

Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge

S. A. Mathie^{1,*}, K. L. Dixon^{2,*}, S. A. Walker¹, V. Tyrrell³, M. Mondhe³, V. B. O'Donnell³,
L. G. Gregory¹ & C. M. Lloyd¹

¹Leukocyte Biology, NHLI, Faculty of Medicine, Imperial College London, London; ²UCB Pharma, Slough, Berkshire; ³Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, UK

To cite this article: Mathie SA, Dixon KL, Walker SA, Tyrrell V, Mondhe M, O'Donnell VB, Gregory LG, Lloyd CM. Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge. *Allergy* 2015; **70**: 80–89.

Keywords

Alveolar macrophage; homeostasis; house dust mite; interleukin-13; lung.

Correspondence

Professor Clare M. Lloyd, Leukocyte Biology Section, NHLI, Imperial College London, London SW7 2AZ, UK.
Tel.: 0207 5943102
Fax: 0207 5943119
E-mail: c.lloyd@imperial.ac.uk

*Authors contributed equally to the work

Accepted for publication 17 October 2014

DOI:10.1111/all.12536

Edited by: Hans-Uwe Simon

Abstract

Background: Alveolar macrophages are sentinels of the pulmonary mucosa and central to maintaining immunological homeostasis. However, their role in governing the response to allergen is not fully understood. Inappropriate responses to the inhaled environment manifest as asthma.

Methods: We utilized a mechanistic IL-13-driven model and a house dust mite allergen mucosal sensitization model of allergic airway disease to investigate the role of alveolar macrophages in regulating pulmonary inflammation.

Results: IL-13-dependent eosinophilic and Th2 inflammation was enhanced in mice depleted of alveolar macrophages using clodronate liposomes. Similarly, depletion of alveolar macrophages during house dust mite sensitization or established disease resulted in augmented Th2 immunity and increased allergen-specific IgG1 and IgE. Clodronate treatment also delayed the resolution of tissue inflammation following cessation of allergen challenge. Strikingly, tissue interstitial macrophages were elevated in alveolar macrophage-deficient mice identifying a new homeostatic relationship between different macrophage subtypes. A novel role for the macrophage-derived immunoregulatory cytokine IL-27 was identified in modulating Th2 inflammation following mucosal allergen exposure.

Conclusions: In summary, alveolar macrophages are critical regulators of Th2 immunity and their dysregulation promotes an inflammatory environment with exacerbation of allergen-induced airway pathology. Manipulating IL-27 may provide a novel therapeutic strategy for the treatment of asthma.

Allergic asthma is a chronic inflammatory disease which has traditionally been attributed to atopy, eosinophilia, and excessive Th2 mediators (1). However, clinical trials to target pro-inflammatory Th2 pathways have had limited success (2–4). Alveolar macrophages (AMs) are thought to play a central role in maintaining immunological homeostasis and host defense in the lung (5–8). Critical to their function, they are situated in the airway lumen, in close proximity to the mucosal surface where typically, they are the first immune cell to encounter the myriad particles that are contained within the inhaled environment. Human studies have demonstrated that the frequency of AMs in asthmatics and healthy individuals is comparable (9); however, some functionality may be altered in disease. AMs retrieved from asthmatics induce higher levels of IL-5 from T-lymphocyte co-cultures compared to those from nonatopic individuals (10). Additionally,

AMs from pediatric asthmatics have impaired phagocytic ability and demonstrate greater apoptosis (11). In experimental systems, prior allergen exposure renders the lungs more susceptible to bacterial infection through desensitization of toll-like receptors (TLRs) and upregulation of endogenous negative regulators of TLR signalling expressed by AMs (12). Furthermore, GM-CSF, a mediator known to be upregulated in asthmatic bronchial epithelium (13), has been shown to inhibit the suppressive capacity of alveolar macrophages in animal studies (14, 15). Thus, it is possible that the development of asthma, its lack of resolution, and associated exacerbations may be intimately linked with a breakdown in AM-mediated homeostasis.

Although inappropriate responses to airborne antigens are thought to contribute to the chronic inflammatory environment observed in asthma, the particular role that AMs play

in controlling these responses is not well understood. Experimental studies utilizing rodents sensitized peripherally with OVA and the adjuvant alum prior to aerosolized OVA challenge have shown that depletion of AMs prior to challenge results in exacerbation of airway hyper-reactivity (16–19). However, AMs are resident within the airway mucosa and sample the inhaled environment; thus, peripheral sensitization models do not mimic how these cells encounter allergen in human disease. We have investigated the role of AMs at disease inception, during established inflammation and in promoting resolution of allergic airways disease (AAD) induced by repeated intranasal administration of house dust mite extract (HDM)—a clinically relevant allergen to which 50–85% of asthmatics are allergic to (20). We demonstrate that AMs are necessary to regulate mucosal sensitization to inhaled allergen. The macrophage-derived immune-regulatory cytokine IL-27 is implicated in maintaining pulmonary homeostasis. Moreover, evidence of a novel interaction between alveolar and interstitial macrophages was demonstrated. By identifying deficiencies in pulmonary defense and homeostatic mechanisms, we may be able to intervene to promote efficient resolution of inflammation and restore immune pulmonary homeostasis.

Methods

Female BALB/c mice (Charles River, Bicester, UK) 6–8 weeks old received intratracheal administration of either IL-13 (5 µg; Gibco, Frederick, MD, USA) or vehicle (PBS + 1% FCS) on three consecutive days. Mice receiving liposomes were dosed 2 days prior to the first IL-13 administration and killed 15 h after the last administration. Additionally, mice received either 25 µg HDM extract *Der-matophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC, USA) or PBS via intranasal instillation, 3 times per week for three weeks. Mice were killed either 4 h or 7 or 13 days postfinal HDM instillation. 50-µl liposome-encapsulated clodronate or PBS was administered intratracheally either once weekly, commencing 2 days prior to HDM exposure (sensitization phase) or twice during the final week of HDM exposure (challenge phase) or 1 and 5 days after the last HDM challenge (resolution phase). All mice were housed in specific pathogen-free conditions and provided food and water *ad libitum*. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Serum, bronchoalveolar lavage (BAL), lung tissue, and lung cells were collected. Differential counts were performed on Wright-Geimsa (Sigma, Poole, UK)-stained cytopins.

Disaggregated lung and BAL cells were stained with CD45, CD3, CD4, Siglec F, Gr-1, CD11c, CD11b (BD Biosciences, Oxford, UK), T1/ST2 (MD Biosciences, St. Paul, MN, USA), MOMA2, CD68 (AbD Serotec, Kidlington, UK) and F4/80 (eBiosciences, Hatfield, UK), or relevant isotype controls for 20 min at 4°C. Fixed cells were analyzed on a FACSCalibur™ using CellQuest (BD Biosciences).

Lung tissue supernatant and BAL were analyzed by ELISA; IL-4, IL-5 and IL-10 (PharMingen, Oxford, UK), IL-13 (eBioscience) and IL-27 (R&D systems, Abingdon,

UK), or ultra-sensitive MSD kits (Meso Scale Discovery, Rockville, MD, USA). Paired antibodies for IgE and IgG1 (R&D systems) were used to measure serum Ig levels.

Tissue lipids were extracted, and lipoxin A4 was quantified by LC/MS/MS.

F4/80+ CD11c+ AMs and F4/80+ CD11c- interstitial macrophages were cell-sorted from lung tissue and BALF. RNA was extracted and IL-27 levels determined by qPCR using specific primers (Applied Biosystems, Paisley, UK).

Additional details on the methods utilized in this study are provided in the Supporting Information (Data S1).

Data were analyzed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as single data points with bars representing group medians, unless otherwise stated. A two-tailed *P* value was determined by the Mann–Whitney *U*-test when comparing between 2 groups. Data shown represent means ± SEM of at least 2 independent experiments (*n* = 8–16).

Results

IL-13-induced Th2 airway pathology is amplified following the depletion of alveolar macrophages

To assess the role of AMs in the development of mucosal Th2 immunity, a short-term model of IL-13-induced lung inflammation was employed (21, 22) in macrophage-replete mice and animals devoid of AMs. Clodronate liposomes administered 2 days prior to IL-13 instillation (Fig. 1A) resulted in 80% depletion of AMs in BAL (Fig. 1B), without affecting tissue-resident interstitial macrophages (Fig. S1). The proinflammatory effects of IL-13 were amplified in the absence of AMs, demonstrated by increased accumulation of eosinophils, neutrophils, and Th2 cells in BAL (Fig. 1C–F). In addition, BAL IL-4 and IL-5 levels were elevated in AM-depleted mice (Fig. 1G and H), although no enhancement of IL-10 was observed (Fig. S2). These data suggest a protective role for AMs against development of Th2 immunity.

The absence of alveolar macrophages augments HDM-induced airway Th2 inflammation

To explore the role of AMs during allergen sensitization and the development of AAD, clodronate was administered prior to HDM exposure and throughout the allergen sensitization period. AMs were also depleted during the final week of allergen challenge to investigate the role of AMs in perpetuating established AAD (Fig. 2A).

House dust mite exposure increased accumulation of eosinophils, neutrophils and Th2 cells, and Th2 cytokines including IL-4, IL-5, and IL-10 in the BAL (Fig. 2B–H). Depletion of AMs prior to allergen sensitization or during challenge significantly exacerbated HDM-induced lung inflammation. The number of BAL leukocytes in clodronate-treated mice was at least twofold greater than in HDM-exposed mice receiving control liposomes (Fig. 2B). The absence of AMs resulted in increased numbers of both eosinophils and neutrophils (Fig. 2C and D). Concomitant with the enhanced

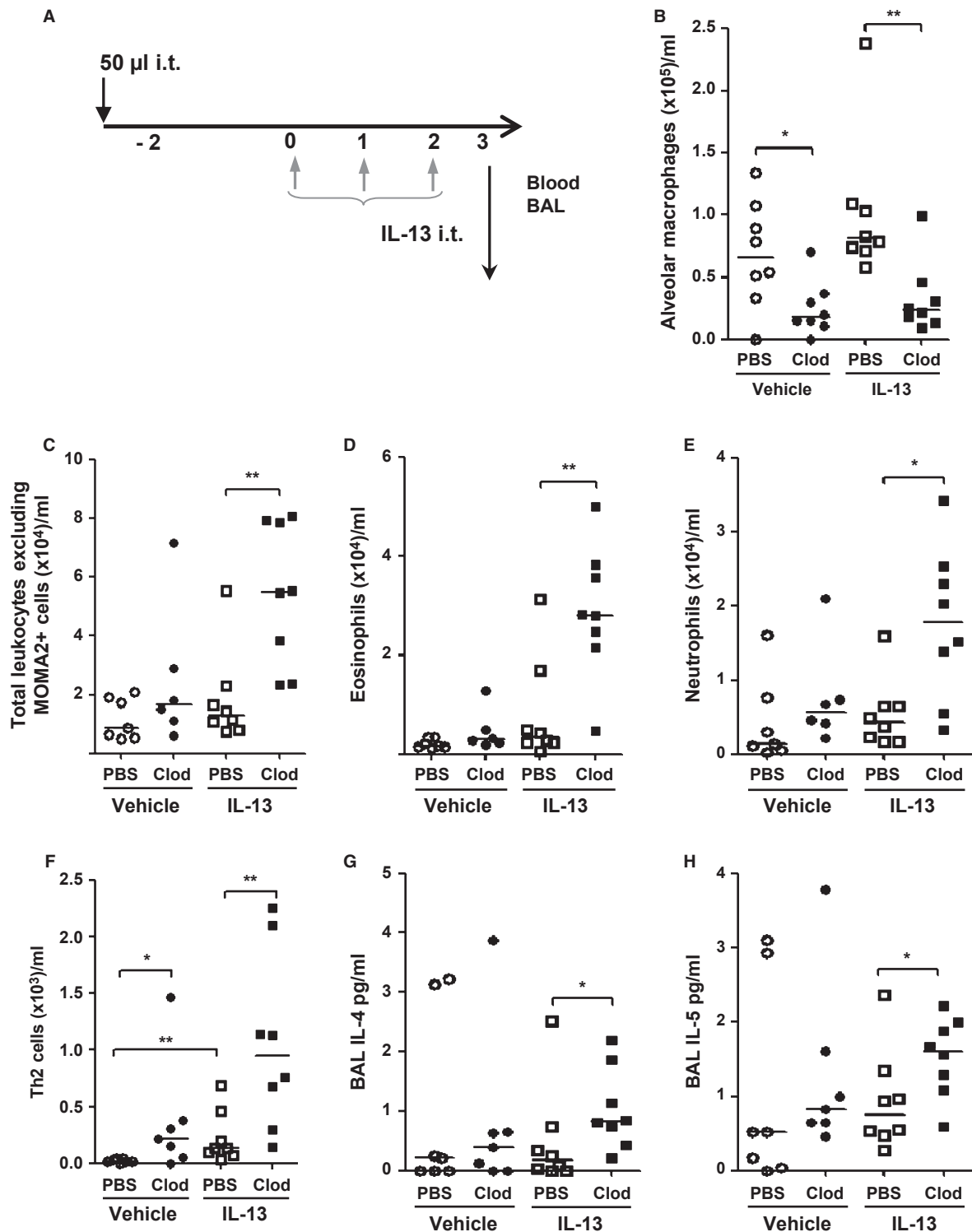


Figure 1 IL-13-induced Th2 airway pathology is amplified following the depletion of alveolar macrophages (A) Schematic depicting either PBS or clodronate (Clod) liposome and IL-13 administration protocol. Mice received either clodronate-containing liposomes (Clod) or PBS-containing liposomes (PBS). 48 hours later, mice received either 5 μ g of IL-13 or PBS + 1% FCS (vehicle) for 3 consecutive

days. Mice were culled 15 h later. (B) alveolar macrophages (C) total airway leukocytes (D) eosinophils (E) neutrophils (F) Th2 lymphocytes retrieved following broncho-alveolar lavage and quantified by flow cytometry. BAL fluid was analyzed for inflammatory cytokines by ELISA (G) IL-4 and (H) IL-5. * $P < 0.05$ and ** $P < 0.01$ relative to PBS control group by Mann–Whitney U -test, $n = 6$ –8.

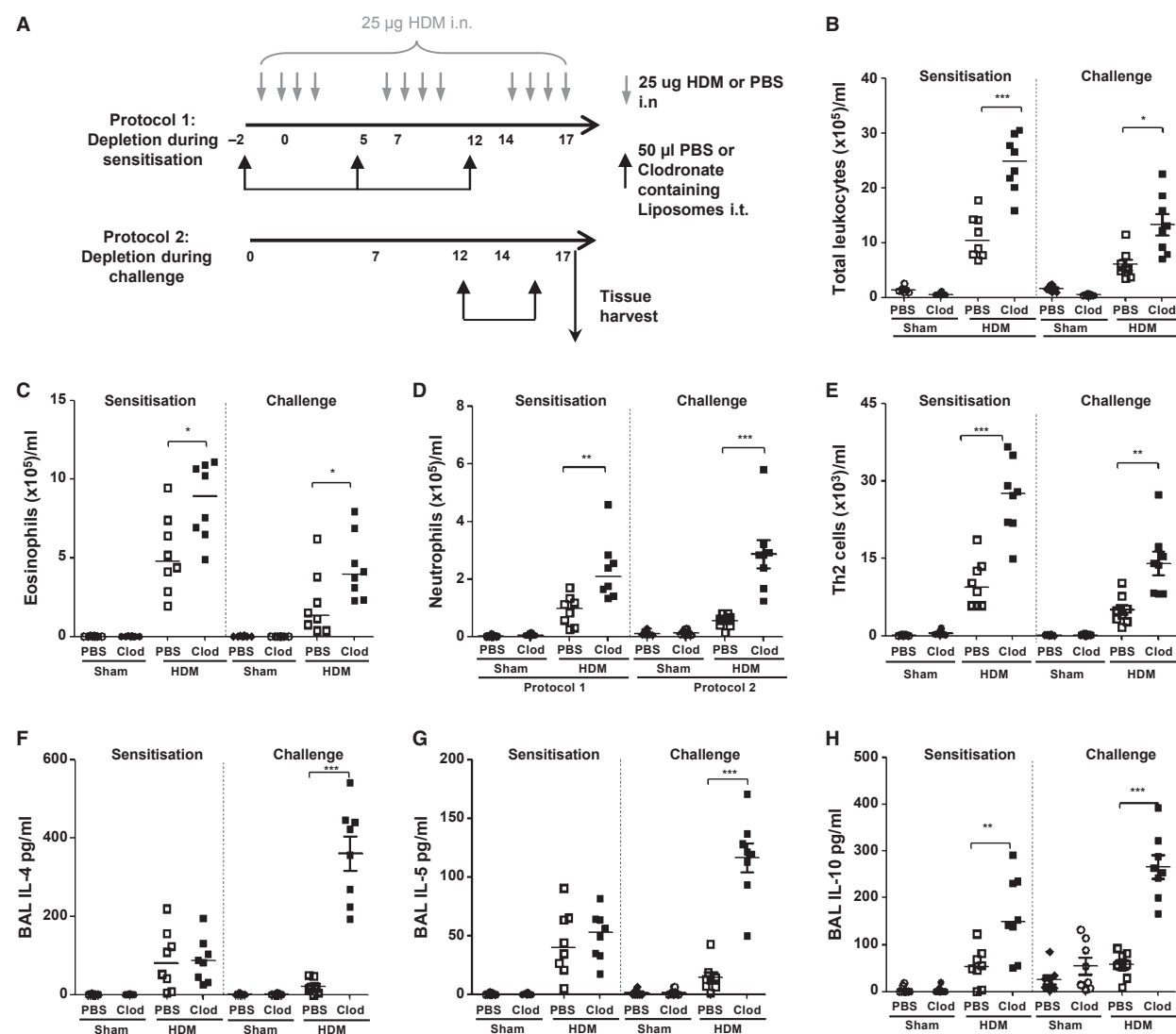


Figure 2 The absence of alveolar macrophages during mucosal allergen sensitization and challenge augments house dust mite-induced Th2 inflammation in the airways (A) Schematic depicting either PBS or clodronate (Clod) liposome and house dust mite (HDM) administration. (B) Total airway leukocytes, (C) eosinophils,

(D) neutrophils, and (E) Th2 lymphocytes retrieved following bronchoalveolar lavage and quantified by flow cytometry. BAL fluid was analyzed for inflammatory cytokines by ELISA (F) IL-4, (G) IL-5, and (H) IL-10. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ relative to PBS control group by Mann–Whitney U -test, $n = 6$ –8.

eosinophilia, eotaxin1/CCL11 was also increased in the BAL in the absence of AMs (Fig. S3A). In contrast, the neutrophil chemoattractant, KC/CXCL1, was only elevated when AMs were depleted during HDM challenge (Fig. S3B). In addition, AM deficiency during the sensitization or challenge phases significantly increased the number of Th2 cells in BAL (Fig. 2E). However, exaggerated production of the Th2 cytokines IL-4 and IL-5 was only observed when AMs were depleted during allergen challenge (Fig. 2F and G). No changes in HDM-induced IL-13 were detected (data not shown). Surprisingly, depletion of AMs during HDM sensitization or challenge further enhanced HDM-induced

production of IL-10, IL-12, and IFN γ (Fig. 2H and S3C and D). These findings indicate AMs are important for regulating the pulmonary immune response to inhaled allergen in the airways.

Alveolar macrophage depletion influences the leukocyte composition of lung tissue and augments systemic immune responses following mucosal HDM exposure

Depletion of AMs during either allergen sensitization or challenge significantly increased the number of tissue leukocytes recruited to the lung in response to HDM exposure

(Fig. 3A). Tissue-resident interstitial macrophages have also been proposed to play a key role in maintaining immune homeostasis in the respiratory tract (23). Therefore, it was important to verify that this population of macrophages was not eliminated by the clodronate treatment. AM depletion actually increased the number of interstitial macrophages in response to HDM (Fig. 3B and C), which was coincident with elevated levels of IL-10 (Fig. 2H). Thus, the augmentation of Th2 airway pathology in HDM-exposed clodronate-treated mice is not due to the deletion of regulatory tissue interstitial macrophages.

The absence of AMs during HDM sensitization specifically enhanced the systemic Th2 antibody response. Total IgE and HDM-specific IgE and IgG1 were significantly elevated in HDM-exposed mice pretreated with clodronate liposomes (Fig. 3D–F), whereas HDM-specific IgG2a was not modulated (Fig. 3G). The absence of AMs in the respiratory tract has far-reaching consequences both in the lung parenchyma and on systemic Th2 responses following allergen exposure.

Alveolar macrophages are elevated concomitant with the resolution of HDM-induced inflammation

We next sought to ascertain whether AMs play an active role in the resolution of HDM-induced AAD. Mediators of allergic disease were measured 7 and 13 days after the last allergen challenge and compared to peak inflammation, 4 h post-HDM (Fig. 4A). HDM-induced inflammation remained significantly elevated 7 days postfinal challenge and resolved to baseline levels by 13 days (Figs 4B and S4A). Tissue eosinophils remained at least threefold higher at 7 days, resolving by 13 days (Fig. 4C). In the BAL, inflammation was also predominantly eosinophilic. Numbers were significantly reduced by 13 days, but some residual eosinophils remained (Fig. S4B). HDM-induced neutrophilia, evident at peak inflammation, resolved more rapidly and returned to baseline by 7 days (Figs 4D and S4C). HDM-induced Th2 lymphocytes and IL-5 and IL-13 levels remained elevated 7 days post-HDM and resolved by day 13 (Fig. 4E–G). Lipoxin A4 was induced following HDM exposure, and elevated levels were measured throughout the resolution time course indicating that lipoxin A4 is involved in the active resolution of inflammation (Fig. 4H). FoxP3⁺ T-regulatory cells remained significantly elevated at 7 days and returned to baseline levels by 13 days after the final allergen exposure (Fig. 4I). AMs were significantly elevated following cessation of allergen challenge (Fig. 4J) implying that these cells play an active role in directing the return to pulmonary homeostasis.

Return to homeostasis is delayed following the removal of alveolar macrophages

Alveolar macrophages were depleted during the resolution phase (Fig. 5A), and parameters of airway inflammation were assessed 14 days postfinal HDM exposure. Administration of clodronate liposomes during the resolution phase prevented the clearance of HDM-induced airway inflammatory cells (Fig. 5B). Eosinophilia, which resolves by day 14, was

not altered by macrophage depletion (Fig. 5C); however, HDM-induced neutrophilia and Th2 cells failed to resolve in the absence of AMs (Fig. 5D and E). No change in the profile of IL-4, IL-5, or IL-13 levels was observed as a result of depleting AMs during resolution (data not shown). However, levels of KC/CXCL1 and MDC/CCL22 remained elevated at 14 days correlating with the neutrophil and T-cell numbers in clodronate-treated mice (Fig. 5F), whereas eotaxin1/CCL11 levels returned to baseline with resolution of eosinophilia.

The interstitial macrophage population was not modulated by exposure to HDM or AM depletion alone; however, numbers of pulmonary interstitial macrophages were increased in HDM-exposed mice treated with clodronate during the resolution phase. (Fig. 5G).

Exacerbation of HDM-induced disease is associated with reduced levels of IL-27

We sought to determine whether immunoregulatory mediators were modulated as a consequence of macrophage depletion. TGF- β was not significantly altered following alveolar macrophage manipulation (data not shown), and we demonstrated elevated IL-10 levels (Fig. 2H). Thus, we hypothesized that there may be a role for the myeloid-derived, immunoregulatory cytokine IL-27 in our models. To confirm AMs were a source of IL-27, we analyzed FACS-sorted cells and demonstrated the expression of IL-27 in this population (Fig. 6A).

In the lung tissue, levels of IL-27 were upregulated in response to IL-13 or repeated HDM exposure (Fig. 6B and C). Interestingly, the depletion of AMs also increased baseline levels of IL-27 in nonallergic mice. However, clodronate treatment during the sensitization phase resulted in a reduction of HDM-induced IL-27 concomitant with exacerbation of Th2 pathology. Depletion of AMs during the resolution phase did not modulate tissue levels of IL-27 which return to baseline by 14 days. These data demonstrate that AMs are a source of IL-27 and suggest a specific temporal role for IL-27 in regulating Th2 responses at mucosal surfaces.

Discussion

This work characterizes for the first time the immunomodulatory role of AMs in a model of inhaled allergen-induced AAD. It is evident that AMs are critical for the maintenance of pulmonary homeostasis and disruption of this axis results in uncontrolled Th2 inflammation in response to HDM and IL-13. We also show that AMs drive the resolution of inflammation following allergen challenge. In healthy individuals, the primary role of AMs is in maintaining or restoring homeostasis; however, in asthmatics, inappropriate or insufficient AM activity may perpetuate chronic inflammation by lowering the immune activation threshold.

Activation of mucosal epithelial cells in response to inhaled particulates can lead to the infiltration of granulocytes, which release harmful cytotoxic mediators intended to protect host tissues; however, left unchecked, these are potentially deleterious to the local microenvironment (24). Thus, granulocyte

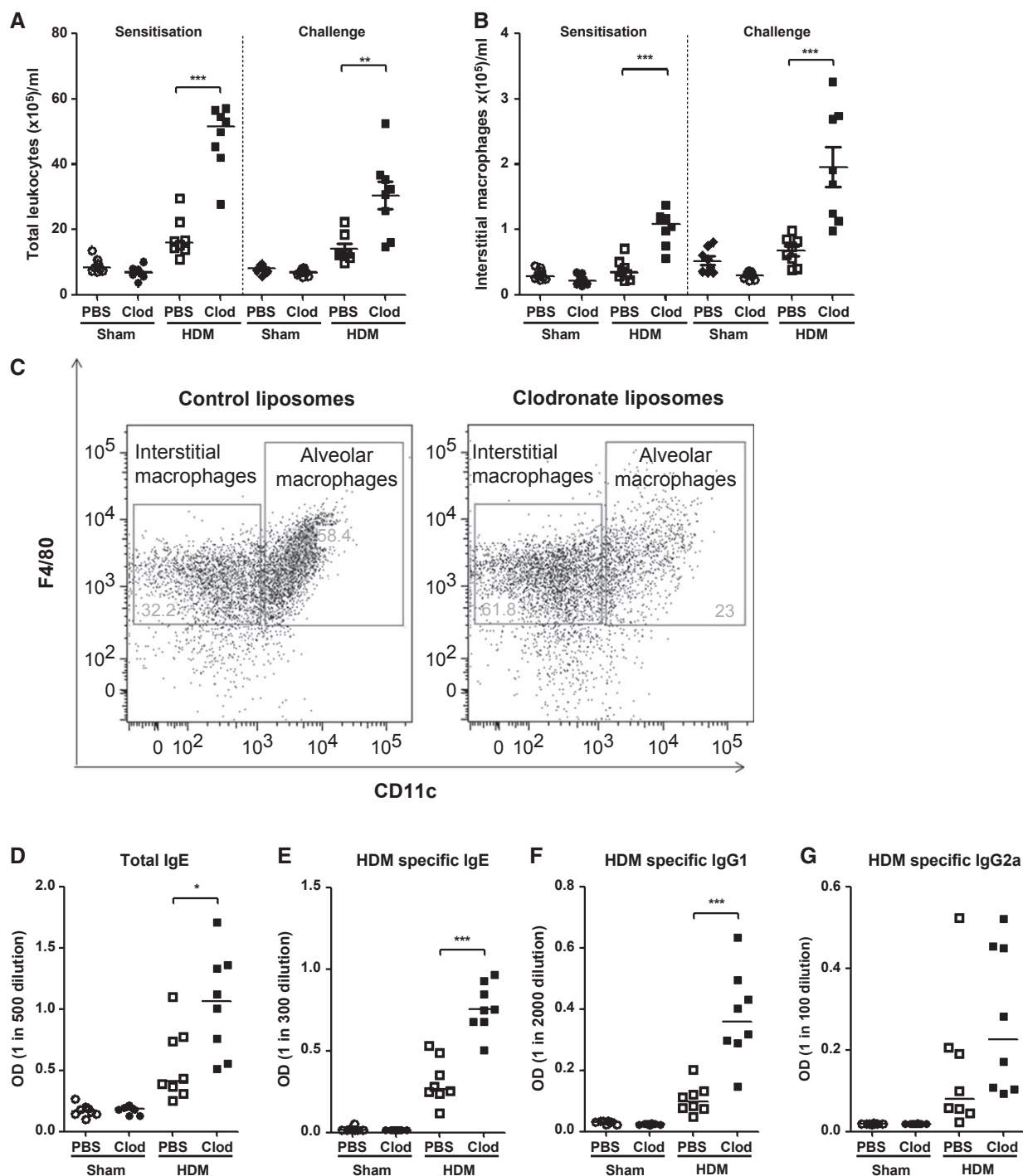


Figure 3 Disruption of alveolar macrophage-directed homeostasis promotes an increase in interstitial lung tissue macrophages and enhances the systemic immune response following HDM mucosal challenge (A) Total tissue leukocytes and (B) lung interstitial macrophages quantified by flow cytometry. (C) FACS plots depicting lung

alveolar and interstitial macrophage populations. (D) Total IgE, (E) HDM-specific IgE, (F) HDM-specific IgG1, and (G) HDM-specific IgG2a in serum following clodronate treatment during sensitization quantified by ELISA. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ relative to PBS control group by Mann-Whitney U -test, $n = 6-8$.

accumulation warrants tight regulation to preserve tissue integrity (25, 26). Signals from apoptotic granulocytes induce their efferocytosis by local tissue macrophages (26), and our

data show that macrophages are essential to limit the accumulation of eosinophils and neutrophils in response to HDM exposure, promoting a return to tissue homeostasis.

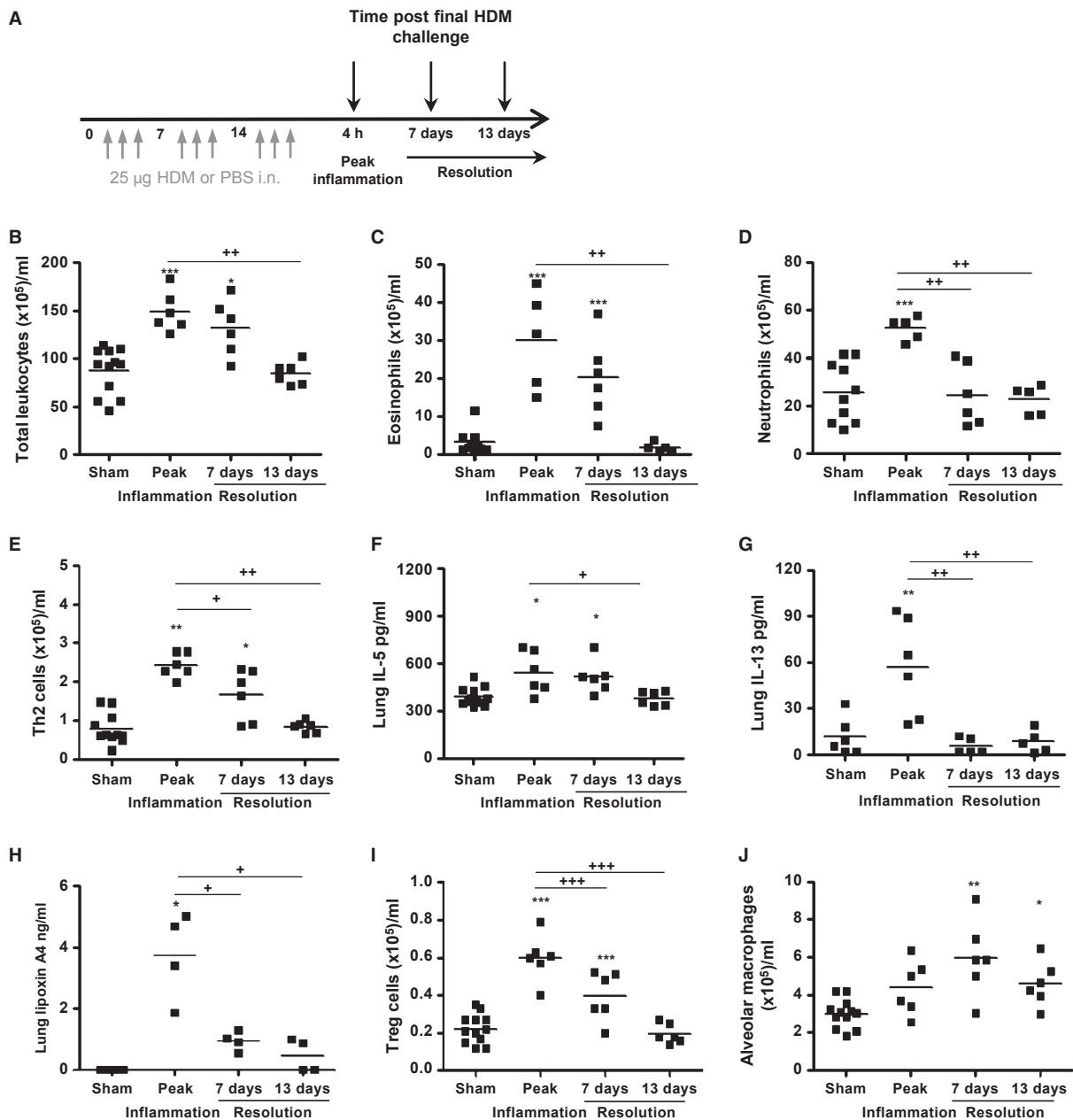


Figure 4 Alveolar macrophages are elevated concomitant with the resolution of mucosal inflammation (A) Schematic depicting PBS or HDM, (B) total tissue leukocytes, (C) eosinophils, and (D) neutrophils were quantified by differential counting. (E) Th2 lymphocytes quantified by flow cytometry. Th2 cytokine levels in lung tissue homogenate were quantified by ELISA (F) IL-5 and (G) IL-13. (H) LC/MS/MS was used to quantify lung tissue lipoxin A4.

House dust mite-challenged mice-lacking AMs during the sensitization phase had increased Th2 cell recruitment compared to macrophage-replete mice. When clodronate was administered during the HDM challenge phase, once inflammation was established, Th2 immunity was also augmented, with increased accumulation of Th2 cells within

(I) $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ T-regulatory lymphocytes and (J) alveolar macrophages were quantified by flow cytometry. At each time point $n = 4\text{--}12$ mice/group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ relative to PBS control group by Mann-Whitney U -test. $+P < 0.05$, $++P < 0.01$, and $+++P < 0.001$ relative to peak inflammation, 4-h time point group by Mann-Whitney U -test.

the airways and elevated IL-4 and IL-5. Cytokines were quantified 5 days and 24 h after the last clodronate treatment in the sensitization and challenge protocols, respectively. Thus, the dysregulation of Th2 cytokines following AM depletion appears to be transient and wanes as AMs begin to repopulate the airways. Our data corroborate the studies

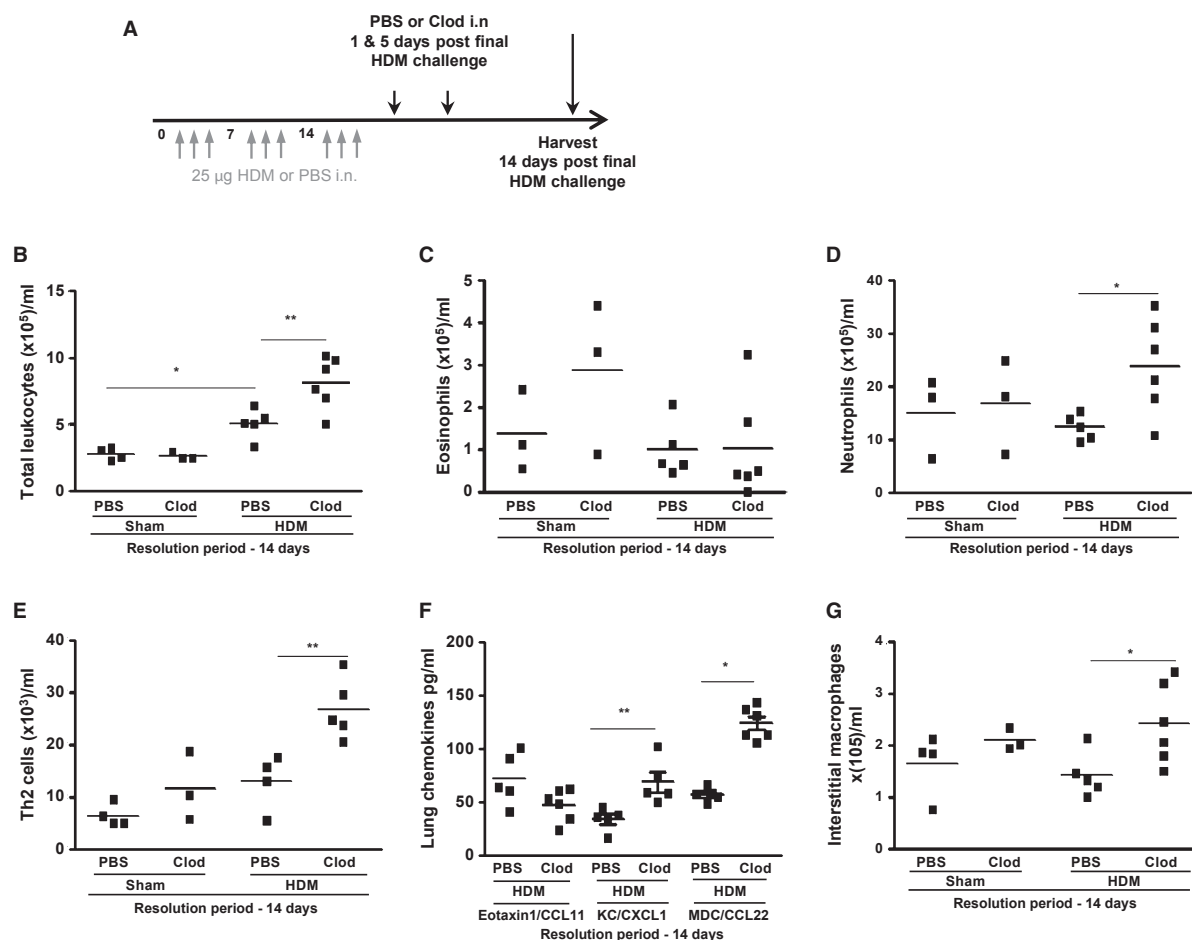


Figure 5 The return to homeostasis after allergen challenge is disrupted following the removal of alveolar macrophages (A) Schematic depicting PBS or clodronate (Clod) liposome administration protocol following the induction of allergic airways disease. (B) airway leukocytes retrieved following broncho-alveolar lavage and (C) tissue eosinophils; (D) tissue neutrophils deter-

mined by differential counting. (E) Lung tissue Th2 lymphocytes quantified by flow cytometry. (F) lung tissue chemokines measured by ELISA. (G) interstitial macrophages determined by flow cytometry, $n = 3-6$ mice/group, bar represents median. * $P < 0.05$ and ** $P < 0.01$ relative to PBS control group by Mann-Whitney U -test, $n = 3-6$.

utilizing the OVA model in which augmented airway pathology was observed following AM removal (17–19). However, our work further demonstrates that AMs constrain granulocyte and effector T-cell accumulation in response to inhaled allergen exposure irrespective of prior sensitization. In addition, we have established a critical role for AMs during the different stages of disease pathogenesis.

Our data demonstrate that the immune activation threshold is reduced in the absence of AMs and that these cells limit HDM-induced allergic inflammation during established disease suggesting that AMs maintain a regulatory phenotype throughout acute HDM exposure. In contrast, adoptive transfer of AMs from OVA-sensitized mice fails to restore homeostasis compared to transfer of naive AMs (19). It was proposed that OVA sensitization and challenge altered the phenotype of AMs to produce inflammatory cytokines and lose their suppressive capacity. However, *ex vivo* culture of allergen-sensitized AMs were shown to downregulate their pro-

inflammatory phenotype and restore their homeostatic functions when subsequently transferred (27, 28). This exploitation of macrophage plasticity makes these cells an interesting potential target for therapy (29).

We investigated the role of AMs in promoting resolution and restoring homeostasis by depleting AMs after cessation of HDM exposure. This resulted in delayed resolution of Th2 immunity, indicating that AMs are involved in the clearance of T-cell infiltrates recruited to the lung as a result of allergen exposure.

Augmented inflammation was apparent within the lung tissue and airway lumen as a result of the loss of AM-directed homeostasis. Perhaps unexpectedly, the number of interstitial macrophages in lung tissue was significantly increased during exacerbated AAD. This expansion of interstitial macrophages following the depletion of AMs was apparent in all three HDM protocols indicating a direct relationship between these two regulatory macrophage cell types. Interstitial macrophages

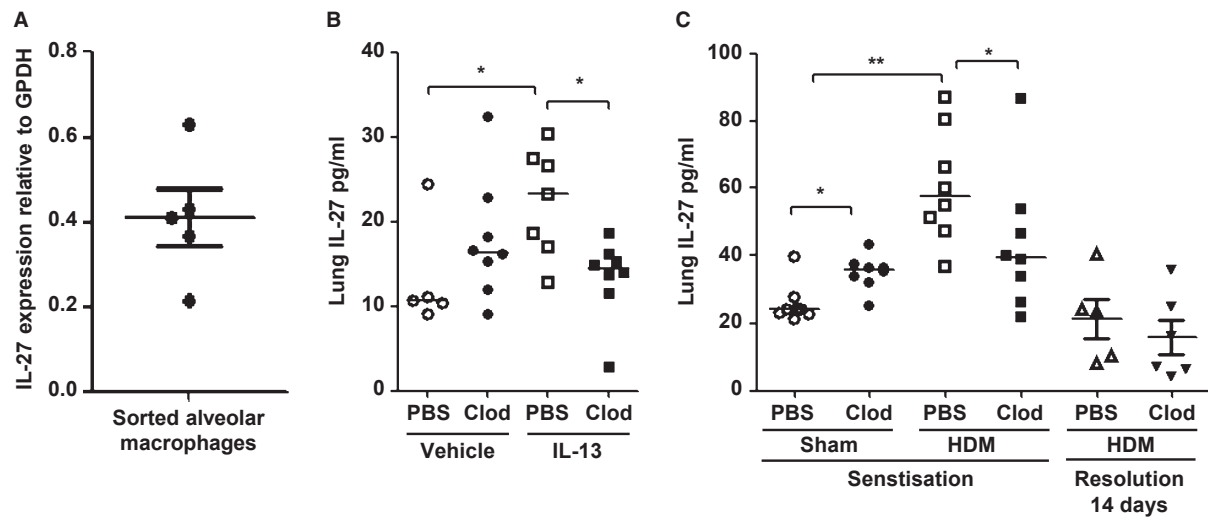


Figure 6 Exacerbation of IL-13- and HDM-induced disease following alveolar macrophage depletion is associated with reduced levels of the immuno-regulatory cytokine IL-27. IL-27 mRNA expression in lung FACS-sorted macrophages analyzed by qPCR (A)

F4/80⁺ CD11c⁺ alveolar macrophages, (B) IL-13-treated mice, and (C) HDM-treated mice with alveolar macrophage depletion during sensitization and during resolution * $P < 0.05$ and ** $P < 0.01$ relative to PBS control group by Mann–Whitney U -test, $n = 5$ –8.

have been reported to regulate airway inflammation via TLR4-dependent IL-10 secretion (23). Additionally, interstitial macrophages have been shown to express high levels of eotaxin1/CCL11 (30). HDM extract contains TLR4 agonists (31), and enhanced levels of IL-10 and eotaxin1/CCL11 were apparent in our studies concomitant with the increased number of interstitial macrophages. This suggests that interstitial macrophage functionality remains intact during exacerbated AAD and that the regulation of this subset is impaired in the absence of AMs. The role of interstitial macrophages remains unclear in the context of asthma. Production of eotaxin1/CCL11 and IL-10 suggests they have the potential to exert a dual role in an allergic setting and further investigation is required. From our studies, we clearly demonstrate that an important axis exists between airway and tissue macrophage subsets.

The potent effects of removing AMs led us to investigate potential mediators responsible for this dysregulated homeostasis. IL-27 is predominantly produced by macrophages and dendritic cells. This cytokine is upregulated in steroid refractory asthmatics (32) and has been shown to modulate airway hyper-reactivity via downregulation of glucocorticoid receptor signalling in macrophages (32). IL-27 also dampens Th2 cell polarization and cytokine production in naïve CD4⁺ T-cell cultures and ameliorates OVA-induced allergic inflammation when administered *in vivo* (33). In the gut, IL-27 has been shown to mediate intestinal epithelial cell barrier protection via transcriptional activation of anti-inflammatory and antibacterial pathways (34). We demonstrate for the first time that IL-27 is upregulated in the lung following the administration of either exogenous IL-13 or HDM and that AMs are a source of this cytokine. These data demonstrate that IL-27 is a macrophage-derived immune-regulatory mediator which regulates the pulmonary mucosal response to allergen.

In summary, we show that AMs are integral in the regulation of inflammatory responses to inhaled allergen. We also show for the first time a direct relationship between airway alveolar and tissue interstitial macrophage populations. Thus, macrophages play a critical role in the maintenance and restoration of homeostasis following allergen exposure at the airway mucosa.

Acknowledgments

The authors thank Lorraine Lawrence for histological sectioning and staining. This work was supported by the Wellcome trust (grant number 087618/Z/08/Z).

Author contributions

SAM and KLD conducted the majority of the experiments, analyzed the data, and wrote the manuscript; SAW, VD, MM, and VBO'D performed some of the experimental work; LGG performed some of the experimental work and edited the article; and CML designed the study, supervised the project, and edited the article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alveolar macrophages are depleted following clodronate treatment.

Figure S2. IL-10 levels are not modulated in response to IL-13 and are unaffected by macrophage depletion.

Figure S3. Alveolar macrophage depletion augments house dust mite induced chemokine and cytokine secretion.

Figure S4. Resolution of inflammation following cessation of allergen challenge.

Data S1. Materials and methods.

References

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodelling. *Am J Respir Crit Care Med* 2000;**161**:1720–1745.
- Holgate ST, Davies DE. Rethinking the pathogenesis of asthma. *Immunity* 2009;**31**:362–367.
- Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;**13**:345–350.
- Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;**18**:716–725.
- Wissinger E, Goulding J, Hussell T. Immune homeostasis in the respiratory tract and its impact on heterologous infection. *Semin Immunol* 2009;**21**:147–155.
- Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 2008;**8**:142–152.
- Naessens T, Vander Beken S, Bogaert P, Van Rooijen N, Lienenklaus S, Weiss S et al. Innate imprinting of murine resident alveolar macrophages by allergic bronchial inflammation causes a switch from hypoinflammatory to hyperinflammatory reactivity. *Am J Pathol* 2012;**181**:174–184.
- Thepen T, Kraal G, Holt PG. The role of alveolar macrophages in regulation of lung inflammation. *Ann NY Acad Sci* 1994;**725**:200–206.
- St-Laurent J, Turmel V, Boulet LP, Bissonnette E. Alveolar macrophage subpopulations in bronchoalveolar lavage and induced sputum of asthmatic and control subjects. *J Asthma* 2009;**46**:1–8.
- Tang C, Rolland JM, Li X, Ward C, Bish R, Walters EH. Alveolar macrophages from atopic asthmatics, but not atopic nonasthmatics, enhance interleukin-5 production by CD4⁺ T cells. *Am J Respir Crit Care Med* 1998;**157**:1120–1126.
- Fitzpatrick AM, Holguin F, Teague WG, Brown LA. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. *J Allergy Clin Immunol* 2008;**121**:1372–1378.
- Habibzay M, Saldana JI, Goulding J, Lloyd CM, Hussell T. Altered regulation of Toll-like receptor responses impairs antibacterial immunity in the allergic lung. *Mucosal Immunol* 2012;**5**:524–534.
- Sousa AR, Poston RN, Lane SJ, Nakhosteen JA, Lee TH. Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am Rev Respir Dis* 1993;**147**:1557–1561.
- Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP et al. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J Clin Invest* 1998;**102**:1704–1714.
- Bilyk N, Holt PG. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1993;**177**:1773–1777.
- Thepen T, Hoebe K, Breve J, Kraal G. Alveolar macrophages down-regulate local pulmonary immune responses against intratracheally administered T-cell-dependent, but not T-cell-independent antigens. *Immunology* 1992;**76**:60–64.
- Careau E, Bissonnette EY. Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness. *Am J Respir Cell Mol Biol* 2004;**31**:22–27.
- Careau E, Turmel V, Lauzon-Joset JF, Bissonnette EY. Alveolar macrophages reduce airway hyperresponsiveness and modulate cytokine levels. *Exp Lung Res* 2010;**36**:255–261.
- Bang BR, Chun E, Shim EJ, Lee HS, Lee SY, Cho SH et al. Alveolar macrophages modulate allergic inflammation in a murine model of asthma. *Exp Mol Med* 2011;**43**:275–280.
- Gregory LG, Causton B, Murdoch JR, Mathie SA, O'Donnell V, Thomas CP et al. Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clin Exp Allergy* 2009;**39**:1597–1610.
- Tomlinson KL. Mechanisms of IL-13 inhibition of allergic inflammation. PhD. Imperial College London, 2010.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;**282**:2258–2261.
- Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada CF, Henry E et al. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest* 2009;**119**:3723–3738.
- Nathan C. Points of control in inflammation. *Nature* 2002;**420**:846–852.
- Leitch AE, Duffin R, Haslett C, Rossi AG. Relevance of granulocyte apoptosis to resolution of inflammation at the respiratory mucosa. *Mucosal Immunol* 2008;**1**:350–363.
- Savill J, Haslett C. Granulocyte clearance by apoptosis in the resolution of inflammation. *Semin Cell Biol* 1995;**6**:385–393.
- Careau E, Proulx LI, Pouliot P, Spahr A, Turmel V, Bissonnette EY. Antigen sensitization modulates alveolar macrophage functions in an asthma model. *Am J Physiol Lung Cell Mol Physiol* 2006;**290**:L871–L879.
- Pouliot P, Spahr A, Careau E, Turmel V, Bissonnette EY. Alveolar macrophages from allergic lungs are not committed to a pro-allergic response and can reduce airway hyperresponsiveness following ex vivo culture. *Clin Exp Allergy* 2008;**38**:529–538.
- Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodeling. *J Pathol* 2013;**229**:176–185.
- Siddiqui S, Secor ER Jr, Silbart LK. Broncho-alveolar macrophages express chemokines associated with leukocyte migration in a mouse model of asthma. *Cell Immunol* 2013;**281**:159–169.
- Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;**457**:585–588.
- Li JJ, Wang W, Baines KJ, Bowden NA, Hansbro PM, Gibson PG et al. IL-27/IFN- γ induce MyD88-dependent steroid-resistant airway hyperresponsiveness by inhibiting glucocorticoid signaling in macrophages. *J Immunol* 2010;**185**:4401–4409.
- Yoshimoto T, Yoshimoto T, Yasuda K, Mizuguchi J, Nakanishi K. IL-27 suppresses Th2 cell development and Th2 cytokines production from polarized Th2 cells: a novel therapeutic way for Th2-mediated allergic inflammation. *J Immunol* 2007;**179**:4415–4423.
- Diegelmann J, Olszak T, Goke B, Blumberg RS, Brand S. A novel role for interleukin-27 (IL-27) as mediator of intestinal epithelial barrier protection mediated via differential signal transducer and activator of transcription (STAT) protein signaling and induction of antibacterial and anti-inflammatory proteins. *J Biol Chem* 2012;**287**:286–298.

Figure S1.

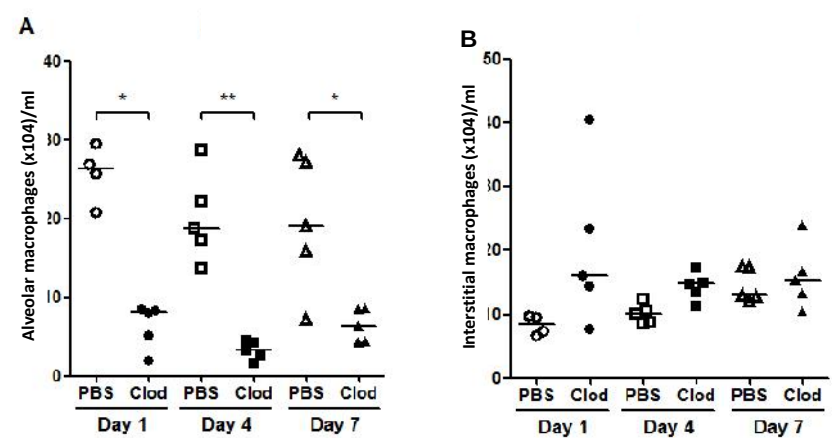


Figure S2.

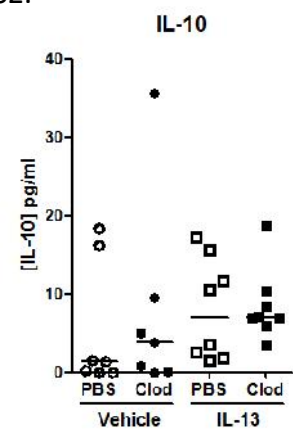


Figure S3.

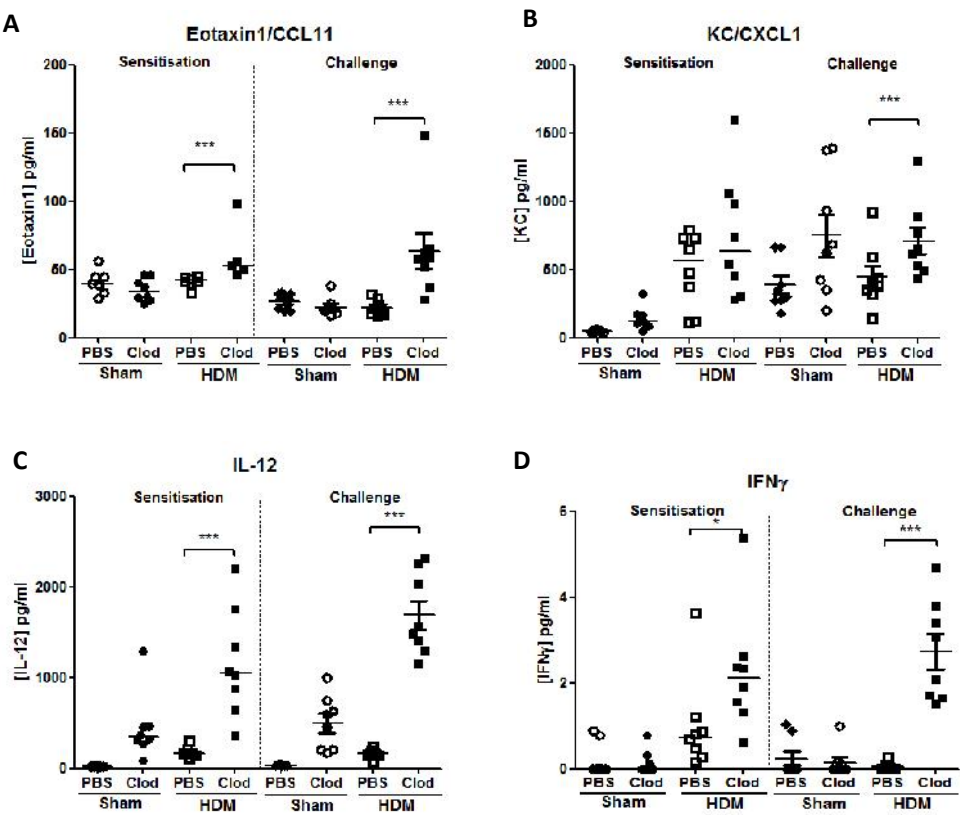
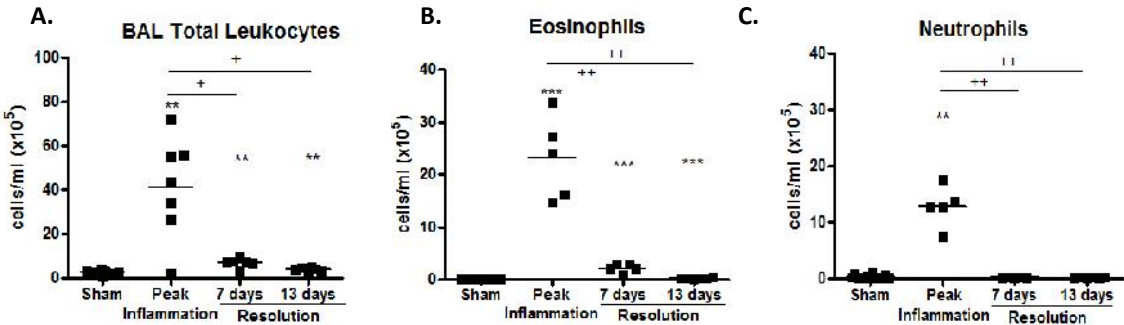


Figure S4.



Supplementary data

Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge.

Sara A. Mathie, Kate L. Dixon, Simone A. Walker, Victoria Tyrrell, Madhav Mondhe, Valerie B. O'Donnell, Lisa G. Gregory, & Clare M. Lloyd

Materials and Methods

Measurement of AHR

Measurements of dynamic resistance and compliance were performed using The Flexivent system (Scireq, Montreal, Canada). After induction of anaesthesia with an i.p. injection of Pentobarbitone (50 mg/Kg) (Sigma, UK) and i.m. injection of Ketamine (200 mg/Kg) (Fortdodge Animal Health Ltd, Southampton, UK), mice were tracheostomised and connected to the Flexivent ventilator via a blunt-ended 19-gauge needle. Mice were ventilated using the following settings; tidal volume of 7 ml/kg body weight, 150 breaths/minute; positive end-expiratory pressure approximately 2 cm H₂O. Standardisation of the lung volume history was done by performing two deep inflations. Subsequently, measurements of airway function were made. Measurements of resistance and compliance were determined from a user defined protocol using the snapshot-150 perturbation, which is a single frequency sinusoidal waveform. Resultant data was fitted using multiple linear regression to the single compartment model to determine the above parameters. The resultant data was fitted to the Constant-Phase model to determine the above parameters. Changes in lung/airway function parameters were measured in response to increasing concentrations of nebulised MCh from 3 - 100 mg/ml, delivered into the inspiratory line of the flexivent ventilator. Airway resistance

was expressed as cmH₂O.s/ml, elastance was expressed as cmH₂O/mL and compliance was expressed as mL/cmH₂O.

Collection and preparation of samples

Bronchoalveolar lavage (BAL) was collected by lavage of the lungs three times with 0.4 mL of PBS via a tracheal cannula. BAL fluid was centrifuged (700 *g*, 5 min, 4°C) and cells were recovered and resuspended in 0.5ml RPMI. BAL cell supernatants were removed and analyzed for cytokines by ELISA. To disaggregate the cells from the lung tissue, one lobe (~100 mg) of lung was incubated at 37°C for 1 h in digest reagent (0.15 mg/ml collagenase type D, 25 µg/ml DNase type I) in complete RPMI media. The recovered cells were filtered through a 70-µm nylon sieve, washed twice, resuspended in 1ml complete media, and counted in a hemocytometer prior to cytocentrifugation. Lung and BAL cells were applied to glass slides by centrifugation (5x10⁴ cells/slide) and stained with Wright-Giemsa (Thermo Fisher Scientific Inc, Waltham, MA). Percentages of macrophages, lymphocytes/mononuclear cells, eosinophils and neutrophils were determined under 40x magnification by counting cells in 8 randomly selected fields and dividing this number by the total number of cells counted. To obtain absolute numbers, this percentage was multiplied by the total number of cells recovered in 1 ml of lavage fluid and lung digest suspension which were normalised for the weight of the lung.

Lipid extraction and quantitation

For quantification, 10ng of each 12-HETE-*d*₈ and PGE2-*d*₄ was added to BALF or lung homogenate before extraction as deuterated internal standards. Lipids were extracted by

adding a solvent mixture [1 M acetic acid, 2-propanol, hexane (2:20:30, v/v/v)] to the sample at a ratio of 2.5mL solvent mixture/1mL sample, vortexing and then adding 2.5 mL hexane. After centrifugation, lipids were recovered in the upper hexane layer. The samples were then re-extracted by addition of an equal volume of hexane. The combined hexane layers were dried under vacuum and analyzed for free eicosanoids using liquid chromatography-coupled to electrospray ionisation tandem mass spectrometry (LC-MS/MS ABI SCIEX 4000 QTRAP) as follows: Lipids were separated on a C₁₈ Spherisorb ODS2 column (150 x 4.6 mm, 5µm particle, Waters Ltd, Elstree, UK) using a gradient of 50–90% B over 30 min, followed by 5 min at 90% B (A, water : acetonitrile : acetic acid, 75 : 25 : 0.1, B, methanol : acetonitrile : acetic acid, 60 : 40 : 0.1) with a flow rate of 1 mL/min. Eicosanoid species were monitored with specific parent to daughter ion transitions in negative ion mode ([M-H]⁻) for LXA4 (*m/z* 351.2) at 115. 12-HETE-*d*₈ and PGE₂-*d*₄ was monitored at *m/z* 327 to 184 and *m/z* 335.2 to 275 respectively. Products were identified and quantified using primary standards and internal standard run in parallel under the same method conditions.

Macrophage cell sort

Whole lung was digested as previously described. Isolated leukocytes were incubated with FITC conjugated CCR3 and Ly6C, F4/80 (PeCY7), CD11c (PE) and CD11b (APC). Cells were negatively selected for CCR3 and Ly6C to remove eosinophils and neutrophils. F4/80+ cells were sorted for CD11c and CD11b expression. Alveolar macrophages were defined as CD11c+ and IMs as CD11c-. Cells were sorted into RLT buffer for RNA extraction.

Quantitative PCR

Quantitative PCR was performed on FACS sorted macrophages. Briefly, RNA was prepared from cell isolated as per manufacturer's instructions (Qiagen). cDNA was synthesised using oligo(dT)-primed reverse transcriptase reactions. (Applied Biosystems). Quantitative PCR

was performed using taqman probes for IL-27 and GAPDH. Levels of IL-27 mRNA were normalised to GAPDH.

Figure Legends

Figure S1. Alveolar macrophages are depleted following clodronate treatment

Mice received either clodronate containing liposomes (Clod) or PBS containing liposomes (PBS). (A) Alveolar macrophage and (B) Interstitial macrophages numbers 1, 4 and 7 days post PBS or Clod administration quantified by flow cytometry. * $p < 0.05$ and ** $p < 0.01$ relative to PBS control group by Mann Whitney test, $n = 4-5$.

Figure S2. IL-10 levels are not modulated in response to IL-13 and are unaffected by macrophage depletion

BAL fluid was analysed for IL-10 by ELISA. $n = 7-8$.

Figure S3. Alveolar macrophage depletion augments house dust mite induced chemokine and cytokine secretion

BAL fluid was analysed for inflammatory cytokines (A) eotaxin, (B) KC, (C) IL-12 and (D) IFN- γ by ELISA. * $p < 0.05$ and *** $p < 0.001$ relative to PBS control group by Mann Whitney test, $n = 5-8$.

Figure S4. Resolution of inflammation following cessation of allergen challenge

(A) Total leukocytes (B) eosinophils and (C) neutrophils recovered from the BALF were quantified by differential counting. ** $p < 0.01$ and *** $p < 0.001$ relative to PBS control group

by Mann Whitney test. ⁺ p<0.05 and ⁺⁺ p<0.01 relative to peak inflammation, 4 hour time point group by Mann Whitney test, n=5-8.