

The transcription factor PU.1 promotes alternative macrophage polarization and asthmatic airway inflammation

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Abstract

The transcription factor PU.1 is involved in regulation of macrophage differentiation and maturation. However, the role of PU.1 in alternatively activated macrophage (AAM) and asthmatic inflammation has yet been investigated. Here we report that PU.1 serves as a critical regulator of AAM polarization and promotes the pathological progress of asthmatic airway inflammation. In response to the challenge of DRA (dust mite, ragweed, and *Aspergillus*) allergens, conditional PU.1-deficient (PU/ER(T)^{+/-}) mice displayed attenuated allergic airway inflammation, including decreased alveolar eosinophil infiltration and reduced production of IgE, which were associated with decreased mucous glands and goblet cell hyperplasia. The reduced asthmatic inflammation in PU/ER(T)^{+/-} mice was restored by adoptive transfer of IL-4-induced wild-type (WT) macrophages. Moreover, after treating PU/ER(T)^{+/-} mice with tamoxifen to rescue PU.1 function, the allergic asthmatic inflammation was significantly restored. *In vitro* studies demonstrate that treatment of PU.1-deficient macrophages with IL-4 attenuated the expression of chitinase 3-like 3 (Ym-1) and resistin-like molecule alpha 1 (Fizz-1), two specific markers of AAM polarization. In addition, PU.1 expression in macrophages was inducible in response to IL-4 challenge, which was associated with phosphorylation of signal transducer and activator of transcription 6 (STAT6). Furthermore, DRA challenge in sensitized mice almost abrogated gene expression of Ym-1 and Fizz-1 in lung tissues of PU/ER(T)^{+/-} mice compared with WT mice. These data, all together, indicate that PU.1 plays a critical role in AAM polarization and asthmatic inflammation.

Keywords: PU.1, alternatively activated macrophage, asthmatic inflammation, Th2, IL-4

Introduction

Asthma is a chronic Th2-mediated lung disease that inflames and narrows the airways with eosinophilic inflammation, goblet cell hyperplasia, smooth muscle hypertrophy, and peribronchial fibrosis (Besnard et al., 2012; Lloyd and Saglani, 2013; Apter, 2014).

There are various immune cells in the asthmatic airway, such as lymphocytes, dendritic cells, and macrophages, which potentially contribute to asthmatic inflammation (Kiss et al., 2013). Recent evidence indicates that macrophages change genetic susceptibility to allergic asthma by modulating airway hyper-responsiveness such as cell proliferation, epithelial integrity, and immune responses (Careau and Bissonnette, 2004).

Differentiation and accumulation of alternatively activated macrophage (AAM) appears to be a hallmark of allergic lung disease (Holtzman, 2012). Presumably, the Th2 cytokine profile in a asthmatic mouse model contributes to polarization of AAM, which exhibits decreased phagocytosis and impaired innate immunity that contributes to the pathogenesis of the allergic inflammatory process (Alexis et al., 2001). Thus, within the context of asthmatic inflammation, alveolar macrophages are differentiated and polarized within a Th2-mediated immune microenvironment towards a M2 pro-asthmatic inflammatory phenotype. Mechanistically, there are several possibilities. For example, IL-33, released by endothelial or epithelial cells, can synergize with IL-13/IL-4R α signals to polarize alveolar macrophages towards AAM via inducing the expression of ST2L, which contributes to airway inflammation and eosinophilia in the lung (Kurowska-Stolarska et al., 2009). Also, serum amyloid P attenuates AAM polarization through inhibiting STAT6 phosphorylation and expression of Arginase-1 and Ym-1, and protects against fungal spore-induced allergic airway disease (Moreira et al., 2010). Although

these data collectively suggest that AAMs are associated with allergic inflammation, the mechanism of AAM polarization and how to maintain AAM phenotype in asthmatic inflammation are still unknown.

There is a growing appreciation that AAM is involved in inflammation, infection, metabolism, atherosclerosis, and tumorigenesis (Martinez et al., 2009; Cai et al., 2012). However, the roles of AAM in modulating the pathogenesis and resolution of allergic inflammatory responses have remained controversial. It has been reported that AAM has survival benefits through enhancing host defense against parasite infection (Anthony et al., 2006), promoting wound healing (Loke et al., 2007), and improving insulin sensitivity (Odegaard et al., 2007). However, it has also been reported that AAM in the context of tumorigenesis and allergic inflammation can exacerbate these diseases (Zhu et al., 2004; Gallina et al., 2006). AAMs are characterized by abundant expression of mannose receptor (MR/CD206), CD163, arginase, chitinase-like molecules (Ym-1/2), and resistin-like molecule α (RELMA/Fizz-1) upon stimulation with Th2-type cytokines such as IL-4 and IL-13 (Nair et al., 2005). Furthermore, chemokines including CCL17 (TARC), CCL22 (MDC), and CCL11 (eotaxin-1) have been associated with AAM activation (Mantovani et al., 2004). These signature markers in combination are used to determine mechanisms of AAM in different pathogenesis. Arginase-1 is considered as a useful indicator of IL-4 and IL-13-induced AAM polarization. Ym-1, one chitinase-like molecule, and Fizz-1, a small-secreted cysteine-rich protein, are strongly induced in AAM upon IL-4 or IL-13 stimulation (Raes et al., 2002; Nair et al., 2005). Arginase-1, Ym-1, and Fizz-1 can be regulated by signal transducer and activator of transcription 6 (STAT6)-mediated signaling pathways (Martinez et al., 2009) and modulate Th2-type

immune responses that trigger the asthmatic inflammation (Nair et al., 2009; Pesce et al., 2009).

The transcription factor PU.1 belongs to E-twenty-six (ETS) family, which is most prominent in myeloid cells and involved in the development and maturation of B cells, macrophages, and neutrophils (Scott et al., 1997; DeKoter and Singh, 2000; Dahl et al., 2003; Lawrence and Natoli, 2011). In addition, PU.1 can enhance granule protein expression by eosinophils, MHC class II expression by dendritic cells, and IL-4 production by mast cells (Henkel and Brown, 1994; Gombart et al., 2003; Kitamura et al., 2012). The competitive antagonism between PU.1 and GATA controls the fate of lymphoid-myeloid progenitors (Cantor and Orkin, 2001). In macrophages, PU.1 is necessary for maintaining macrophage differentiation and maturation by formatting and regulating the whole complement of genomic regions (Lawrence and Natoli, 2011). Our previous studies have identified that PU.1 is also required for neutrophilic lung inflammation via modulating classically activated macrophage (CAM) (Karpurapu et al., 2011). However, it is still unknown whether PU.1 can regulate AAM and plays a role in Th2-type asthmatic airway inflammation.

In the present study, PU/ER(T)^{+/-} mice with partial deficiency of PU.1 displayed an extremely attenuated asthmatic airway inflammation, reduced eosinophil infiltration, and decreased IgE production in both acute and chronic asthmatic inflammation induced by dust mite, ragweed, and *Aspergillus* (DRA), which was restored by conditional induction of PU.1 expression via treating PU/ER(T)^{+/-} mice with tamoxifen (TMX). Moreover, the reduced asthmatic inflammation in PU/ER(T)^{+/-} mice was restored to the WT level by adoptive transfer of IL-4-induced wild-type (WT) macrophages that show an AAM

phenotype. Additionally, the expression of Ym-1 and Fizz-1, two markers of AAM polarization, was remarkably attenuated in lung tissues and macrophages from PU/ER(T)^{+/-} mice treated with DRA or IL-4, respectively. These results, all together, demonstrate that PU.1 is an important regulator of AAM polarization and asthma pathogenesis and thus is a potential drug target for the therapeutic intervention.

Results

PU/ER(T)^{+/-} mice show impaired development of DRA-induced acute allergy airway inflammation and asthmatic response

Because the transcription factor PU.1 plays an essential role in hematopoiesis, PU.1 deficiency-caused embryonic lethality has been a barrier for animal models. Here, we use PU/ER(T)^{+/-} mice that show normal capability of behavior and fertility and normal myeloid cell development. In these mice, a single PU.1 locus is transcriptionally inactivated by fusing with the modified estrogen receptor (ER) ligand binding domain. The fusion molecule PU.1-ER is retained in a transcriptionally inactive form in the cytoplasm, and can be reactivated when treated with TMX via translocating to the nucleus and binding to its cognate DNA sequence in the enhancer regions of key genes (Karpurapu et al., 2011). Our previous studies have shown an attenuation of the acute lung inflammation in LPS-challenged PU/ER(T)^{+/-} mice (Karpurapu et al., 2011). Although PU.1 is known to play roles in T cells (Chang et al., 2010) and dendritic cells (Kitamura et al., 2012), its role in AAM polarization and asthmatic inflammation has not been previously defined. To address this gap in the literature, we investigated whether PU.1 is involved in asthmatic inflammation in a newly described triple allergen DRA-

induced acute asthma model (Figure 1A). As shown by H&E staining, DRA induced severe asthmatic airway inflammation and inflammatory cell infiltration in WT mice, which was significantly attenuated in PU/ER(T)^{+/-} mice (Figure 1B). In response to DRA challenge, total IgE in plasma of WT mice was 1114.82 ± 55.6 ng/ml, while that in PU/ER(T)^{+/-} mice was extremely reduced to 368.96 ± 56.15 ng/ml (Figure 1C). Total cells and eosinophils in BAL fluid were increased in challenged WT and PU/ER(T)^{+/-} mice. However, the numbers of total cells and eosinophils in PU/ER(T)^{+/-} mice were reduced by 35.2% and 63.3%, respectively, compared with that in WT mice (Figure 1D and E). Interestingly, total numbers of alveolar macrophages were not significantly different between WT and PU/ER(T)^{+/-} mice (Figure 1F). The reduced eosinophil infiltration in PU/ER(T)^{+/-} mice was also observed by cytopsin slides with HEMA 3 staining (Figure 1G, eosinophils are indicated with black arrowheads). Although lymphocyte infiltration was also observed in BAL fluid, there was no difference in numbers of infiltrated lymphocytes between WT and PU/ER(T)^{+/-} mice in response to DRA challenge (data not shown). Based on the premise that alveolar macrophages lack CD11b and express high levels of CD11c and Siglec-F (Lambrecht and Hammad, 2012), while eosinophils are typically identified as Siglec-F⁺CD11c⁻ (Stevens et al., 2007), DRA mediated abundant eosinophil infiltration in BAL fluid (84.8%) in WT mice, which was decreased in PU/ER(T)^{+/-} mice (20.9%) (Figure 1H). In all, these data indicate that functional PU.1 is required for DRA-induced acute asthmatic inflammation.

Adoptive transfer of WT macrophages restores DRA-induced allergic inflammation in PU/ER(T)^{+/-} mice

Although PU.1 is required for the development and activation of B cells, dendritic cells, eosinophils, mast cells, and T cells that are all involved in regulation of asthma, it is still unknown whether PU.1 promotes the asthmatic inflammation via modulating macrophage polarization. To address this concern, we carried out adoptive transfer of polarized AAMs into PU/ER(T)^{+/-} mice. WT and PU/ER(T)^{+/-} mice were intraperitoneally injected with 2 µg of IL-4 twice, and peritoneal cells were collected for adoptive transfer. Meanwhile, PU/ER(T)^{+/-} mice were used as recipient mice and subjected to DRA-induced acute asthma as described in Figure 1. One day before DRA challenge, PU/ER(T)^{+/-} mice received IL-4-induced WT or PU/ER(T)^{+/-} macrophages. Asthmatic inflammation was analyzed on Day 15 as illustrated in Figure 2A. PU/ER(T)^{+/-} mice receiving WT macrophages developed an enhanced inflammatory cell infiltration into the lavagable airspace (Figure 2B), which was attributable to a 3.6-fold increase in eosinophils compared with mice receiving PU/ER(T)^{+/-} macrophages (Figure 2C). Flow cytometry analysis confirmed that the percentage of eosinophils in BAL fluid was increased in PU/ER(T)^{+/-} mice receiving WT macrophages, compared with those receiving PU/ER(T)^{+/-} macrophages (Figure 2D). We also examined lung tissues with Periodic acid-Schiff (PAS) staining that detects mucus glycol conjugates in goblet cells within the asthmatic airway epithelium. PU/ER(T)^{+/-} mice receiving PU/ER(T)^{+/-} macrophages had a low number of PAS-positive goblet cells, whereas PU/ER(T)^{+/-} mice receiving WT macrophages showed an increase in PAS-positive cells (Figure 2E), which was similar to the response upon DRA challenge in sensitized WT mice. Thus,

these data show that adoptive transfer of WT macrophages restores the inflammatory response to DRA allergen challenge in PU/ER(T)^{+/-} mice, demonstrating that PU.1 can promote the allergic inflammation, at least in part, through modulating macrophage polarization.

PU/ER(T)^{+/-} mice display impaired polarization of AAM during acute allergic airway inflammation

Given that PU.1 is important in regulating monocyte to macrophage differentiation, we hypothesized that PU.1 may be involved in the development of asthmatic inflammation through regulating macrophage polarization. Lung tissues isolated from WT and PU/ER(T)^{+/-} mice on Day 15 after DRA challenge were analyzed for the expression of AAM feature genes Ym-1, Fizz-1, and Arginase-1 by real-time PCR. In WT mice, DRA challenge resulted in a 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 3A). Furthermore, DRA challenge resulted in a 49-fold induction of Fizz-1 in WT mice, and a 17-fold induction in PU/ER(T)^{+/-} mice (Figure 3B). Interestingly, we did not observe any significant differences in Arginase-1 induction between WT and PU/ER(T)^{+/-} mice challenged with DRA (Figure 3C). Collectively, these data indicate that acute challenge of DRA can induce AAM polarization with significant increase in marker gene expression, which can be reduced by partial loss of PU.1 function in PU/ER(T)^{+/-} mice and restored by adoptive transfer of IL-4-treated macrophages.

PU.1 is required for AAM polarization in vitro

It is well known that PU.1 functions in a genome-wide manner to control the formation and accessibility of whole complement of macrophage-specific regulatory genomic regions (Lawrence and Natoli, 2011); however, the role of PU.1 in macrophage polarization is not defined. We stimulated WT BMDMs with 5 ng/ml of IL-4, which is Th2-type cytokine to induce AAM activation via activating STAT6 (Ruffell et al., 2009). As expected, phosphorylation of STAT6 was increased by 10–12-fold from 15 min to 1 h (Figure 4A and B). Meanwhile, PU.1 expression was significantly induced by treatment with IL-4 for 30 and 60 min (Figure 4A and C). These data suggest that PU.1 expression is associated with AAM polarization that can be induced by IL-4.

To further investigate PU.1 function in AAM polarization, we challenged BMDMs from WT or PU/ER(T)^{+/-} mice with 5 ng/ml of IL-4 or PBS for 24 h and performed real-time PCR and immunoblot assays. In response to IL-4 stimulation, BMDMs from PU/ER(T)^{+/-} mice, compared with those from WT mice, displayed markedly attenuated expression two AAM markers Ym-1 and Fizz-1 (Figure 5A and B). But another M2 marker Arginase-1 showed equivalent expression in WT and PU/ER(T)^{+/-} mice (Figure 5C). In addition, protein levels of Ym-1 and Fizz-1 were significantly attenuated in PU/ER(T)^{+/-} mice with or without IL-4 treatment (Figure 5D and E). These data suggest that PU.1 is required for IL-4-induced AAM polarization.

Recovery of PU.1 reverses the attenuated asthmatic inflammation in PU/ER(T)^{+/-} mice

Since TMX can force PU.1 to enter into nuclei and become functional, which converts PU.1-deficient PU/ER(T)^{+/-} mice to the WT phenotype, we placed TMX wafer or placebo

in the subcutaneous tissue of neck before sensitization to determine whether functional PU.1 reverses the attenuated asthmatic inflammation in PU/ER(T)^{+/-} mice. The amounts of total cells and eosinophils in BAL fluid were dramatically increased in TMX-treated PU/ER(T)^{+/-} mice compared with placebo-treated mice (Figure 6A and B), while total number of macrophages in BAL fluid was not significant different between treated or non-treated PU/ER(T)^{+/-} mice (Figure 6C). In contrast, the eosinophil infiltration in lungs was increased from 21% to 69% in TMX-treated PU/ER(T)^{+/-} mice compared with non-treated groups (Figure 6D). Total IgE in plasma was markedly increased with the treatment of TMX (Figure 6E). These data indicate that functional PU.1 is necessary for the development of asthmatic inflammation.

PU/ER(T)^{+/-} mice show impaired development of DRA-induced chronic airway inflammation

Considering that most allergic asthma patients are chronically exposed to multiple allergens, we use a chronic murine asthma model, in which mice were challenged with DRA chronically, for better understanding the pathologic process of asthma. The chronic model differs from the acute model in that it does not include an adjuvant sensitization process, which more naturally mimics human asthma (Figure 7A). Although previous data showed *in vitro* and *in vivo* evidence of PU.1 regulating AAM polarization, we further assessed whether PU.1 also plays an important role in chronic asthmatic inflammation. In response to instillation of DRA for 8 weeks, inflammatory cell infiltration in BAL fluid was increased in both WT and PU/ER(T)^{+/-} mice. But the amounts of total cells (Figure 7B), eosinophils (Figure 7C), and macrophages (Figure 7D) were

significantly reduced in PU/ER(T)^{+/-} mice than that in WT mice. In contrast to the acute asthma model, macrophage accumulation in PU/ER(T)^{+/-} mice was obviously less than that in WT mice in the chronic model (Figure 7D). To investigate whether PU.1 is involved in regulating AAM polarization in the chronic asthma model, we isolated RNA from lung homogenates and measured the expression of AAM-specific markers. We found increased expression of Ym-1 (Figure 7E), Fizz-1 (Figure 7F), and Arginase-1 (Figure 7G) in lungs of DRA-challenged WT mice, which was dramatically attenuated in PU/ER(T)^{+/-} mice. All together, these data confirm that PU.1 is critical for driving macrophages towards AAM polarization in DRA-induced chronic asthma model. In order to carry out unbiased histologic analyses, we used the Genie system, which is a novel digital pathology method for automated classification of tissue on whole slide images (Park et al., 2013). Upper panel shows H&E staining slides from the entire left lungs from WT and PU/ER(T)^{+/-} mice that were subjected to chronic DRA challenge. Middle panel shows a colorized version of lungs where alveolus in blue, airway lumen in pink, airway epithelium in yellow, and asthmatic inflammatory area in green. Lower panel shows details of a section of the lung that is indicated by a square in the middle panel, which demonstrates less green inflammatory area in PU/ER(T)^{+/-} mouse lung compared with WT lung (Figure 7H). Similarly, WT mice chronically challenged with DRA showed an impressive mucous gland and goblet cell hyperplasia compared with PU/ER(T)^{+/-} mice, as indicated by PAS staining (Figure 7I, black arrowhead). These histological data support the conclusion that PU.1 plays a critical role in chronic airway inflammation and asthmatic airway remodeling.

Discussion

The transcription factor PU.1 plays an essential role in both myeloid and lymphoid development and differentiation (Singh et al., 1999; Turkistany and DeKoter, 2011), PU.1 whole-body knockout would cause embryonic lethality (Scott et al., 1994; Anderson et al., 1998). In the present study, conditional partial PU.1-deficient mice were used to delineate the functional role of PU.1 in regulating asthmatic inflammation and AAM polarization. Although our previous study has proved that PU.1 modulates LPS-induced classic macrophage activation (Karpurapu et al., 2011), this study demonstrates for the first time that PU.1 is also involved in IL-4-mediated AAM polarization and eosinophilic asthmatic inflammation.

Asthma is the most common complicated chronic pulmonary disease; however, an appropriate mouse model for comprehensively mimicking clinic symptoms is still lacking (Nials and Uddin, 2008). Since mice do not develop asthma spontaneously, ovalbumin (OVA), generated from chicken egg as a foreign protein, has been broadly used as an allergen to induce allergic lung inflammation acutely or chronically. Although the OVA model is characterized as “Type 2”-mediated immune responses including CD4⁺ Th2 cell activation and proliferation, OVA-specific IgE generation, and “Type 2” cytokine production, it lacks the impressive asthmatic airway remodeling or chronic changes that are found in human asthma (Swirski et al., 2002). In contrast, common allergens that lead to human asthma, include dust mite, ragweed, and *aspergillus*, are used in a DRA-induced asthma model. This model better recapitulates many pathological features of the human asthmatic response, including airway smooth muscle hypertrophy, peribronchial cell infiltration, and prominent goblet cell hyperplasia (Lee et al., 2014).

The DRA-induced chronic asthma model was carried out by continuous instillation of DRA for 8 weeks without pre-treatment of adjuvant alum, to avoid the effect of adjuvant. Compared with DRA-induced acute asthma model, the chronic model displayed an increased macrophage infiltration in pulmonary alveoli, which resembles the pathological process in asthma patients showing markedly increased pulmonary macrophage infiltration (Poston et al., 1992). In the present study, we observed abundant airway inflammation and eosinophil recruitment in both acute and chronic models, as well as goblet cell hyperplasia in the chronic model. Taking advantage of both models, we found that asthmatic inflammation in PU/ER(T)^{+/-} mice was significantly attenuated, suggesting that PU.1 plays a critical role in promoting the allergic airway inflammation.

Th2 lymphocytes and dendritic cells have been considered as critical immune cells for promoting eosinophilic inflammation and consequent immune responses (Busse and Lemanske, 2001). Although alveolar macrophages are the predominant immune cells in the lung, their roles in the asthma paradigm has been underscored for long time, which are so called 'forgotten cell in asthma' (Peters-Golden, 2004). However, our recent study has proved that macrophages contributed to the pathologic process of allergic asthmatic inflammation (Lee et al., 2014). PU/ER(T)^{+/-} mice displayed a reduced expression of AAM markers including Ym-1, Fizz-1, and Arginase-1 (Zimmermann et al., 2003; Nair et al., 2005; Homer et al., 2006) in lung tissues from both DRA-induced acute and chronic asthma models, which was associated with decreased eosinophil infiltration and goblet cell hyperplasia. Given that asthmatic immune responses lead to pulmonary Th2 microenvironment that can polarize macrophages to AAMs, we

hypothesized that PU.1 promotes allergic asthmatic inflammation via regulating AAMs. DRA-induced chronic asthma model is associated with eosinophil infiltration and increased proliferation of CD4 T cells (Goplen et al., 2009). Our results not only confirm that chronic treatment with DRA can induce eosinophil accumulation, but further show that macrophage infiltration is also increased, indicating that macrophages are involved in asthmatic immune responses.

Adoptive transfer of AAMs could enhance the mouse allergic pulmonary inflammation via IL-4 receptor α (IL-4R α)-mediated signal (Ford et al., 2012). Moreira et al. (2010) also found that adoptive transfer of AAMs into mice with *Aspergillus fumigatus* exacerbated asthmatic inflammation and airway remodeling. These results indicate that AAMs are required for asthmatic inflammation. Several inflammatory factors from AAMs can regulate Th2 responses, eosinophil accumulation, and airway remodeling. AMM-derived Fizz-1 inhibits CD4 Th2 cell activation and ameliorates Th2 cytokine-mediated lung inflammation (Nair et al., 2009). Ym-1 belongs to a family of chitinase-like molecules that are pertinent to the severity of human asthma (Chupp et al., 2007). In OVA-induced mouse allergic inflammation, Ym-1/2 accelerates the production of Th2 cytokines and enhances allergic inflammation (Cai et al., 2009). Interestingly, as a general AAM marker, Arginase-1 deficiency does not attenuate OVA-induced asthma (Barron et al., 2013), suggesting that Arginase-1 is not involved in pathological changes of asthma. Our results have shown that the expression of Ym-1 and Fizz-1, which was greatly induced by DRA in WT mice, was extremely attenuated in lung tissues of partial PU.1-deficient mice in both acute and chronic models. Therefore, PU.1-dependent Ym-

1 and Fizz-1 production may mainly contribute to asthmatic inflammation and airway remodeling, which might be interdicted as potential drug targets for asthma.

In addition to macrophages, PU.1 has functional roles in regulation of B lymphocytes, T lymphocytes, and dendritic cells, which are all involved in allergic inflammation.

Although expressed in B cells and regulating the development of pro-B cells in the embryo (Klemsz et al., 1990), PU.1 has no effect on the development and function of mature B cells (Polli et al., 2005). Mice with PU.1 deficiency in T cells have shown defects in OVA-mediated allergic inflammation and oxazolone-induced colitis by regulating Th9 cells (Chang et al., 2010). Additionally, PU.1 enhances MHC class II expression in dendritic cells (Kitamura et al., 2012). Because both T cells and dendritic cells are involved in Th2-mediated immune responses, the involvement of PU.1-deficient T cells and dendritic cells cannot be excluded in our asthmatic models.

Our previous results indicate that PU.1 is dispensable for survival of mature macrophages and is required for LPS-induced classical macrophage activation (Karpurapu et al., 2011). We also found that depletion of macrophages resulted in significant attenuation of DRA-induced asthmatic inflammation by using Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice (Lee et al., 2014), indicating a non-redundant role of macrophages in asthmatic inflammation. It was further confirmed by the *in vitro* experiment that BMDMs isolated from PU/ER(T)^{+/-} mice displayed an attenuated induction of Ym-1 and Fizz-1 in response to IL-4 treatment. These results indicate that PU.1 is required for AAM polarization that maintains the homeostasis of Th2 cell activation, eosinophil filtration, and asthmatic airway remodeling.

Typically, AAM polarization is mediated by IL-4 and IL-13-mediated phosphorylation of STAT6, which results in the generation of Ym-1, Fizz-1, and Arginase-1 during macrophage polarization (Mikita et al., 1996). We found that the expression of PU.1 was induced after IL-4 treatment for 30 min, which was associated with STAT6 phosphorylation. In B cell activation, Ig heavy chain class switching to IgE can be induced by IL-4 and IL-13, and the interaction and cooperation between STAT6 and PU.1 can further facilitate IgE transcription and maturation (Stutz and Woisetschlager, 1999; Pesu et al., 2003). Moreover, during the activation of B cells, PU.1 co-binds with the transcription factor IRF4 and then regulates immunoglobulin class switch, recombination, and cell differentiation (Ochiai et al., 2013). Because recent publications have shown that IRF4 can promote AAM polarization in inflammation related to obesity (Eguchi et al., 2013) and helminth (Satoh et al., 2010), it is possible that PU.1 binds to STAT6 and IRF4 and cooperatively regulates the expression of Ym-1 and Fizz-1. In summary, our studies identify a previously unrecognized role for PU.1 in type 2 immune responses, acting as an important transcription factor in the development of allergic inflammation via polarizing AAMs and inducing the expression of pro-allergic factors Ym-1 and Fizz-1. Although our approach cannot exclude a functional role of PU.1 in activation of dendritic cells, B cells, and T cells, based on our data, it is without doubt that PU.1 plays a non-redundant role in AAM polarization. Since AAMs are involved in a broad range of diseases such as asthma, atherosclerosis, diabetes, obesity, and cancer (Ahmad, 2010), the interdiction in PU.1 gene expression or function could be an effective therapeutic strategy for the treatment of AAM-mediated human diseases such as asthma.

Materials and methods

Mice

The 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 receptor (ER) ligand-binding domain that is TMX responsive (Karpurapu et al., 2011). Functional PU.1 can be induced by treatment with TMX 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 and The Ohio State University. Mice at age of 8–12 weeks were used in this study. All experiments involving mice were conducted with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Chicago and The Ohio State University.

Allergens

Triple allergens include extracts of dust mite (*Dermatophagoides farina*), ragweed (*Ambrosia artemisifolia*), and *Aspergillus fumigates* (Greer Laboratories, Lenoir, NC). Aluminum (Inject Alum; Thermo Scientific) was used for adjuvant. Quantities of allergens for intraperitoneal (200 µl) per mouse were used 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) (Goplen et al., 2009). Quantities of allergens for intranasal injection (50 µl) were used as follows: *D. farina* (8.3 µg), ragweed (83.4 µg), and *Aspergillus fumigates* (8.3 µg) for the acute model, and *D. farina* (25 µg), ragweed (250 µg), and *Aspergillus fumigates* (25 µg) for the chronic model.

Murine asthma models

WT and PU/ER(T)^{+/-} mice at age of 8–10 weeks were sensitized by an intraperitoneal (i.p.) injection of 200 µl alum-precipitated antigens containing triple allergen mix (60 µg of DRA) in PBS vehicle. Five days after the first immunization (D0), mice were given a second intraperitoneal booster of the same dose of allergens in alum adjuvant (D5), followed by exposure to DRA (100 µg) combination (50 µl) or PBS (50 µl) 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 on Day 15, and bronchoalveolar lavage (BAL) fluid and lung tissues were collected for further analysis (Goplen et al., 2009). For tamoxifen recovery experiments, PU/ER(T)^{+/-} mice were anesthetized with ketamine and xylazine, then a 21-day-release TMX wafer or placebo was implanted into the skin pocket made on the lateral side of the neck (D-1). Twenty-four hours later, mice were subjected to acute DRA sensitization and challenge. Alternatively, chronic murine asthma model was conducted for mimicking human asthmatic inflammation. WT and PU/ER(T)^{+/-} mice at age of 8–10 weeks were chronically exposed to triple allergens (60 µg of 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 before analyses (Goplen et al., 2009). Timelines of the acute and chronic DRA models are shown in Figures 1A and 7A, respectively.

Adoptive transfer of macrophages

In order to induce AAMs, WT and PU/ER(T)^{+/-} mice were i.p. injected with 2 µg of IL-4 twice at three days (D-3) and one day (D-1) before collection. IL-4-induced peritoneal macrophages were lavaged and washed with Ca²⁺/Mg²⁺-free HBSS and suspended at a concentration of 1×10^7 cells/ml in PBS. Then 50 µl (5×10^5 cells/sample) of donor cells were intratracheally (i.t.) injected into a DRA (60 µg)-sensitized recipient PU/ER(T)^{+/-} mouse at one day (D11) before DRA challenge. On Day 15, recipient mice were sacrificed and the allergic asthmatic inflammation was analyzed. The protocol of adoptive transfer of macrophages is shown in Figure 2A.

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). BAL fluid on cytospin slides was stained with HEMA 3 (Thermo Scientific) for differential cell counts. The number of macrophages and eosinophils was quantitated and compared for statistical significance.

Measurement of IgE

Plasma collected from mice were used for detecting the level of total IgE with the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience) according to manufacturer's instructions.

Lung histology and airway mucus expression

To easily handle the lung tissues 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 (Halbower et al., 1994). Both hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining were conducted by the Research Histology and Tissue Imaging Core at the University of Illinois at Chicago. The digital pathology assay was performed by using the Genie system (Aperio Technologies Inc.), which is an unbiased approach to analyze the whole lung asthmatic inflammation based on the standards that we published recently (Park et al., 2013).

Isolation of total RNA and Real-time PCR

Frozen right lobes of lungs were homogenized with the TissueLyser system (Qiagen). Total cellular RNA was extracted from BMDMs or lung tissues homogenates with miRNeasy Mini Kit (Qiagen) following the instruction. cDNA was prepared by qScript cDNA supermix (Quanta Biosciences) and amplified by Real-time PCR on LightCycler 480 instrument (Roche Diagnosis) with primer sets for Arginase-1 (forward, CAATGAAGAGCTGGCTGGTGT; reverse, GTGTGAGCATCCACCCAAATG; 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).

Flow cytometry assay

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

Preparation of BMDMs

Femoral and tibia bone marrow was isolated from WT and PU/ER(T)^{+/-} mice as previously described (Deng et al., 2012). Mouse bone marrow cells flushed from femurs and tibias were washed with Ca²⁺/Mg²⁺-free HBSS and then cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 ng/ml M-CSF for 6 days. Then BMDMs were stimulated for 24 h with 5 ng/ml of IL-4 or PBS. Culture supernatants were discarded and cells were collected for RNA and protein expression analysis. The expression of Ym-1 and Fizz-1 was analyzed by western blot and quantitative real-time PCR.

Western blot analysis

Western blot was conducted to detect the protein expression as previously described (Qian et al., 2012). BMDMs were plated in 6-well plates at 2×10⁶/well overnight and challenged with 5 ng/ml of IL-4 for 15, 30, and 60 min, or stimulated with 5 and 10 ng/ml of IL-4 or PBS for 24 h. Cells were then collected and lysed in loading buffer (Bio-Rad

Laboratories). The cell lysate was analyzed by immunoblot using antibodies against PU.1, phospho-STAT6, STAT6, GAPDH (Cell Signaling Technology), Ym-1 (Stemcell Technologies), and Fizz-1 (Abcam). Quantification of western blots was performed with ImageJ software (NIH).

Statistical analysis

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

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Figure legends

Figure 1 PU/ER(T)^{+/-} mice show an impaired development of DRA-induced acute allergic airway inflammation. **(A)** The schematic timeline show that mice were sensitized with DRA on Days 0 and 5 and challenged with DRA on Days 12, 13, and 14. On Day 15, mice were sacrificed for analysis of asthmatic inflammation. **(B)** Histopathology was performed based on H&E staining to determine the asthmatic inflammation in PBS- or DRA-treated WT or PU/ER(T)^{+/-} mice (original magnification 200×). **(C)** Total IgE level in mouse plasma was detected by ELISA. Total infiltrated cells **(D)**, eosinophils **(E)**, and macrophages **(F)** in BAL fluid were counted based on total amount of BAL cells and percentage of different cell types on cytopsin slides. **(G)** Representative cytopsin slides with BAL fluid showed DRA-induced eosinophil infiltration. Macrophages (white arrowhead) and eosinophils (black arrowhead) were indicated. **(H)** Cell staining for markers of eosinophils (Eos, Siglec F⁺CD11c⁺) and macrophages (Mac, Siglec F⁺CD11c⁺) in BAL fluid was analyzed by flow cytometry. Results represent mean ± SEM (*n* = 5 mice for each group). ND stands for “not detected”. * *P* < 0.05, *** *P* < 0.001 between the indicated two groups.

Figure 2 Adoptive transfer of wild-type macrophages restores the attenuated allergic inflammation in PU/ER(T)^{+/-} mice treated with DRA. **(A)** The protocol of adoptive transfer of IL-4-induced peritoneal macrophages into PU/ER(T)^{+/-} mice. WT and PU/ER(T)^{+/-} mice were intraperitoneally (i.p.) injected with 2 µg of IL-4 twice at 3 days and 1 day before collection. Donor peritoneal macrophages from IL-4-treated WT and PU/ER(T)^{+/-} mice were intratracheally (i.t.) instilled into PU/ER(T)^{+/-} mice on Day 11 after asthma was induced. Then the mice received transferred macrophages were intranasally (i.n.)

challenged with DRA on Days 12, 13, and 14. The allergic inflammation was detected on Day 15. Total numbers of infiltrated cells (**B**) and eosinophils (**C**) in BAL fluid were detected. (**D**) Infiltrated cells in BAL fluid were stained with PE-anti-Siglec F and APC-anti-CD11c antibodies. The percentage of eosinophils (Eos, Siglec F⁺CD11c⁻) and macrophages (Mac, Siglec F⁺CD11c⁺) was analyzed by flow cytometry. (**E**) Lung lobes were fixed and PAS staining was performed for identification of goblet cells in the epithelium. Black arrowheads indicate PAS-positive goblet cells. Results represent mean $\pm$ SEM ($n = 5$ mice for each group). * $P < 0.05$, and *** $P < 0.001$ between the two indicated groups.

Figure 3 PU/ER(T)^{+/-} mice display an impaired polarization of AAM during acute allergic airway inflammation. Total RNAs were isolated from lung homogenates, and mRNA expression levels for Ym-1 (**A**), Fizz-1 (**B**), and Arginase-1 (**C**) were quantified using real-time PCR. $n = 3$ for each group. * $P < 0.05$, *** $P < 0.05$ between the indicated two groups.

Figure 4 IL-4 induces the expression of PU.1 and phosphorylation of STAT6. BMDMs from WT mice were cultured and stimulated with 5 ng/ml of IL-4 for 15, 30, and 60 min. (**A**) The phosphorylated STAT6, total STAT6, PU.1, and GAPDH were identified by western blot. The ratio of phosphorylated and total STAT6 (**B**) and the ratio of PU.1 and GAPDH (**C**) in densitometry were analyzed by ImageJ software. Results represent mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$ between the indicated two groups.

Figure 5 PU.1 is required for AAM *in vitro*. BMDMs were stimulated with 5 ng/ml of IL-4 or PBS for 24 h. Real-time PCR was performed to measure fold changes of mRNA expression levels for Ym-1 (**A**), Fizz-1 (**B**), and Arginase-1 (**C**), all compared with

GAPDH. **(D)** BMDMs isolated from WT and PU/ER(T)^{+/-} mice were challenged with 5 or 10 ng/ml of IL-4 for 24 h. Immunoblotting was performed to detect protein levels of Ym-1 and Fizz-1. Densitometry analysis shows ratios of Ym-1 **(E)** and Fizz-1 **(F)** to GAPDH. Data are mean $\pm$ SEM and are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ between IL-4-treated WT and PU/ER(T)^{+/-} BMDMs.

Figure 6 Recovery of PU.1 function exacerbates the asthmatic inflammation in PU/ER(T)^{+/-} mice. PU/ER(T)^{+/-} mice were implanted tamoxifen (TMX), which forces PU.1 to translocate into nuclei and serve as a functional transcription factor. Acute asthmatic inflammation was induced by DRA as indicated in Figure 1. Total cells **(A)**, eosinophils **(B)**, and macrophages **(C)** in BAL fluid were detected. **(D)** Cell staining for markers of eosinophils (Siglec F⁺CD11c⁻) and macrophages (Siglec F⁺CD11c⁺) in BAL fluid was analyzed by FACS. **(E)** Total IgE in plasma was measured by ELISA. Results represent mean $\pm$ SEM ($n = 5$ mice for each group). ND represents “not detected”. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the two indicated groups.

Figure 7 PU/ER(T)^{+/-} mice show an attenuated asthmatic inflammation and decreased AAM polarization in DRA-induced chronic asthma model. **(A)** The timeline of allergen exposure, rest period, and time of analysis in the chronic mouse model without alum as adjuvant. Amounts of total cells **(B)**, eosinophils **(C)**, and macrophages **(D)** in BAL fluid were detected. Total RNAs were isolated from lung homogenates, and the expression of Ym-1 **(E)**, Fizz-1 **(F)**, and Arginase-1 **(G)** were quantified based on real-time PCR. **(H)** Upper panel shows H&E staining for the entire left lungs. Middle panel shows a colorized version of lungs where blue indicates alveoli, pink indicates airway lumen, yellow indicates airway epithelium, and green indicates inflammatory area by using

Genie system. Lower panel shows a zoomed section of the lung as indicated by a square in the middle panel. (I) PAS-stained lung sections from the mice exposed to PBS or DRA chronically. Black arrowheads indicate PAS-positive goblet cells (original magnification 200×). Results represent mean $\pm$ SEM ($n = 5$ mice for each group). ND stands for “not detected”. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the two indicated groups.

Figure 1

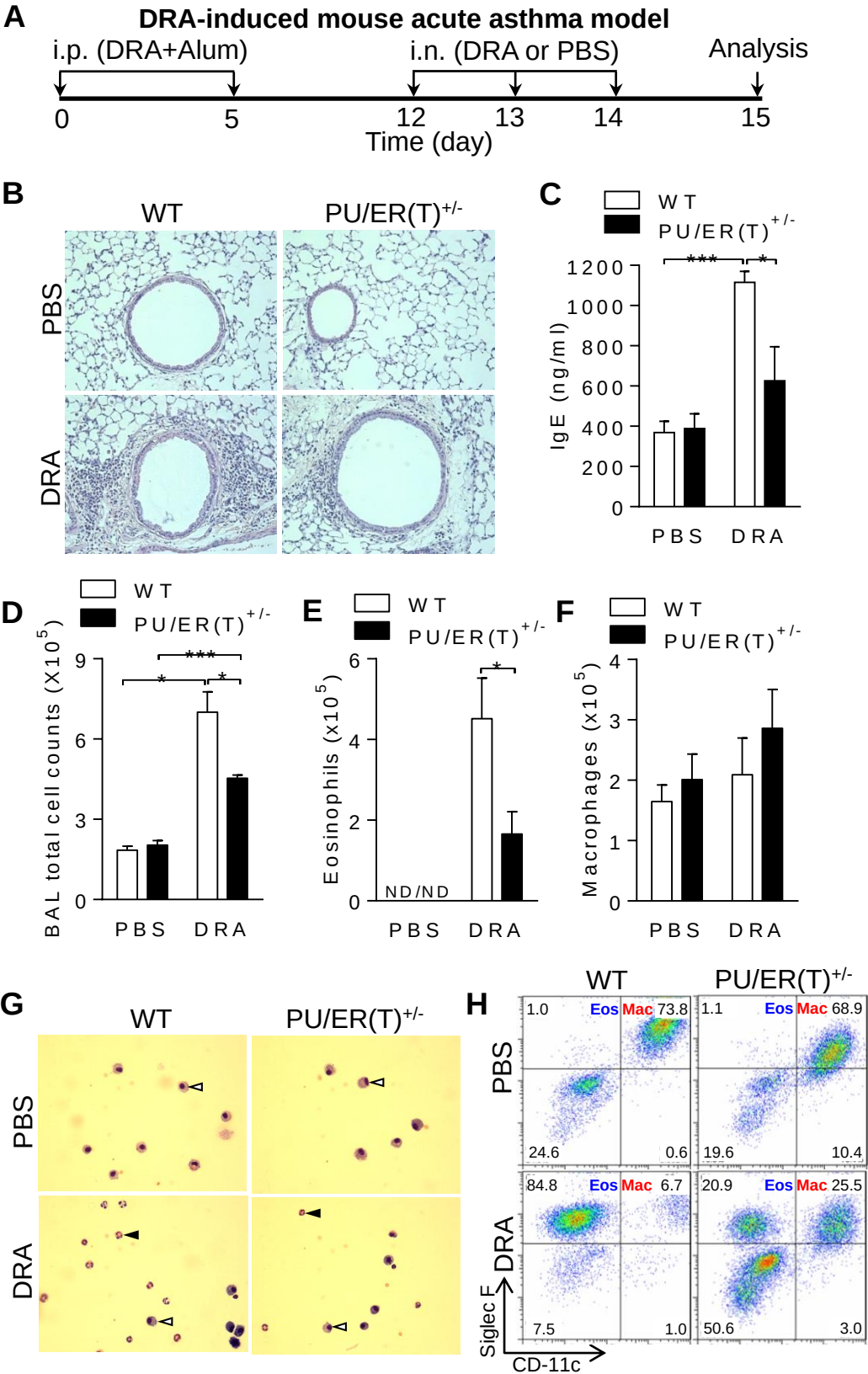


Figure 2

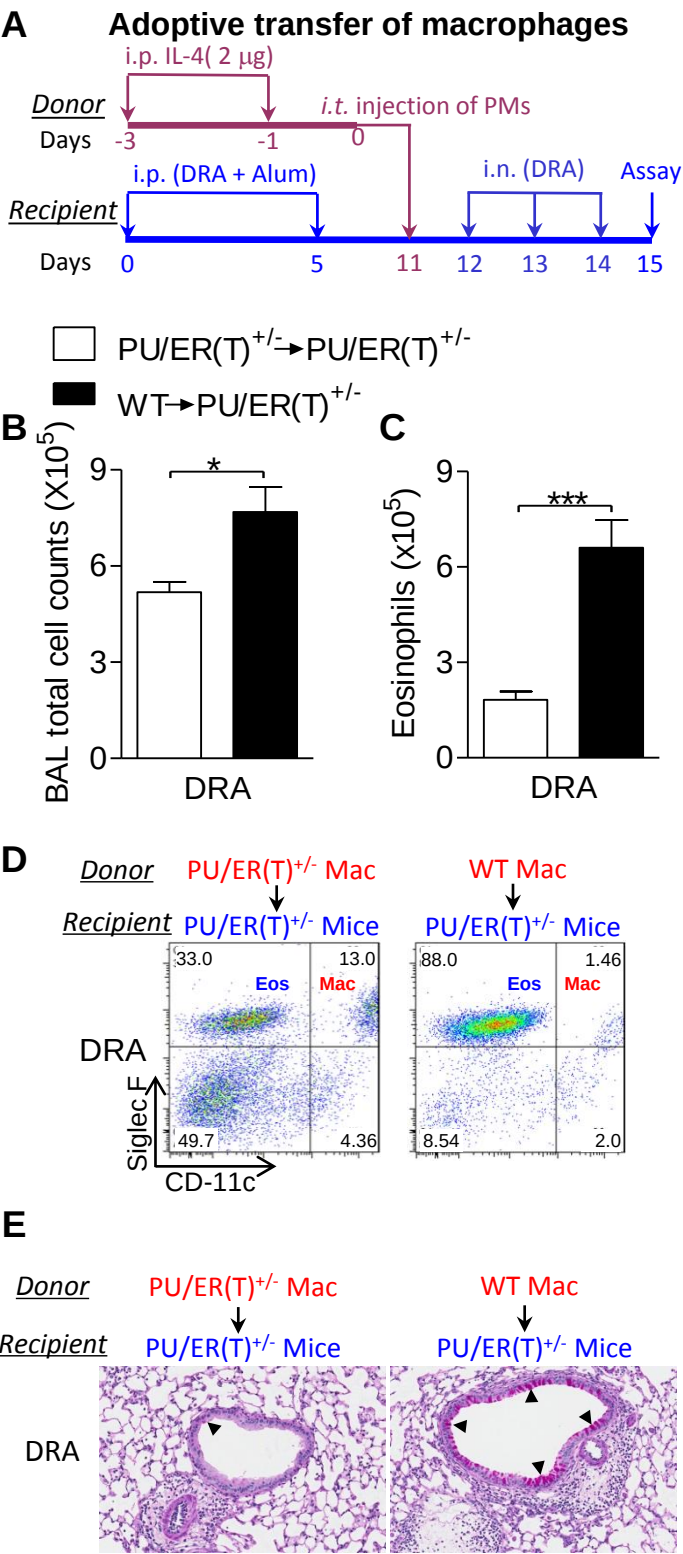


Figure 3

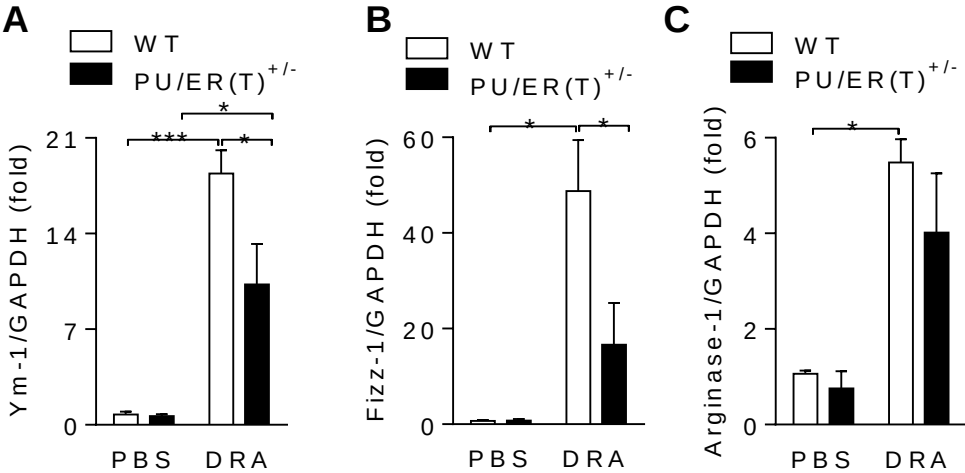


Figure 4

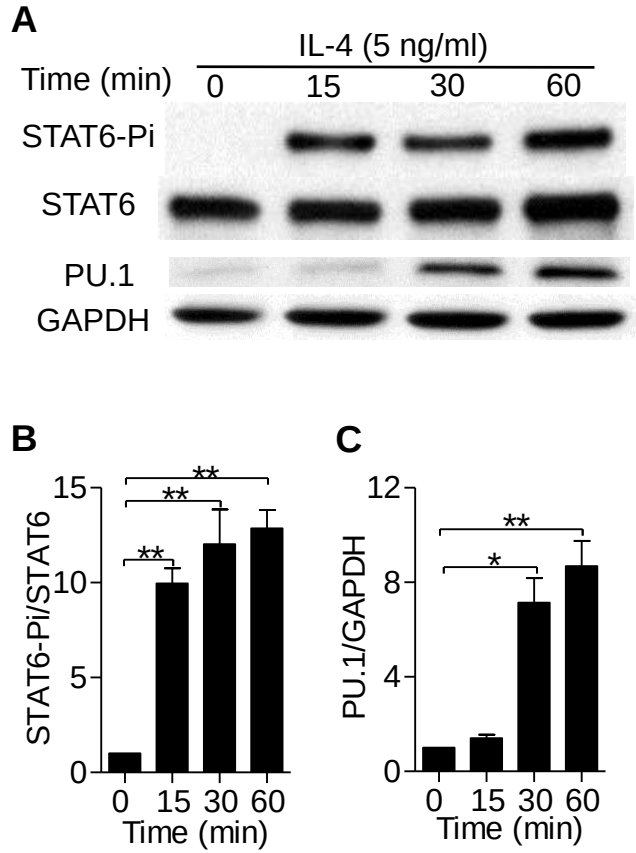


Figure 5

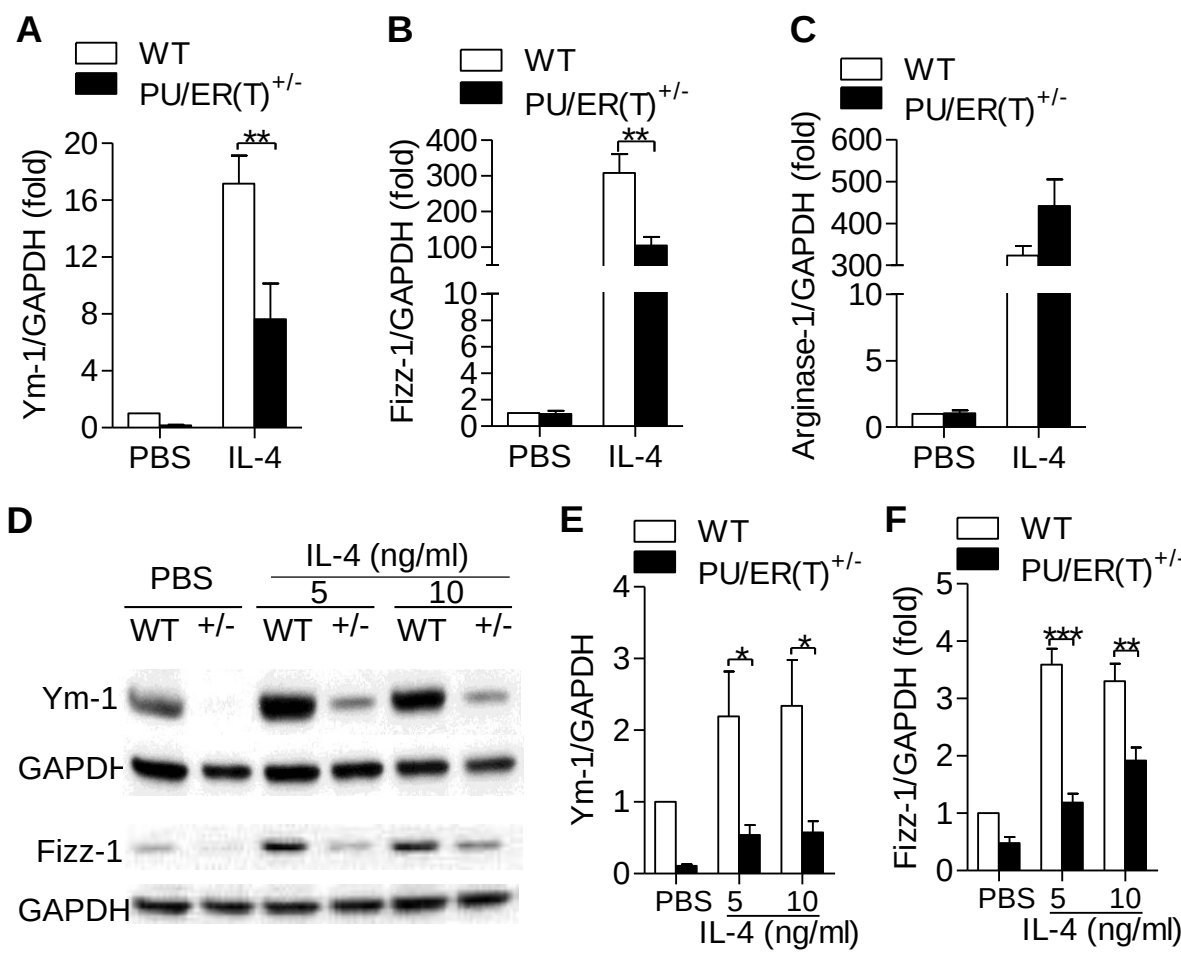


Figure 6

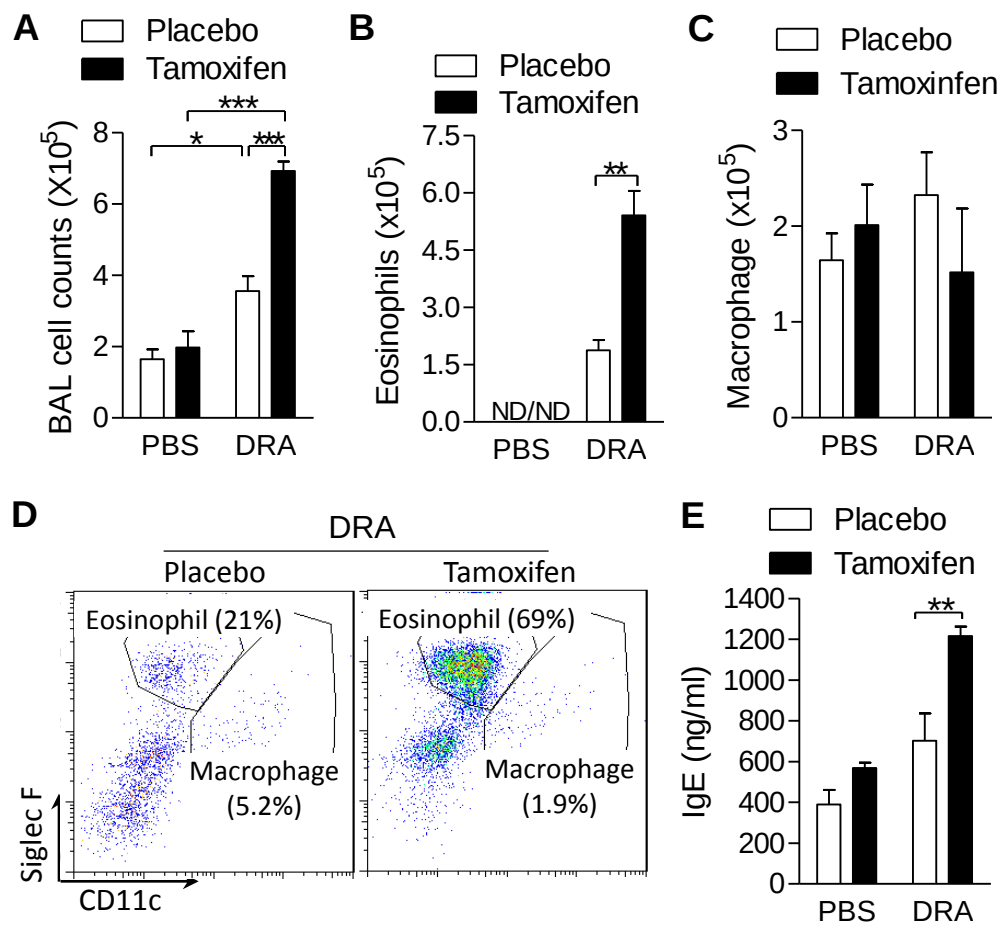


Figure 7

A DRA-induced mouse chronic asthma model

