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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	30% accomplished. This will be a focus of the upcoming year, **but was delayed due to COVID19**.
Subtask 3: Examine the role of the mast cell/cysteinyl leukotriene/GPR99 axis in mucin release elicited by several secretogogues	100% accomplished. • 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, including ATP.
Subtask 4: Examine how GPR99 controls Alternaria-induced goblet cell metaplasia in the lung	100% accomplished. This work will be the subject of future studies. • We found that cysLTs generated from brush cells drive proliferation and differentiation of basal stem

	like epithelial progenitor cells into goblet cells. This happens in a STAT6-independent fashion.
Milestone(s) Achieved: Presentation of project data at a national meeting	• This work was the basis of a planned symposia presentation by Dr. Lora Bankova on cysteinyl leukotrienes at the annual American Academy of Asthma, Allergy, and Immunology meeting. It was cancelled due to COVID19. • Dr. Barrett was invited to speak about these findings in the airway epithelium at the annual American Academy of Asthma, Allergy, and Immunology meeting plenary (virtual recording delivered), and at the Asthma Keystone meeting (rescheduled to virtual meeting in December 2020).
Milestone(s) Achieved: Determination of the role of GPR99 in epithelial function and development; publication of 1-2 peer reviewed papers	• This work was published in the manuscript *Airway brush cells generate cysteinyl leukotrienes through the ATP sensor P2Y2* by Ualiyeva et al, Science Immunology in January 2020.
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 research narrative. • We have done sequencing on DCs from WT and MC-deficient mice, which was not revealing.
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.	100% accomplished. • Done in prior reporting period.
Subtask 4: Examine ILC2 expansion and type 2 inflammation elicited by repeated doses of intranasal LTE4 in mice	100% accomplished. • Done in prior reporting period.
Milestone(s) Achieved: Presentation of project data at a national meeting	• As noted in prior progress reports, this work 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." • As noted in prior progress reports, this work 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	• As noted in prior progress reports, this work was published in the manuscript *The cysteinyl leukotriene 3 receptor regulates expansion of IL-*

	25–producing airway brush cells leading to type 2 inflammation by Bankova et al in Science Immunology October 2018

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.

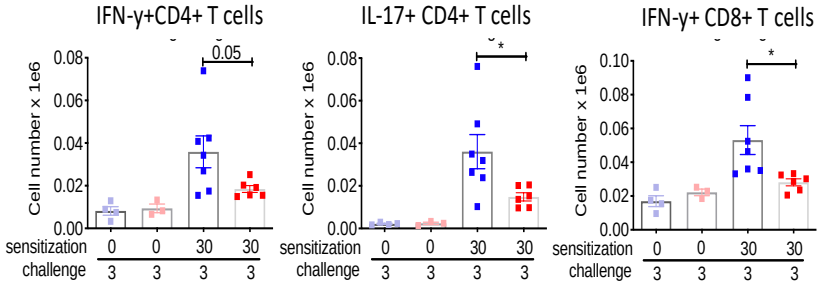

Fig. 1. MC Regulation of *Alternaria*-elicited Th1 and Th17 lung inflammation. WT (blue) and MC-deficient Mcpt5/DTA mice (red) were sensitized with inhaled *Alternaria* at 0 or 30 ug/ml, challenged with 3 ug of inhaled *Alternaria* on days 9 and 10 and euthanized on day 12. Flow cytometric enumeration of the lung T cell infiltrate demonstrating reduced Th1 (left), Th17 (middle), and cytotoxic T cells (right) in the lungs of MC-deficient mice, as compared to WT. Data are means ± SEM, across 3 experiments. * p<0.01.

This work was published this year (4) so it is summarized briefly. Using MC-deficient mice, we demonstrated that MCs were not the source of cysLTs driving lung inflammation. 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. We then flow cytometrically isolated these cells from the nasal mucosa and stimulated them with the calcium ionophore A23187 to show that they generated robust levels of CysLTs. **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, which was inhibited by the P2Y2 inhibitor (AR-C118925) and by MK886. Finally, brush cell-deficient mice had impaired cysLT generation in response to intranasal allergen challenge with Alternaria. **These results demonstrate that P2Y2 is a novel BrC receptor regulating CysLT generation.**

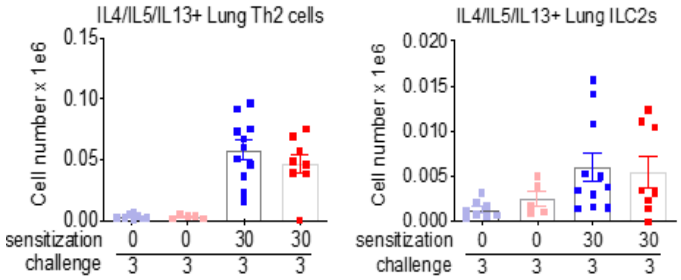

Fig. 2. MCs do not regulate Alternaria-elicited type 2 inflammation. WT (blue) and MC-deficient Mcpt5/DTA mice (red) were sensitized with inhaled *Alternaria* as in Fig. 1. Flow cytometric enumeration of the lung T cell infiltrate demonstrating comparable levels of TCRβ+CD4+IL4/5/13+ Th2 cells (left) and comparable levels of CD45+lin−Thy1.2+CD44+ICOS+ type 2 lymphoid cells (ILC2s) (right) in WT and MC-deficient mice. Data are means ± SEM, across 3 experiments.

In summary, we have had to revise our hypotheses that 1) MCs were the primary source of 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 and that this pathway is operative in allergen recognition in vivo.

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 allergen-elicited adaptive lung inflammation, we sensitized and challenged mice with inhaled *Alternaria.* WT mice developed robust lung inflammation, including CD4+ and CD8+ T cell infiltrates assessed by flow cytometry (**Fig. 1-2**). MC-deficient mice had decreased total CD45+

cell counts (not shown) and decreased numbers of pulmonary Th1, Th17, and cytotoxic CD8+ T cells (**Fig. 1**). By contrast, Mcpt5/DTA mice had no reduction in lung Th2 cells and no reduction in lung ILC2 numbers (**Fig. 2**). To understand this, 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. 3**). 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***.

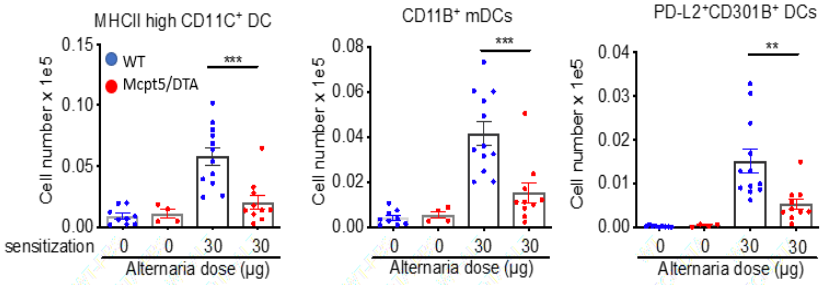

Fig. 3. MC-dependent DC migration. WT (blue) and MC-deficient Mcpt5/DTA mice (red) were treated with inhaled *Alternaria* for a single dose. 24 h later, the lung draining LN were evaluated for total DC cell count (left), CD11b+ myeloid DCs (middle), and PDL2+CD301b+ DCs (right). Data are means ± SEM,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

To understand whether the 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. This demonstrated marginal changes in gene expression (not shown).

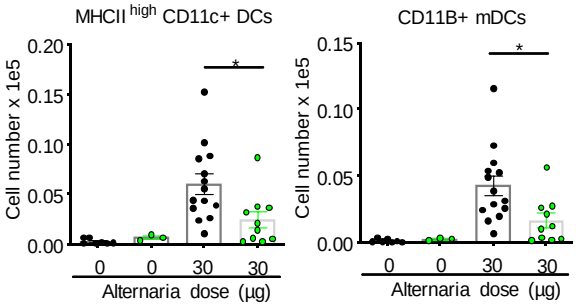

Fig. 4 CysLT-dependent DC migration. WT and CysLT-deficient LTC4S-null mice were treated with inhaled *Alternaria* for a single dose. 24 h later, the lung draining LN were evaluated for total DC cell count (left), CD11b+ myeloid DCs (middle), and PDL2+CD301b+ DCs (right). Data are means ± SEM, across 3 experiments. *p = 0.0.1

We next focused on mediators that can drive DC migration and are reported to be generated in MCs. 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. 4**). Here we found that DC migration was reduced. We also used intranasal blocking antibody to TNF-α over the course of sensitization and found that this also reduces DC migration to regional LNs (**Fig. 5**). These studies point to several mediators MCs generate with potential to act as an adjuvant. Currently, we are generating Mcpt5-Cre mice crossed to floxed strains to validate a role for MC mediators in this response.

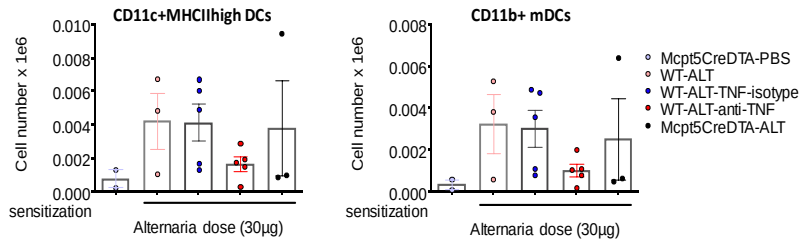

Fig. 5. TNFα-dependent DC migration. WT mice and MC-deficient mice were treated with 10 ug intranasal monoclonal antibody to TNF-α. 0 or 30 ug inhaled *Alternaria* for a single dose in the presence of monoclonal antibody-mediated blockade of TNF-alpha 24 h later, the lung draining LN were evaluated for total DC cell count (left) and CD11b+ myeloid 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. 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 validate this conclusion.

C. Opportunities for training and professional development

While this grant is not specifically designed to provide professional development, it did support work that allowed investigators to get invitations to present at national meetings.

- Dr. Lora Bankova was invited to give at talk at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2020. This was cancelled due to COVID19.

- Dr. Nora Barrett was invited to give a plenary talk at the annual meeting of the American Academy of Allergy Asthma and Immunology in 2020. Her plenary talk was recorded for a virtual seminar in July 2020.

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 our epithelial cell secretory function studies (Aim 1A).

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.
  - Mediators derived from tissue-resident mast cells can serve as an airway alarm that triggers adaptive Th1 and Th17 immunity. Future studies will examine whether this is a steroid resistant pathway that may be implicated in severe neutrophilic asthma.

- 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 performing animal experiments due to COVID 19.
- 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, 2020.
  - McGinty JW, Ting H, Billipp TE, Nadjsombati MS, Khan DM, **Barrett NA**, Liang H, Matsumoto I, von Moltke J. Tuft-Cell-Derived Leukotrienes Drive Rapid Anti-helminth Immunity in the Small Intestine but Are Dispensable for Anti-protist Immunity. Immunity, 2020.
  - Derakhshan T, Samuchiwal SK, Hallen N, Bankova L, Boyce JA, **Barrett NA**, Austen KF, Dwyer DF. Lineage-specific transcriptomes define differential expansion and development of inducible and constitutive mast cells. J Exp Med, 2020; *accepted*
- 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; 2020.
- Websites.
  - We have developed a laboratory website 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.

- “Airway Epithelial Remodeling” was presented at the Northwestern University Allergy and Immunology seminar series, Division of Allergy and Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL in 2019.

- “Epithelial remodeling in the airway mucosa: from form to function” was presented by Dr. Nora Barrett at the annual immunology retreat at the Washington University Immunology Program, St. Louis MO in 2019.

- “A Single-Cell Expression Atlas of Epithelial Cells – Mechanistic Insights into Inflammatory and Allergic Airway Diseases” was presented as an online plenary for the annual meeting of the AAAAI in a recorded session due to COVID19.

- “Revisiting Airway Epithelial Remodeling in T2 Inflammation” will be presented by Dr. Nora Barrett at the Keystone Symposia 2020, Asthma: Making New Discoveries into Better Therapies. *this was rescheduled due to COVID19 to December 2, 2020.

- 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:	2.9 mos
Contribution to the Project:	Dr. Barrett is responsible for the design and conduct of all experiments.
Funding Support:	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

- Changes to Other Support: Dr. Barrett's effort on NIH grant with ID: 5R01HL120952 and title: "Allergic Pulmonary Inflammation through the Dectin-2 Pathway" decreased to 0 in December 2019 due to the grant ending and effort discontinuing.
- There are no other organizations involved as a partner.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report

10. REFERENCES

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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, Frank Austen K, 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
4. Ualiyeva S, Hallen N, Kanaoka Y, Ledderose C, Matsumoto I, Junger WG, Barrett NA, Bankova LG. 2020. Airway brush cells generate cysteinyl leukotrienes through the ATP sensor P2Y2. *Sci Immunol* 5