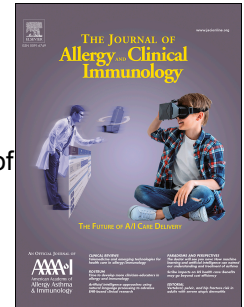


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Clearance of Apoptotic cells by Lung Alveolar Macrophages Prevents Development of House Dust Mite-induced Asthmatic Lung Inflammation

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Clearance of Apoptotic cells by Lung Alveolar Macrophages Prevents Development of House Dust Mite-induced Asthmatic Lung Inflammation

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Abstract

Background: Poor clearance of apoptotic cells has been suggested to contribute to severe asthma, but whether uptake of apoptotic cells by lung phagocytes might dampen House Dust Mite (HDM)-induced lung inflammation has not been shown.

Objective: We investigated if apoptotic cell engulfment in the murine lung impacts the development of allergen-induced asthmatic airway inflammation and which immune modulating mechanisms were activated.

Methods: Apoptotic cells were infused into the lungs of mice challenged with HDM allergen and lung inflammation, expression of suppressive molecules, and induction of Treg were monitored. Additionally, an adenosine receptor agonist was tested to study the mechanism of suppression elicited by apoptotic cells.

Results: Apoptotic cell uptake by lung alveolar macrophages suppressed HDM-driven allergic asthma. This was associated with promoting the Treg-inducing molecule retinoic acid, inhibiting inflammatory cytokine production, and making macrophages more susceptible to receiving suppressive signals from adenosine. Correspondingly, adenosine receptor agonist treatment also limited HDM-driven allergic airway inflammation through an action on alveolar macrophages.

Conclusion: These data provide insight into the mechanisms by which lung macrophages

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dampen allergen-induced airway inflammation. They suggest that targeting lung macrophages to increase their phagocytic capacity, enhance their ability to make retinoic acid, dampen their capacity to make inflammatory cytokines, and increase their responsiveness to adenosine, could be useful to suppress allergic responses.

Clinical Implications/Key Messages

Targeting lung macrophages in asthmatic patients to increase their ability to promote Treg and increase their responsiveness to adenosine could be useful to suppress allergic responses.

Capsule summary

Apoptotic cell clearance by lung alveolar macrophages limits allergic asthma induced by House Dust Mite (HDM) through promoting regulatory T cell-inducing molecules, inhibiting inflammatory cytokine production, and via receiving suppressive signals from adenosine.

Key Words

Apoptotic cell clearance, Asthma, Alveolar macrophage, Regulatory T cell, Adenosine

Abbreviations

HDM: House Dust Mite; Treg: regulatory T cells; OVA: Ovalbumin; DC: Dendritic cells; BALF:

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55 Bronchoalveolar lavage fluid; Apo: Apoptotic cells; CL: Clodronate

56 **Introduction**

57 House dust mite (HDM) is one of the most common aeroallergens, inducing type 2
58 cytokine responses which promote eosinophil recruitment to the airways. Allergenicity is
59 associated with both mite antigenic proteins and proteases, and with ligands derived from
60 mite-associated bacterial and fungal products (1). Understanding mechanisms to suppress these
61 insults in the lungs could then provide insights into how to treat allergic asthma.

62 We previously reported that macrophages in the mouse lung can drive the generation of
63 Foxp3⁺ regulatory T cells (Treg) that promote airway tolerance (2), suggesting that enhancing
64 macrophage suppressive activity could limit allergenicity of house dust mites. How macrophages
65 differentiate into this regulatory state or how it can be augmented is still largely unclear (3).
66 Impaired apoptotic cell clearance has been associated with lung disease (4-6), and reduced
67 efferocytosis of macrophages from asthmatics has been noted (7, 8). Moreover, DNA release
68 from secondary necrosis of apoptotic cells can directly accelerate Th2 responses (9, 10). In line
69 with this, mice deficient in Mer, a molecule important for clearing apoptotic cells by macrophages,
70 exhibited delayed resolution of Th2 lung inflammation induced by ovalbumin (11). Therefore, as
71 macrophages are thought to be the primary lung phagocyte, their regulatory ability might be
72 related to uptake of apoptotic cells. We investigated this by intratracheally infusing apoptotic cells

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into the lungs of mice to determine their impact on macrophage activity and HDM-induced airway inflammation. We report that apoptotic cell clearance by alveolar macrophages can limit HDM allergic asthma, through promoting molecules that induce peripheral Treg, as well as inhibiting inflammatory cytokine production, and making macrophages more susceptible to adenosine-derived suppressive signals.

Methods

For detailed methods, please see the Methods section in this article's Online Repository at www.jacionline.org.

Results and Discussion

Mice given apoptotic thymocytes intratracheally, compared to viable cells, showed decreased eosinophil recruitment, Th2 cytokine production, and overall reduced histologic signs of lung inflammation, upon exposure to HDM allergen (**Fig. 1A-C**). Infusion of apoptotic Jurkat cells also inhibited development of this asthmatic phenotype (**Fig. 1D**). Moreover, after sensitization with allergen, apoptotic cell infusion still reduced airway inflammation upon rechallenge with allergen (**Fig. 1E**). Through adoptive transfer of naïve Foxp3-negative OVA-reactive CD4 T cells and challenge with OVA, we correlated this suppression with a

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significant increase in the number of antigen-specific Foxp3⁺ Treg generated in vivo (**Fig. 1F**). TGF- β and retinoic acid are two primary factors that promote the development of Foxp3⁺ Treg. In accordance, after apoptotic cell injection, lung tissue mRNA for RALDH1 and RALDH2, enzymes that drive retinoic acid production, was significantly up-regulated, and TGF- β was high, albeit unchanged (**Fig. 1G**). These results show that apoptotic cells can suppress HDM-driven lung inflammation and suggested that modifying antigen-presenting cells (APC) to induce Treg could be central to the action of engulfing apoptotic cells.

Alveolar macrophages display a strong capacity to phagocytose apoptotic cells compared to macrophages found in the lung interstitium, spleen, or liver (12). CFSE-labeling showed that intratracheally injected apoptotic cells were taken up by ~70% of BAL and ~30% of lung tissue alveolar macrophages (CD11b⁺SiglecF^{high}CD11c⁺) (**Fig. 2A**). This was not modified significantly by intranasal HDM (data not shown). How uptake of apoptotic cells by alveolar macrophages might control airway homeostasis is not understood (3, 13), but one report linked this with impaired signaling through TLR9 (12). We analyzed sorted alveolar macrophages for Treg-inducing molecules after in vivo engulfment of CFSE-labeled apoptotic cells. They expressed mRNA for TGF- β and RALDH2 at high, albeit similar levels, compared to macrophages from mice not injected with apoptotic cells, but showed significant up-regulation of RALDH1 and also IL-10 (**Fig. 2B**). Correspondingly, the in vitro co-culture of alveolar

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macrophages with apoptotic cells also showed up-regulation of RALDH1 with a trend to greater IL-10 (**Fig. 2C**). We then sorted CFSE-labeled vs. unlabeled lung macrophages and co-cultured them with OVA and naïve OVA-reactive CD4 T cells, and found they were significantly better at driving the upregulation of Foxp3 in vitro (**Fig. 2D**). Therefore, uptake of apoptotic cells by alveolar macrophages is associated with inhibition of allergic lung inflammation and a greater propensity for these APC to promote formation of regulatory T cells. Analysis of M1 or M2 markers found that the alveolar macrophages overall had a more M2 phenotype (Arg1, Ym1, Fizz1) which was unchanged with uptake of apoptotic cells (data not shown).

We next depleted alveolar macrophages by i.t. injection of clodronate liposomes. In these mice, suppression of HDM-driven lung inflammation by apoptotic cells was not observed (**Fig. 2E**). In addition, we sorted lung macrophages that engulfed apoptotic cells in vivo, and found that they suppressed eosinophilia when transferred into the lungs of HDM-challenged mice (**Fig. 2F**). Similar trends were seen when analyzing BAL Th2 cytokines. This strongly suggests that apoptotic cell uptake by alveolar macrophages was required for reducing the inflammation and Th2 responses caused by the allergen.

Previously, it was reported that airway epithelial cells could phagocytose apoptotic cells (14), but in our model, we did not find any significant number of CD45 negative lung cells that engulfed CFSE-labeled apoptotic cells (data not shown). However, a small population of

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CD11b⁺CD8⁺ DC took up apoptotic cells (**Fig. 2A**). Similar splenic CD8⁺ DC are thought to be specialized to capture dying cells and induce Treg (15). The CD8⁺ DC were undetectable in the naïve lung, and appeared in the lungs ~24 hours after apoptotic cell infusion (data not shown), likely recruited by signals from the lung macrophages that phagocytosed apoptotic cells. In support of this, the macrophages showed strong induction of XCL1, the chemokine ligand for XCR1 which is expressed on CD8⁺ DC (**Fig. 2G**). Moreover, the lung CD8⁺ DC showed higher expression of RALDH2 and IL-10 suggesting they may also reinforce suppression and Treg induction downstream of the alveolar macrophages (**Fig. 2H**).

In addition to upregulation of RALDH1, the in vitro co-culture of sorted alveolar macrophages and apoptotic cells also led to reduced TNF and IL-6 expression when macrophages were stimulated with HDM (**Fig. 3A**). Furthermore, CFSE⁺ lung macrophages that engulfed apoptotic cells in vivo exhibited significantly lower levels of mRNA for TNF and IL-6 (**Fig. 3B**). These cytokines can promote multiple inflammatory activities, and can also antagonize Treg induction, with their neutralization restoring the Treg-promoting activity of differentiated lung macrophages (2). This then represents a complementary way in which apoptotic cells may lead to suppression of allergen-induced inflammation. Expanding this notion, we found that mRNAs for the suppressor of cytokine signaling molecule, SOCS3, and adenosine A2A and A2B receptors, *Adora2a* and *Adora2b*, were significantly up-regulated in CFSE-labeled macrophages

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(**Fig. 3C**). SOCS3 expression and adenosine signaling in macrophages both can limit inflammatory cytokines, and have been associated with enhanced generation of Treg (3, 16-18).

Further reinforcing the idea that enhancing adenosine receptor expression could be of significance to suppression, we treated macrophages that engulfed apoptotic cells (CFSE+) with an A2A receptor agonist (ATL), and found that this significantly increased their ability to generate Treg in vitro (**Fig. 4A**). This correlated with an increase in mRNA for RALDH1 (**Fig. 4B**). Moreover, ATL administered intranasally to mice reduced airway inflammation induced by HDM (**Fig. 4C-E**). Lastly, when alveolar macrophages were depleted in vivo by chlodronate liposome treatment, we found that suppression of airway inflammation by ATL was abrogated (**Fig. 4F**). These data are in line with our previous report that adenosine agonist treatment attenuated neutrophilic lung inflammation induced by OVA and LPS, and increased Treg numbers, via an action on lung macrophages (19).

In summary, our results indicate that apoptotic cell clearance by lung alveolar macrophages can limit airway inflammation to HDM, and is associated with enhanced adenosine activity, suppressed inflammatory cytokine production, and the generation of Treg. While a therapeutic approach with apoptotic cell infusion is being considered for inflammatory diseases, and can also prevent inflammation in experimental models as diverse as GvHD, sepsis, Type1 diabetes, arthritis, EAE, and CHS (20), understanding the mechanistic basis for how apoptotic

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163 cells induce suppression may be more important for formulating practical therapies. Targeting
164 lung alveolar macrophages in severe asthmatic patients to increase their phagocytosis of
165 apoptotic cells and increase their responsiveness to adenosine could be useful to suppress
166 allergic responses.

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References

1. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends in immunology*. 2011;32(9):402-11.
2. Soroosh P, Doherty TA, Duan W, Mehta AK, Choi H, Adams YF, et al. Lung-resident tissue macrophages generate Foxp3⁺ regulatory T cells and promote airway tolerance. *The Journal of experimental medicine*. 2013;210(4):775-88.
3. Grabiec AM, Hussell T. The role of airway macrophages in apoptotic cell clearance following acute and chronic lung inflammation. *Seminars in immunopathology*. 2016;38(4):409-23.
4. McCubbrey AL, Curtis JL. Efferocytosis and lung disease. *Chest*. 2013;143(6):1750-7.
5. Szondy Z, Garabuczi E, Joos G, Tsay GJ, Sarang Z. Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications. *Frontiers in immunology*. 2014;5:354.
6. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nature reviews Immunology*. 2014;14(3):166-80.
7. Erriah M, Pabreja K, Fricker M, Baines KJ, Donnelly LE, Bylund J, et al. Galectin-3 enhances monocyte-derived macrophage efferocytosis of apoptotic granulocytes in asthma. *Respiratory research*. 2019;20(1):1.
8. Fernandez-Boyanapalli R, Goleva E, Kolakowski C, Min E, Day B, Leung DY, et al. Obesity impairs apoptotic cell clearance in asthma. *The Journal of allergy and clinical immunology*.

Miki et al.,

185 2013;131(4):1041-7, 7 e1-3.

186 9. Imanishi T, Ishihara C, Badr Mel S, Hashimoto-Tane A, Kimura Y, Kawai T, et al. Nucleic acid
187 sensing by T cells initiates Th2 cell differentiation. *Nature communications*. 2014;5:3566.

188 10. Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, et al. DNA released from
189 dying host cells mediates aluminum adjuvant activity. *Nature medicine*. 2011;17(8):996-1002.

190 11. Felton JM, Lucas CD, Dorward DA, Duffin R, Kipari T, Vermeren S, et al. Mer-mediated
191 eosinophil efferocytosis regulates resolution of allergic airway inflammation. *The Journal of*
192 *allergy and clinical immunology*. 2018;142(6):1884-93 e6.

193 12. Roberts AW, Lee BL, Deguine J, John S, Shlomchik MJ, Barton GM. Tissue-Resident
194 Macrophages Are Locally Programmed for Silent Clearance of Apoptotic Cells. *Immunity*.
195 2017;47(5):913-27 e6.

196 13. Draijer C, Peters-Golden M. Alveolar Macrophages in Allergic Asthma: the Forgotten Cell
197 Awakes. *Current allergy and asthma reports*. 2017;17(2):12.

198 14. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, et al. Apoptotic
199 cell clearance by bronchial epithelial cells critically influences airway inflammation. *Nature*.
200 2013;493(7433):547-51.

201 15. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, et al. CD8⁺ CD205⁺
202 splenic dendritic cells are specialized to induce Foxp3⁺ regulatory T cells. *Journal of immunology*.

Miki et al.,

2008;181(10):6923-33.

16. Carow B, Rottenberg ME. SOCS3, a Major Regulator of Infection and Inflammation. *Frontiers in immunology*. 2014;5:58.

17. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. *Nature reviews Immunology*. 2014;14(2):81-93.

18. Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature reviews Drug discovery*. 2008;7(9):759-70.

19. Pei H, Linden J. Adenosine influences myeloid cells to inhibit aeroallergen sensitization. *American journal of physiology Lung cellular and molecular physiology*. 2016;310(10):L985-92.

20. Saas P, Daguindau E, Perruche S. Concise Review: Apoptotic Cell-Based Therapies-Rationale, Preclinical Results and Future Clinical Developments. *Stem cells (Dayton, Ohio)*. 2016;34(6):1464-73.

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Figure Legends

Fig. 1. Apoptotic cells suppress HDM-driven airway inflammation. **A-D**, Viable or apoptotic (Apo) thymocytes (A-C) or Jurkat cells (D) were given i.t. to mice prior to HDM sensitization and challenge. BAL total cells, eosinophils, Th2 cytokines. Lung H&E and inflammation score. n = 3-9 mice/group. **E**, Viable or apoptotic thymocytes were given to mice on day 14 after sensitization followed by HDM challenge. BAL eosinophils. Lung inflammation score. n = 8 mice/group. **F**, CD45.1 Foxp3⁻ OT-II T cells were transferred into CD45.2 recipients subsequently exposed to OVA with viable or apoptotic thymocytes. Foxp3⁺ OT-II T cell accumulation in the lung draining LN. n = 3-4 mice/group. **G**, Total lung mRNA after apoptotic cell transfer. n = 3 mice/group. All data representative of 3 experiments.

Fig. 2. Apoptotic cells trigger Treg-associated activity in alveolar macrophages. **A**, CFSE⁺ apoptotic thymocytes were given i.t. BAL and lung cells analyzed for uptake (black lines). **B**, mRNA in CFSE⁺ macrophages from BAL and lung 4hr after transfer of apoptotic cells. **C**, mRNA in alveolar macrophages co-cultured with apoptotic cells in vitro. **D**, Foxp3⁻ OT-II T cells were cultured in vitro with OVA and sorted CFSE⁺ or CFSE⁻ macrophages. Level and % Foxp3⁺ T cells. **E**, Alveolar macrophages were depleted with clodronate liposomes (left) before sensitization with HDM. BAL eosinophils and Th2 cytokines. n = 4-5 mice/group. **F**, Naïve or CFSE⁺ alveolar

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macrophages (Apo) were given to recipients prior to sensitization with HDM. BAL eosinophils and Th2 cytokines. n = 6-9 mice/group. **G**, Xcl1 mRNA expression in CFSE⁺ macrophages. **H**, mRNA in CFSE⁺ vs. naive CD8⁺ DC. All data representative of 3 experiments.

Fig. 3. Uptake of apoptotic by alveolar macrophages results in anti-inflammatory activities.

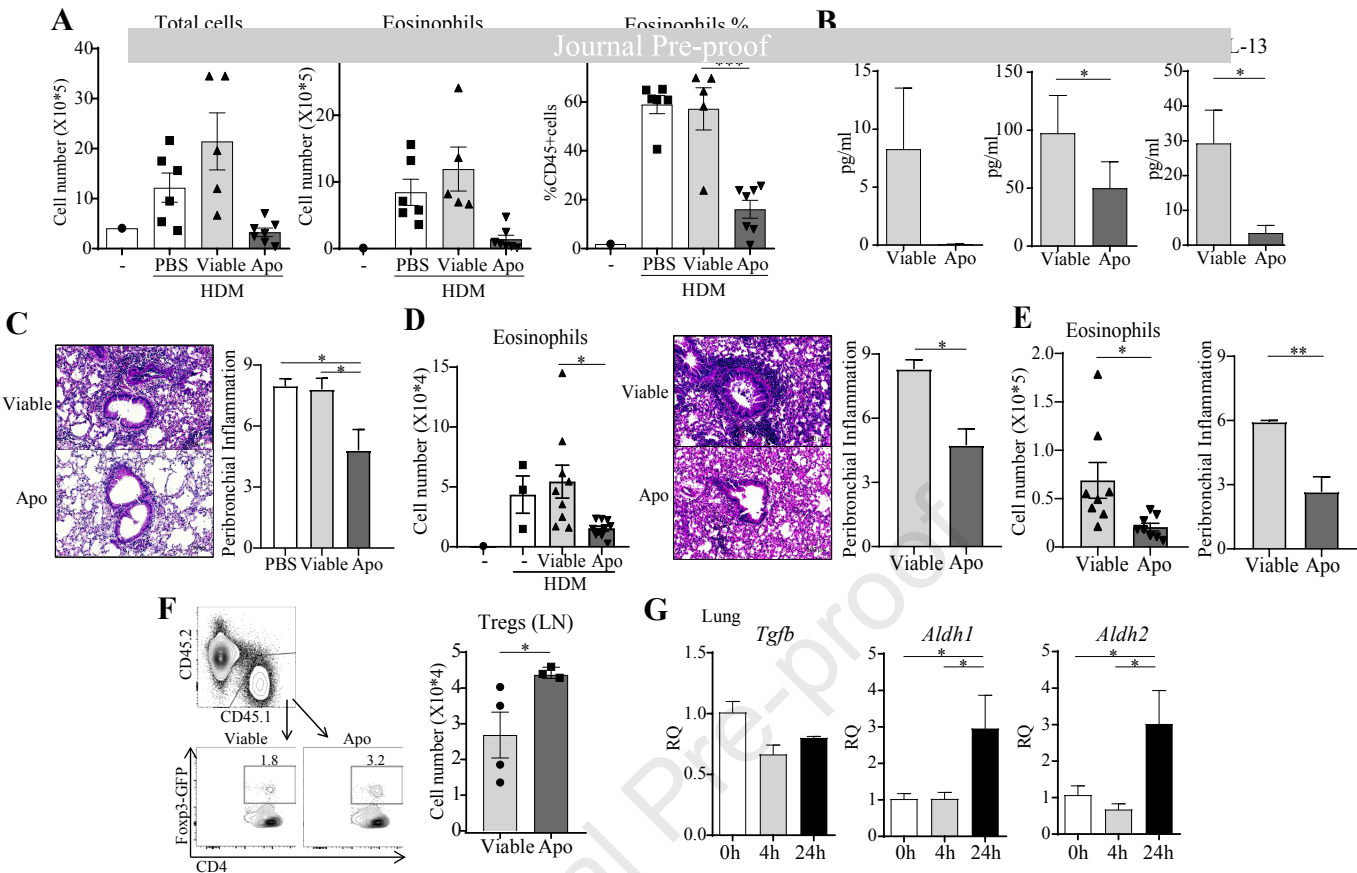
A, mRNA in naive alveolar macrophages co-cultured with apoptotic cells in vitro with or without HDM. **B-C**, mRNA in CFSE⁻ or CFSE⁺ macrophages sorted from BAL and lung, compared to macrophages from mice given PBS. n = 6 mice/group. All data representative of 2-3 experiments.

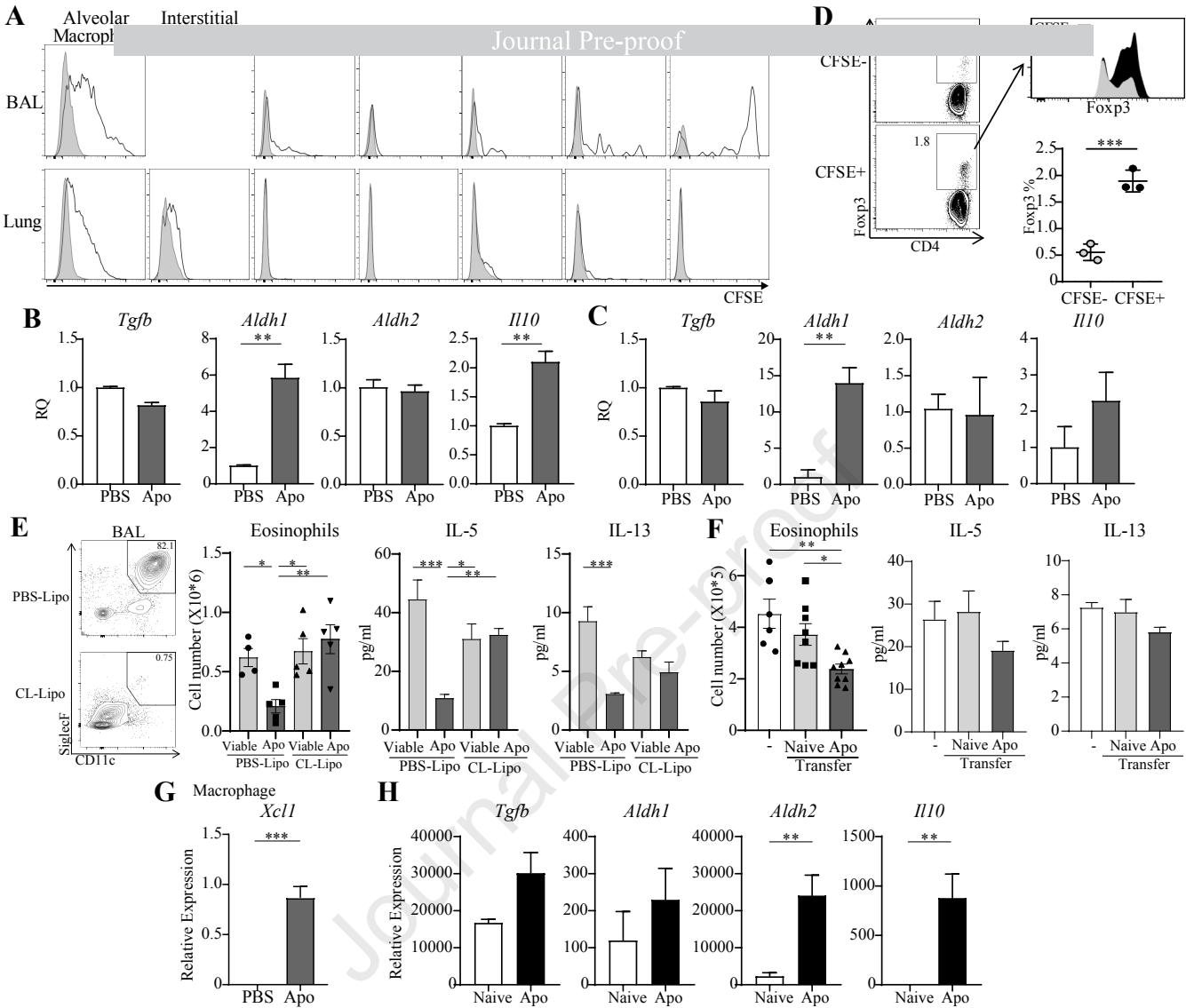
Fig. 4. Adenosine receptor signaling promotes suppressive activity in alveolar

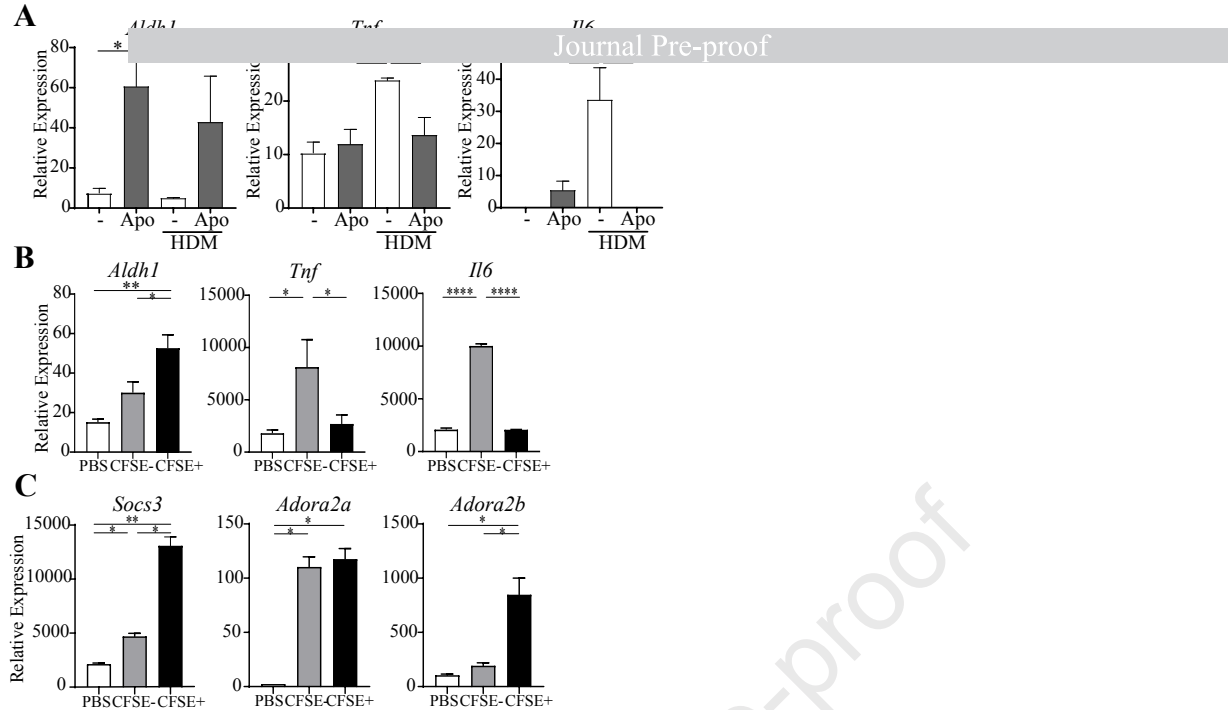
macrophages. A, Foxp3⁺ OT-II T cells were cultured with OVA and naïve or CFSE⁺ macrophages sorted from BAL and lung, in the presence or absence of ATL. % Foxp3⁺ T cells. **B**, mRNA in alveolar macrophages cultured with ATL in vitro. **C-E**, Mice were given ATL and sensitized and challenged as in Fig. 1 with HDM. BAL total cells, eosinophils, Th2 cytokines, lung H&E, and inflammation score. n = 6-9 mice/group. **F**, Alveolar macrophages were depleted with clodronate liposomes before treatment with ATL and sensitization with HDM. BAL eosinophils and Th2 cytokines. n = 5 mice/group. All data representative of 2-3 experiments.

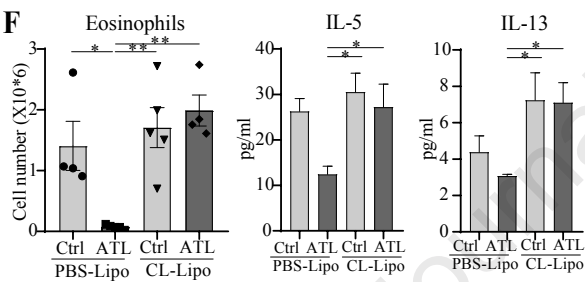
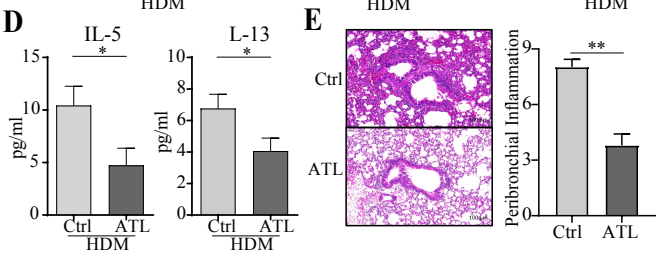
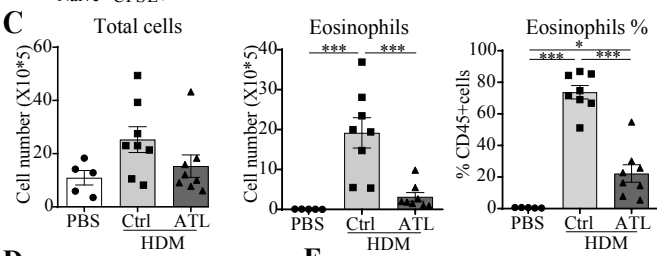
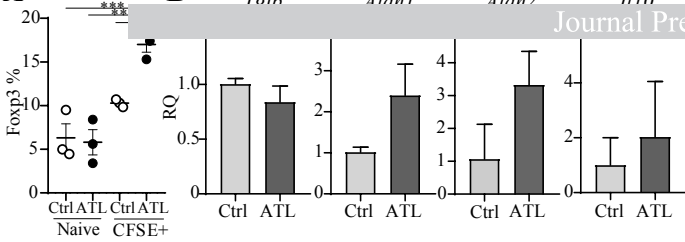
TableE1

Gene		Sequence
<i>TGFβ</i>	forward	5'-CCCTATATTTGGAGCCTGGA-3'
<i>TGFβ</i>	reverse	5'-GTTGGTTGTAGAGGGCAAGG-3'
<i>Aldh1</i>	forward	5'-ATGGTTTAGCAGCAGGACTCTTC-3'
<i>Aldh1</i>	reverse	5'-CCAGACATCTTGAATCCACCGAA-3'
<i>Aldh2</i>	forward	5'-GACTTGTAGCAGCTGTCTTCACT-3'
<i>Aldh2</i>	reverse	5'-TCACCCATTTCTCTCCCATTTC-3'
<i>Il10</i>	forward	5'-TGAATTCCTGGGTGAGAAG-3'
<i>Il10</i>	reverse	5'-GCTCCACTGCCTTGCTCTTA-3'
<i>TNF</i>	forward	5'-AACTAGTGGTGCCAGCCGAT-3'
<i>TNF</i>	reverse	5'-CTTCACAGAGCAATGACTCC-3'
<i>Il6</i>	forward	5'-GACAAAGCCAGAGTCCTTCAGAGAG-3'
<i>Il6</i>	reverse	5'-CTAGGTTTGCCGAGTAGATCTC-3'
<i>Adora1</i>	forward	5'-ATCCCTCTCCGGTACAAGACAGT-3'
<i>Adora1</i>	reverse	5'-ACTCAGGTTGTTCCAGCCAAAC-3'
<i>Adora2a</i>	forward	5'-CCGAATTCCACTCCGGTACA-3'
<i>Adora2a</i>	reverse	5'-CAGTTGTTCCAGCCCAGCAT-3'
<i>Adora2b</i>	forward	5'-TCTTCCTCGCCTGCTTCGT-3'
<i>Adora2b</i>	reverse	5'-CCAGTGACCAAACCTTTATACCTGA-3'
<i>Socs3</i>	forward	5'-ATGGTCACCCACAGCAAGTTT-3'
<i>Socs3</i>	reverse	5'-TCCAGTAGAATCCGCTCTCCT-3'
<i>Xcl1</i>	forward	5'-TTCTCCTCCTGACTTTCCTG-3'
<i>Xcl1</i>	reverse	5'-AACTGAGATGAGCTGCTTAAC-3'









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Online Methods

Mice

6-8 weeks old, male C57BL/6J (CD45.2+) mice were purchased from the Jackson Laboratory.

Foxp3/GFP reporter C57BL/6-SJL (CD45.1+) OT-II TCR transgenic mice were bred in house (1).

All experiments were conducted according to the guidelines of the La Jolla Institute for Immunology's Institutional Animal Care and Use Committee.

Apoptotic cell induction and transfer

Thymocytes from 4-6 week old C57BL/6J mice, or Jurkat T cells, were irradiated with 1mJ/cm² of UV-C followed by incubation in RPMI (Thermo Fisher Scientific, Waltham, MA) containing 10%FBS at 37°C for 4 hours. Annexin V and PI were stained using an AnnexinV Apoptosis Detection Kit (Thermo Fisher Scientific) to determine the frequency of apoptotic cells by flow cytometry. Apoptotic cells were labeled with CFSE using with Vybrant™ CFDA SE Cell Tracer Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Freshly isolated thymocytes or Jurkat cells were used as viable cell controls.

Allergic airway inflammation

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Mice were sensitized by administration of 50 µg HDM protein (Greer Laboratory, Lenoir, NC) given intranasally on day 0, and challenged with 25 µg HDM protein intranasally on days 7 and 14. 10×10^6 viable or apoptotic cells were transferred intratracheally in 50 µl PBS to the mice 4hr before the sensitization on day 0. Lung inflammation was then assessed on day 17. Alternatively, 10×10^6 viable or apoptotic cells were transferred intratracheally to already sensitized mice on day 14, followed by 3 consecutive challenges with 25 µg HDM protein on days 14, 15, and 16, with analysis on day 17. For adenosine agonist treatment, 30 µg/kg of ATL-146e (Adenosine therapeutics LLC, Charlottesville, VA) or control vehicle was administered intranasally with each HDM dose on days 0, 7 and 14.

Bronchoalveolar lavage and lung histology

BAL cells were spun and collected for differential cell count by flow cytometric analysis. BALF was examined for cytokine content by ELISA. For histologic analysis, whole lung lobes were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E and analyzed by light microscopy. Five sections stained with H&E were randomly selected per lung and scored for peribronchial inflammatory cell infiltration, as described previously (2).

Adoptive transfer of naïve CD4⁺ T cells and alveolar macrophages

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1 × 10⁶ naïve CD4⁺ T cells (CD44^{lo}CD62L^{hi}CD25⁻Foxp3⁻) purified from CD45.1⁺ OT-II Foxp3/GFP TCR transgenic mice were adoptively transferred intravenously into CD45.2⁺ mice. Mice were exposed to inhaled OVA protein (Worthington Biochemical, Lakewood, NJ) over 3 consecutive days. On day 5, CD45.1⁺ OT-II cells in draining LN were analyzed for Foxp3/GFP by flow cytometry. 2-5 × 10⁴ sorted naïve or CFSE⁺ BAL and lung alveolar macrophages (from mice injected with CFSE-labeled apoptotic cells) were intratracheally injected into recipients before intranasal HDM administration.

Alveolar Macrophage depletion

75 µl of clodronate liposomes or PBS liposomes (FormuMax, Sunnyvale, CA) were intratracheally injected 48 hours before apoptotic cell transfer or HDM sensitization.

In vitro culture of macrophages with apoptotic cells and naïve CD4⁺ T cells

5 × 10⁴ sorted CFSE⁺ or CFSE⁻ BAL and lung alveolar macrophages, from mice injected with CFSE-labelled apoptotic cells, were co-cultured with 5 × 10⁵ naïve splenic CD4⁺ T cells from OT-II Foxp3/GFP mice in the presence of 10 µM of OVA peptide 257-264 (InnoPep, San Diego, CA) and or 1 µM of ATL-146e for 5 days. CD4⁺ T cells were analyzed for Foxp3/GFP induction by

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flow cytometry. In some experiments, naïve alveolar macrophages were sorted and co-cultured with apoptotic cells and analyzed for mRNA expression.

Flow cytometry

BAL, lung, and LN cells from individual mice were collected and treated with red blood cell lysing buffer (Sigma). Lungs were dissociated using with Lung Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and Gentle MACS dissociator (Miltenyi Biotec). After Fc block with the 2.4G2mAb (eBioscience, San Diego, CA), cells were stained with the antibodies: CD45.1-APC-Cy7 (A20, BioLegend, San Diego, CA), CD45.2-PerCPCy5.5 (30-F11, BioLegend), CD11b-APC-Cy7 (M1/70, BD Biosciences, San Jose, CA), CD11c-PE-Cy7 (HL3, BD Biosciences), SiglecF-BV421 (E50-2440, BD Biosciences), Ly6G-Alexa700 (1A8, BioLegend), MHCII-APC (M5/114.15.2, BioLegend), CD103-BV605 (M290, BD Biosciences), CD4-BV570 (RM4-5, BioLegend), CD8a-PEeFluor610 (53-6.7, eBioscience), CD44-Alexa700 (17A2, BioLegend), CD62L-APC (MEL-14, BioLegend), CD25-PE (PC61, BioLegend). Flow cytometry analysis was performed on a Fortessa (BD Biosciences) and data were analyzed using FlowJo Software (version 10, FlowJo, LLC, Ashland, OR). Lung immune cells were separated into eosinophils (CD11c⁻SiglecF⁺), alveolar macrophages (CD11b⁺CD11c⁺SiglecF⁺), interstitial macrophages (CD11b⁺MHCII⁺SiglecF⁻CD64⁺), neutrophils (CD11b⁺Ly6G⁺), CD103⁺ DC

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(CD11c⁺MHCII⁺CD11b⁻CD103⁺), CD11b⁺ DC (CD11c⁺MHCII⁺CD11b⁺CD64⁺CD103⁻), and CD8⁺ DC (CD45⁺CD11c⁺MHCII⁺CD11b⁻CD8⁺).

ELISA

Cytokines in BALF were assayed by ELISA with paired antibodies, according to the manufacturers' recommendations. 96-well ELISA plates (Thermo Scientific scientific) were coated with anti-mouse cytokine antibody; anti-IL-4, anti-IL-5, anti-IL-13 (eBioscience), as a capture antibody, followed by detection with biotinylated anti-mouse cytokine antibody (eBioscience) and Avidin-HRP (eBioscience).

Reverse transcription and real-time quantitative PCR

Lung tissue was homogenized with TISSUE MASTER (OMNI International) and total RNA was isolated with TRIzol (Life Technologies, Grand Island, NY). Macrophages were isolated by sorting and total RNA isolated using RNeasy kit (Qiagen, Hilden, Germany). cDNA was prepared using a SuperScript IV reverse transcriptase kit (Thermo Fisher). Real-time PCR was performed with LightCycler (Roche, Indianapolis, IN) using PowerUp SYBR Green master mixes (Applied Biosystems, Foster City, CA). Data are normalized with housekeeping gene GAPDH

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107 presented as relative expression or relative quantification to control samples. The
108 oligonucleotide primer sequences are listed in the **Table E1**.

109

110 **Statistical analysis**

111 Statistical significance was analyzed by unpaired *t*-test for comparison of means between two
112 independent groups or by one-way ANOVA followed by Tukey's post hoc multiple comparison
113 test for differences of means between multiple groups using Prism 8 software (version 8,
114 GraphPad, San Diego, CA). *p* value <0.05 was considered statistically significant. **P*<0.05,
115 ***P*<0.01, ****P*<0.001, ****, *P*<0.0001.

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References

1. Soroosh P, Doherty TA, Duan W, Mehta AK, Choi H, Adams YF, et al. Lung-resident tissue macrophages generate Foxp3⁺ regulatory T cells and promote airway tolerance. *The Journal of experimental medicine*. 2013;210(4):775-88.
2. Doherty TA, Soroosh P, Broide DH, Croft M. CD4⁺ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. *American journal of physiology Lung cellular and molecular physiology*. 2009;296(2):L229-35.