Pathogenic T_H17 inflammation is sustained in the lungs by conventional dendritic cells and Toll-like receptor 4 signaling

Karim H. Shalaby, PhD,[a] Miranda R. Lyons-Cohen, BSc,[a] Gregory S. Whitehead, MSc,[a] Seddon Y. Thomas, PhD,[a] Immo Prinz, PhD,[b] Hideki Nakano, PhD,[a] and Donald N. Cook, PhD[a] *Research Triangle Park, NC, and Hannover, Germany*

Background: Mechanisms that elicit mucosal T_H17 cell responses have been described, yet how these cells are sustained in chronically inflamed tissues remains unclear.

Objective: We sought to understand whether maintenance of lung T_H17 inflammation requires environmental agents in addition to antigen and to identify the lung antigen-presenting cell (APC) types that sustain the self-renewal of T_H17 cells.

Methods: Animals were exposed repeatedly to aspiration of ovalbumin alone or together with environmental adjuvants, including common house dust extract (HDE), to test their role in maintaining lung inflammation. Alternatively, antigen-specific effector/memory T_H17 cells, generated in culture with $CD4^+$ T cells from *Il17a* fate-mapping mice, were adoptively transferred to assess their persistence in genetically modified animals lacking distinct lung APC subsets or cell-specific Toll-like receptor (TLR) 4 signaling. T_H17 cells were also cocultured with lung APC subsets to determine which of these could revive their expansion and activation.

Results: T_H17 cells and the consequent neutrophilic inflammation were poorly sustained by inhaled antigen alone but were augmented by inhalation of antigen together with HDE. This was associated with weight loss and changes in lung physiology consistent with interstitial lung disease. The effect of HDE required TLR4 signaling predominantly in lung hematopoietic cells, including $CD11c^+$ cells. $CD103^+$ and $CD11b^+$ conventional dendritic cells interacted directly with T_H17 cells *in situ* and revived the clonal expansion of T_H17 cells both *ex vivo* and *in vivo*, whereas lung macrophages and B cells could not.

Conclusion: T_H17-dependent inflammation in the lungs can be sustained by persistent TLR4-mediated activation of lung conventional dendritic cells. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *T_H17 cells, dendritic cells, chronic inflammation, house dust extract, Toll-like receptor 4*

Inhaled compounds in the environment that stimulate innate immune pathways are critical for the priming of antigen-specific T-cell responses in the lungs. Yet it is unclear how such responses are sustained in chronically inflamed tissues after the onset of inflammation. IL-17 family member–producing T_H17 cells have been implicated in patients with numerous chronic inflammatory diseases, including respiratory diseases, such as chronic obstructive pulmonary disease,[1] hypersensitivity pneumonitis,[2] cystic fibrosis,[3,4] and asthma.[5,6]

T_H17 cells produce IL-17A and IL-17F, both of which signal through a heterodimeric receptor comprising IL-17 receptors A and C. In the lung, IL-17A binding to its receptor on airway epithelial cells induces neutrophil-attracting chemokines, such as IL-8, in human subjects and CXCL1 and CXCL5 in mice.[7] In addition, IL-17A can also act directly on airway smooth muscle,[8] epithelial cells,[9] fibrocytes,[10] and fibroblasts[11,12] to elicit the synthesis of proinflammatory and profibrotic mediators. Consequently, IL-17A can promote pathologies, such as airway hyperresponsiveness, fibrosis, and remodeling. Unlike T_H2 cells, which are associated with predominantly eosinophilic asthma, T_H17 cells are resistant to glucocorticoids,[13] and there is an unmet need for new therapeutic strategies that target T_H17-dependent inflammation. An improved understanding of how T_H17 cells are maintained and reactivated in the diseased lung would help to achieve this goal.

The development of antigen-specific T_H17 responses depends on activation of lung antigen-presenting cells (APCs) by inhaled environmental adjuvants, including microbial products, such as LPS, which signals through Toll-like receptor (TLR) 4.[14] Inhalation of house dust extract (HDE) can also promote T_H17 development, and this activity is largely dependent on TLR4. Studies suggest that TLR4 signaling specifically in hematopoietic cells of the lungs is important for T_H17 cell development.[15,16] However, once T_H17 cells occupy the lungs, it is unclear whether they require continued stimulation by adjuvants or whether antigen alone is sufficient to maintain their self-renewal.

It is also unknown which MHC class II–displaying APCs in the lungs are responsible for sustaining tissue-resident T_H17 cells. Conventional dendritic cells (cDCs) in the lungs are characterized by their dependence on the cytokine FMS-like tyrosine kinase 3 ligand (Flt3l) and can be divided into 2 distinct populations: transcription factor basic leucine zipper ATF-like transcription factor 3 (BATF3)–dependent $CD103^+CD11b^-$ cDCs and interferon regulatory factor (IRF) 4–dependent $CD11b^+CD103^-$ cDCs. Interstitial macrophages, which are sometimes also referred to as monocyte-derived dendritic cells (DCs), constitute

From [a]the Immunogenetics Group, Immunity, Inflammation and Disease Laboratory, Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, and [b]Institut für Immunologie, Medizinische Hochschule, Hannover.

Supported by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences (ZIA ES102025-09).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication June 5, 2017; revised September 21, 2017; accepted for publication October 2, 2017.

Corresponding author: Donald N. Cook, PhD, Immunity, Inflammation and Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, 111 T.W. Alexander Dr, Rall Building, Room E224, Research Triangle Park, NC 27709. E-mail: cookd@niehs.nih.gov.

0091-6749

Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

https://doi.org/10.1016/j.jaci.2017.10.023

Abbreviations used
APC: Antigen-presenting cell
Asp: Protease extract from *Aspergillus oryzae*
BAL: Bronchoalveolar lavage
BATF3: Basic leucine zipper ATF-like transcription factor 3
cDC: Conventional dendritic cell
cIMDM: Complete Iscove's modified Dulbecco's medium
DC: Dendritic cell
Flt3l: FMS-like tyrosine kinase 3 ligand
HDE: House dust extract
IRF: Interferon regulatory factor
MFI: Median fluorescence intensity
OVA: Ovalbumin
Sftpc: Surfactant protein C
TCR: T-cell receptor
TLR: Toll-like receptor
WT: Wild-type
YFP: Yellow fluorescent protein

another subset of lung CD11b^{hi} APCs. Finally, alveolar macrophages and B cells in the lungs might also be capable of presenting antigen to and sustaining T_H17 cells. cDCs, monocytic cells, and B cells have all been reported to contribute to T_H17 responses in various tissues, such as the lung, gastrointestinal tract, skin, or central nervous system.[17-24]

In the present report we show that chronic antigen challenges alone, after cessation of adjuvant exposures, poorly sustain lung T_H17-dependent inflammation. T_H17 adoptive transfer and fate-mapping experiments reveal that HDE sustains the self-renewal of antigen-specific T_H17 cells, promoting severe neutrophilic inflammation, morbidity, and impaired lung function reminiscent of chronic hypersensitivity pneumonitis. This effect is dependent on TLR4 signaling in multiple cell types, although primarily in hematopoietic and CD11c-expressing cells. cDCs are the only APCs capable of reviving T_H17 responses *ex vivo*, and both subsets sustain T_H17 responses in the lungs.

METHODS

Animals

Mice were bred and housed in specific pathogen-free conditions at the National Institute of Environmental Health Sciences and used between 6 and 12 weeks of age in accordance with guidelines provided by the Institutional Animal Care and Use Committees. The following mouse strains were purchased from the Jackson Laboratory (Bar Harbor, Me): C57BL/6J, OT-II (B6.Cg-Tg[TcraTcrb]425Cbn/J), B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J, *Nur77*^{GFP} (C57BL/6-Tg[Nr4a1-EGFP/cre]820Khog/J), *Cd11c-cre* (B6.Cg-Tg[Itgax-cre]1-1Reiz/J), *Tlr4*$^{fl/fl}$ (B6(Cg)-Tlr4$^{tm1.1Karp}$/J, *Irf4* $^{fl/fl}$ (B6.129S1-Irf4^{tm1Rdf}/J), *Batf3*$^{-/-}$ (B6.129S[C]-Batf3tm1Kmm/J), *Il12p35*$^{-/-}$ (B6.129S1-*Il12a*^{tm1Jm}/J), and *Il12p40*$^{-/-}$ (B6.129S1-Il12btm1Jm/J). *Il17ra*$^{-/-}$ mice (B6.129-Il17ra^{tm1Koll}) were obtained from Taconic Farms (Germantown, NY) and used with permission from Amgen (Thousand Oaks, Calif). *Flt3l*$^{-/-}$ mice (C57BL/6-flt3Ltm1Imx) were also purchased from Taconic Farms. *Il17a/f*$^{-/-}$ (C57BL/6-Il17a/Il17f$^{tm1.1Impr}$)[25] and *Tlr4*$^{-/-}$ (B6.129P2-Tlr4^{tm1Aki}) mice were provided by Immo Prinz (Hannover, Germany) and Shizuo Akira (Osaka University, Japan), respectively.

We generated *Il17a* fate-mapping mice in which DNA encoding a Cre recombinase/yellow fluorescent protein (YFP) fusion protein was inserted into the 3′ untranslated region of *Il17* downstream of an engineered internal ribosome entry site (*Il17-cre* mice; see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). These animals were crossed to mice having a *Cre*-inducible Tomato transgene at the *Rosa26* locus (see Fig E1, *B*). The resulting animals (see Fig E1, *C*) were in turn crossed to OT-II mice to generate ovalbumin (OVA)–specific T_H17 fate-mapping mice whose *Il17*-expressing cells (and their progeny) permanently acquire Tomato fluorescence (see Fig E1, *D*). Fluorescence resulting from the Cre/YFP fusion gene was undetectable and therefore not used in the current study.

Bone marrow chimeric mice

Reciprocal bone marrow chimeric mice were generated by using C57Bl/6J or *Tlr4*$^{-/-}$ mice, as described in the Methods section in this article's Online Repository at www.jacionline.org. Before their use in experiments, chimeric mice were rested for 12 to 16 weeks to allow for hematopoietic reconstitution. Mice selectively lacking *Tlr4* in lung epithelial cells were generated by crossing *Tlr4*$^{fl/fl}$ mice to surfactant protein C *(Sftpc)-cre* mice, provided by Brigid Hogan (Duke University, Durham, NC). Mice lacking TLR4 in *Cd11c*-expressing cells were generated by crossing *Tlr4*$^{fl/fl}$ mice to *Cd11c-cre* mice.

HDEs

Sterile HDEs were prepared from dust collected from North Carolinian homes, as described previously.[26] Allergens in the extracts were evaluated by using the multiplex array for indoor allergens (Indoor Biotechnologies, Charlottesville, Va), and the endotoxin concentration was determined to be 10^{-1} μg of LPS/20 μL of HDE, as determined by using a Limulus Amebocyte Lysate assay (Lonza, Karlsruhe, Germany).

Chronic antigen exposure

Naive CD4$^+$ cells were isolated from the spleens and skin-draining lymph nodes of OT-II mice by using antibody-labeled magnetic bead–mediated negative selection (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells (1×10^5) were transferred by means of retro-orbital injection to naive C57BL/6J animals, which were sensitized on days 0 and 7 by oropharyngeal instillation of 60 μL of sterile PBS containing 10 μg of LPS-free OVA (Worthington Biochemical, Lakewood, NJ), either alone or together with an adjuvant. The adjuvants tested included 100 ng of LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St Louis, Mo), 20 μg of protease from *Aspergillus oryzae* (Sigma-Aldrich), or 10 μL of HDE. Similar exposures were repeated on days 14 to 16 ("acute challenge") to establish airway inflammation. This was followed by a 6-week period of chronic challenge, during which the animals were given either OVA alone or OVA together with the same adjuvant to which they had previously been exposed, 3 times per week. Mice were harvested 3 days after the final challenge (Fig 1, *A*). Bronchoalveolar lavage (BAL) was performed, and cells were analyzed by the Diff-Quik method (Medical Diagnostics, Düdingen, Germany). Left lungs were excised and incubated for 24 hours at 37°C in complete Iscove's modified Dulbecco's medium (cIMDM) containing 10 μg/mL OVA to evaluate the antigen-stimulated lung explant cytokine response *ex vivo*.

Analysis of cytokines in BAL fluid, lung explants, and culture supernatants

Levels of IL-17A and other mediators were measured by means of ELISA (BioLegend, San Diego, Calif) or multiplex fluorescent bead–based immunoassays, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, Calif).

OVA-specific immunoglobulin

OVA-specific IgG_1 and IgG_{2a} from serum were evaluated by using commercial ELISA kits purchased from Thermo Fisher Scientific (Waltham, Mass) and Invitrogen BD Biosciences (San Diego, Calif), respectively. Briefly, plates were coated overnight at room temperature with 10 μg/mL OVA Grade V (Sigma) in coating buffer containing 1.94 g/L $NaHCO_3$ and 3.52 g/L Na_2CO_3 in deionized H_2O (pH 9.6). The plates were washed 3 times with