PU.1 Is Required for Macrophage Polarization and the Development of Allergic Inflammation

BY

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THESIS

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Jonna Frasor, Chair John W. Christman, Advisor Primal De Lanerolle Lei Xiao, Medicine This thesis is dedicated to my supportive family: my parents and mother-in-law, my loving husband, Feng Qian, and lovely daughter, Olivia, without whom it would have never been accomplished.

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LIST OF ABBREVIATIONS

α	Alpha
AHR	Airway hyperresponsiveness
AAM	Alternative Activated Macrophages
ANOVA	Analysis of Variance
Ab	Antibody
BMDMs	Bone marrow derived macrophages
BSA	Bovine Serum Albumin
BAL	Bronchoalveolar lavage
CCL5	Chemokine (C-C motif) ligand 5
CAM	Classically Activated Macrophages
CXCL-10	C-X-C motif chemokine 10
d	Days
DRA	D. farina, ragweed, Aspergillus fumigates
FBS	fetal bovine serum
GM-CSF	Granulocyte-macrophage colony- stimulating factor
HBSS	Hanks' balanced salt solution
H&E staining	Hematoxylin and eosin staining
h	Hour
iNOS	Inducible NO synthase
IRF	Interferon-regulatory factor
IFN-γ	Interferon-y
IL-12	Interleukin-12
i.n.	Intranasal injection

i.p.	Intraperitoeal injection
M-CSF	Macrophage colony stimulating factor
min	Minutes
MR	Mannose receptor
ΡΡΑRγ	Peroxisome proliferator-activated receptor- γ
PAS	Periodic acid-Schiff staining
PBS	Phosphate-buffered solution
RELMa	Resistin-like molecule α
SAP	Serum amyloid P
STAT	Signal transducer and activator of transcription
Th	T helper
TLRs	Toll-like receptors
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
WT	Wide type

SUMMARY

Macrophages are a group of heterogeneous cells derived from bone marrow and well identified as sentinels in innate and acquired immunity against microbial products or other environmental stimuli through signal presenting, phagocytosis, clearance of pathogens and apoptotic cells. Macrophages are highly plastic and can be functionally polarized towards M1 or M2 macrophages. Classically activated M1 macrophages are induced by IFN-γ and LPS to increase host defense against microorganisms and enhance the specific M1 marker gene expression including inducible NO synthase (iNOS) and interleukin-12 (IL-12); whereas alternatively activated M2 macrophages are driven by IL-4 and IL-13 to present anti-inflammatory and homeostatic functions with M2 feature marker gene high expression, such as arginase-1, Ym-1, and Fizz-1. Recent studies show that one subtype of M2 macrophages (M2a, AAM) is critical in Th2 dependent immune responses, exhibiting enhanced phagocytic activity and expression of phenotypic pro-allergic factors such as Ym-1, and Fizz-1to promote asthmatic lung inflammation and airway remodeling in murine asthma models or human asthma patients.

Macrophage differentiation is under the regulation of macrophage-specific transcription factors, especially requiring high concentration of transcription factor PU.1. Although it has been reported that PU.1 is essential for macrophage differentiation and maturation by controlling the whole macrophage-specific enhancer repertoire in a genome-wide manner, not much is known about its transcriptional regulation in macrophage polarization. In current knowledge, M1 phenotype is promoted by signal transducer and activator of transcription 1 (STAT1) and interferon-regulatory factor 5 (IRF5), while STAT6, IRF4 and peroxisome proliferator-activated receptor- γ (PPAR γ) are required for M2 phenotype. Our previous studies have shown that PU.1

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is necessary for mature macrophage function and has an important role in the NF- κ B activation and neutrophilic lung inflammation during endotoxemia by using a specific knock-out PU/ER(T)^{+/-} mice with partial PU.1 functional deficiency. Here, we extended the study of PU.1 functions in Th2 type inflammation.

Evidence from our current study shows that AAM inducer IL-4 significantly increased the expression of PU.1 in cultured bone marrow derived macrophages (BMDMs) and the expression of AAM markers such as Ym-1 and Fizz-1 were attenuated in PU/ER(T)^{+/-} mice compared to wild type (WT). This suggests that PU.1 is associated with AAM polarization and also led us to determine whether PU.1 is involved in the Th2 dependent immune response. Recently, we have well established an improved asthma model by challenge with triple allergens (DRA: dust mite, ragweed and *aspergillus*). After repeated airway challenge with DRA, the bronchoalveolar lavage (BAL) from PU/ER(T)^{+/-} mice showed fewer inflammatory cells and attenuated eosinophilic infiltration compared to WT mice. Lung histology in PU/ER(T)^{+/-} mice showed attenuated peribronchial inflammation and less or lack of goblet cell hyperplasia, which are characteristics of airway inflammation and remodeling in asthma. Furthermore, lung homogenates from DRA-induced acute and chronic asthma models showed that abundant Fizz-1 and Ym-1 mRNA expression in WT mice but abrogated in PU/ER(T)^{+/-} mice, which indicates deficiency of PU.1 impairs the AAM polarization as well as asthma development.

In summary, AAM polarization can be induced by IL-4 as shown abundant expression of Ym-1 and Fizz-1 in WT mice, whereas limited in PU/ER(T)^{+/-} mice. Moreover, acute and chronic asthma models show PU.1 is necessary for cell infiltration and airway hyperplasia as well as AAM polarization in response to multiple allergens. However, further studies are needed to determine the mechanism how PU.1 regulates AAM polarization and asthma process.

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I. BACKGROUND

A. Macrophages

1. General information

Macrophages are tissue immune-effector cells that arise from bone marrow myeloid progenitor cells; those are released to blood stream as monocytes and recruited into the lung parenchyma and airspace (1). Monocytes migrate into tissues in response to chemoattrractants are differentiated into macrophages to replenish long-lived tissue-specific macrophages such as bone, alveoli, central nervous system, connective tissue, gastrointestinal tract, liver, spleen and peritoneum (2). Macrophages have different morphologies and phenotypes in the various organs or tissues. In response to infection, inflammation or trauma, macrophage assume an inflammatory phenotype in tissues that is associated with an enhanced migration of monocytes from the blood stream that are susceptible to polarization into different inflammatory phenotypes (3). Macrophages express a broad array of cell surface receptors that recognize and remove apoptotic cells as well as microorganisms. Macrophages also secret various products, including cytokines, such as interleukin-10 (IL-10), transforming growth factor- β (TGF- β), and tumor necrosis factor (TNF), chemokines, and growth factors, as well as reactive oxygen species (4). In general, macrophages are specialized hematopoietic cells present in almost all tissues playing a key role in embryonic development, homeostasis, tissue remodeling, and host defense. Usually, macrophages function to recognize, phagocytize, and clear invading pathogens but also have a role in tissue repair. However, excessively activated or dysregulated macrophages may also contribute to tissue damage.

2. Macrophage activation and polarization

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Macrophages display plasticity in response to different stimuli through various mechanisms modulating their differentiation, tissue distribution, and responsiveness (3). In 1986, the concept of different subtypes of CD4+ T cells came out based on the secreted cytokine profile (5). T helper 1 (Th 1) lymphocytes are characterized by expressing and secreting interferon- γ (IFN- γ) and IL-12, which are thought to be involved in host defense against viruses and bacteria. In contrast, Th2 lymphocytes, which express and secret IL-4, IL-5, and IL-13, are critical for the initiation and development of allergic airway diseases and tissue remodeling. Mirroring the concept of Th1/Th2 cells, activated macrophages are divided as M1 and M2 macrophages (see Figure 1). Classically activated M1 macrophages (CAM) express most toll-like receptors (TLRs) and inducible NO synthase (iNOS), characterized by secreting IL-12, TNF- α , IL-1 β , IL-23, IL-6, C-X-C motif chemokine 10 (CXCL10), chemokine (C-C motif) ligand 5 (CCL5/RANTES)(4). They are differentiated by type 1 inflammatory cytokines and microbial products such as lipopolysaccharide (LPS, the ligand of toll-like receptor 4). M2 macrophages, in contrast, are characterized by their low secretion of IL-12 and can be further subdivided into three groups: M2a (alternatively activated macrophages, AAM), induced by IL-4 or IL-13; M2b, induced by immune complexes and agonists of TLRs or IL-1 receptors; and M2c, induced by IL-10, TGF- β , or glucocorticoids (3)(6). Generally, M1 contribute to innate immunity against microorganisms and tumor cells as well as inflammation, whereas M2 macrophages promote and regulate type 2 inflammatory immune responses, anigogenesis, and tissue repair.

Interestingly, unlike the Th1/Th2 paradigm, macrophage polarization is dynamic and exhibits reversible phenotypes between M1 and M2 macrophages due to changes of stimuli. Growth factors GM-CSF and M-CSF individually can drive macrophage polarization in opposite directions as GM-CSF gives rise to M1 macrophages and M-CSF to M2 macrophages based on



Figure 1. Macrophage differentiation and polarization

This figure shows that macrophages are derived from myeloid progenitor cells in the bone marrow depending on lineage-determining cytokines, such as M-CSF and GM-CSF, and transcription factors such as PU.1. Once released into the blood stream, monocytes enter all tissue compartments throughout the whole body. Tissue-resident macrophages undergo local activation in response to various inflammatory and immune stimuli which are driven to M1 or M1 polarization to play different roles in diseases.

the patterns of cytokine expression (7). Macrophage polarization is subjected to transcriptional regulation with the key transcription factor signal transducer and activator of transcription (STAT) family. For instance,

IFN γ binding to its receptor triggers Janus kinase (JAK) –mediated tyrosine phosphorylation of STAT1, which further binds the promoters of the genes encoding NOS2, IL-12 and other M1 macrophage markers (8). In comparison, IL-4 and IL-13 bind to their receptors and trigger M2 phenotype through JAK-STAT6 signal pathway (9). The interferon-regulatory factor 5 (IRF5) has a dual role in macrophage polarization, either enhancing GM-CSF derived M1 macrophage polarization or suppressing M-CSF induced M2 phenotype in human monocytes (10), whereas another transcription factor IRF4 and peroxisome proliferator-activated receptor- γ (PPAR γ) specifically drive M2 macrophage polarization (11) (12).

3. Mechanism and functions of AAM

There is a growing appreciation that the alternative pathway of macrophage activation is involved in inflammation, infection, metabolism, atherosclerosis, and tumorigenesis. However, the roles of AAM in modulating the pathogenesis and resolution of inflammatory responses have remained controversial. It has been reported that AAM is beneficial through enhancement of host defense against parasite infection (13), promoting wound healing (14), and attenuating diabetes by improving insulin resistance (15). However, it has also been reported that AAM in the context of tumorogenesis and allergic inflammation may exacerbate disease (16) (17). AAM are characterized by abundant expression of mannose receptor (MR/CD206), CD163, arginase, chitinase- like molecules Ym-1/2, resistin-like molecule α (RELM α /Fizz-1) upon stimulation with Th2 type cytokines (18). Furthermore, chemokines including CCL17 (TARC), CCL22 (MDC), and eotaxin, have been associated with AAM activation (19). These signature markers in combination are used to determine mechanisms of AAM in different pathogenesis. Arginase-1 can be upregulated by many signal pathways, served as a useful indicator of IL-4 and IL-13 effects on macrophages. Ym-1 belongs to a family of chitinase-like molecules, which is strongly induced in AAM compared with CAM upon IL-4 stimulation (20). Fizz-1 is a small, secreted cysteine-rich protein recently identified predominantly expressed in antigen presenting cells activated in Th2 type cytokines in response to IL-4 and IL-13(18). These gene products that are associated with AAM are all regulated by STAT6 signaling pathway upon IL-4 or IL-13 binding to their receptors: type 1 IL-4R and type 2 IL-4R or IL-13R (21). However, the influence of these molecules on the development, progression, or resolution of diseases is poorly understood.

B. Asthma

1. General information

Asthma is a complex syndrome characterized as a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness (AHR) and reversible airflow obstruction due to swelling of the mucosal membrane lining the airways and excessive amounts of mucus (22). However, despite remarkable advances in asthma research, many asthmatic patients have persistent symptoms due to unrelieved inflammation and progressive airway remodeling. To date, the etiology of asthma and underlying pathophysiology are still not very well defined. It is well considered that many environmental factors, such as viral infection (23), allergen exposure (24), and occupational exposures (25) are implicated. What we have learned from animal models is that allergic asthma involves both innate and adaptive immune systems. The involvement of adaptive immunity is associated with a Th2 type immune response in that Th2 type cytokines such as IL-4, IL-5, and IL-13 are known to cause many of the features of asthma (26). Current asthma paradigm includes the concept that macrophages and macrophage-like dendritic cells are

essential for antigen presentation to T cells, and then Th2 cells are activated to recruit eosinophils and consequently B cells generate allergen-specific IgE, leading to activation of mast cells to recruitment of Th2 effector cells to the airway (see Figure 2)(27).

2. Macrophage involvement in allergic asthma

There are many cell types present in the asthmatic airway, among which macrophages have been investigated as an important factor involved in asthma pathogenesis in that they are activated and polarized during both inflammatory and repair phases. Alveolar macrophages seem to change genetic susceptibility to allergic asthma by suppressing one or more of the pathogenic components to AHR such as cell polarization, epithelial integrity, and immune responses (28). More promising evidence show that differentiation and recruitment of AAM could be hallmark of allergic lung disease recently. Studies show that the Th2 cytokine profile in asthmatic mouse model is contributory to the polarization of AAM, which exhibit enhanced phagocytosis and impaired innate immunity (29). AAM generate various proallergic factors, such as chemokines, arginase-1, Ym-1, and Fizz-1, all in favor of the airway inflammatory and remodeling responses during asthma (30) (31). Moreover, IL-33 released by endothelial or epithelial cells can act on alveolar macrophages and synergize with IL-13/IL-4R α signal to polarize macrophages towards AAM by inducing the expression of ST2L, which contribute to airway inflammation and eosinophilia in the lungs (32). Serum amyloid P (SAP) attenuates M2 macrophage activation through the inhibition of STAT6 phosphorylation and the expression of arginase-1 and Ym-1, and protects against fungal spore-induced allergic airway disease (33).



Figure 2. Cells involved in the immune pathways lead to allergic lung disease.

The figure depicts the current asthma paradigm is that macrophages and macrophage-like dendritic cells are essential for antigen presentation to CD4⁺ Th2 cells, and then Th2 cells are activated to secret IL-5 to recruit eosinophils and IL-4 and IL-13 polarize macrophages to M2 phenotype. Consequently B cells generate allergen-specific IgE, leading to activation of mast cells to recruitment of Th2 effector cells to the airway and resulting excessive mucus generation and airway spasm.

C. Transcription factor PU.1

1. General information

In the past 20 years, the ETS family transcription factor PU.1 has been found to express specifically in hematopoietic cell types with concentration dependent to decide the fates of cells, as higher expression of PU.1 is needed for macrophage development while lower expression is for B lymphocyte development (34) as well as neutrophil development (35), which indicates that PU.1 is a potent link between innate and adaptive immunity. PU.1 was identified to be critical in hematopoietic differentiation and in particular, macrophage differentiation and maturation in a cell-autonomous manner (36).

2. Function and signaling

Appropriate expression of PU.1 has been related to healthy status whereas altered expression levels are associated with disease development. PU.1 deficient mice have shown attenuated inflammatory gene expression and inflammatory cytokine secretion associated with survival benefit in LPS-induced sepsis (37). Mutations or chromosomal translocations with lower expression of PU.1 gene are associated with the pathogenesis of acute myeloid leukemia and acute lymphoblastic leukemia(38). It would possibly be greatly beneficial to understand and intervene in disease pathogenesis by interdiction in the PU.1 regulatory signal pathways.

PU.1 could modulate lymphoid-myeloid progenitors by binding to another transcription factor GATA-1, countering each other's ability to regulate myeloid lineage-specific genes (39). PU.1 and GATA-1 also bind the mast cell-specific enhancer of IL-4 and stimulate its activity cooperatively (40). PU.1 is also necessary for cooperative activation of eosinophil granule proteins with C/EBP family (41). More importantly, high expression of PU.1 is necessary for maintaining macrophage differentiation in that PU.1 is macrophage specific transcription factor by formatting and regulating the whole complement of macrophage-specific regulatory genomic regions (12). The expression of PU.1 in Th2 cells could bind to another transcription factor IRF4 and limit its function by decreasing the binding to its target genes such as IL-10 (42). IRF4 was identified to be key for controlling M2 macrophage polarization through Jmjd3-mediated H3K27 demethylation (11) and contributed to Th2 heterogeneity (42). Therefore, PU.1 could be a potent candidate in regulating macrophage polarization in Th2 type immune response.

Although broad studies on PU.1 have put it as a key regulator in the hematopoiesis, the embryonic lethality from PU.1 deficiency has restrained further studies in animal models. PU/ER(T)^{+/-} mice that we used were generated by Dr. Edward Scott from University of Florida. Their behavior and fertility are as normal as WT mice. Those heterozygote mice are partial PU.1 deficient mice where a single PU.1 locus is transcriptionally inactive by fused with the modified estrogen (E) receptor (R) ligand binding domain but can be reactivated by tamoxifen (37), which provides us a useful tool to study the function of PU.1. In another words, without administration of tamoxifen, the PUER fusion protein in heterozygotes will be stuck in the cytoplasm and transcriptionally inactive, which causes to partially knock out the function of PU.1. Our group has shown attenuated lung and systemic inflammatory phenotype associated with survival benefit in LPS-challenged PU/ER(T)^{+/-} mice (37); however, the role of PU.1 in AAM polarization and in the pathogenesis of asthmatic lung has not been investigated yet.

D. <u>Hypothesis and specific aims</u>

Taking our previously mentioned results into consideration, we hypothesize that PU.1 is critical for M2 macrophage polarization and the development of asthmatic inflammation and airway remodeling, in part through modulation of M2 skewing. As discussed, PU.1 is essential for mature macrophage function and AAM is involved in the development of asthmatic process,

II. MATERIALS AND METHODS

A. Materials

1. Allergens and adjuvant

Triple allergens include extracts of dust mite (*Dermatophagoides farina*), ragweed (*Ambrosia artemisifolia*), and *Aspergillus fumigates* (Greer Laboratories, Lenoir, NC). Adjuvant was aluminum and magnesium hydroxide (Inject Alum; Thermo Scientific, Waltham, MA). Quantities of allergens for intraperitoneal (200 μl) and intranasal injection (50 μl) are as follows: *D. farina* (5 μg, 3-35 EU by means of LAL assay), ragweed (50 μg, 5 EU), and *Aspergillus fumigates* (5 μg, 0.1 EU) (43).

2. <u>Reagents</u>

Ca^{2+/}Mg²⁺⁻free Hanks' balanced salt solution (HBSS), Phosphate-buffered solution (PBS), DMEM and fetal bovine serum (FBS) were purchased from Thermo Scientific. M-CSF was purchased from Peprotech (Rocky Hill, NJ). Nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Interleukin-4 (IL-4) was purchased from Sigma (St. Louis, MO). SYBR Green and cDNA synthesis kit were obtained from Quanta Biosciences (Gaithersburg, MD). All primers used in real-time PCR were designed based on literatures and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The antibodies for PU.1, Ym-1, Fizz-1, phospho-STAT6, STAT6, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA), STEMCELL Technologies (Vancouver, Canada), Abcam (Cambridge, MA), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

B. Methods

1. <u>Mice</u>

PU/ER(T) ^{+/-} transgenic mice generated by Dr. Edward Scott (University of Florida, Gainesville), were heterozygous with a single PU.1 locus fused to the modified estrogen (E) receptor (R) ligand binding domain that is tamoxifen responsive (37). Therefore functional PU.1 can be induced by treatment with tamoxifen in these mice, while homozygote is embryo lethal. WT and PU/ER(T) ^{+/-} mice were bred, maintained in the animal facility of University of Illinois at Chicago. All animal studies were performed in accordance with the guidelines of the Animal Care Committee of the University of Illinois at Chicago and all animal experimental protocols were approved by the Animal Care Committee of the University of Illinois at Chicago.

2. <u>Acute murine asthma model</u>

WT and PU/ER(T) ^{+/-} mice at age of 8 to 10 weeks were sensitized by an intraperitoneal injection (i.p.) of 200 μ l alum-precipitated antigens containing triple allergen mix (DRA) (*D. farina* 5 μ g; **R**agweed 50 μ g; *Aspergillus fumigates* 5 μ g) in PBS vehicle. Five days after the first immunization (D0), the mice were given a second intraperitoneal booster of the allergens in alum adjuvant (D5) followed by exposure to DRA combination (50 μ l) or PBS (50 μ l) as control on days 12, 13, and 14 by intranasal injection (i.n.) after anesthesia with ketamine and xylazine. Then mice were sacrificed by humane CO2 suffocation and collected bronchoalveolar lavage (BAL) fluid and lung tissue for further analysis on Day 15 (43).

3. <u>Chronic murine asthma model</u>

WT and PU/ER(T) ^{+/-} mice at age of 8 to 10 weeks were chronic exposed to triple allergens (DRA) intranasally twice per week for 8 weeks without subcutaneous immunization in alum. After the 8-week exposure period, these mice were rested for 3 weeks and then harvested samples for analyses (43). A timeline for performance of the acute and chronic triple allergen DRA models is shown in Figure 3A and Figure 10A, respectively.

4. Bronchoalveolar lavage (BAL) differential cell count

BAL fluid was collected by lavaging the lung with 900 µl of PBS twice via a tracheal catheter and analyzed for total cell counts by countess automated cell counter (Life Technologies, Grand Island, NY). BAL fluid on cytospin slides was stained with HEMA 3 (Thermo Scientific, Waltham, MA) for differential cell counts. The number of macrophages and eosinophils were quantitated and compared for statistical significance.

5. Lung histology and airway mucus expression

To easily handle the lung tissue and maintain lung architecture during cryoprotection for further histological analysis, we infiltrated left lungs with 0.5% low temperature agarose and fixed them in paraformaldehyde solution immediately (44). Lungs were harvested and analyzed for hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining.

6. Bone marrow derived macrophages (BMDMs) culture

Femoral and tibia bone marrow were isolated from WT and PU/ER(T) ^{+/-} mice, by methods previously described(45). Mouse bone marrow cells flushed from femurs and tibias were washed with $Ca^{2+/}Mg^{2+-}$ free HBSS and then cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 ng/ml M-CSF for 6 d. Then BMDMs were stimulated for 24 h with IL-4, LPS, IFN- γ , or PBS. Discard culture supernatants and collect cells for RNA amd protein expression analysis. The expression of Ym-1 and Fizz-1 were analyzed by western blot. The mRNA expression of arginase-1, Ym-1 and Fizz-1 were analyzed by quantitative realtime PCR.

7. Western blot analysis

BMDMs were plated in 6-well plates at 2×10^6 /well overnight and challenged with 5 ng/ml and 10 ng/ml of IL-4 or PBS for 24 h, or stimulated with 5 ng/ml of IL-4, or 100 ng/ml of

LPS, or 20 ng/ml of IFN- γ for 24 h. The cells were then collected and lysed. The cell lysate was analyzed by immunoblot using antibodies against PU.1, phospho-STAT6, STAT6, Y-m1, Fizz-1 and GAPDH. Quantification of western blots were analyzed with ImageJ software.

8. Isolation of total RNA and Real-time qPCR

Frozen right lobes of lungs were homogenized with the TissueLyser system (Qiagen, Germantown, MD). Total-cellular RNA extracted from BMDMs or lung tissue homogenates was isolated with miRNeasy Mini Kit (Qiagen) following the instruction. cDNA was prepared by qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD) and amplified by Real-time qPCR on LightCycler 480 instrument (Roche Diagnosis, Indianapolis, IN) with primer sets for arginase-1(forward, CAATGAAGAGCTGGCTGGTGT; reverse, GTGTGAGCATCC ACCCAAATG; Integrated DNA Technologies), Ym-1(forward, GGGCATACCTTTAT CCTGAG; reverse, CCACTGAAGTCATCCATGTC; Integrated DNA Technologies), Fizz-1(forward, GAGACCATAGAGATTATCGTGGA; reverse, CACACCCAGTAGCAGTCATC; Integrated DNA Technologies), and GAPDH (forward, TGCGACTTCAACAGCAACTC; reverse, CTTGCTCAGTGTCCTTGCTG; Integrated DNA Technologies).

9. Flow cytometry

Cells from the BAL fluid were incubated with Fc blocking anti-mouse CD 16/32 antibodies (BD Bioscience, Walthma, MA) followed by PE-conjugated anti-Siglec F (BD Bioscience), APC-conjugated anti-CD11c (Biolegend, San Diego, CA). Cells were analyzed on a Beckman Coulter CyAn ADP flow cytometer in our Research Resources Center and data were analyzed with FlowJo software.

10. <u>Statistical analysis</u>

Data were expressed as means \pm SEM. Differences between groups of mice were evaluated with two way-ANOVA between indicated groups, others using Student's *t* test. A statistical software GraphPad Prism was used for the analysis. P value < 0.05 was considered statistically significant.

III. RESULTS

A. Specific aim 1: determine the role of PU.1 on the pathogenesis of asthma

1. Establish the acute DRA-induced murine asthma model

Our previous studies of the function of transcription factor PU.1 in macrophages demonstrated that PU.1 is required for macrophage differentiation and maturation and elucidated the *in vivo* function of PU.1 in mediating mature macrophage inflammatory phenotype *via* NF- κ B activation (37). However, the precise contribution of PU.1 in allergic inflammation remains unraveled. Since PU.1 deficient mice are embryonically lethal (46), we used partial PU.1 knockout mice designated as PU/ER(T)^{+/-} mice to investigate the potential function of PU.1 in the pathogenesis of asthmatic lung inflammation.

Ovalbumin (OVA)-induced murine asthma models have been well accepted and established; however, the airway pathology and hyperreactivity subsides within 1 to 2 weeks after allergen challenge and is not associated with chronic airway remodeling which is a prominent feature of severe asthma (47). In case of chronic exposure to the same allergen, mice become tolerant. Therefore, we used the newly described DRA triple allergen induced asthma model to break through tolerance and induce persistent airway pathologies which better outlines human asthma (43). To establish this triple allergen induced asthma model, we used 4 doses of DRA for challenge in WT mice (Figure 3A). The total cell counts in the BAL fluid significantly increased in challenge groups compared with PBS control group in a dose-dependent pattern (Figure 3B), which indicates acute DRA challenge can induce asthmatic lung inflammation.

2. Role of PU.1 on DRA-induced acute asthmatic lung inflammation

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To investigate whether PU.1 is involved in DRA-induced acute asthmatic lung inflammation, WT and PU/ER(T)^{+/-} mice were challenged with 20 μ g of DRA in 50 μ l of PBS or 50 μ l of PBS alone based on our previous study. The total cell counts in the BAL fluid increased dramatically in both WT and PU/ER(T)^{+/-} mice compared to PBS controls, while was attenuated by about 25% in the PU/ER(T)^{+/-} mice versus WT (Figure 4A). In addition, analysis of cytospin slides revealed more abundant alveolar eosinophils in the BAL fluid from WT mice compared to those from PU/ER(T)^{+/-} mice (Figure 4B). Interestingly, there were no differences in macrophage counts between WT and PU/ER(T)^{+/-} mice with or without DRA challenge although it showed increased trend after challenge (Figure 4C). BAL cytospins also showed the morphology of eosinophils and macrophages as alveolar macrophages are present characteristic foamy and opaque appearance with less dense chromatin in nuclei than lymphocytes (Figure 4D, white arrowhead) while eosinophils appear intense eosinophilic granule staining in the cytoplasm with multiple-lobed nuclei (Figure 4D, black arrowhead).

To analyze the cell population after allergen challenge, the BAL fluid is usually used to study the presence of cells by flow cytometry combined with morphologic analysis and manual counting on cytospin slides. Alveolar macrophages lack CD11b and express high levels CD11c and Siglec-F, while dendritic cells in the BAL fluid are either CD11c^{hi}CD11b⁺or CD11c^{hi}CD11b⁻ (48). Eosinophils are typically identified as Siglec-F⁺ but CD11c⁻ (49), whose recruitment from the blood stream into the airway is a conspicuous feature of allergic asthma. Therefore, the way to distinguish alveolar macrophages and dendritic cells as well as eosinophils is by flow cytometric analysis can be simply based on expression of Siglec-F and CD11c. Surface staining for markers of eosinophils (Siglec F⁺CD11c⁻) and macrophages (Siglec F⁺CD11c⁺) revealed significant eosinophil accumulation (84.8%) in DRA-challenged WT mice while the percentage of macrophages decreased from 73.8 to 6.7 after challenge. In contrast, PU/ER(T)^{+/-} mice had an impaired development of DRA-induced acute eosinophilic lung inflammation compared with WT mice (Figure 5). Together these data indicate that functional PU.1 is required for the development of DRA-induced acute allergic airway.

B. Specific aim 2: investigate how PU.1 regulates asthmatic inflammation

Given that PU.1 is important in regulating macrophage differentiation and previous reports of AAM involved in Th2 cytokine-mediated allergic responses, we hypothesized that PU.1 deficiency may be involved in the development of asthmatic inflammation through regulating AAM polarization. We have shown that PU/ER(T)^{+/-} mice developed impaired allergic airway inflammation compared with WT mice. To examine AAM responses after DRA acute challenge, lungs isolated from WT and $PU/ER(T)^{+/-}$ mice at day 15 after challenged with DRA or PBS were analyzed for expression of the AAM feature genes Ym-1, Fizz-1, and arginase-1 by real-time PCR. In WT mice, DRA acute challenge resulted in an 18-fold induction of Ym-1 mRNA expression over PBS controls. In contrast, there was only a 10-fold induction of Ym-1 in DRA-challenged $PU/ER(T)^{+/-}$ mice (Figure 6A). Furthermore, although DRA-challenged WT mice resulted in a 49-fold induction of Fizz-1, there was as low as a 17-fold induction of Fizz-1 in PU/ER(T)^{+/-} mice (Figure 6B). Interestingly, we did not observe any significant differences in induction of arginase-1 between WT and $PU/ER(T)^{+/-}$ mice challenged with DRA (Figure 6C). Together, these data demonstrate DRA acute

challenge could induce AAM polarization with significant increase in marker gene expression, which was impaired in the absence of PU.1.

C. <u>Specific aim 3: determine whether PU.1 plays a pivotal role in macrophage</u> <u>polarization</u>

1. PU.1 is associated with AAM polarization

It has been well studied that PU.1 functions in a genome-wide manner to control the formation and accessibility of the whole complement of macrophage-specific regulatory genomic regions (12); however, whether PU.1 is associated with macrophage polarization is unraveled. Our hypothesis is PU.1 not only plays an important role in regulation of macrophage differentiation, but also is essential for macrophage polarization. To investigate our hypothesis, we stimulated WT BMDMs with IL-4 (known Th2 type cytokine to induce AAM activation). Given the well-established contribution of IL-4 mediated signaling in M2 macrophage activation and the M2 polarization is regulated primarily by STAT6 (50), we reasoned that STAT6 should be phosphorylated by IL-4. As expected, the ratio of STAT6 phosphorylation to total STAT6 significantly increased in a 4.8- to 7.0-fold from 15 min to 1 h following the similar pattern of PU.1 expression (Figure 7A). Similarly, significantly increasing the expression of PU.1 in BMDMs was at 30 min by IL-4 (Figure 7B). Taken together, these data demonstrate that PU.1 is associated with AAM polarization induced by IL-4.

2. PU.1 is required for AAM polarization

To investigate whether PU.1 plays an important role in AAM polarization, BMDMs from WT or PU/ER(T)^{+/-} mice were cultured in the presence of M-CSF. After 6 days, BMDMs were stimulated for 24 h with IL-4 (5 ng/ml, 10 ng/ml) or PBS. Western blot showed Ym-1 protein expression was significantly attenuated in PU/ER(T)^{+/-} mice with or without stimulated by IL-4 (Figure 8A). Densitometry analysis was shown in the mean ratios of Ym-1 to GAPDH (Figure 8B). In addition, BMDMs were stimulated with M1 macrophage inducer IFN- γ or LPS, and M2 macrophage inducer IL-4 for 24 h. BMDMs from PU/ER(T)^{+/-} mice, compared to those from WT mice, displayed markedly attenuated expression of the AAM markers, Ym-1 and Fizz-1 by both western blot and real-time PCR assay (Figure 8C and 9) in response to IL-4 stimulation. Even in resting status, the protein levels of Ym-1 and Fizz-1 were still lower in PU/ER(T)^{+/-} mice (Figure 8). LPS could induce slight expression of Ym-1 but not Fizz-1 while IFN- γ had no effects on both expressions (Figure 8C); suggesting PU.1 is required for the development of IL-4 induced AAM phenotype. Similar to acute asthma model, another M2 marker arginase-1 showed equivalent mRNA expression in both mice (Figure 9C).

<u>3. Deficiency of PU.1 attenuates the polarization towards AAM during DRA-</u> induced chronic allergic airway inflammation and airway remodeling

Since most allergic asthmatic patients are chronically exposed to multiple allergens, we used a chronic asthma model challenged with a combination of 3 common allergens (DRA) chronically for better understanding the pathologic process of asthma in order to further intervene in this process in patients with asthma. This chronic model differs from the acute model shown above in that it does not include an adjuvant sensitization process, which more naturally mimics human asthma. Above data show *in vitro* and *in vivo* evidence of PU.1 regulating AAM polarization, we further accessed whether PU.1 is also important in the chronic asthmatic process. Therefore, we exposed WT and PU/ER(T)^{+/-} mice to DRA for 8 weeks, which induced severe airway inflammation and airway remodeling in WT mice while slightly changes in PU/ER(T)^{+/-} mice (Figure 10, 12, and 13). Analysis of BAL showed greater cellular infiltration in chronically challenged WT and PU/ER(T)^{+/-} mice than in unchallenged mice, but total cell counts (Figure 10B), eosinophil (Figure 10C) and macrophage cell counts (Figure 10D) were lower in PU/ER(T)^{+/-} mice than in WT mice. Not like acute asthma model, chronic model showed macrophage accumulation in WT mice obviously more than PU/ER(T)^{+/-} mice, indicating macrophages are also pivotal for the development of asthmatic inflammation that is regulated by functional PU.1 in long-term allergic response.

To investigate whether PU.1 is critical for regulating the generation of proasthmatic macrophage phenotype in chronic asthma model, we isolated RNA from lung homogenates and measured M2 specific marker gene expression. We found dramatically increased expression of Ym-1(Figure 11A), Fizz-1(Figure 11B), and arginase-1 (Figure 11C) mRNA in DRA-challenged lungs, while the expression were attenuated in PU/ER(T)^{+/-} mice compared to WT mice. Previously both *in vitro* and acute *in vivo* experiments showed no difference in arginase-1 mRNA expression between WT and PU/ER(T)^{+/-} mice. The possible explanation for this discrepancy is that arginase-1 expression could need longer and stronger stimulation compared to other markers. All together, these data confirmed that functional PU.1 is critical to drive macrophages towards M2 phenotype.

Analysis of hematoxylin and eosin (H&E) stained lung sections isolated from WT and PU/ER(T)^{+/-} mice revealed no pathological change in the absence of PU.1 in PBS control groups (Figure 12, top). Strikingly, WT mice challenged with DRA exhibited

remarkable cellular infiltration in peribronchial areas which attenuated in DRAchallenged PU/ER(T)^{+/-} mice (Figure 12, bottom). Similarly, WT mice challenged with DRA showed an impressive mucous gland and goblet cell hyperplasia by PAS staining compared to DRA-challenged PU/ER(T)^{+/-} mice (Figure 13, bottom, black arrowhead). In contrast, there were no PAS-positive goblet cells in PBS control groups (Figure 13, top). Those histological data suggest that PU.1 is necessary for chronic airway inflammation and airway remodeling.

Collectively, functional PU.1 was confirmed to be an important transcription factor that is essential for the pathogenesis of allergic asthmatic lung inflammation and airway remodeling, in partially through regulating alternative macrophage polarization. Α



Figure 3. Triple allergen DRA induced acute allergic airway inflammation.

A. A protocol for immunization, allergen exposure and time of analysis in the acute model. WT mice were sensitized (i.p.) with 50 μ g of DRA in 200 μ l alum on day 0 and day 5, and then challenged (i.n.) on day 12, 13, and 14 with 4 different doses of DRA or PBS control. B. Mice were killed on day 15. Bronchoalveolar lavage (BAL) cells were counted and shown in 4 doses of DRA. N=3 each group. *, p<0.05, **, p<0.01, DRA challenged *vs* PBS control WT mice.



Figure 4. PU/ER(T)^{+/-} mice show an impaired development of DRA-induced acute allergic airway inflammation.

WT and PU/ER(T)^{+/-} mice were challenged (i.n.) three times with 20 µg of DRA or PBS. Mice were killed 24 hours after the third DRA challenge and BAL cells were counted and dispersed onto microscope slides using a cytospin. Total cell counts (A), eosinophils counts (B), and macrophages (C) in the BAL fluid were performed. N=3 each group. *, p<0.05, DRA challenged WT vs PU/ER(T)^{+/-} mice. D. Differential cell types including macrophages (black arrowhead) and eosinophils (white arrowhead) showed on the cytospin slides.



Figure 5. PU/ER(T)^{+/-} mice show an impaired development of DRA-induced acute eosinophilic lung inflammation.

WT and PU/ER(T)^{+/-} mice were sensitized (i.p.) with 50 μ g of DRA in 200 μ l alum on day 0 and day 5, and then challenged (i.n.) on day 12, 13, and 14 with 20 μ g of DRA or PBS. Twenty four hours later, BAL fluid was collected. Surface staining for markers of eosinophils (Siglec F⁺CD11c⁻) and macrophages (Siglec F⁺CD11c⁺) in the BAL fluid were analyzed by FACS. Siglec F single positive indicated eosinophils, while Siglec F and CD 11c double positive indicated macrophages.



Figure 6. PU/ER(T)^{+/-} mice show an impaired polarization of AAM during acute allergic airway inflammation.

WT and PU/ER(T)^{+/-} mice were sensitized (i.p.) with 50 µg of DRA in 200 µl alum on day 0 and day 5, and then challenged (i.n.) on day 12, 13, and 14 with 20 µg of DRA or PBS. On day 15 mice were killed and lungs were collected, followed by RNA isolation and mRNA expression levels for Ym-1 (A), Fizz-1 (B), and Arginase-1 (C) were quantified using real-time qPCR. N=3 each group. *, p<0.05, DRA challenged WT vs PU/ER(T)^{+/-} mice.



Figure 7. PU.1 is associated with AAM polarization.

A. BMDMs from WT mice were cultured in the presence of M-CSF (10 ng/ml). After 6 days, BMDMs were stimulated with 5 ng/ml of IL-4 for 15, 30, and 60 min. The levels of phosphorylated STAT6 and total STAT6 were analyzed by western blot. Below lane showed the ratio of phosphorylated and total STAT6 in densitometry.

B. The protein levels of PU.1 were analyzed using western blot.





A, BMDMs from WT or PU/ER(T)^{+/-} mice were cultured in the presence of M-CSF (10 ng/ml). After 6 days, BMDMs were stimulated for 24 h with IL-4 (5 ng/ml, 10 ng/ml) or PBS. Western blot showed Ym-1 protein expression.

B, Densitometry analysis was shown in the mean ratios of Ym-1 to GAPDH. Data are representative of three independent experiments shown in means \pm SEM and *, *p*<0.05. C, BMDMs from WT or PU/ER(T)^{+/-} mice were cultured and stimulated with IL-4 (5 ng/ml), or LPS (100 ng/ml), or IFN- γ (20 ng/ml), or PBS as control for 24 h. Western blot showed the protein expression of Ym-1 and Fizz-1 with GAPDH as control.



Figure 9. Deficiency of PU.1 attenuates the polarization of AAM *in vitro*. BMDMs from WT or PU/ER(T)^{+/-} mice were cultured in the presence of M-CSF (10 ng/ml). After 6 days, BMDMs were stimulated for 24 h with 5 ng/ml of IL-4 or PBS. RNA was isolated 24 h later and real-time quantitative PCR was used to measure mRNA fold changes for Ym-1 (A), Fizz-1 (B) and Arginase-1 (C) compared to GAPDH. Data are means \pm SEM and are representative of three independent experiments. **, *p*<0.01, WT vs PU/ER(T)^{+/-} mice.

Α





A. A timeline of allergen exposure, rest period, and time of analysis in the chronic mouse model without alum as adjuvant. WT and PU/ER(T)^{+/-} mice were chronically exposed to 60 μ g of DRA in 50 μ l PBS intranasally twice a week for 8 weeks and rested for 3 weeks before analysis. Bronchoalveolar lavage (BAL) cells were counted and dispersed onto microscope slides using a cytospin. Total cell counts (B), eosinophils counts (C), and macrophages (D) in the BAL fluid were performed. N=3 each group. *, *p*<0.05, **, *p*<0.01, and ***, *p*<0.001, DRA challenged PU/ER(T)^{+/-} vs WT mice.





WT and PU/ER(T)^{+/-} mice were exposed to 60 µg of DRA in 50 µl PBS twice weekly for 8 weeks and were rested for 3 weeks. On week 12, mice were sacrificed and lungs were collected, followed by RNA isolation and mRNA levels for Ym-1, Fizz-1 and Arginase-1 were quantified using real-time qPCR. N=3 each group. *, p<0.05, **, p<0.01, DRA challenged PU/ER(T)^{+/-} vs WT mice.



Figure 12. PU/ER(T)^{+/-} mice shows less peribronchial inflammation in DRA-induced chronic asthma model.

WT and PU/ER(T)^{+/-} mice were exposed to 60 μ g of DRA in 50 μ l PBS twice weekly for 8 weeks and were rested for 3 weeks. On week 12, mice were sacrificed and left lungs were infiltrated with 0.5% low temperature melting agarose and fixed in 1% paraformaldehyde solution. 200X hematoxylin and eosin-stained lung sections from the mice exposed to PBS or DRA chronically.



Figure 13. PU/ER(T)^{+/-} mice shows less mucous gland and goblet cell hyperplasia in DRAinduced chronic asthma model.

WT and PU/ER(T)^{+/-} mice were exposed to 60 µg of DRA in 50 µl PBS twice weekly for 8 weeks and were rested for 3 weeks. On week 12, mice were sacrificed and left lungs were infiltrated with 0.5% low temperature melting agarose and fixed in 1% paraformaldehyde solution. 200X periodic acid-Schiff (PAS)-stained lung sections from the mice exposed to PBS or DRA chronically. Black arrows indicate PAS positive goblet cells.

IV. DISCUSSION

There is uncertainty regarding the etiology and molecular pathogenesis of severe asthma which is increasing in frequency and severity in our population. Animal models, in particular, murine asthma models have been used to unfold pathophysiology of asthma and to pinpoint novel therapeutic targets. Since mice do not develop asthma spontaneously, OVA, generated from chicken egg as a foreign protein, has been broadly used as allergen to induce allergic lung inflammation acutely or chronically. However, the OVA model does not replicate human asthma as chronic exposure to OVA shows loss of allergic response through the development of tolerance (51). Although no animal model can completely recapitulate all features of human asthma, chronic DRA asthma model has better clinical implication by reproducing more of the features of clinical asthma. This model has been shown to have following advantages compared to traditional OVA models: components including extracts of dust mite which is a well-known human asthma trigger, resistance to chronic tolerance, persistence of eosinophilic inflammation, and induction of chronic inflammation and airway remodeling without the need for adjuvants (43). We observed abundant airway inflammation and eosinophil recruitment in both acute and chronic DRA mouse model, as well as goblet cell hyperplasia in the chronic model. Furthermore, we used this novel DRA-induced asthma model in PU/ER(T) WT and heterozygous mice to study the role of PU.1 in the development of allergic inflammation. Similarly, we have observed impaired development of allergic inflammation and airway remodeling in mice with partial loss of functional PU.1, suggesting functionally intact PU.1 is required for the development of allergic airway inflammation.

Currently, our knowledge about asthma focuses on Th2 lymphocytes generated cytokines to promote eosinophilic inflammation and consequent responses (22). Yet it is not clear in our

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model what types of cells affected by PU.1 are attributed to the impaired development of asthma. In our hands, the major component of the increased cellular infiltration was due to a massive increase in eosinophils in the WT mice challenged with DRA and this was reduced more than half in the DRA treated $PU/ER(T)^{+/-}$ mice. Surprisingly, WT mice had obviously increased macrophage recruitment into lungs upon DRA chronic challenge and this also was markedly attenuated in $PU/ER(T)^{+/-}$ mice, which raised a hypothesis that macrophages may play a critical role in the allergic asthma and this role is under regulation of PU.1. In fact, alveolar macrophages are above 80% in the BAL fluid in healthy non-smokers. We also observed almost 100% of alveolar macrophages in BAL from non-challenged mice. Paradoxically, the importance of abundant alveolar macrophages as the first immune cells in the lung has been underscored for long time and they are so called 'forgotten cell in asthma' (52) that is not considered as a specific cell type in the asthma paradigm. DRA chronic asthma model is associated with higher level of dendritic cell signaling and increased proliferation of CD4 T cells; however, no investigation of macrophages in this model has been reported (43). Recently, mice with PU.1-deficient T cells have shown defects in the development of allergic inflammation induced by OVA but normal Th2 responses without effects on dendritic cells (53). Furthermore, it demonstrated that PU.1 is required for the development of IL-9-producing T cells and allergic inflammation by promoting the expression of IL-9 and proallergic chemokines in T cells. Therefore, the involvement of PU.1-deficiency T cells cannot be excluded in our asthma model. It has been reported that mice harboring PU.1-deficient B cells have normal numbers of peripheral B cells and normal immune responses, suggesting not like the development of B cells in the embryo, the development of functional B cells beyond the pre-B stage does not require PU.1(54). Based on this information, we deduce that B cells might not be affected by deficiency of PU.1 in our model. Our previous

report demonstrated that PU.1 is dispensable for survival of mature macrophages as there was no difference in either total number or apoptosis of alveolar macrophages between WT and PU.1-deficient mice but inflammatory phenotype of mature macrophages was changed depend on functional PU.1 (37). Therefore, one explanation could be deficiency of PU.1 attenuates the development of allergic inflammation at least in part through regulating macrophage's phenotype. Without proper macrophage phenotype, T cells and eosinophils cannot function appropriately.

In addition to the well-established role in differentiation of macrophages and B lymphocytes (46), PU.1 is required for the M-CSF dependent macrophage proliferation by regulating M-CSF receptors expression on macrophages via binding to the promoter of the M-CSF receptors (55). Many cytokines, chemokines, growth factors, or transcription factors are involved in regulation of macrophage polarization. It is well known that M-CSF drives macrophages to M2 phenotype (7). Typically, AAM polarization is mediated by IL-4 and IL-13 dependent phosphorylation of STAT6, which results in the generation of Ym-1, Fizz-1, and arginase-1 in macrophages (56). Moreover, PU.1 antagonized another key transcription factor IRF4 in Th2 cell differentiation, which was identified to drive AAM polarization against helminth infection. Although many roles for PU.1 have been established, the way it regulates macrophage polarization is barely known. In this present study, *in vitro* data show that PU.1 strongly expressed in M-CSF cultured macrophages upon IL-4 stimulation through STAT6 activation. The depletion of PU.1 in mature macrophages resulted in less expression of Ym-1 and Fizz-1 in either resting or activated state, leading to the hypothesis that PU.1 may be important for promoting AAM polarization interacting with other transcription factors such as IRF4 via IL-4/STAT6 signal pathways.

It is not clear whether AAM are beneficial or harmful to the host in allergic inflammation. M2 macrophage specific markers arginase, Ym-1 and Fizz-1 are all upregulated and each important in the development of allergic inflammation (18) (31) (57). Ym-1 belongs to a family of chitinase-like molecules, which are shown to correlate with the severity of human asthma (58). Although it has been shown that Fizz-1 promoted early stages of airway remodeling in OVAinduced asthma by inducing fibroblasts to express myofibroblast differentiation markers (59); conflictingly, Fizz-1 produced by AAM, epithelium, and eosinophils in type 2 inflammations in the lung appeared to inhibit inflammation through negative regulation of CD4⁺ T cell responses (60). Adoptive transfer of M2 macrophages into mice with A *fumigates* allergy exacerbated airway inflammation and remodeling (33). Here, we did observe dramatic increase of arginase-1, Ym-1 and Fizz-1 in allergen-challenged lungs, which all contribute to the airway inflammation and airway remodeling during asthma. Furthermore, we have confirmed that deficiency of PU.1 resulted in attenuated allergic inflammation in chronic asthma model and this observation correlated with less production of arginase-1, Ym-1 and Fizz-1. One explanation is that PU.1dependent proallergic factors produced by AAM are responsible for the impaired allergic inflammation of PU/ER(T)^{+/-} mice. AAM participate in the repair of lungs during inflammation or injury; however, the persistence of these cells to release excessive proallergic factors or inflammatory factors apparently attributes to abundant cell recruitment, airway mucus secretion, and airway hyper-responsiveness that characterize asthma. Those AAM either have impaired ability to present antigens to T cells to elicit asthma cascaded responses or generate molecules to interact with Th2 cells by decreasing Th2 type cytokine levels.

In summary, our studies identify a previously unrecognized role for PU.1 in type 2 immunity, acting as an important transcription factor in the development of allergic

inflammation, in part through polarizing AAM by regulating the expression of proallergic factors Ym-1 and Fizz-1 in allergic inflammation. Since AAM are involved in a broad range of diseases such as asthma and cancer, the manipulation of PU.1 expression could be a promising therapeutic strategy for the treatment of human diseases induced by persistent AAM activation.

CITED LITERATURE

- 1. Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad, and K. Ley. 2010. Development of monocytes, macrophages, and dendritic cells. *Science* 327:656-661.
- 2. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.
- 3. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.
- 4. Gordon, S., and F. O. Martinez. 2010. Alternative activation of macrophages: mechanism and functions. *Immunity* 32:593-604.
- 5. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.
- 6. Benoit, M., B. Desnues, and J. L. Mege. 2008. Macrophage polarization in bacterial infections. *J Immunol* 181:3733-3739.
- 7. Fleetwood, A. J., T. Lawrence, J. A. Hamilton, and A. D. Cook. 2007. Granulocytemacrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J Immunol* 178:5245-5252.
- 8. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415-1421.
- 9. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380:627-630.
- Krausgruber, T., K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, and I. A. Udalova. 2011. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* 12:231-238.
- Satoh, T., O. Takeuchi, A. Vandenbon, K. Yasuda, Y. Tanaka, Y. Kumagai, T. Miyake, K. Matsushita, T. Okazaki, T. Saitoh, K. Honma, T. Matsuyama, K. Yui, T. Tsujimura, D. M. Standley, K. Nakanishi, K. Nakai, and S. Akira. 2010. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol* 11:936-944.
- 12. Lawrence, T., and G. Natoli. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol* 11:750-761.
- 13. Anthony, R. M., J. F. Urban, Jr., F. Alem, H. A. Hamed, C. T. Rozo, J. L. Boucher, N. Van Rooijen, and W. C. Gause. 2006. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med* 12:955-960.
- Loke, P., I. Gallagher, M. G. Nair, X. Zang, F. Brombacher, M. Mohrs, J. P. Allison, and J. E. Allen. 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol* 179:3926-3936.
- Odegaard, J. I., R. R. Ricardo-Gonzalez, M. H. Goforth, C. R. Morel, V. Subramanian, L. Mukundan, A. Red Eagle, D. Vats, F. Brombacher, A. W. Ferrante, and A. Chawla. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 447:1116-1120.

- 16. Zhu, Z., T. Zheng, R. J. Homer, Y. K. Kim, N. Y. Chen, L. Cohn, Q. Hamid, and J. A. Elias. 2004. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 304:1678-1682.
- Gallina, G., L. Dolcetti, P. Serafini, C. De Santo, I. Marigo, M. P. Colombo, G. Basso, F. Brombacher, I. Borrello, P. Zanovello, S. Bicciato, and V. Bronte. 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 116:2777-2790.
- 18. Nair, M. G., I. J. Gallagher, M. D. Taylor, P. Loke, P. S. Coulson, R. A. Wilson, R. M. Maizels, and J. E. Allen. 2005. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect Immun* 73:385-394.
- 19. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 25:677-686.
- 20. Raes, G., P. De Baetselier, W. Noel, A. Beschin, F. Brombacher, and G. Hassanzadeh Gh. 2002. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol* 71:597-602.
- 21. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 27:451-483.
- 22. Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. N Engl J Med 344:350-362.
- Stein, R. T., D. Sherrill, W. J. Morgan, C. J. Holberg, M. Halonen, L. M. Taussig, A. L. Wright, and F. D. Martinez. 1999. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 354:541-545.
- 24. Halonen, M., D. A. Stern, C. Lohman, A. L. Wright, M. A. Brown, and F. D. Martinez. 1999. Two subphenotypes of childhood asthma that differ in maternal and paternal influences on asthma risk. *Am J Respir Crit Care Med* 160:564-570.
- 25. Venables, K. M., and M. Chan-Yeung. 1997. Occupational asthma. *Lancet* 349:1465-1469.
- 26. Georas, S. N., J. Guo, U. De Fanis, and V. Casolaro. 2005. T-helper cell type-2 regulation in allergic disease. *Eur Respir J* 26:1119-1137.
- 27. Holtzman, M. J. 2012. Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens. *J Clin Invest* 122:2741-2748.
- 28. Careau, E., and E. Y. Bissonnette. 2004. Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness. *Am J Respir Cell Mol Biol* 31:22-27.
- 29. Alexis, N. E., J. Soukup, S. Nierkens, and S. Becker. 2001. Association between airway hyperreactivity and bronchial macrophage dysfunction in individuals with mild asthma. *Am J Physiol Lung Cell Mol Physiol* 280:L369-375.
- 30. Vercelli, D. 2003. Arginase: marker, effector, or candidate gene for asthma? *J Clin Invest* 111:1815-1817.
- 31. Homer, R. J., Z. Zhu, L. Cohn, C. G. Lee, W. I. White, S. Chen, and J. A. Elias. 2006. Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. *Am J Physiol Lung Cell Mol Physiol* 291:L502-511.
- 32. Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C. J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I. B. McInnes, D. Xu, and F. Y. Liew. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol* 183:6469-6477.

- 33. Moreira, A. P., K. A. Cavassani, R. Hullinger, R. S. Rosada, D. J. Fong, L. Murray, D. P. Hesson, and C. M. Hogaboam. 2010. Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease. *J Allergy Clin Immunol* 126:712-721 e717.
- 34. Dahl, R., J. C. Walsh, D. Lancki, P. Laslo, S. R. Iyer, H. Singh, and M. C. Simon. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol* 4:1029-1036.
- 35. DeKoter, R. P., and H. Singh. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 288:1439-1441.
- Scott, E. W., R. C. Fisher, M. C. Olson, E. W. Kehrli, M. C. Simon, and H. Singh. 1997. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity* 6:437-447.
- 37. Karpurapu, M., X. Wang, J. Deng, H. Park, L. Xiao, R. T. Sadikot, R. S. Frey, U. A. Maus, G. Y. Park, E. W. Scott, and J. W. Christman. 2011. Functional PU.1 in macrophages has a pivotal role in NF-kappaB activation and neutrophilic lung inflammation during endotoxemia. *Blood* 118:5255-5266.
- 38. DeKoter, R. P., M. B. Kamath, and I. B. Houston. 2007. Analysis of concentrationdependent functions of PU.1 in hematopoiesis using mouse models. *Blood Cells Mol Dis* 39:316-320.
- 39. Cantor, A. B., and S. H. Orkin. 2001. Hematopoietic development: a balancing act. *Curr Opin Genet Dev* 11:513-519.
- 40. Henkel, G., and M. A. Brown. 1994. PU.1 and GATA: components of a mast cellspecific interleukin 4 intronic enhancer. *Proc Natl Acad Sci U S A* 91:7737-7741.
- 41. Gombart, A. F., S. H. Kwok, K. L. Anderson, Y. Yamaguchi, B. E. Torbett, and H. P. Koeffler. 2003. Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBP epsilon and PU.1. *Blood* 101:3265-3273.
- 42. Ahyi, A. N., H. C. Chang, A. L. Dent, S. L. Nutt, and M. H. Kaplan. 2009. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J Immunol* 183:1598-1606.
- 43. Goplen, N., M. Z. Karim, Q. Liang, M. M. Gorska, S. Rozario, L. Guo, and R. Alam. 2009. Combined sensitization of mice to extracts of dust mite, ragweed, and Aspergillus species breaks through tolerance and establishes chronic features of asthma. *J Allergy Clin Immunol* 123:925-932 e911.
- 44. Halbower, A. C., R. J. Mason, S. H. Abman, and R. M. Tuder. 1994. Agarose infiltration improves morphology of cryostat sections of lung. *Lab Invest* 71:149-153.
- 45. Deng, J., X. Wang, F. Qian, S. Vogel, L. Xiao, R. Ranjan, H. Park, M. Karpurapu, R. D. Ye, G. Y. Park, and J. W. Christman. 2012. Protective role of reactive oxygen species in endotoxin-induced lung inflammation through modulation of IL-10 expression. *J Immunol* 188:5734-5740.
- 46. Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265:1573-1577.
- 47. Whitehead, G. S., J. K. Walker, K. G. Berman, W. M. Foster, and D. A. Schwartz. 2003. Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 285:L32-42.

- 48. Lambrecht, B. N., and H. Hammad. 2012. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu Rev Immunol* 30:243-270.
- 49. Stevens, W. W., T. S. Kim, L. M. Pujanauski, X. Hao, and T. J. Braciale. 2007. Detection and quantitation of eosinophils in the murine respiratory tract by flow cytometry. *J Immunol Methods* 327:63-74.
- 50. Ruffell, D., F. Mourkioti, A. Gambardella, P. Kirstetter, R. G. Lopez, N. Rosenthal, and C. Nerlov. 2009. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A* 106:17475-17480.
- 51. Swirski, F. K., D. Sajic, C. S. Robbins, B. U. Gajewska, M. Jordana, and M. R. Stampfli. 2002. Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J Immunol* 169:3499-3506.
- 52. Peters-Golden, M. 2004. The alveolar macrophage: the forgotten cell in asthma. *Am J Respir Cell Mol Biol* 31:3-7.
- 53. Chang, H. C., S. Sehra, R. Goswami, W. Yao, Q. Yu, G. L. Stritesky, R. Jabeen, C. McKinley, A. N. Ahyi, L. Han, E. T. Nguyen, M. J. Robertson, N. B. Perumal, R. S. Tepper, S. L. Nutt, and M. H. Kaplan. 2010. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* 11:527-534.
- 54. Polli, M., A. Dakic, A. Light, L. Wu, D. M. Tarlinton, and S. L. Nutt. 2005. The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood* 106:2083-2090.
- 55. Celada, A., F. E. Borras, C. Soler, J. Lloberas, M. Klemsz, C. van Beveren, S. McKercher, and R. A. Maki. 1996. The transcription factor PU.1 is involved in macrophage proliferation. *J Exp Med* 184:61-69.
- 56. Mikita, T., D. Campbell, P. Wu, K. Williamson, and U. Schindler. 1996. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. Mol Cell Biol 16:5811-5820.
- 57. Zimmermann, N., N. E. King, J. Laporte, M. Yang, A. Mishra, S. M. Pope, E. E. Muntel, D. P. Witte, A. A. Pegg, P. S. Foster, Q. Hamid, and M. E. Rothenberg. 2003. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. J Clin Invest 111:1863-1874.
- 58. Chupp, G. L., C. G. Lee, N. Jarjour, Y. M. Shim, C. T. Holm, S. He, J. D. Dziura, J. Reed, A. J. Coyle, P. Kiener, M. Cullen, M. Grandsaigne, M. C. Dombret, M. Aubier, M. Pretolani, and J. A. Elias. 2007. A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med 357:2016-2027.
- Dong, L., S. J. Wang, B. Camoretti-Mercado, H. J. Li, M. Chen, and W. X. Bi. 2008. FIZZ1 plays a crucial role in early stage airway remodeling of OVA-induced asthma. J Asthma 45:648-653.
- 60. Nair, M. G., Y. Du, J. G. Perrigoue, C. Zaph, J. J. Taylor, M. Goldschmidt, G. P. Swain, G. D. Yancopoulos, D. M. Valenzuela, A. Murphy, M. Karow, S. Stevens, E. J. Pearce, and D. Artis. 2009. Alternatively activated macrophage-derived RELM-{alpha} is a negative regulator of type 2 inflammation in the lung. J Exp Med 206:937-952.

APPENDIX



2/16/2012

Office of Animal Care and Institutional Biosafety Committee (OACIB) (M/C 672) Office of the Vice Chancellor for Research 206 Administrative Office Building 1737 West Polk Street Chicago, Illinois 60612

John Christman Medicine/Pulmonary, Critical Care & Sleep Medicine M/C 719

Dear Dr. Christman:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and renewed on 2/16/2012.

Title of Application:	Macrophage Gene Expression in Acute Lung Inflammation
ACC NO:	10-006
Original Protocol Approval:	2/19/2010 (3 year approval with annual continuation required).
Current Approval Period:	2/16/2012 to 2/16/2013

Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

Number of funding sources: 5				
Funding Agency	Grant Title		Portion of Grant Matched	
NIH	Macrophage Gene Expression in Acute Lung Unflammation		Matched	
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL075557-06A1	Funded	2008-06721	UIC	John W. Christman
Funding Agency	Grant Title			Portion of Grant Matched
NIH	Nadph Oxidase Regulation of the Macrophage Inflammatory		Matched	
	Phenotype in Sepsi	s		
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL103643-01	Funded	2010-01635	UIC	John W. Christman
Funding Agency	Grant Title			Portion of Grant Matched
VA- Veteran's	Regulation of Neutrophilic Lung Inflammation		Matched	
Administration				
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
VA Merit award	Funded		UIC	John W. Christman

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD Chair, Animal Care Committee

BM/kg cc: BRL, ACC File, Jing Deng

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	Feng Qian, Jing Deng, Benjamin Gantner, Chen Dong, John Christman, and Richard Ye. MAP Kinase Phosphatase 5 Protects Against Sepsis- induced Acute Lung Injury. <i>J Immunol</i> , 188:112.10, 2012.
	Jing Deng , Feng Qian, Sangwoon Chung, Gye Young Park, Ravi Ranjan, Manjula Karpurapu, Lei Xiao, John W. Christman. Critical role of glycogen synthase kinase (GSK)-3 beta in differential regulation of macrophage microRNAs (miRNAs) and cytokine expression. <i>Am. J. Respir. Crit. Care Med.</i> 185: A1370, 2012.
	Ravi Ranjan, Manjula Karpurapu, Jing Deng , Lei Xiao, Sangwoon Chung, Gye Young Park, John W. Christman. Epigenetic regulation of KLF4 during macrophage maturation and inflammatory signaling. <i>Am. J. Respir. Crit. Care Med.</i> 185: A1062, 2012.
	Zhang C, Zhao G., Xu X, Zhao Q, Deng J , Xiao L: Role of NADPH oxidase subunit p67phox in regulation of pressure-overload-induced cardiac hypertrophic oxidative stress and antioxidant gene expression. <i>Circulation</i> . 124(21):A17477, 2011.
	Zhao Q, Xu X., Zhao G, Zhang C, Deng J , Christman JW, Xiao L: Myocardial-specific overexpression of a dominant-negative mutant of

NADPH oxidase subunit p67phox attenuates pressure-overload-induced cardiac hypertrophy in adult transgenic mice. *Circulation*. 124(21): A17329, 2011.

Thomas S, Zhao G, Yu R, **Deng J**, Christman JW, Xiao L: Critical Role Of Reactive Oxygen Species (ROS) In Differential Regulation Of Lipopolysaccharide (LPS)-Induced Prostaglandins (PG) Production In Mouse Bone Marrow-Derived Macrophages (BMDM). *Am. J. Respir. Crit. Care Med.* 183: A1076, 2011.

Zhao G, Thomas S, **Deng J**, Christman JW, Xiao L: Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) mediate lipopolysaccharide (LPS)-induced prostaglandin E synthase-1 (PGES-1) expression via an autocrine mechanism in mouse bone marrow-derived macrophages (BMDM). *Am. J. Respir. Crit. Care Med.* 183: A4541, 2011.

Karpurapu M, Wang M, **Deng J**, Park H, Xiao L, Sadikot RT, Frey RS, Maus UA, Park GY, Scott E, Christman JW: Lung inflammation and inflammatory gene expression in response to endotoxemia are attenuated in bone marrow chimera mice with functionally inactive PU.1. *Am. J. Respir. Crit. Care Med.* 183: A5768, 2011.

Park G, Fu P, Park H, **Deng J**, Maybruck B, Nyenhuis S, Berdyshev E, Morris AJ, Ackerman SJ, Christman JW, Natarajan V: Over-Expression Of Autotaxin Accentuates Allergic Asthmatic Inflammation. *Am. J. Respir. Crit. Care Med.* 183:A2833, 2011.

Gye Young Park, Hyesuk Park, **Jing Deng**, Manjula Karpurapu, Ulrich A. Maus, John W. Christman. Phenotypic Characterization Of Alveolar Macrophages In The Triple Antigen-Induced Murine Model Of Allergic Asthma. *Am. J. Respir. Crit. Care Med.* 183: A1321, 2011.

Zhao Q, Xu X, Shi Y., Zhao G, **Deng J**, Thomas S, Christman J.W, Ornatowska M, Xiao L.: Selective inhibition of myocardial NADPH oxidase attenuates pressure-overload-induced cardiac hypertrophy in aged transgenic mice with cardiac-specific overexpression of a dominant-negative mutant of p67phox. *Circulation*. 122(21): A20592, 2010.

Frey, F.S., **Deng J.**, Wang, X., Joo, M., Park, G.Y., Xiao L., Christman J.W.: NADPH oxidase-derived reactive oxygen species serve a protective role in endotoxin regulated immune cell function. *Am. J. Respir. Crit. Care Med.* 181:A2692, 2010.

Zhao Q., Xu X., Shi Y., Zhao G., **Deng J.**, Thomas S., Christman J.W., Ornatowska Zhao G., Thomas S., Kowalsky G., Christman J.W., Qian F., Levitan I., **Deng J.**, Xiao L.: Lipopolysaccharide (LPS) stimulation upregulates the expression of NADPH oxidase (NOX) in mouse bone marrow-derived macrophages (BMDM). *Am. J. Respir. Crit. Care Med.* 181: A1277, 2010.

Zhao Q., Xu X., Shi Y., Zhao G., Deng J., Thomas S., Christman J.W.,

Ornatowska M., Xiao L.: Selective inhibition of myocardial NADPH oxidase attenuates pressure-overload-induced cardiac hypertrophy in aged transgenic mice with cardiac-specific overexpression of a dominant-negative mutant of p67^{phox}. *Circulation*. 122(21): A20592, 2010.

Zhao G., Thomas S., Kowalsky G., Christman J.W., Qian F., Levitan I., **Deng J.**, Xiao L.: Lipopolysaccharide (LPS) stimulation up-regulates the expression of NADPH oxidase (NOX) in mouse bone marrow-derived macrophages (BMDM). *Am. J. Respir. Crit. Care Med.* 181: A1277, 2010.

Qian F., **Deng J**., Welch E.J., Malik A.B., Flavell R.A., Dong C., Ye R.: A Nonredundant Role for MAP Kinase Phosphatase 5 in Protecting Against Oxidant-Mediated Vascular Injury. *Arterioscler Thromb Vasc Biol.* 29(7): e60, 2009.

Qian F., **Deng J.**, Cheng N., Welch E.J., Zhang Y., Malik A.B., Flavell R.A., Dong C., Ye R.: A Nonredundant Role for MAPKP5 in Limiting ROS Production and Preventing LPS-induced Vascular Injury. *Proceedings of the 38th Annual Autumn Immunology Conference*. 38:64, 2009.

PUBLICATIONS: Guiqing Zhao, Rui Yu, **Jing Deng**, Qiong Zhao, Yongchao Li, Myungsoo Joo, Richard B. van Breemen, John W. Christman, Lei Xiao. Pivotal role of reactive oxygen species in differential regulation of lipopolysaccharide-induced prostaglandins production in macrophages. *Mol Pharmacol.* 2013; 83(1):167-78. PMID: 23071105.

Lei Xiao, Magdalena Ornatowska, Guiqing Zhao, Hongmei Cao, Rui Yu, **Jing Deng**, Yongchao Li, Qiong Zhao, Ruxana T. Sadikot, John W. Christman. Lipopolysaccharide-induced expression of microsomal prostaglandin E synthase-1 mediates late-phase PGE2 production in bone marrow derived macrophages. *PLoS One*. 2012; 7(11):e50244.

Jing Deng, Xuerong Wang, Feng Qian, Stephen M. vogel, Lei Xiao, Ravi Ranjan, Hyesuk Park, Manjula Karpurapu, Richard D. Ye, Gye Young Park, John W. Christman. Protective role of reactive oxygen species in endotoxin-induced lung inflammation through modulation of IL-10 expression. *J Immunol.* 2012; 188(11):5734-40. PMID: 22547702.

Feng Qian, **Jing Deng**, Benjamin N. Gantner, Richard R. Flavell, Chen Dong, John W. Christman, Richard D Ye, MAP Kinase Phosphatase 5 protests against sepsis-induced acute lung injury. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 2012; 302: L866-74. PMID: 22307906 (co-first author).

Feng Qian, Guy C. Le Breton, Jia Chen, **Jing Deng**, John W. Christman, Dianqing Wu, Richard D. Ye. A critical role for the guanine nucleotide exchange factor P-Rex1 in platelet secretion and aggregation.

Arterioscler Thromb Vasc Biol. 2012; 32: 768-777. PMID: 22207728.

Manjula Karpurapu, Xuerong Wang, **Jing Deng**, Hyesuk Park, Lei Xiao, Ruxana T. Sadikot, Randall S. Frey, Ulrich A. Maus, Gye Young Park, Edward W. Scott, John W. Christman. Functional PU.1 in macrophages has a pivotal role in NF- κ B activation and neutrophilic lung inflammation during endotoxemia. *Blood.* 2011; 118: 5255-5266. PMID: 21937699.

Feng Qian, **Jing Deng**, Ni Cheng, Emily J. Welch, Yongliang Zhang, Asrar B. Malik, Richard A. Flavell, Chen Dong, Richard D. Ye. A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury. *EMBO J.* 2009; 28: 2896-2907. PMID: 19696743.

Jing Deng, Dian-min Li, Yu-qing Chen, Li-nian Huang, Hua Gao. Pazufloxacin sodium chloride injection in treatment of lower respiratory infection. *Journal of Bengbu Medical College*. 2005; 30: 247-249.

Jing Deng, Yu-qing Chen, Yang Lu, Ai-hua Wang. Application and clinical value of impulse oscillometry in chronic obstructive pulmonary disease. *Journal of Bengbu Medical College*. 2005; 30: 24-26.

Jun-feng Hu, Yu-qing Chen, Dian-min Li, Xuemei Xia, **Jing Deng**, Linian Huang. Apoptosis of lung cancer cell line induced by all-transretinoic acid. *Journal of Bengbu Medical College*. 2005; 30: 104-107.

Jing Deng, Yu-qing Chen, Ying-yun Cai. Progress of epidermal growth factor receptor inhibitors in targeted therapeutic strategies of cancer. *Foreign Medical Sciences* · *Section of Internal Medicine*. 2004; 31: 277-280. (Review)