An Alternate STAT6-Independent Pathway Promotes Eosinophil Influx into Blood during Allergic Airway Inflammation

Wan Wang, Philip M. Hansbro, Paul S. Foster*, Ming Yang*
Centre for Asthma and Respiratory Disease, School of Biomedical Sciences and Pharmacy, Faculty of Health, University of Newcastle and Hunter Medical Research Institute, Callaghan, New South Wales, Australia

Abstract

**Background:** Enhanced eosinophil responses have critical roles in the development of allergic diseases. IL-5 regulates the maturation, migration and survival of eosinophils, and IL-5 and eotaxins mediate the trafficking and activation of eosinophils in inflamed tissues. CD4+ Th2 cells are the main producers of IL-5 and other cells such as NK also release this cytokine. Although multiple signalling pathways may be involved, STAT6 critically regulates the differentiation and cytokine production of Th2 cells and the expression of eotaxins. Nevertheless, the mechanisms that mediate different parts of the eosinophilic inflammatory process in different tissues in allergic airway diseases remain unclear. Furthermore, the mechanisms at play may vary depending on the context of inflammation and microenvironment of the involved tissues.

**Methodology/Principal Findings:** We employed a model of allergic airway disease in wild type and STAT6-deficient mice to explore the roles of STAT6 and IL-5 in the development of eosinophilic inflammation in this context. Quantitative PCR and ELISA were used to examine IL-5, eotaxins levels in serum and lungs. Eosinophils in lung, peripheral blood and bone marrow were characterized by morphological properties. CD4+ T cell and NK cells were identified by flow cytometry. Antibodies were used to deplete CD4+ and NK cells. We showed that STAT6 is indispensable for eosinophilic lung inflammation and the induction of eotaxin-1 and -2 during allergic airway inflammation. In the absence of these chemokines eosinophils are not attracted into lung and accumulate in peripheral blood. We also demonstrate the existence of an alternate STAT6-independent pathway of IL-5 production by CD4+ and NK cells that mediates the development of eosinophils in bone marrow and their subsequent movement into the circulation.

**Conclusions:** These results suggest that different points of eosinophilic inflammatory processes in allergic airway disease may be differentially regulated by the activation of STAT6-dependent and -independent pathways.

Introduction

Eosinophilic inflammation is a hallmark feature of allergic diseases of the lung (asthma), gastrointestinal tract (allergic eosinophilic gastroenteritis), skin (eczema), other systemic diseases (idiopathic hypereosinophilic syndrome and eosinophilic pneumonia) and parasitic helminth infection [1]. Eosinophils play an important pathogenetic role in the processes that lead to the precipitation of these diseases by releasing a wide range of cytotoxic products and proinflammatory factors [1,2]. A substantial body of research has elucidated the major molecular processes that regulate the development of eosinophilic inflammation. Eosinophils differentiate in the bone marrow from pluripotent stem cells and IL-3, IL-5 and GM-CSF are particularly important factors that promote their development [1,3].

IL-5 is the most important factor that regulates the expansion, growth and survival of eosinophils although it is dispensable for eosinophil development under homeostatic conditions [4]. This cytokine also directly promotes allergic airway disease by mediating eosinophilic inflammation [5]. Indeed many diseases that have accompanying eosinophilic inflammation are often associated with increased expression of IL-5 [6]. Importantly, this cytokine provides a critical signal for the eosinophilic response in bone marrow and the subsequent release of this cell into peripheral blood in response to inflammatory stimulation [5,7]. Mice deficient in IL-5 have reduced numbers of eosinophils in peripheral blood and bone marrow and mice over-expressing IL-5 have increased infiltrations of eosinophils into many tissues (e.g. spleen, bone marrow, lung and lymph nodes) [4,8]. Nevertheless, the cellular and molecular mechanisms that mediate the production of IL-5 and the subsequent development of eosinophilic responses have not been fully elucidated.

Once eosinophils are produced specific chemotactic factors, namely the chemokines eotaxin-1, -2 and -3, cooperate with IL-5
to critically regulate their migration and activation during allergic inflammation [1]. These chemokines possess common biologic functions but regulate different phases of eosinophil recruitment during allergic inflammation in humans, although only eotaxin-1 and -2 have been identified in mice [1]. Eotaxins also induce rapid and transient actin polymerization, upregulate integrin function, and modulate respiratory burst in eosinophils [1].

Many immune cells, in particular CD4+ T-helper type 2 lymphocytes (Th2 cells), CD8+ T cells, and NK cells but also mast cells and eosinophils produce IL-5. Of these cells, Th2 cells are the predominant source of IL-5 during allergic responses [9–11]. NK cells have also been demonstrated to secrete IL-5 and actively regulate the development of eosinophilic inflammation in human and animal studies [9,12]. Although NK cells are well known to critically regulate both Th1 and Th2 responses [13], their roles in the regulation of eosinophilic responses in bone marrow during allergic inflammation remains incompletely understood.

Clinical and experimental investigations have demonstrated the obligatory role of Th2 cells in the pathogenesis of eosinophilic inflammation and allergic disorders [14–16]. STAT6 is a critical factor for efficient Th2 polarization [17,18] and the expression of eotaxins [19–21]. Indeed, STAT6-deficient mice do not develop AHR or eosinophilic airway inflammation in mouse models of allergic airway disease [22–24]. By contrast, in other systems, STAT6 is not required for tissue eosinophilia [25,26]. Furthermore, there is evidence that STAT6-independent IL-5 production is involved in eosinophilic inflammation of the intestine during Nippostrongylus brasiliensis infection in mice [27]. The role of STAT6 in different parts of the eosinophilic inflammatory processes in allergic airway disease is not understood. Furthermore, the contribution and roles of STAT6 in mediating the production of IL-5 and eotaxins and in the development of eosinophils in the bone marrow, their release into blood and in the progression of eosinophilic inflammation remains poorly characterized.

In this study we assessed the role of STAT6 in the development of eosinophilic inflammation in a mouse model of allergic airways inflammation using wild type (WT) and STAT6-deficient mice. We also determined the roles of IL-5, eotaxins, CD4+ and CD8+ T cells and NK cells in the development of STAT6-independent eosinophilic inflammation.

Materials and Methods

Animals
Specific pathogen free WT and STAT6-deficient BALB/c mice (male and female, 6–8 weeks) were obtained from the University of Newcastle and Australian National University. STAT6-deficient mice were backcrossed for 12 generations onto the BALB/c background. All experiments were performed with approval from the animal ethics committees of The University of Newcastle (ID 899 and 974) and the Australian National University (The early part of experiments was conducted at ANU and ANU ID was expired and not archived).

Induction of allergic airways inflammation
Mice were sensitized at 6–8 wk of age by i.p. injection (day 0) with 50 µg of ovalbumin (OVA) (fraction V, Sigma, St Louis, MO, USA) admixed with 1 mg Alhydrogel (Reheis Inc., Berkeley Heights, NJ, USA) in 200 µl of 0.9% sterile saline on day 0 and 12. Non-sensitized mice were injected with 1 mg Alhydrogel in 200 µl of 0.9% sterile saline. On days 24, 26, 28 and 30, all groups of mice were challenged with aerosolized OVA (10 mg/ml in 0.9% saline) for 3×30 minutes with 30 minutes break using an ultrasonic nebulizer. On day 23, 25, and 27, 29 and 31, eosinophils as a percentage of leukocytes in peripheral blood were assessed. On day 31 inflammatory responses in bronchoalveolar lavage, airway sections and lungs tissue, and eosinophil numbers in the bone marrow were assessed as previously described [28–30].

Bronchoalveolar lavage and histopathology
Bronchoalveolar lavage was collected, cells isolated and stained and differential inflammatory cell counts performed as previously described [30]. Lungs were fixed in 10% phosphate-buffered formalin, sectioned, and stained with carbol chromotrope and eosinophils enumerated as previously described [31].

ELISA Analysis
Blood was collected by heart bleed and cell-free serum prepared. IL-5, eotaxin-1 and -2 were determined by ELISA cytokine according to the instructions of the manufacturer (BD Pharmingen, San Diego, CA, USA) [30].

Quantitative PCR
The method for quantitative PCR has been described in detail previously [32]. Briefly, RNA was prepared from cells or tissue using the TRIzol RNA isolation buffer following the protocol of the manufacturer (Invitrogen Life Technologies). cDNA was synthesized by an oligo(dT) primed reverse transcriptase reaction using 0.5 µg of RNA from each sample. Quantitative PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following primers: murine IL-5 (forward, CTCTGTTAGACAAGGAATGAGCC and reverse, TCTTCAGATGTCTAGCCCGCG; eotaxin-1 (forward, CCCAACACACTCTGAAGAGTCACAA, and reverse, TTGTGCAACACCTGGTCTTG); eotaxin-2 (forward, TAGGCTGGCGGTGTGGATCTTCG, and reverse, TAAACCTCGGTGCTATTTGCACAGG); and GAPDH (forward, CAGGTGTCTCCTCGCCGACCTGCT, and reverse, CCCTCTTGTCTGGACCTTA). SYBR-green was used to detect changes in amplicon levels with each sequential amplification cycle. The fluorescence intensity was normalized to the rhodamine derivative ROX as a passive reference label, which was present in the buffer solution. The level of mRNA, normalized to GAPDH, was calculated as fold change.

Flow cytometry
Bone marrow was isolated as previously described [33]. Samples were dissociated into single cell suspensions, and depleted of erythrocytes using 0.86% (w/v) ammonium chloride. Cells were washed and stained for surface marker expression using fluorescent monoclonal antibodies. Anti-CD3, anti-CD4 and anti-CD8 (Biolegend, San Diego, CA, USA) were used to detect CD4+ or CD8+ T cells and anti-CD3, anti-CD49b and anti-FceRII (Biolegend) were used to detect NK cells (CD3–CD19–/FceRII+). All samples were analysed using a FACSCanto flow cytometer and associated software (BD Bioscience, San Jose, CA, USA).

Depletion of IL-5, CD4+ and CD8+ T cells and NK cells
Mice were injected i.p. with anti-IL-5 (200 µg, TFK5, rat anti-mouse monoclonal IgG1, ATCC, Manassas, VA, USA) or isotype control (rat IgG1) monoclonal antibody (mAb) on days 22, 26 and 30 during the OVA challenge as previously described [34]. Depletion of IL-5 was confirmed in serum samples by ELISA.

CD4+ or CD8+ cells were depleted by i.p. injection with 500 µg anti-CD4 (Clone GKL1.5, rat anti-mouse monoclonal IgG2b) [35] or anti-CD8 (Clone 2H7, rat anti-mouse monoclonal IgG2b) mAbs [36] or the corresponding isotype control (rat IgG2b), on
Depletion of CD4+ or CD8+ T cells was confirmed in spleens by FACS analysis.

NK cells were depleted by i.v. injection with 50 μl anti-ASIALO GM1 polyclonal antibody (Wako Chemicals, Osaka, Japan) or rabbit serum on day 22, 26 and 30, according to manufacturer’s instructions as previously described [37]. Depletion of NK cells was determined in spleens by FACS analysis. In some experiments, both CD4+ cells and NK cells were depleted by combined treatment with anti-CD4 and anti-ASIALO GM1 antibodies.

Data analysis
An initial one-way analysis of variance (or a Kruskal-Wallis test for non-parametric data) was followed by appropriate comparisons to test for differences between means of groups. Values are reported as the mean ± SEM for each experimental group. The number of mice in each group ranged from 8–12. Differences in means were considered significant if P<0.05.

Results

STAT6 is required for the influx of eosinophils into the lungs during allergic airway inflammation

We first investigated the role of STAT6 in eosinophil accumulation in the lung. WT and STAT6-deficient mice were sensitized and challenged with OVA/OVA or SAL/OVA and leukocyte numbers were determined in the BALF. Pronounced infiltrations of inflammatory cells were detected in the BALF of OVA/OVA treated WT mice, compared to SAL/OVA treated controls (Figure 1A). In particular, the levels of eosinophils were substantially increased (20 fold). By contrast, the numbers of eosinophils in OVA/OVA treated STAT6-deficient mice were not significantly increased compared to SAL/OVA treated controls, and were similar to those in SAL/OVA treated WT mice. Neutrophils in OVA/OVA treated STAT6-deficient mice were increased compared to SAL/OVA treated controls but were significantly decreased compared to OVA/OVA treated WT mice. The infiltrations of lymphocytes and macrophages were increased and were similar in both OVA/OVA treated WT and STAT6-deficient groups.

Histological examination of lung tissue showed that a significant infiltration of eosinophils occurred into the peribronchial and perivascular regions of OVA/OVA treated WT mice compared to SAL/OVA treated controls (Figure 1B). Consistent with the observations in BALF, eosinophil accumulation in lung tissue was absent in OVA/OVA treated STAT6-deficient mice, and was similar to that in SAL/OVA treated WT mice. These results demonstrate that STAT6 is required for eosinophilic inflammation in the lung.

Deficiency in STAT6 results in enhanced eosinophil accumulation in peripheral blood but impaired development in bone marrow

To determine the point of the inflammatory pathways where eosinophil influx in the lung was disrupted in STAT6-deficient
mice, eosinophil levels in the blood and bone marrow were assessed. The percentages of eosinophils in peripheral blood of OVA/OVA treated WT and STAT6-deficient mice were significantly greater than in respective SAL/OVA treated controls (Figure 2A). In the absence of STAT6, there was an increase in the accumulation of eosinophils in peripheral blood in response to OVA/OVA treatment compared to WT mice. No increase of eosinophil percentages in the blood was observed in SAL/OVA treated WT or STAT6-deficient mice.

OVA/OVA treatment of WT and STAT6-deficient mice resulted in increased numbers of eosinophil in bone marrow compared to the respective SAL/OVA controls (Figure 2B). The number of eosinophils in the bone marrow of OVA/OVA treated STAT6-deficient mice was significantly lower than in OVA/OVA treated WT mice. Eosinophils numbers in SAL/OVA treated WT and STAT6-deficient mice were not significantly different.

**STAT6 contributes to IL-5 production and is required for the expression of eotaxins**

IL-5 and eotaxins are critical regulators of the expansion and chemotaxis of eosinophils [38]. Therefore, we assessed whether the lack of eosinophilic inflammation in the absence of STAT6 resulted from reduced IL-5 or eotaxin responses. OVA/OVA treatment of WT mice increased the levels of IL-5 in serum compared to SAL/OVA controls (Figure 3A). OVA/OVA treatment of STAT6-deficient mice also increased IL-5 levels but to a lower level than in WT mice. No increased levels of eotaxin-1 and -2 were detected in the serum of all groups (unpublished data). OVA/OVA treatment of WT mice profoundly increased the mRNA expression of IL-5, eotaxin-1 and -2 in lung tissues (Figure 3B–D). Levels of IL-5 in the lung of OVA/OVA treated STAT6-deficient mice were markedly decreased compared to OVA/OVA treated WT mice but were still significantly higher than that of respective SAL/OVA group (Figure 3B). However, no increase of eotaxin-1 and -2 was observed in OVA/OVA treated STAT6-deficient mice or SAL/OVA treated controls. These results indicate that STAT6 does contribute to IL-5 production and eosinophil accumulation in the lung tissue. However, IL-5 production and eosinophil development and influx into peripheral blood may also occur via a STAT6-independent pathway. These results also suggest that STAT6-dependent production of eotaxins is required for the influx of eosinophils into lung tissue.

**STAT6-independent IL-5 production contributes to eosinophil accumulation in peripheral blood and development and expansion in bone marrow**

We then assessed whether the accumulation of eosinophils in blood and development in bone marrow in the absence of STAT6 was associated with the STAT6-independent production of IL-5. OVA/OVA treated STAT6-deficient mice were administered IL-5 neutralizing mAb or isotype control every four days from day 22 d of the OVA/OVA treatment regime. Anti-IL-5 mAb treatment completely abolished the increases in the percentage of eosinophils in the peripheral blood of OVA/OVA treated STAT6-deficient mice (Figure 4A). The hematopoietic expansion of eosinophils in bone marrow in OVA/OVA treated STAT6-deficient mice was also completely abolished by neutralization of IL-5 (Figure 4B). Thus, STAT6 independent IL-5 production mediates eosinophil accumulation in the blood and development in bone marrow.

**CD4+ cells and NK cells cooperatively regulate the STAT6-independent development of eosinophils in bone marrow**

CD4+ and CD8+ cells and NK cells produce IL-5 and may regulate the expansion of eosinophils in bone marrow. Therefore, we then assessed the contribution of these cells on the STAT6-independent development of eosinophils. OVA/OVA treated STAT6-deficient mice were administered CD4+, NK, CD4+ / NK or CD8+ cell neutralizing antibodies or isotype control every four days from day 22 d of the OVA/OVA treatment regime. Some studies have suggested that anti-ASIALO GM1 antibody may target other cells (e.g. CD8+ T cells or NK cells) [39,40], however, many other investigations have found that this antibody specifically neutralizes NK cells without affecting other cells [41–43]. We have assessed the specificity and efficacy of NK cell depletion in our models. This was achieved by examining the levels of NK cells in the spleens of animals treated with anti-ASIALO GM1 antibody one day after the last OVA aero-challenge. The frequency of NK cells was 0.3 ± 0.25% (mean ± SEM) compared with 4.1 ± 0.5% (mean ± SEM) in isotype-treated

![Figure 2. Eosinophil levels in peripheral blood and bone marrow.](image-url)
animals. By contrast, the frequency of NKT cells, CD4+ and CD8+ T cells remained unchanged. This indicates that NK cells, but not NKT or T cell populations, were specifically depleted [41–43]. Depletion of CD4+ cells and NK cells significantly reduced the numbers of eosinophils in the bone marrow of OVA/OVA treated STAT6-deficient mice, however, numbers were still greater than in SAL/OVA treated controls (Figure 5A and B). However, importantly, combined treatment of antibodies against CD4 and NK cells completely abolished the increase in the numbers of eosinophil in the bone marrow of OVA/OVA treated STAT6-deficient mice, which were similar to the levels in SAL/OVA controls (Figure 5C). Furthermore, the increase in IL-5 was abolished by the combined administration of anti-CD4 and anti-NK cell antibodies (5.6±0.6 pg/ml, data expressed as mean ± SEM, n = 6, P<0.05), compared to isotype control (59±0.8 pg/ml), anti-CD4 (32.7±0.6 pg/ml) or anti-NK cell (34.3±0.4 pg/ml).

Figure 3. The levels of IL-5 in serum and expression of eotaxin-1 and -2 mRNA in lungs. OVA/OVA treated WT mice had significantly increased levels of (A) serum IL-5 and mRNA encoding (B) IL-5, (C) eotaxin-1 and (D) eotaxin-2 in lung tissue compared to SAL/OVA treated controls. OVA/OVA treated STAT6-deficient mice also had significantly increased levels of IL-5 in serum and lung but these levels were lower than OVA/OVA treated WT mice. There was no increase the expression of eotaxin-1 and -2 in OVA/OVA treated STAT-6 deficient or SAL/OVA treated controls. #P<0.05 compared to other groups. *P<0.05 compared to respective SAL/OVA treated control. doi:10.1371/journal.pone.0017766.g003

Figure 4. The effects of neutralization of IL-5 on eosinophil levels in peripheral blood and bone marrow in the absence of STAT6. The increased (A) percentages of eosinophils in peripheral blood and (B) numbers of eosinophils in bone marrow in response to OVA/OVA treatment were completely abolished by neutralization of IL-5 with anti-IL-5 antibody in STAT6-deficient mice. #P<0.05 compared to other groups. doi:10.1371/journal.pone.0017766.g004
Indeed, the levels were reduced to those in SAL/OVA treated WT (6.0±1.3 pg/ml) and STAT6-deficient mice (5.9±0.4 pg/ml). Administration of anti-CD8 antibody had no effect on eosinophil numbers (Figure 5D). These data suggest that STAT6-independent IL-5 production from CD4⁺ T cells and NK cells contributes to the development of eosinophil responses in allergic airway inflammation.

Discussion

Here we demonstrate that STAT6 is a critical mediator of eosinophil influx into the lung. However, we also demonstrate that an alternative pathway that is independent of STAT6 controls the movement of eosinophils into the blood and partially mediates their development in the bone marrow during allergic airway inflammation. These effects were associated with reduced but still elevated levels of IL-5 in serum and the inhibition of the expression of eotaxins in the lung. We also show that IL-5 production, most likely from CD4⁺ and NK cells, may mediate STAT6-independent eosinophil accumulation in the blood and development in the bone marrow.

Other studies by Kuperman et al., using STAT6-deficient mice on a BALB/c background showed only a 50% reduction in eosinophil influx into the BALF during allergic airway inflammation [22]. These results are in direct contrast to our own and those of others that used STAT6-deficient mice on C57BL/6 or B6/129 backgrounds, which had >90% reduction in eosinophil influx [23,24]. The differences of reduction of eosinophil influx observed by Kuperman et al., may be due to the differences in sensitization/challenge regime or mouse strain. By profiling gene transcripts of lung tissue from mice with two forms of experimental asthma (e.g. OVA- and *Aspergillus*-induced models), Zimmermann et al., found that a large number of genes are uniquely expressed independently of STAT6, including chemokines, membrane receptors, transcriptional regulators and enzymes [44]. The authors deduced that the regulation of some genes may be associated with, but are not necessarily restricted by STAT6 signalling. This study clearly indicates that alternative STAT6-independent signalling pathways exist that may contribute to asthma pathogenesis.

In our study, STAT6 deficiency led to reduced levels of eosinophils in bone marrow (Figure 2B) and IL-5 in serum (Figure 3A) in response to OVA/OVA treatment. Anti-IL-5
different cell types is controlled by variety of pathways. These cells also produce this cytokine [48]. The expression of IL-5 by [5]. However, other cells such as CD8

References

1. Rothenberg ME, Hogan SP (2006) The eosinophil. Annu Rev Immunol 24: 147–74.

2. Gleich GJ (2000) Mechanisms of eosinophil-associated inflammation. J Allergy Clin Immunol 105: 651–63.

3. Radlinger M, Lovell J (2009) Eosinophil progenitors in allergy and asthma - do they matter? Pharmacol Ther 121: 174–94.

4. Kopf M, Brennacher F, Hodgkin PD, Ramsey AJ, Melbourne EA, et al. (1996) IL-5-deficient mice have a developmental defect in CD8+ B cells and lack eosinophils but have normal antibody and T cell responses. Immunol 4: 15–24.

5. Foster PS, Hogan SP, Ramsey AJ, Mathiis KL, Young IG (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 183: 195–201.

6. Owen WF, Rothenberg ME, Petersen J, Weller PF, Silberstein D, et al. (1989) Interleukin 5 and phenotypically altered eosinophils in the blood of patients with idiopathic hyper eosinophilic syndrome. J Exp Med 167: 943–8.

7. Collins PD, Marceau S, Griffiths-Johnson DA, Jose PJ, Williams TJ (1995) Cooperation between interleukin-3 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med 182: 1169–74.

8. Deut LA, Strath M, Mello AL, Sanderson CJ (1990) Eosinophilia in transgenic mice expressing interleukin 5. J Exp Med 172: 1425–31.

9. Walker C, Checkel J, Camielski S, Leeson PJ, Gleich GJ (1998) IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. J Immunol 161: 1962–9.

10. Rothenberg HF, Phillips S, Foster PS (2007) Eosinophil trafficking in allergy and asthma. J Allergy Clin Immunol 119: 1303–10; quiz 11-2.

11. Chong LK, Aicheler RJ, Llewellyn-Lacey S, Tomasec P, Brennan P, et al. (2008) Eosinophilic inflammation is differentially regulated by the activation of STAT6-dependent and -independent pathways at different parts of the inflammatory process. J Exp Med 205: 1931–42.

12. Warren HS, Kinnear BF, Phillips JH, Lanier LL (1995) Production of IL-5 by human CD4+ T cells and NK cells and highlights the potential differential regulation of eosinophilic responses that is mediated by the surrounding inflammatory environment.

13. Yokoyama WM, Kim S, French AR (2004) The dynamic life of natural killer cells. Annu Rev Immunol 22: 405–29.

14. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, et al. (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 326: 298–304.

Author Contributions

Conceived and designed the experiments: PSF MY. Performed the experiments: WW MY. Analyzed the data: WW PMH PSF MY. Contributed reagents/materials/analysis tools: PMH PSF MY. Wrote the paper: PMH PSF MY.
