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14. ABSTRACT

We previously demonstrated that inhalation of common aeroallergens such as the house dust mite *Dermatophagoides pteronyssinus* and the mold *Alternaria alternata* elicits the generation of a potent proinflammatory lipid mediator, leukotriene E4 (LTE4), which is part of the cysteinyl leukotriene (CysLT) family. LTE4 promotes lung inflammation and release of mucus into the airway through its action on an epithelial G protein-coupled receptor called CysLT3R or GPR99 or OXGR1. During this period we have found that:

1) Epithelial brush cells (BrCs) are a dominant cell type making LTE4 in the airway in several important clinical settings.
2) Allergen-elicited BrC CysLT generation depends on a GPCR called P2Y2.
3) GPR99 drives expansion of BrC number which is a key step in promoting BrC-dependent pulmonary inflammation.

15. SUBJECT TERMS

Lipid mediators, Leukotrienes, G protein-coupled receptors, Lung, Inflammation, Epithelial cell,

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Annual Report

1. INTRODUCTION

Cysteinyl leukotrienes (CysLTs), leukotriene C_4 (LTC_4), LTD_4, and LTE_4, are lipid mediators that elicit lung inflammation and bronchoconstriction in asthma. CysLTs are not normally detected in biologic fluids, but are generated from membrane lipids through the 5-lipoxygenase/LTC_4 synthase (LTC_4S) pathway when leukocytes are activated. LTC_4, the terminal product of intracellular CysLT generation, is exported extracellularly, and rapidly metabolized to LTD_4 and to LTE_4, the stable CysLT detected in the bronchoalveolar lavage and the urine of patients with active asthma.

CysLTs act at three receptors, $CysLT_1R$, $CysLT_2R$, and $CysLT_3R$ (also known as GPR99 or Oxgr1). Our group previously defined $CysLT_3R$ as the high affinity receptor for LTE_4 (1), and we found that $CysLT_3R$ mediated the development of airway goblet cells and their release of mucus (2). In subsequent studies, we have demonstrated that $CysLT_3R$ can control the generation of IL-25 and type 2 inflammation through a distinct epithelial pathway (3). This proposal will dissect how $CysLT_3R$ controls type 2 inflammation and epithelial plasticity and secretory function in mouse models of allergic asthma using null strains for each CysLT receptor.

2. KEYWORDS

Lipid mediators, Leukotrienes, G protein-coupled receptors, Inflammation, Epithelial cells, Lung

3. ACCOMPLISHMENTS

A. Table 1: Research Accomplishment Summary

RESEARCH SPECIFIC TASKS (AS PROPOSED IN SOW)	ACCOMPLISHMENTS IN THIS REPORTING PERIOD
Major Task 1: Mouse studies on epithelial cell function and development	
Subtask 1: Submit documents for ACURO approvals	Done
Milestone(s) Achieved: Obtain ACURO approval	Done
Subtask 2: Define how GPR99 regulates secretory epithelial cell function in the nasal and bronchial mucosa of mice	We have not begun this work yet. This will be a focus of the upcoming year.
Subtask 3: Examine the role of the mast cell/cysteinyl leukotriene/GPR99 axis in mucin release elicited by several secretogogues	50% accomplished. This work is ongoing. • We hypothesized that activated mast cells generated CysLTs which triggered mucus release in response to several agonists. We found instead that a rare epithelial cell called a brush cell was the source of CysLTs (not mast cells). Brush cell CysLT generation can be activated by several secretogogues proposed in this Aim, including ATP. See Fig. 1-5.
Subtask 4: Examine how GPR99 controls Alternaria-induced goblet cell metaplasia in the lung	10% accomplished. This work is ongoing. • We are finding that GPR99 activates basal stem cells to differentiate into goblet cells and brush cells in a STAT6-independent fashion.

Milestone(s) Achieved: Presentation of project data at a national meeting	• This work was presented at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019 by Saltanat Ualiyeva in a talk entitled “Nasal epithelial brush cells generate cysteinyl leukotrienes in response to aeroallergens and stress signals”.
Milestone(s) Achieved: Determination of the role of GPR99 in epithelial function and development; publication of 1-2 peer reviewed papers	This work is part of a manuscript in revision at Science Immunology.
Major Task 2: Mouse studies on *Alternaria*-elicited type 2 pulmonary inflammation	
Subtask 1: Submit documents for ACURO approvals	Done
Milestone(s) Achieved: Obtain ACURO approval	Done
Subtask 2: Generation of adaptive immunity in Alternaria-sensitized and challenged mice. Comparison between wild-type and null strains	50% accomplished. This work is ongoing. • To understand how the MC/CysLT axis may lead to the development of adaptive immunity we have continued to assess DC migration and activation in the lung and lymph node of naïve mice. See Fig. 6-7. • We have done sequencing on DCs from WT and MC-deficient mice, the results of which are pending.
Subtask 3: Examine innate type 2 pulmonary inflammation in the first week after a single Alternaria exposure in mice. Comparison between wild-type and null	Done in prior reporting period.
Subtask 4: Examine ILC2 expansion and type 2 inflammation elicited by repeated doses of intranasal LTE4 in mice	Done in prior reporting period.
Milestone(s) Achieved: Presentation of project data at a national meeting	• This was presented at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019 by Dr. Sachin Samuchiwal in a talk entitled “MC-dependent adjuvant activity is a key component of the respiratory immune response to inhaled Alternaria.” • This was presented at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019 by Dr. Lora Bankova in a talk entitled “Leukotriene-Dependent Regulation of Epithelial-Dependent Innate Type 2 Immunity”.
Milestone(s) Achieved; publication of 1-2 peer reviewed papers	•

B. Research Accomplishment Narrative

Specific Aim 1. To define the role of MCs and the LTE4 receptor GPR99 in respiratory EpC secretory function and differentiation in the murine nasal and lung mucosa.

A central hypothesis in this grant was that mast cells (MCs) generate CysLTs which activate the epithelial cell (EpC) receptor GPR99, facilitating mucin secretion. To understand the role of MCs in CysLT-dependent secretory epithelial cell functions, we assessed mucin release from the nasal mucosa of WT, GPR99/$CysLT_3R$-null, and MC-deficient mice (not shown). While each strain had reduced release of mucin in response to *Alternaria*, surprisingly the MC-deficient mice did not have a reduction in CysLTs detected in the nasal or lung lavage, indicating that there was one or more additional cell types which generate CysLTs. After assessment of macrophage and dendritic cell-deficient strains (which also had intact CysLT generation), we turned to epithelial cells (EpCs). We isolated several EpC subtypes from the nasal and lung mucosa. We noted that the transcriptional profile of olfactory, nasal and tracheal brush cells (BrCs) was enriched for the enzymes that generate CysLTs (**Fig. 1**). We further assessed transcript levels for each enzyme in the CysLT pathway, validating high levels of expression in these BrC subsets (**Fig. 2**). We then flow cytometrically isolated these cells from the nasal mucosa and stimulated them with the calcium ionophore A23187 (**Fig. 3**). BrCs generated robust levels of CysLTs, which was reduced by the conventional inhibitor of CysLT generation, MK886. **These results demonstrate that BrCs generate CysLTs.**

After assessment of candidate cell surface receptors that may mediate CysLT generation, we found that BrCs from most sites expressed several members of the P2Y receptor family including P2Y2, which recognizes ATP (not shown). Ex-vivo stimulation of isolated nasal BrCs with the stable ATP analogue ATPγS elicited dose-dependent CysLT generation (**Fig. 4**), which was inhibited by the P2Y2 inhibitor (AR-C118925) and by MK886.

These results demonstrate that P2Y2 is a novel BrC receptor regulating CysLT generation. In summary, we have had to revise our hypotheses that 1) MCs were the primary source of

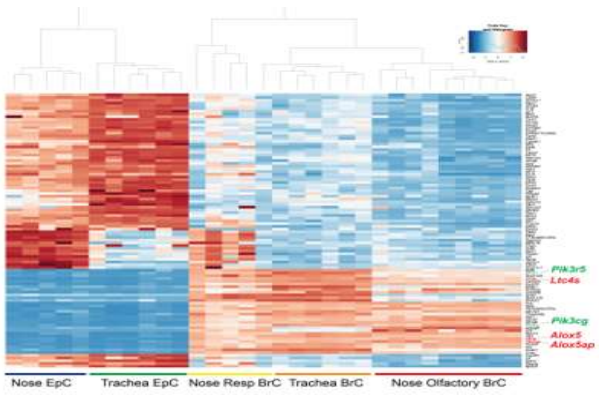

Fig. 1 CysLT biosynthetic enzymes are expressed in airway BrCs. Hierarchical clustering of the top 100 most variable genes using DeSeq2. Transcripts of enzymes in the CysLT biosynthetic pathway are highlighted in red.

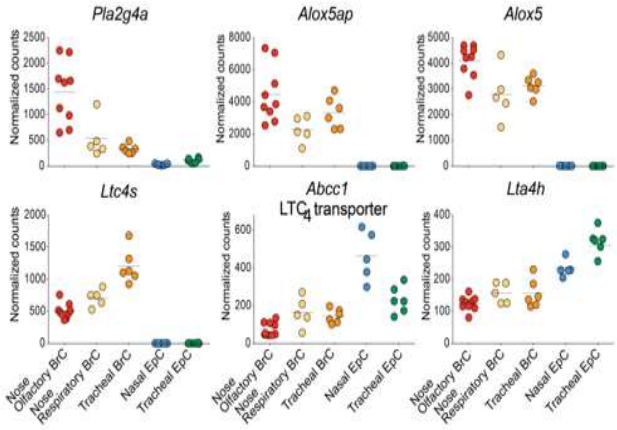

Fig. 2. The CysLT biosynthetic pathway is expressed at high levels. Normalized counts of transcripts encoding CysLT biosynthetic enzymes and transporters across BrC subsets, as compared to other EpCs.

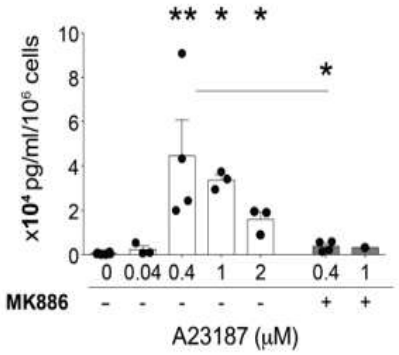

Fig. 3. BrCs generate CysLTs. BrCs were isolated flow cytometrically and stimulated *ex vivo* with the calcium ionophore A23187. Where indicated, cells were pre-treated for 15 min with the FLAP inhibitor MK886. CysLTs in the supernatants were measured by enzyme immunoassay at 30 min. Data are means + SEM, from 3 expts, * p<0.05, ** p<0.01.

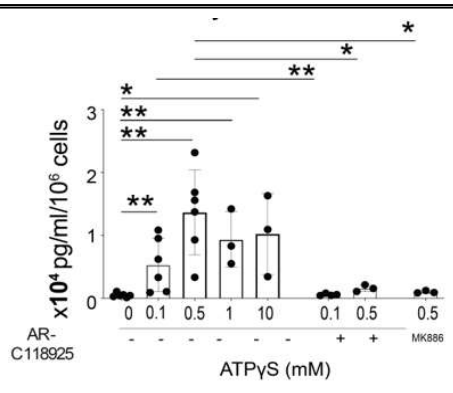

Fig. 4. P2Y2 signaling elicits BrC CysLT generation. BrCs were isolated from the nasal mucosa (nose) and stimulated *ex vivo* with the indicated doses of ATPγS for 30 min. Where indicated, BrCs were pre-treated for 15 min with the P2RY2-specific inhibitor AR-C118925, the FLAP inhibitor MK886 or HBSS and subsequently stimulated with ATPγS. Data are means ± SEM, from 3 expts, * p<0.05, ** p<0.01.

CysLTs regulating epithelial biology and that 2) they were the primary responders to alarmins such as ATP. We instead have found that the ATP/P2Y2 axis that regulates CysLT generation from Brush cells.

To understand whether this pathway might be part of a recognition system for aeroallergens, we stimulated isolated BrCs with *Alternaria* and assessed CysLTs in the supernatant (**Fig. 5**). *Alternaria* stimulated BrCs generated significant CysLTs. This was absent in BrCs from CysLT-deficient (Ltc4s-/-) mice and was reduced by the P2Y2 inhibitor. **These findings demonstrate that our novel pathway for BrC activation may be operative in the response to aeroallergen.**

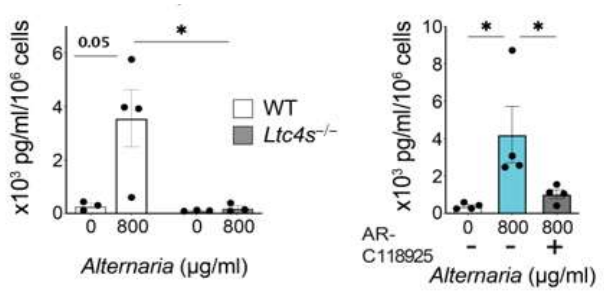

Fig. 5. The P2Y2 receptor pathway regulates BrC CysLT generation elicited by Alternaria. WT or CysLT-deficient (LTC4S-null) BrCs were isolated from the nasal mucosa and stimulated *ex vivo* with the indicated doses of Alternaria for 30 min. Where indicated, BrCs were pre-treated for 15 min with the P2RY2-specific inhibitor AR-C118925. Data are means ± SEM, from 2 expts. *, p<0.05.

Specific Aim 2. To define the role of MCs, GPR99, and other CysLT receptors in driving type 2 pulmonary inflammation elicited by *Alternaria alternata*.
To define the role of MCs and CysLTs in the early events leading to conventional adaptive type 2 lung inflammation (Th2 immunity) we sensitized mice with a single dose of *Alternaria* and assessed dendritic cell (DC) activation in the lung and migration to the lung-draining lymph nodes (LNs). WT mice treated with *Alternaria* had a robust migration of DCs to the lung-draining LNs (**Fig. 6**). This included CD11B+ myeloid DCs (mDCs) and PD-L2+CD301B+ DCs, a subset reported to elicit type 2 immunity in the skin. By contrast, MC-deficient Mcpt5/DTA mice had dramatically reduced DC migration to the LN, across all DC subsets. **These results indicate that MCs control airway DC migration in response to *Alternaria***.

To understand whether the failure of DCs to migrate to the regional LN reflected a baseline DC abnormality or a failure to be activated, we assessed lung DC activation (CD80, CD86, OX40L expression) at 0, 6, and 24 h after Alternaria inhalation. We found no difference in the lung DC response between WT and Mcpt5/DTA at baseline or after *Alternaria* (not shown). To use a more sensitive technique to assess DC activation, lung DCs were isolated from each genotype after *Alternaria* challenge and sent for sequencing, which is currently pending.

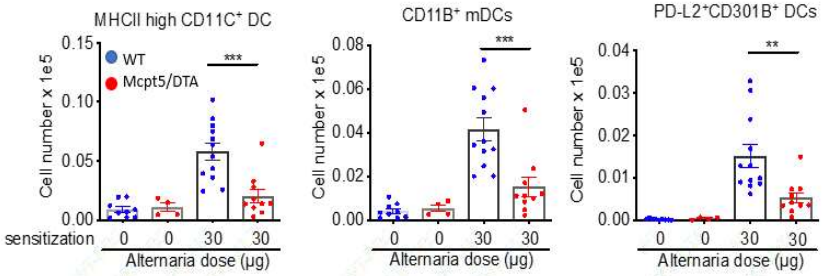

Fig. 6 MC-dependent DC migration. WT and MC-deficient Mcpt5/DTA mice were treated with inhaled *Alternaria* for a single dose. 24 h later, the lung draining LN were evaluated for total DC cell count (left), CD11b+ myeloid DCs (middle), and PDL2+CD301b+ DCs (right). Data are means ± SEM, across 3 experiments. ** p<0.01, *** p<0.001.

To understand whether the reduction in DC migration seen in MC-deficient mice might be due to MC-derived CysLTs we performed Alternaria inhalation in WT and CysLT-deficient (LTC_4S-null) mice (**Fig. 7**). Here again we found that DC migration was reduced in two experiments with additional ones ongoing, suggesting that **MC-derived CysLTs likely act as an endogenous danger signal to drive DC migration.**

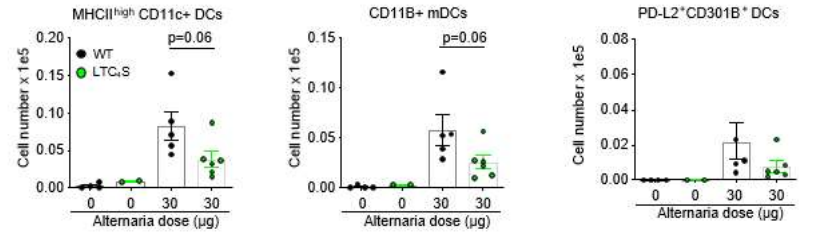

Fig. 7 CysLT-dependent DC migration. WT and CysLT-deficient LTC4S-null mice were treated with inhaled *Alternaria* for a single dose. 24 h later, the lung draining LN were evaluated for total DC cell count (left), CD11b+ myeloid DCs (middle), and PDL2+CD301b+ DCs (right). Data are means ± SEM, across 2 experiments.

Conclusions.
In sum, our findings demonstrate that: 1) Several cells in the naïve respiratory mucosa can generate CysLTs including

MCs and BrCs. 2) BrC CysLT generation can be elicited by *Alternaria* and by danger signals such as ATP. This likely contributes to the CysLT-dependent mucus release we have previously reported. Future studies will be needed to secure this. 3) MC CysLT generation in the submucosa can also be elicited by *Alternaria*. This likely contributes to the CysLT-dependent migration of DCs from the lung to the LN. Studies are ongoing to determine the mechanism by which this happens.

C. Opportunities for training and professional development

While this grant is not specifically designed to provide professional development, it did support work that allowed junior investigators to present at a national meeting.

- Dr. Lora Bankova presented at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019. Her talk was entitled "Leukotriene-Dependent Regulation of Epithelial-Dependent Innate Type 2 Immunity".

- Dr. Saltanat Ualiyeva spoke at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019. Her talk was entitled "Nasal epithelial brush cells generate cysteinyl leukotrienes in response to aeroallergens and stress signals".

- Dr. Sachin Samuchiwal spoke at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019. His talk was entitled "MC-dependent adjuvant activity is a key component of the respiratory immune response to inhaled Alternaria."

D. Dissemination to communities of interest

- Nothing to report

E. Plans for the Next Reporting Period

- In the next reporting period we will complete our mast cell studies (Aim 2.A) and focus on additional epithelial cell functions and secretory development from Aim 1.

4. IMPACT

- Impact on the principal discipline. We have made three important discoveries in this work. The first is that the LTE_4 receptor GPR99 drives the generation of airway goblet cells and their mucus release. This can contribute to airflow obstruction in asthma. The second is that the same system drives the generation of a rare specialized lung epithelial cell called the brush cell. We have discovered that this cell has several pro-inflammatory functions including the generation of IL-25 and the generation of CysLTs (including LTE_4). Thus, activation of GPR99 leads to a feedforward loop promoting the generation of more ligand. This circuit is likely designed to stabilize the remodeling of the airway epithelium. As GPR99 appears to influence epithelial cells in several important ways, we are looking to understand its site of action, possibly on lung progenitor cells.

- Impact on additional disciplines, technology transfer, society and behavior. Nothing to report.

5. CHANGES/PROBLEMS

- There was no change in approach over this reporting period.
- There was a delay in animal experiments due to a delay in obtaining ACURO approval. This has been resolved.
- There were no changes in the use of vertebrate animals, biohazards, or select agents.
- This grant does not include human subjects.

6. PRODUCTS

- Journal publications.
  - Ualiyeva S**, Yoshimoto E, **Barrett NA**, Bankova LG. Isolation and Quantitative Evaluation of Brush Cells from Mouse Tracheas. J Vis Exp. 2019; (148), e59496, doi:10.3791/59496 PMID: 31259891
  - **Barrett NA**, Shalek S, Revisiting Airway Epithelial Remodeling in Type 2 Immunity: Beyond Goblet Cell Metaplasia. Journal of Allergy and Clinical Immunology. (*accepted*)
  - Ualiyeva S, Hallen N, Kanaoka Y, Ledderose C, Matsumoto I, Junger W, **Barrett NA**, Bankova LG. Airway Brush Cells Generate Cysteinyl Leukotrienes Through the ATP Sensor P2Y2. Science Immunology. (*revised manuscript under review*)
- Book publications.
  - Bankova L**, **Barrett NA**. Chapter 1: Innate Immunity. In: Burks AW, Holgate ST, O'Hehir RE, Bacharier LB, Broide DH, Khurana Hershey G, Peebles RS, eds. Middleton's Allergy, 9th Edition. Amsterdam, Netherlands: Elsevier; 2019 (forthcoming).
- Websites.
  - We have developed a laboratory website this year to help disseminate research to the community. https://barrettlab.bwh.harvard.edu/
- Presentations.
  - "MC-dependent adjuvant activity is a key component of the respiratory immune response to inhaled Alternaria" was presented by Dr. Sachin Samuchiwal at the American Academy of Allergy Asthma and Immunology in 2019.
  - "Leukotriene-Dependent Regulation of Epithelial-Dependent Innate Type 2 Immunity" was presented by Dr. Lora Bankova at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019.
  - "Nasal epithelial brush cells generate cysteinyl leukotrienes in response to aeroallergens and stress signals" was by Dr. Saltanat Ualiyeva at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2019.
  - "Epithelial remodeling in the airway mucosa: from form to function" was presented by Dr. Nora Barrett at the Washington University Immunology Program annual retreat.
- No technologies, inventions, patents, licenses, or other reportable outcomes resulted from this research.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name:	Nora Barrett
Project Role:	PI
Researcher Identifier:	0000-0003-2211-8811
Nearest person month worked:	2.4 mos
Contribution to the Project:	Dr. Barrett is responsible for the design and conduct of all experiments.
Funding Support:	RO1 HL120952, RO1 AI134989, U19 AI095219

Name:	Sachin Samuchiwal
Project Role:	Postdoctoral fellow
Researcher Identifier:	0000-0001-6232-5650
Nearest person month worked:	12 mos
Contribution to the Project:	Dr. Samuchiwal is spearheading our mouse mast cell work to define the mechanism by which they regulate the initiation of adaptive immunity.
Funding Support:	No other grants

Name:	Daniel Dwyer
Project Role:	Postdoctoral fellow
Researcher Identifier:	0000-0001-5029-261X
Nearest person month worked:	1 mos
Contribution to the Project:	Dr. Dwyer is spearheading the work on airway epithelial remodeling in the lung in Aim 1.
Funding Support:	U19 AI095219, RO1 AI134989

- There has been no change in the active other support.
- There are no other organizations involved as a partner.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report

10. REFERENCES

1. Kanaoka Y, Maekawa A, Austen KF. 2013. Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. *J Biol Chem* 288: 10967-72
2. Bankova LG, Lai J, Yoshimoto E, Boyce JA, Austen KF, Kanaoka Y, Barrett NA. 2016. Leukotriene E4 elicits respiratory epithelial cell mucin release through the G-protein-coupled receptor, GPR99. *Proc Natl Acad Sci U S A* 113: 6242-7
3. Bankova LG, Dwyer DF, Yoshimoto E, Ualiyeva S, McGinty JW, Raff H, von Moltke J, Kanaoka Y, K Frank Austen, Barrett NA. 2018. The cysteinyl leukotriene 3 receptor regulates expansion of IL-25-producing airway brush cells leading to type 2 inflammation. *Sci Immunol* 3, 28: eaat9453