **(B)** *Ex vivo* cultures of mediastinal lymph node cells from HDM-challenged WT and *Cd163*$^{-/-}$ mice were re-stimulated with HDM (100 μg/ml) or saline (as a control) and cytokine secretion was quantified (n = 3 – 10 mice). **(C)** Chemokines in BALF and lung homogenates (n = 16 – 18 mice, * $P < 0.01$). Data in Panels A and C are pooled from two independent experiments, while Panel B is representative of two independent experiments that showed similar results. Numbers of mice that were included in each experimental condition are shown.

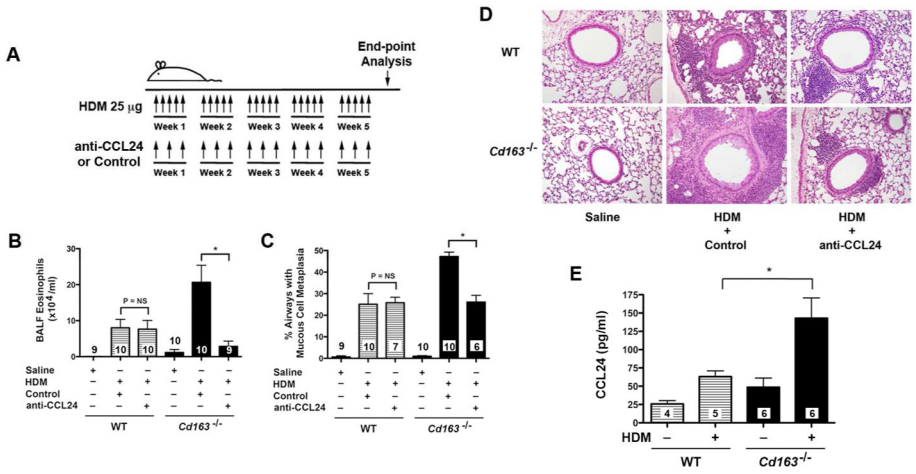

Figure 5. CCL24 neutralization prevents augmented eosinophilic airway inflammation and mucous cell metaplasia (MCM) in HDM-challenged *Cd163*$^{-/-}$ mice

(A) Wild type (WT) and *Cd163*$^{-/-}$ mice were sensitized and challenged by daily intranasal administration of HDM (25 μg) or saline, 5 days a week, for 5 weeks. Mice received concurrent nasal administration of a neutralizing anti-CCL24 antibody or control immunoglobulin, 3 days a week for 5 weeks. End-point analysis was performed 24 h later. (**B**) BALF eosinophils (n = 9 – 10 mice, *P < 0.0001, HDM + control antibody vs. HDM + anti-CCL24 neutralizing antibody). (**C**) Quantification of MCM (n = 6 – 10 mice, *P < 0.0001, HDM + control antibody vs. HDM + anti-CCL24 neutralizing antibody). (**D**) Representative histologic lung sections stained with hematoxylin and eosin (x 200). **(E)** AMΦs were isolated from HDM-challenged WT and *Cd163*$^{-/-}$ mice, cultured *ex vivo* with or without HDM (500 μg/ml) and CCL24 secretion was quantified (n = 3 – 5 mice, *P < 0.01, *Cd163*$^{-/-}$ + HDM vs. WT + HDM). Panels B & C represent pooled data from two independent experiments, while Panel E is representative data from one of two independent experiments. Numbers of mice that were included in each experimental condition are shown.

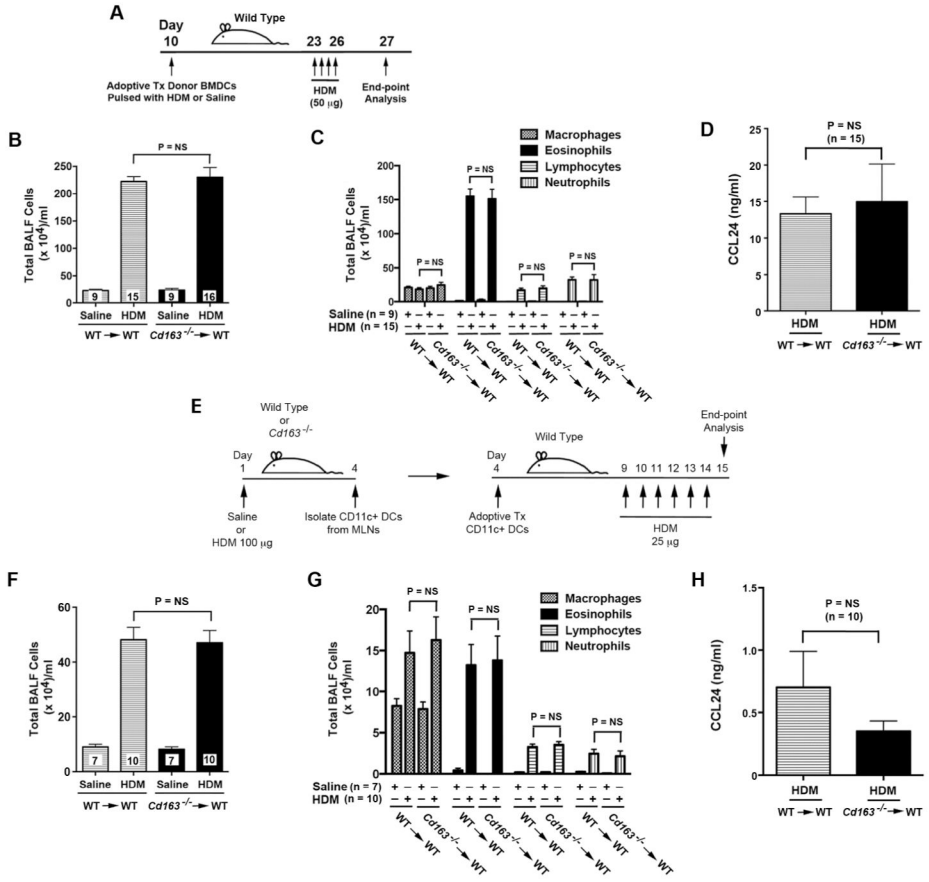

Figure 6. The adoptive transfer of HDM-pulsed dendritic cells (DCs) from *Cd163*$^{-/-}$ mice to recipient wild type mice does not induce increases in eosinophilic airway inflammation or BALF CCL24 levels

(A) Bone marrow-derived dendritic cells (BMDCs) from WT and *Cd163*$^{-/-}$ mice that had been pulsed with HDM or saline, as a control, were adoptively transferred to WT recipient mice on Day 10. All mice received nasal HDM challenges on days 23 through 26 and end-points were analyzed on day 27. (**B and C**) Total BALF inflammatory cells (Panel B) and inflammatory cell types (Panel C) (n = 9 – 16 mice). **(D)** CCL24 in BALF (n = 15 mice, P = NS, Mann-Whitney test). **(E)** CD11c^{+} DCs isolated from MLNs of WT and *Cd163*$^{-/-}$ mice that had been pulsed *in vivo* by intranasal administration of HDM (100 μg) or saline, as a control, were adoptively transferred to WT recipient mice. All mice received nasal HDM challenges on days 9 through 14 and end-points were analyzed on day 15. (**F and G**) Total BALF inflammatory cells (Panel F) and inflammatory cell types (Panel G) (n = 7 – 10 mice). (**H**) CCL24 in BALF (n = 10 mice, P = NS, Mann-Whitney test). Panels B – D and F – H represent pooled data from two independent experiments. Numbers of mice that were included in each experimental condition are shown. The same numbers of mice are shown for Panels B and C and Panels F and G.

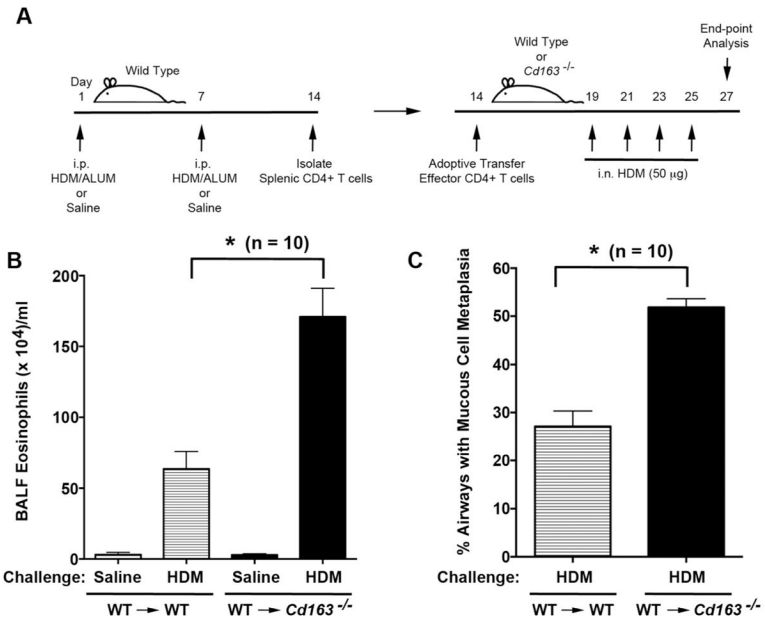

Figure 7. The adoptive transfer of WT effector CD4$^+$ T cells does not modify the phenotype of increased eosinophilic airway inflammation and mucous cell metaplasia (MCM) in HDM-challenged *Cd163*$^{-/-}$ mice

(A) Effector splenic CD4$^+$ T cells were isolated from wild type (WT) mice that had been sensitized by intraperitoneal (i.p.) administration of saline or HDM (100 μg/ml) emulsified in 2% aluminum hydroxide (ALUM) on days 1 and 7. On day 14, 2 × 10^6 CD4$^+$ T cells were purified from the spleens of sensitized mice and adoptively transferred via intraperitoneal administration to recipient WT and *Cd163*$^{-/-}$ mice. All recipient mice received intranasal HDM challenges (50 μg) on days 19, 21, 23 and 25 and endpoints were analyzed on day 27. **(B)** BALF eosinophils (* $P < 0.001$, n = 10, recipient WT vs. recipient *Cd163*$^{-/-}$ mice). **(C)** Quantification of MCM. (* $P < 0.001$, n = 10). Data are pooled from two independent experiments. Numbers of mice that were included in each experimental condition are shown.

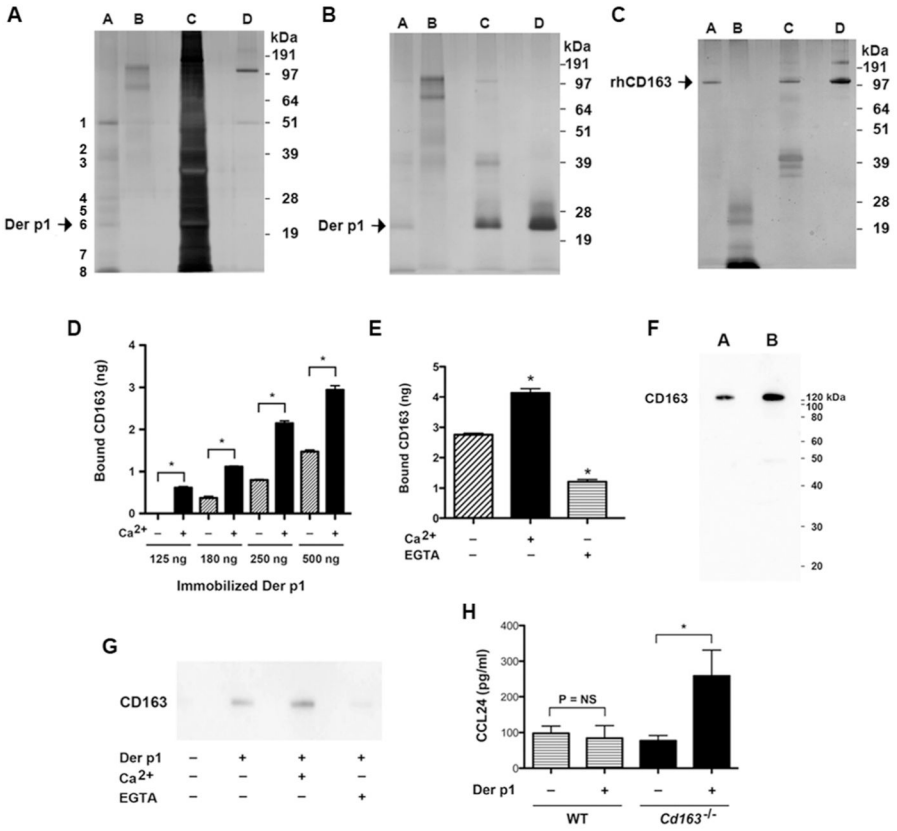

Figure 8. CD163 binds Der p1

(**A**) Proteins in *D. pteronyssinus* extracts that bound to immobilized recombinant human CD163 (rhCD163) were eluted, resolved by SDS-PAGE and visualized by silver staining (Lane A). Band #6 was identified as *D. pteronyssinus* peptidase 1 (Der p1). Also shown are proteins that eluted from the rhCD163 resin in the absence of *D. pteronyssinus* extracts (Lane B), proteins in *D. pteronyssinus* extracts that did not bind to immobilized rhCD163 (Lane C) and rhCD163 alone (Lane D). (**B**) Immobilized rhCD163 was incubated with purified Der p1 and eluted proteins were resolved by SDS-PAGE and visualized by silver staining. A protein corresponding to the molecular weight of Der p1 was eluted from immobilized rhCD163 (Lane A). Proteins that were eluted in the absence of Der p1 (Lane B), purified Der p1 proteins that did not bind to the immobilized rhCD163 resin (Lane C) and Der p1 protein alone (Lane D) are shown. (**C**) Immobilized Der p1 was incubated with rhCD163 and eluted proteins resolved by SDS-PAGE and visualized by silver staining. A protein corresponding to the molecular weight of rhCD163 was eluted from immobilized rhCD163 (Lane A). Proteins that eluted from the immobilized rhCD163 resin in the absence of rhCD163 (Lane B), rhCD163 that did not bind to immobilized Der p1 (Lane C) and rhCD163 alone (Lane D) are shown. **(D)** Increasing amounts of purified Der p1 were bound to plastic and incubated with 120 ng of rhCD163 with or without supplemental calcium (5 mM). The amount of rhCD163 that bound to immobilized Der p1 increased in a dose-responsive fashion ($n = 3$, $P < 0.0001$). Binding of rhCD163 to immobilized Der p1 was calcium-dependent ($n = 3$, * $P < 0.0001$, supplemental calcium vs. no supplemental calcium). **(E)** 250 ng of purified Der p1 was immobilized to plastic and incubated with 120

ng of rhCD163 with or without supplemental calcium (5 mM) or EGTA (5 mM). The amount of rhCD163 that bound immobilized Der p1 was quantified (n = 4, * $P < 0.0001$, vs. PBS alone). **(F)** Immobilized Der p1 was incubated with human monocyte proteins. Bound proteins were eluted and Western blots were performed using an anti-human CD163 antibody (Lane A). For comparison, human monocyte proteins were subjected to Western blotting in Lane B. **(G)** Immobilized Der p1 was incubated with human monocyte proteins with or without supplemental calcium (5 mM) or EGTA (5 mM). Bound proteins were eluted and Western blotting was performed using an anti-human CD163 antibody. **(H)** CCL24 secretion by bone marrow-derived macrophages stimulated with Der p1 (20 μg/ml) (n=6, *$P < 0.05$ vs. medium alone). Data are representative of two or three independent experiments.

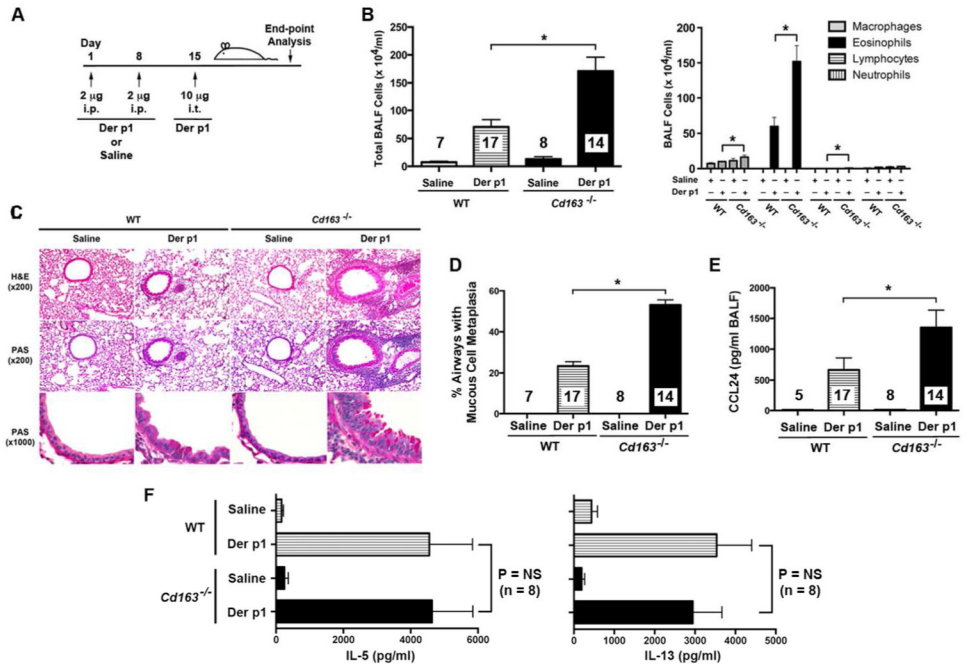

Figure 9. Eosinophilic airway inflammation and mucous cell metaplasia (MCM) are augmented in Der p1-challenged *Cd163*$^{-/-}$ mice

(A) WT and *Cd163*$^{-/-}$ mice were sensitized by intraperitoneal injection of Der p1 or saline on days 1 and 8 and challenged with intratracheal Der p1 on day 15. End-points were analyzed 72 h later. **(B).** Total BALF inflammatory cells and inflammatory cell types (n = 7 – 17 mice, *P < 0.05). **(C)** Representative lung histologic sections from WT and *Cd163*$^{-/-}$ mice that had been challenged with saline or Der p1 and stained with hematoxylin and eosin (H & E) (x 200) or periodic acid Schiff (PAS) (x200 and x1000). (**D**) Quantification of MCM (n = 7 – 17 mice, *P < 0.0001). 30.6 ± 0.9 airways were counted per mouse. **(E)** BALF CCL24 levels (n = 5 – 17 mice, * P < 0.05). (**F**) *Ex vivo* cultures of mediastinal lymph node cells from Der p1-challenged WT and *Cd163*$^{-/-}$ mice were re-stimulated with Der p1 (10 µg/ml) or saline, as a control, and cytokine secretion was quantified (n = 8). Panels B – F are pooled data from two independent experiments. Numbers of mice that were included in each experimental condition are shown.

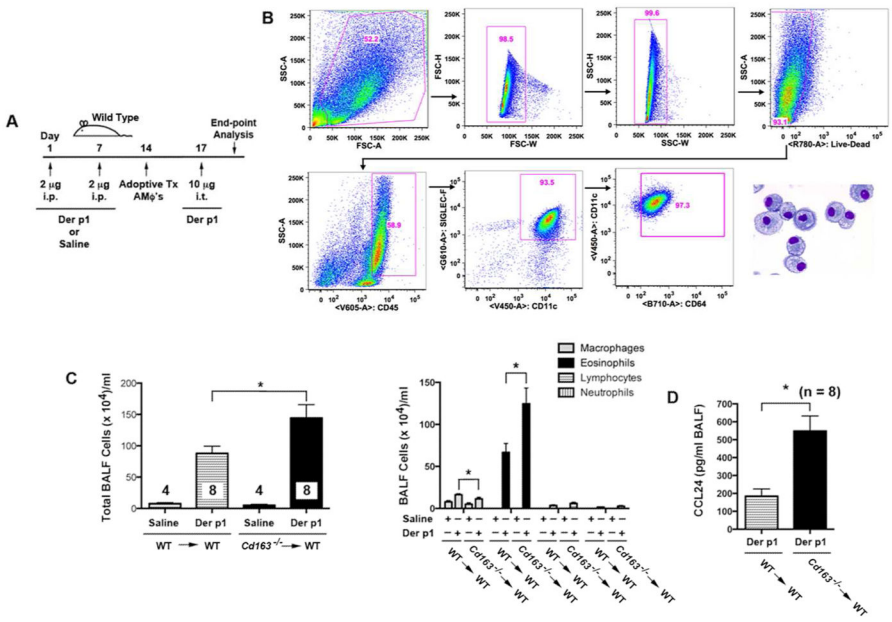

Figure 10. The adoptive transfer of alveolar macrophages (AMΦs) from donor*Cd163*$^{-/-}$ mice enhances Der p1-induced increases in eosinophilic airway inflammation and mucous cell metaplasia (MCM) in recipient wild type mice

(A) WT mice were sensitized by intraperitoneal (i.p.) injection of Der p1 or saline, as a control, on days 1 and 7, followed by the intranasal adoptive transfer of 1×10^5 AMΦ's from naive donor WT or *Cd163*$^{-/-}$ mice on day 14. All mice received an intratracheal Der p1 (10 µg) challenge on day 17 and end-points were analyzed on day 20. **(B)** Gating strategy for sorting of naive murine alveolar macrophages in BALF. Cellular debris was excluded using a forward light scatter/side scatter plot and doublets were excluded using width parameter on FSC and SSC properties. CD45$^+$ cells, that co-expressed CD11c, Siglec-F and CD64 were identified as AMΦs. A microscopic image of sorted CD45$^+$/CD11c$^+$/Siglec-F$^+$/CD64$^+$ cells shows a cellular population possessing typical characteristics of AMΦs. (**C**) Total BALF inflammatory cells and inflammatory cell types (n = 4 – 8 mice, * $P < 0.05$, donor *Cd163*$^{-/-}$ AMΦ's vs. donor WT AMΦ's). (**D**) BALF CCL24 levels from Der p1-challenged recipient mice. (n = 8 mice, $P = 0.0016$, donor *Cd163*$^{-/-}$ AMΦ's vs. donor WT AMΦ's, unpaired *t* test). Panels C and D are representative data from one of three independent experiments. Numbers of mice that were included in each experimental condition are shown. The same numbers of mice are shown for both analyses in Panel C.

Table 1

Clinical characteristics of research subjects.

	Normal	Asthma	
# of Subjects	7	7	
Age (Years)	38.3 ± 5.4	38.4 ± 3.5	P = NS
Gender (M:F)	(2:5)	(2:5)	
Asthma Severity (mild/moderate vs. severe)	n/a	5/2	
Positive Allergy Skin Test[1]	2/7	6/7	
Positive Skin Test to *Dermatophagoides farinae*	1/7	6/7	
FEV1 (liters)	3.51 ± 0.20	2.63 ± 0.14	P = 0.002
FEV1 (% predicted)	103 ± 2.79	82.7 ± 4.67	P = 0.027
Blood Eosinophils (k/ul)	0.137 ± 0.04	0.223 ± 0.05	P = NS
Blood Eosinophils (%)	2.34 ± 0.62	3.46 ± 0.80	P = NS
IgE (IU/ml)	59.17 ± 25.38	3651 ± 3238	P = NS
FeNO (ppb)	19.1 ± 7.4	34.2 ± 10.2	P = NS
Oral Corticosteroids (%)	0	0	
Inhaled Corticosteroids (%)	0	71%	
BALF Macrophages (x 10^4/ml)	1077 ± 253	808 ± 247	P = NS
BALF Eosinophils (x 10^4/ml)	0	14 ± 6	P = NS
BALF Lymphocytes (x 10^4/ml)	118 ± 51	168 ± 89	P = NS
BALF Neutrophils (x 10^4/ml)	65 ± 17	81 ± 22	P = NS

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using a paired two-tailed T test. A P value ≤ 0.5 was considered significant.

[1] Indicates positive skin test reactivity to at least one of six aeroallergens.