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The role of the chitinase-like protein BRP-39 in allergic asthma

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1. ABBREVIATIONS

-/-	Null mutant / knock-out mouse
AHR	Airway hyperresponsiveness
Akt1	Ser/Thr protein kinase
B220	CD45
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Balb/c	Inbred Balb/c mice
BRP-39	Breast regression protein-39
BSA	Bovine serum albumin
CC10	Clara cell 10kDa protein
C57BL/6	Inbred C57 black 6 mice
GP38K	38-kDa heparin-binding glycoprotein
CD	Cluster of differentiation
D-PAS	Diastase-periodic acid-Schiff
Eos	Eosinophil granulocytes
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
Fas	Fas receptor, CD95
Fc	Fragment crystallizable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HcGP-39	Human cartilage glycoprotein-39
H&E	Hematoxylin and Eosin
ICH	Immunhistochemistry
IgG	Immunoglobuline G
IgE	Immunoglobuline E
IL-	Interleukin-
kDa	Kilodalton
mAb	Monoclonal antibody
mDC	Myeloid dentritic cells

MFI	Mean fluorescence intensity
MHCII	Major histocompatibility complex Class II
MMR	Macrophage Mannose Receptor
mRNA	Messenger ribonucleic acid
OVA	Ovalbumin
PBS	Phosphate-buffered-saline
PI	Propidium iodide
PMA	Phorbol myristate acetate
r	Recombinant
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
rtTA	Reverse tetracycline transactivator
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SSC	Side scatter
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphism
TGF-β1	Transforming growth factor beta 1
Th2	T-helper cell type 2
Tg	Transgenic
w/v	Weight/volume

2. INTRODUCTION

2.1 Allergic Asthma

Asthma is the most frequent chronic lung disease in children and has become the most common chronic disease in childhood within the last 30 years [3,4,42]. Asthma is characterized by dry cough, wheezing and exercised-induced dyspnoea. Without early diagnosis and specific treatment, asthma results in loss of lung function, impaired quality of life and the risk to die from uncontrolled asthmatic attacks [47]. Therefore, early diagnosis and specific treatment are required.

Asthma is an atopic disorder and its occurence is mostly associated with a positive family history of allergic diseases [4]. The immune response of allergic asthma is similar to the immune response that is seen in parasitic infections. Both diseases are characterized by a T-helper cell type 2 (Th2) immune response [4,35]. Whereas the Th2 immune response in parasitic infections has a physiologic role for anti-parasitic host defense, the overwhelming Th2 immune response in allergic asthma causes harm to the patient suffering from asthma without any evident beneficial physiologic role. The precise source of the dysregulated Th2 immune response in allergic asthma has not been elucidated as yet [11]. Thus, several lines of research aim to identify key players in the pathogenesis of allergic asthma in order to design specific treatment strategies.

2.2 Chitinases and chitinase-like proteins

Chitinases and chitinase-like proteins are members of the evolutionarily conserved 18-glycosyl-hydrolase family, which contains molecules which are true chitinases and molecules that lack chitinase activity. Much of the research in this area has focused on chitinases like acidic mammalian chitinase which are now known to play critical roles in the life cycle of many parasitic pathogens and the pathogenesis of Th2 and anti-parasite responses [12]. However, the majority of the 18-glycosyl-hydrolase family members are chitinase-like proteins which, as a result of mutations in their highly conserved enzyme sites, do not contain chitinase activity. Enzymatically active

chitinases include acidic mammalian chitinase and chitotriosidase in humans [2]. Chitinase-like proteins include breast regression protein-39 (BRP-39) in the murine system, chitinase-like protein human cartilage glyocoprotein-39 (HcGP-39 / YKL-40), porcine 38-kd heparin-binding glycoprotein (GP38K), and bovine 39-kd whey protein.

2.3 Chitinases and Chitinases-like Proteins in Allergic Asthma

Mammalian chitinase-like proteins (as prototypical example BRP-39) are induced at sites of inflammation, parasitic infections [5] and tissue remodelling [23] . Thus these proteins are believed to play active roles in antiparasite defense and tissue repair responses. While it is believed that mammalian enzymes with true chitinase activity (as prototypical examples acidic mammalian chitinase and chitotriosidase) play a direct role in host responses to chitin-containing pathogens, it is also reasonable that chitinase-like proteins primarily modulate tissue inflammation, immunity and/or remodelling [12] . Host immune responses to parasites include an early innate component to control or eliminate parasitic infestation and an adaptive response which is antigen-specific. The immune response to parasites is frequently Th2 dominated and similar to tissue allergy. Both show an increased production of IL-4, IL-5, IL-13 and IgE and tissue eosinophilia. The Th2 inflammatory response is originally generated to deal with parasites and other pathogens. Based on these notions, it is believed that allergy, atopic asthma and the inflammatory and remodeling responses are triggered in a parasite pathogen-independent fashion. These disorders occur when Th2 responses are poorly controlled [2,20]. As chitinases and chitinase-like proteins are believed to play a key role in the innate immunity to parasites and other infectious agents, it is reasonable to hypothesize that, when produced in a dysregulated fashion, they also play an important role in the pathogenesis of allergy and/or asthma.

The role of acidic mammalian chitinase in murine asthma

To test the hypothesis that chitinases are induced during and play essential roles in Th2 tissue responses, mRNA, protein and bioassay evaluation techniques have been used to assess the expression of acidic mammalian chitinase in lungs from mice with antigen-induced Th2 inflammatory responses and transgenic mice in which IL-13 was overexpressed in a lung-specific fashion. These studies demonstrated that acidic mammalian chitinase mRNA and protein are prominently induced in epithelial cells and macrophages in these murine modeling systems [54]. They also demonstrated that this induction was Th2 specific and that IL-13 was necessary for acidic mammalian chitinase induction in the mouse lung [54]. This inductive event appeared to play an important role in Th2 inflammation because acidic mammalian chitinase-based interventions with anti-acidic mammalian chitinase antiserum or allosamidin decreased bronchoalveolar lavage, tissue inflammation and airways hyperresponsiveness. These findings suggest that acidic mammalian chitinase plays a critical role in allergic asthma. The functional role of BRP-39 in asthma, however, remains to be elucidated.

YKL-40 in human asthma

YKL-40 is the human homologous protein of murine BRP-39. Elevated levels of YKL-40 are found in a variety of human inflammatory diseases [23-26,38]. Nevertheless, their role in allergic inflammation has not been addressed so far. Recent studies from our laboratory demonstrated that the levels of circulating YKL-40 are increased in patients with allergic asthma and correlate directly with asthma severity, airway remodeling and airway YKL-40 expression and inversely with lung function [10]. Further studies revealed that single-nucleotide polymorphisms (SNPs) in the YKL-40 gene affect YKL-40 levels, asthma susceptibility and bronchial hyperresponsiveness [39]. These findings demonstrated that YKL-40 might represent a novel biomarker that can be used to stratify asthmatics on the basis of disease severity and/or airway remodeling. The underlying

mechanism(s) by which YKL-40 modulates the pathogenesis of allergic asthma is unknown so far but is a prerequisite for considering YKL-40 as future therapeutic target.

3. AIMS

Previous studies suggest that chitinases / chitinase-like proteins may play a crucial role in allergic inflammation in mice and men. Recent studies highlight the role of YKL-40 as potential biomarker in allergic asthma in humans. However, the functional roles of chitinases/chitinase-like proteins, in particular BRP-39, are almost completely unknown. This is due, in part, to the fact that mice with null mutations of chitinase-like proteins have not been generated. Therefore, the aim of this study was to define the biology and functional involvement of the chitinase-like protein BRP-39 in allergic asthma. We had the following specific aims in this study:

- Does the genetic deletion of BRP-39 in mice have an effect on ovalbumin-induced airway inflammation or Th2 inflammation in IL-13 overexpressing mice?
- If so, which cells and soluble proteins are affected by the genetic deletion of BRP-39?
- If so, what are the underlying mechansisms by which BRP-39 regulates allergic airway inflammation?

4. MATERIALS AND METHODS

4.1 IL-13 Overexpressing Mice

C57BL/6 and Balb/c wild-type inbred mice were obtained from the Jackson Labs (Bar Harbor, ME, USA). CC10-rtTA-IL-13 transgenic mice were previously generated and characterized in our laboratory (161), bred onto a C57BL/6 background and used in these studies. These mice utilize the Clara cell 10 kDa protein (CC10) promoter and the reverse tetracycline transactivator (rtTA) to target IL-13 to the lung in a doxycycline inducible manner.

4.2 Generation of BRP-39$^{-/-}$ Mice

BRP-39 null mutant mice were generated as described in Figure 1 and used to define the specific role of BRP-39 *in vivo*. Standard gene targeting approaches and homologous recombination were used to generate BRP-39 null embryonic stem cells and BRP-39$^{-/-}$ knockout mice. The targeting vectors that were used eliminated exons 1 to 6 and a part of the BRP-39 promoter (see Figure 1). Western, Southern and mRNA evaluations demonstrated the success of this approach. These mice were generated on a mixed 129/C57BL/6 background and were thereafter consequently bred for over 10 generations onto a C57BL/6 inbred strain mouse background. At baseline the BRP-39$^{-/-}$ mice were viable and fertile and were not able to be differentiated from the wild-type controls based on size, physical appearance, rate of growth or level of physical activity. In addition, autopsy, light microscopic examinations of skin and visceral organs, pulmonary compliance assessments and H&E, trichrome and elastin evaluations showed no differences between wild-type and BRP-39$^{-/-}$ animals (data not shown).

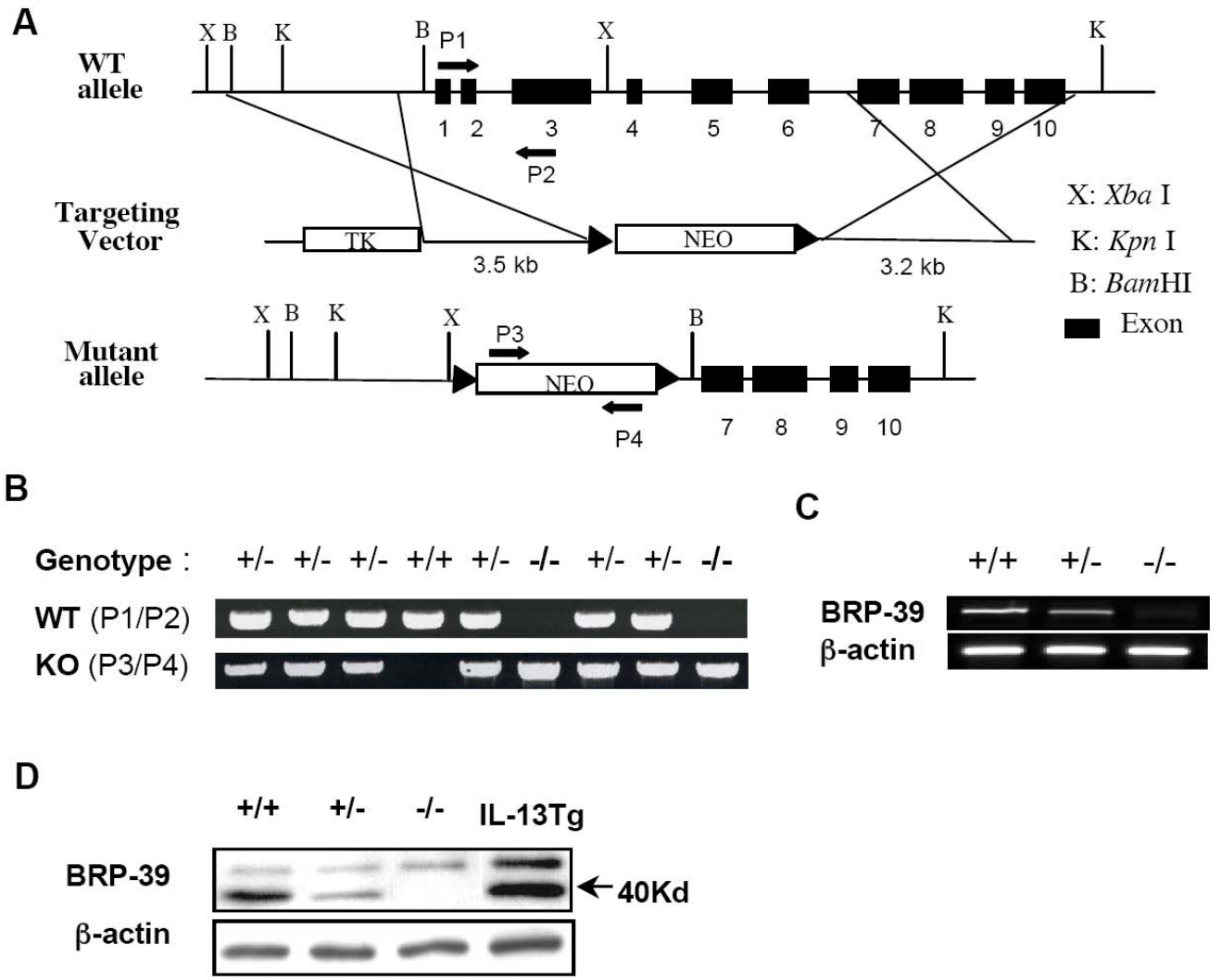


Figure 1. Generation of BRP-39 null mutant mice.

Figure A depicts the constructs to genetically target the promoter and exons 1-6 of BRP-39. Figure B shows the genotyping control using specific primers and conventional PCR (DNA). Figure C shows the confirmation of BRP-39 knock-out on mRNA expression using RT-PCR and Figure D on protein levels using Western blotting.

4.3 OVA Sensitization and Challenge

Six to eight week old BRP-39 null mutant mice and control littermates received i.p. injections containing 20μg of chicken OVA (Sigma, St. Louis, MO) complexed to alumn (Resorptar, Indergen, New York, NY) or alumn alone. This process was repeated 5 days later. After an additional seven days, the animals received three aerosol challenges (40 min a day, 3 consecutive days) with 1% OVA (w/v) in endotoxin-free PBS or PBS alone. The aerosol was generated in a NE-U07 ultrasonic nebulizer (Omron Health care, Vernon Hills, IL). The mice were sacrificed 24, 48 or 72 h after aerosol exposure. In experiments in which the role of BRP-39 was being defined, OVA sensitization and boosting were undertaken as described above. Where indicated, aerosol OVA challenge was then undertaken in the presence and absence of an anti-BRP-39 antibody (engineered by Medimmune Inc.). In these experiments, the mice received 500μl doses of a rabbit polyclonal anti-BRP-39 or pre-immune serum controls via an intraperitoneal route, every other day starting the day before the aerosol challenge.

4.4 BAL and Lung Inflammation

Lung inflammation was assessed by BAL as described previously [49]. Animals were anesthetized, a median sternotomy was performed, the trachea was dissected free from the underlying soft tissues and BAL was performed by perfusing the lungs in situ with 0.8 ml of PBS and gently aspirating the fluid back. This was repeated twice. The samples were then pooled and centrifuged and cell numbers and differentials were assessed. Cell pellets were further used for flow cytometric analyses and other experimental procedures. The cell-free BAL fluid was stored at –70 °C until used.

4.5 Histologic Analysis

The lungs were removed *en bloc*, inflated at 25 cm pressure with PBS containing 0.5% low melting point agarose gel, fixed in Streck solution (Streck Laboratories, La Vista, NE), embedded in paraffin, sectioned and stained. Hematoxylin and Eosin and diastase-periodic acid–Schiff (D-PAS) or MalloryTrichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at the *Yale University School of Medicine*.

4.6 Immunohistochemistry

Immunohistochemistry (ICH) of BRP-39 was performed, using polyclonal rabbit anti-BRP-39 as the primary antibody [52]. The antibody was applied to the lung sections at a 1/100 dilution. The specificity of the antibody was tested using the tissue samples from BRP-39 null mutant mice as a negative control.

4.7 Immunoblot Analysis

BAL (35 μl out of 2ml BAL fluid) and lung lysate (50μg) were subjected to immunoblot analysis using a polycclonal rabbit antiserum against BRP-39. BAL fluid protein or lung lysates were fractionated by SDS-PAGE, transferred to membrane and evaluated [27].

4.8 Quantification of BRP-39

The protein concentration of mouse BRP-39 was measured by ELISA according to the protocol provided by the manufacturer's instructions with modifications. The levels of BRP-39 in BAL or lung lysates were evaluated by ELISA. In these experiments we used the anti-BRP-39 rabbit polyclonal IgG to capture and biotinylated anti-BRP-39 followed by horseradish peroxidase labeled streptavidin (Amersham, GE Healthcare, Piscataway, NJ) for detection. This assay detects as little as 50 pg/ml rBRP-39. This was undertaken with the assay noted above and confirmed with

a commercial assay (Quidel Inc. San Diego, CA). The absence of cross reactivity with other chitnase family members (acidic mammalian chitinase and chitotriosidase) was confirmed using concentrations of purified recombinant proteins upto a concentration of 100 ng/ml.

4.9 mRNA Analysis

mRNA levels were assessed using real-time RT-PCR assays as described previously in detail by our laboratories [33,36]. In these assays, total cellular RNA from the lungs were obtained using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The sequences for the primers used in real-time RT-PCR were obtained from PrimerBank online (http://pga.mgh.harvard.edu/primerbank).

4.10 Flow Cytometry

Whole lung cell suspensions were obtained by a modified method according to Rice WR et al. [44]. In brief, lung tissue was digested using dispase (Stem Cell technologies, 5 mg/ml), collagenase (0.04%; Sigma) and 100U/ml DNAse (Sigma). Both whole lung suspension cells and BAL cells underwent several centrifugations (10 min., 300g-1000g) and hemolysis (precooled hemolysis solution containing 11 mM KHCO3, 152 mM NH4Cl; washing 5 min, 400g at 4°C), were then strained through progressively smaller cell strainers (100-20 µM) and nylon gauze and were finally resuspended in FACS buffer (PBS, 2% BSA, 2% FCS) supplemented with 10 U/ml DNase1. For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) / ionomycin (500 ng/ml) (Merck) in RPMI 1640 in the presence of Brefeldin A (2µM, Sigma) for 6 h at 37°C before extracellular staining. For cell surface staining, the cells were incubated for 30 minutes at room temperature with purified rat anti-mouse CD16/CD32 mAb (1µg/10^5 cells; mouse Fc-Block; BD Biosciences) to prevent non-specific binding of antibodies to Fc receptors. Afterwards, cells were treated for 30 minutes at 4°C with appropriate combinations of specific antibodies for surface

marker staining, namely CD3, CD4, CD8, CD19, CD16, Siglec-F, CCR3, MHCII, CD80, CD86, CD40, CD11b, CD11c, B220, PDCA1, CD206 (MMR) and CD95 (Fas) to characterize specific cell types and analyze their receptors. The corresponding isotype controls were stained in parallel. Gating of leukocyte subtypes was based on light scatter properties and positive expression for characteristic surface markers as described in the respective figures. Propidium iodide (PI, 5 μg/ml; Sigma) and Annexin V-FITC (5 μg/ml; BD Biosciences) were used to detect apoptotic (Annexin V^+, PI^-) and necrotic (Annexin V^+, PI^+) leukocytes. For intracellular staining, cells were fixed with 0.5 ml of ice-cold 2% paraformaldehyde, were permeabilized using 0.5% saponin (Sigma) and stained with anti–IL-4, anti-IL-13, anti-active Caspase 3 or anti-phospho-Akt1 (S473, R&D Systems) or the appropriate isotype control. For anti-phospho-Akt1 a SAP permeabilization buffer (0.1% (w/v) saponin, 0.05% (w/v) NaH_3 in Hanks´ Balanced Salt Solution) was used. For anti-active Caspase 3 or anti-phospho-Akt1 staining, the antibodies were incubated at RT for 60 minutes with the respective cells. All antibodies and FACS reagents were from BD Biosciences except otherwise indicated. Saturating concentrations of the antibodies were used as determined by titration experiments prior to the study. At least 10,000 cells/ sample were analyzed. Isotype controls were subtracted from the respective specific antibody expression and the results are reported as mean fluorescence intensity (MFI). Calculations were performed with Cell Quest analysis software (BD Biosciences). All experiments were performed in triplicate.

4.11 Alternative Macrophage Activation

Alternative macrophage (M2) activation was assessed by using an arginase activity assay (Bioassays Inc) [40] and surface staining of CD206 (macrophage mannose receptor, MMR) [46]. In brief, thioglycollate (3%) was injected via an intraperitoneal route and peritoneal macrophages were harvested after 5 days, were incubated in RPMI 1640 (plus 10% FCS) for 48h with or without rBRP-39 (10μg/ml) or IL-4 (5ng/ml). Afterwards, cells were lysed in 0.5% Triton-X

containing Protease Inhibitor Cocktail Tablets (Roche) before performing the arginase activity assay. CD206 surface expression was performed as described in detail in the flow cytometry section.

4.12 Statistics

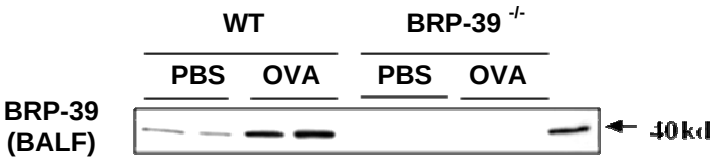
All data was initially checked for normal/parametric distribution (Kolmogorov-Smirnov-Test). If parametric distribution was found, analysis of variance (ANOVA) was applied to screen for differences among at least three groups. To compare two individual groups, Student's *t*-test was applied. If non-parametric distribution was found, the Kruskal-Wallis test was applied to screen for differences among at least three groups, followed by the Mann-Whitney *U* test (Wilcoxon rank-sum test) to compare two individual groups. Statistical analyses were performed using Prism 4.0 (Graph Pad Software) and STATA version 8.2 for Windows (STATA Corporation).

5. RESULTS

5.1 Regulation of BRP-39 by Th2 Inflammation

To define the expression of BRP-39 in mice with acute Th2 inflammation we compared the levels of BRP-39 mRNA and protein expression in lungs from wild-type mice sensitized and challenged with OVA and appropriate non-sensitized and/or non-challenged wild-type controls. The levels of BRP-39 mRNA and protein in lungs from the control mice were near or below the limits of detection in our assays. In contrast, the levels of BRP-39 protein were significantly increased after OVA-sensitization and challenge (Figure 2A). This was readily detected 24 hours after aerosol OVA exposure and persisted at all time points that were assessed. This induction was not seen in mice that received a saline aerosol after OVA sensitization or an OVA aerosol after saline sensitization. In BRP-39 knock-out mice, BRP-39 was not detectable (Figure 2A). In all wildtype mice, immunohistochemistry demonstrated that BRP-39 was induced predominantly in airway epithelial cells and alveolar macrophages (Figure 2B, arrows). This staining was appropriately specific because it was not present when the primary antibody was pre-incubated with BRP-39 peptide and when the primary antibody was not used in the evaluation. When viewed in combination, these studies demonstrate that BRP-39 is induced by Th2 inflammation, predominantly in airway epithelial cells and alveolar macrophages.

A



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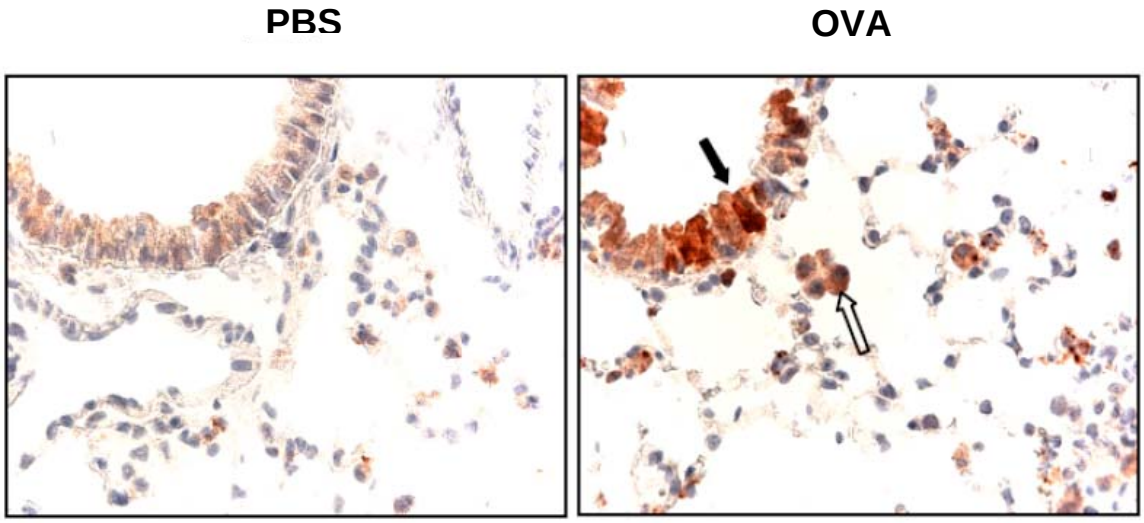


Figure 2. OVA-induced BRP-39 expression in the murine lung. Figure A: Western blot analysis of BRP-39 protein expression in wild-type and BRP-39 knock-out mice challenged with PBS or OVA; Figure B: Immunohistochemistry of BRP-39 staining in lung tissues of PBS or OVA treated wildtype mice. Black arrow indicates airway epithelial cells, white arrow indicates alveolar macrophages.

5.2 Role of BRP-39 in OVA-Induced Inflammation and Physiologic Dysregulation

Next, we evaluated whether the inhibition of BRP-39, achieved by genetic knock-out or antibody blocking, has an effect on aeroallergen-induced Th2 inflammation in mice. In wild-type mice, OVA-sensitization and challenge caused an impressive increase in tissue and BAL total cell, eosinophil, lymphocyte and macrophage numbers. Each of these inductive events was at least partially BRP-39-dependent, because null mutations of BRP-39 caused a significant decrease in each of these parameters (Figure 3A). The changes in cellular BAL content were most impressive for eosinophils. Similar findings were observed in comparison of OVA-sensitized and challenged wild-type mice treated with an anti-BRP-39 antibody (Figure 3B). Further studies were undertaken to determine if BRP-39 played a role in Th2 cytokine response. In contrast to OVA-sensitized and challenged wild-type mice, comparable Th2 cytokine induction was not found in OVA-sensitized and challenged mice with null mutations of BRP-39, because in the BRP-39 knock-out mice BAL levels of the prototypic Th2 cytokines IL-13 and IL-5 were decreased by > 90% compared to wild-type animals. IL-4 was decreased by 50% compared to wild-type animals (Figure 3C). These studies indicate that the absence of BRP-39 reduces Th2 inflammation.

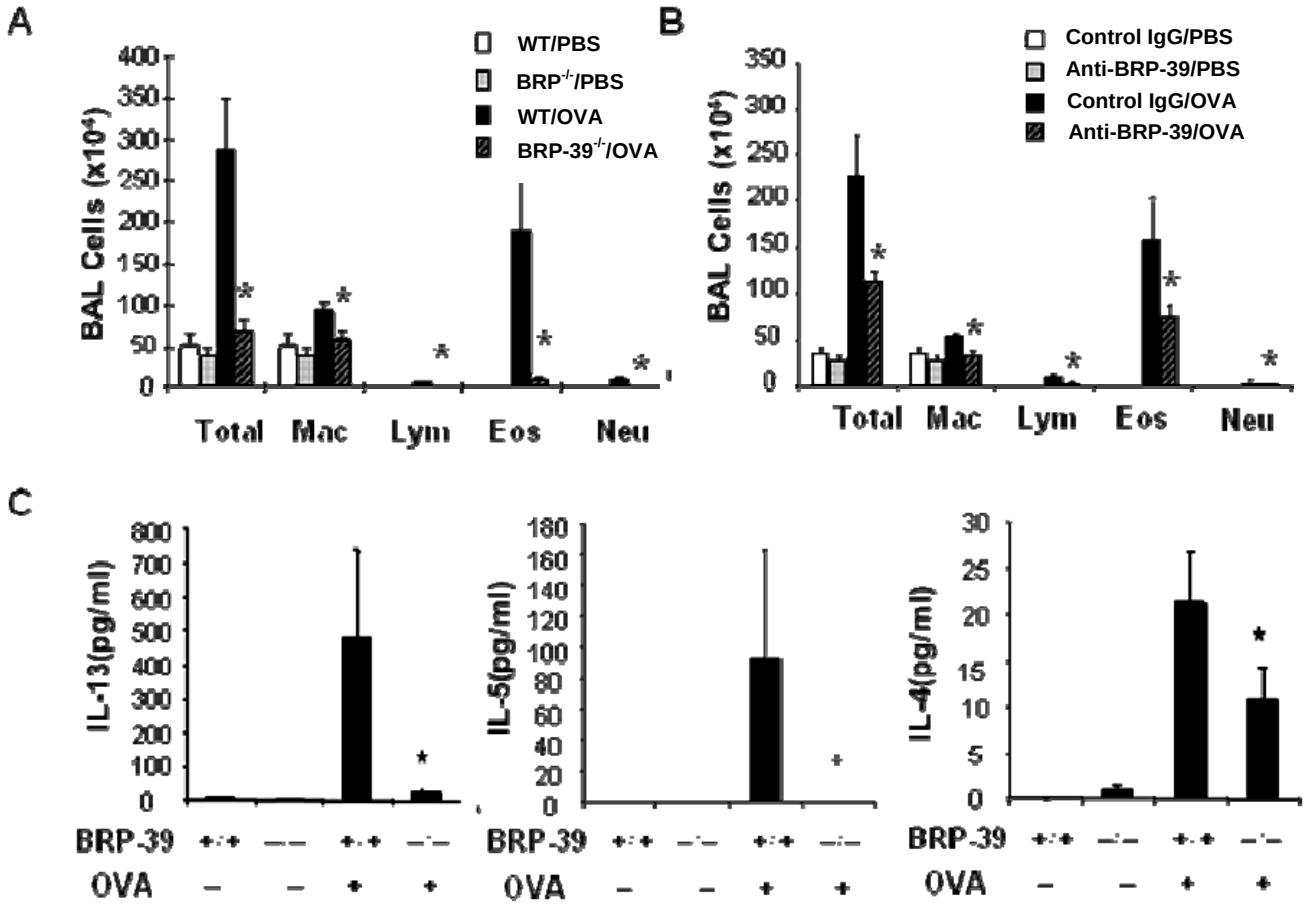


Figure 3. Role of BRP-39 in Th2 inflammation. Figure A and B: Differential cell counts in wild-type, BRP-39$^{-/-}$ and anti-BRP-39 antibody treated wild-type mice in the OVA model. Figure C: Cytokine analysis in BAL of wild-type and BRP-39$^{-/-}$ mice in the OVA model. *$p<0.05$

5.3 BRP-39 Regulation of Dendritic Cells and Alternative Macrophage Activation

To understand the underlying mechanism by which BRP-39 regulates Th2 inflammation, we initially focussed on dendritic cells since dendritic cells are the central initiators and regulators of Th2 inflammation. In particular, myeloid dendritic cells (mDC) are well-known to play an essential role in the pathogenesis of pulmonary Th2 inflammation [31]. Therefore, we analyzed the percentages of mDCs and the activation status of mDCs by means of CD86 and CD40 surface expression using flow cytometry. These experiments showed that the number of mDCs was increased in lungs from wild-type mice that had been sensitized and challenged with OVA compared to non-sensitized or non-challenged mice (Figure 4A). After OVA sensitization and

challenge, mDC showed increased levels of CD86 (Figure 4B) and CD40 (Figure 4C) surface expression compared to cells from PBS sensitized and/or challenged mice. BRP-39 played an important role in these responses because mDC accumulation and mDC expression of CD86 and CD 40 were significantly decreased in lungs from sensitized and challenged BRP-39$^{-/-}$ mice (Figure 4, A-C). These studies showed that BRP-39 stimulates the Th2-induced accumulation and activation of pulmonary mDCs.

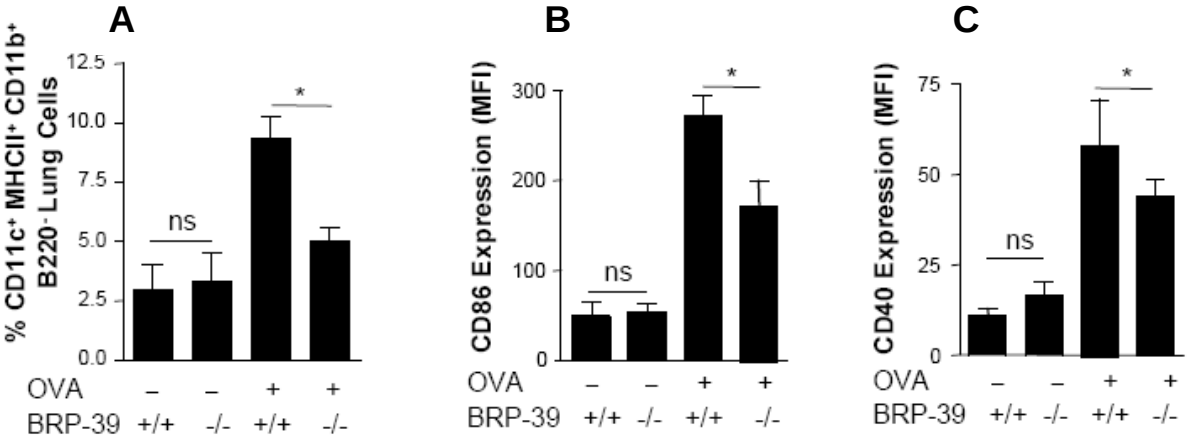


Figure 4. Role of BRP-39 in dendritic cell accumulation and activation. Myeloid dendritic cells (mDCs) in murine lungs were identified by coexpression of CD11c, MHCII and CD11b and negative expression of B220. mDC activation was analyzed by CD86 and CD40 expression. Figure A shows percentages of mDCs in wild-type and BRP-39 KO mice with or without OVA sensitization and challenge. Figure B shows the surface expression of CD86 on murine pulmonary mDCs (MFI = mean fluorescence intensity). Figure C schows CD40 surface expression on mDCs. *$p<0.05$

Another important mechanism in allergic inflammation is the conversion of macrophages towards alternative macrophage (M2) activation. To see if recombinant (r) BRP-39 contributed to the alternative activation of macrophages, we analyzed alternative macrophage activation in BALF measured by surface expression of the macrophage mannose receptor (MMR) on F4/80^{+} alveolar macrophages in wild-type and BRP-39 deficient mice. As shown in Figure 5, OVA sensitization and challenge resulted in an impressive upregulation of MMR surface expression (Figure 5A, left panel) on macrophages and an accumulation of MMR^{+} alveolar macrophages in the lungs from

wild-type mice (Figure 5A, right panel). To investigate and confirm this mechanism *in vitro*, isolated peritoneal macrophages were harvested and incubated with PBS, rBRP-39 or IL-4 as positive control for M2 alternative macrophage activation. Alternative macrophage activation was measured by MMR surface expression on peritoneal macrophages (Figure 5B, left panel) and arginase 1 activity of lysed isolated peritoneal macrophages (Figure 5B, right panel). In these experiments rBRP-39 caused a significant increase in arginase 1 activity and an upregulation of MMR surface expression compared to PBS treatment. This alternative activation was found after 48 hours of macrophage-BRP-39 incubation where it was almost comparable to the effects of the positive control IL-4. These studies demonstrate that BRP-39 induces alternative macrophage activation *in vitro* and suggest that the induction of alternative (M2) macrophage activation may represent a key underlying mechanism by which BRP-39 triggers Th2 airway inflammation *in vivo*.

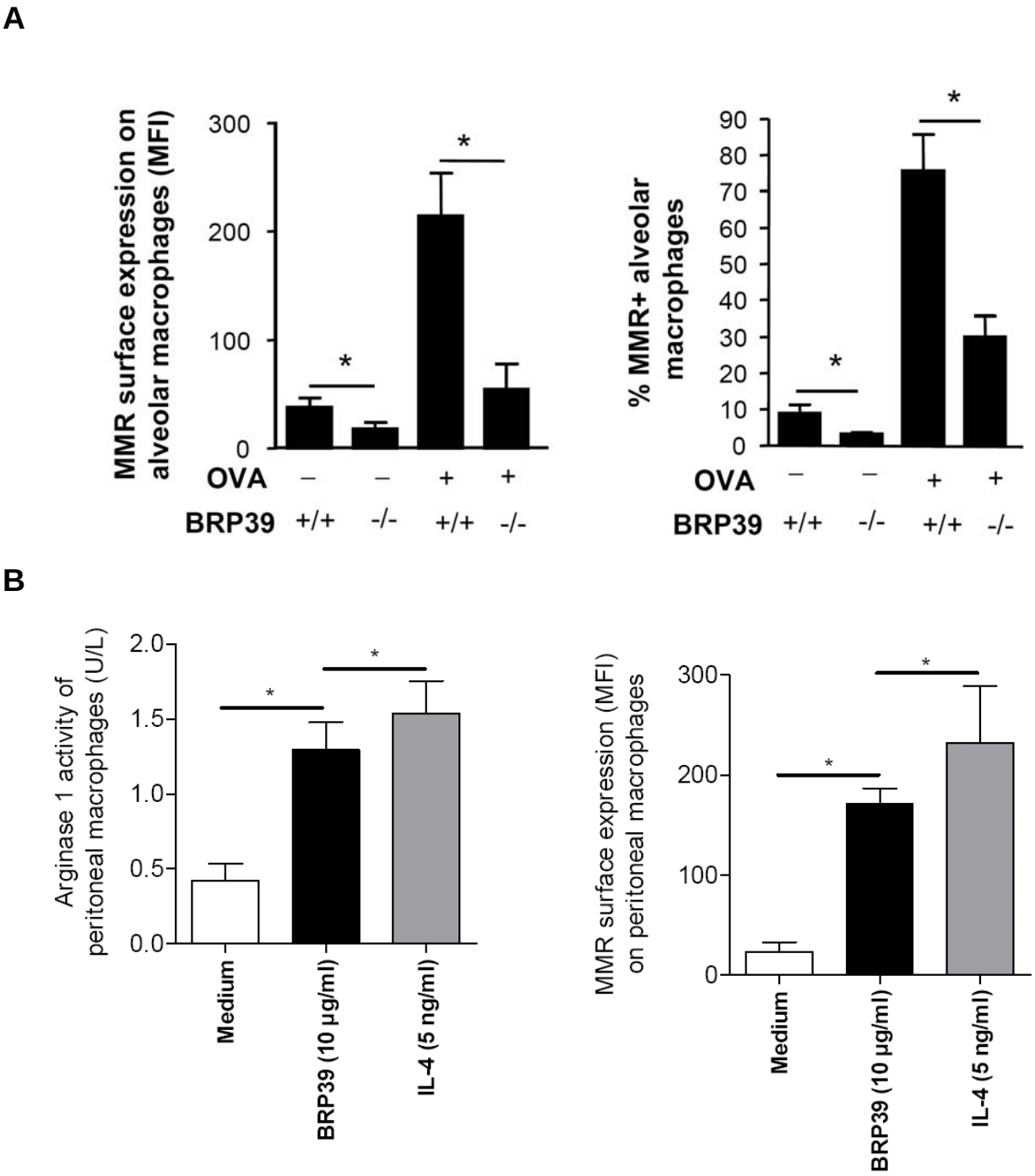
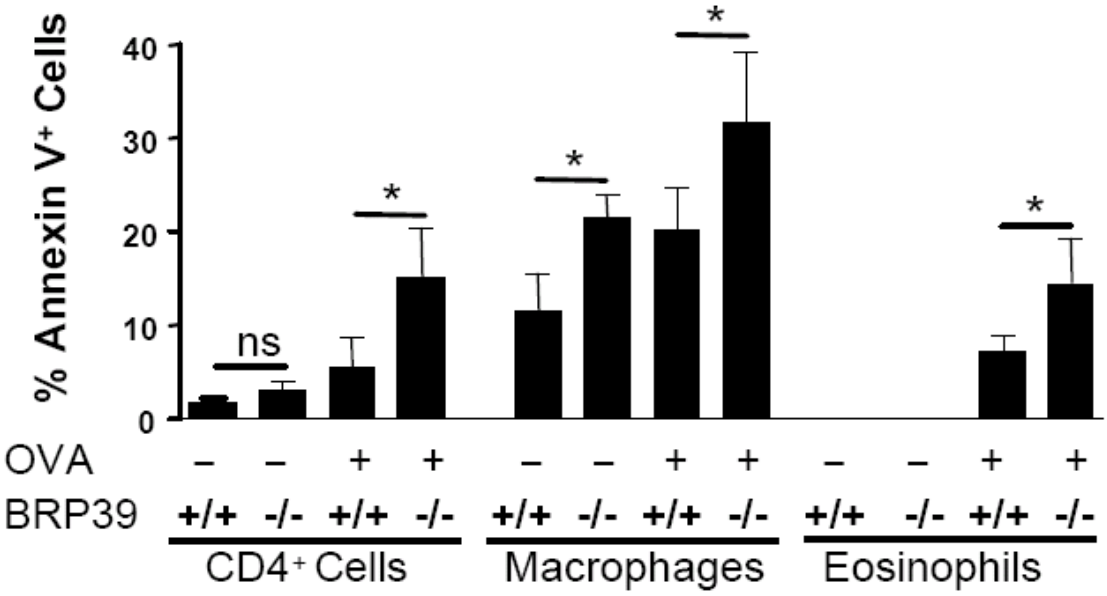


Figure 5. Decreased OVA-induced alternative alveolar macrophage activation in the absence of BRP-39. Figure 5A shows alternative macrophage activation as measured by MMR surface expression on F4/80$^+$ BAL macrophages. The left panel shows the surface expression of MMR (MFI) on F4/80$^+$ BAL macrophages, the right panel the percentage of MMR$^+$ alveolar macrophages in BALF. Where indicated, BRP-39 knock-out mice were used. Where indicated, the OVA sensitization and challenge model was performed. Figure 5B depicts the effect of recombinant BRP-39 (10 µg/ml) or IL-4 (5 ng/ml) on alternative macrophage activation as measured by arginase 1 activity of lysed on isolated peritoneal macrophages (left panel) or by MMR surface expression on isolated peritoneal macrophages (right panel). *p<0.05

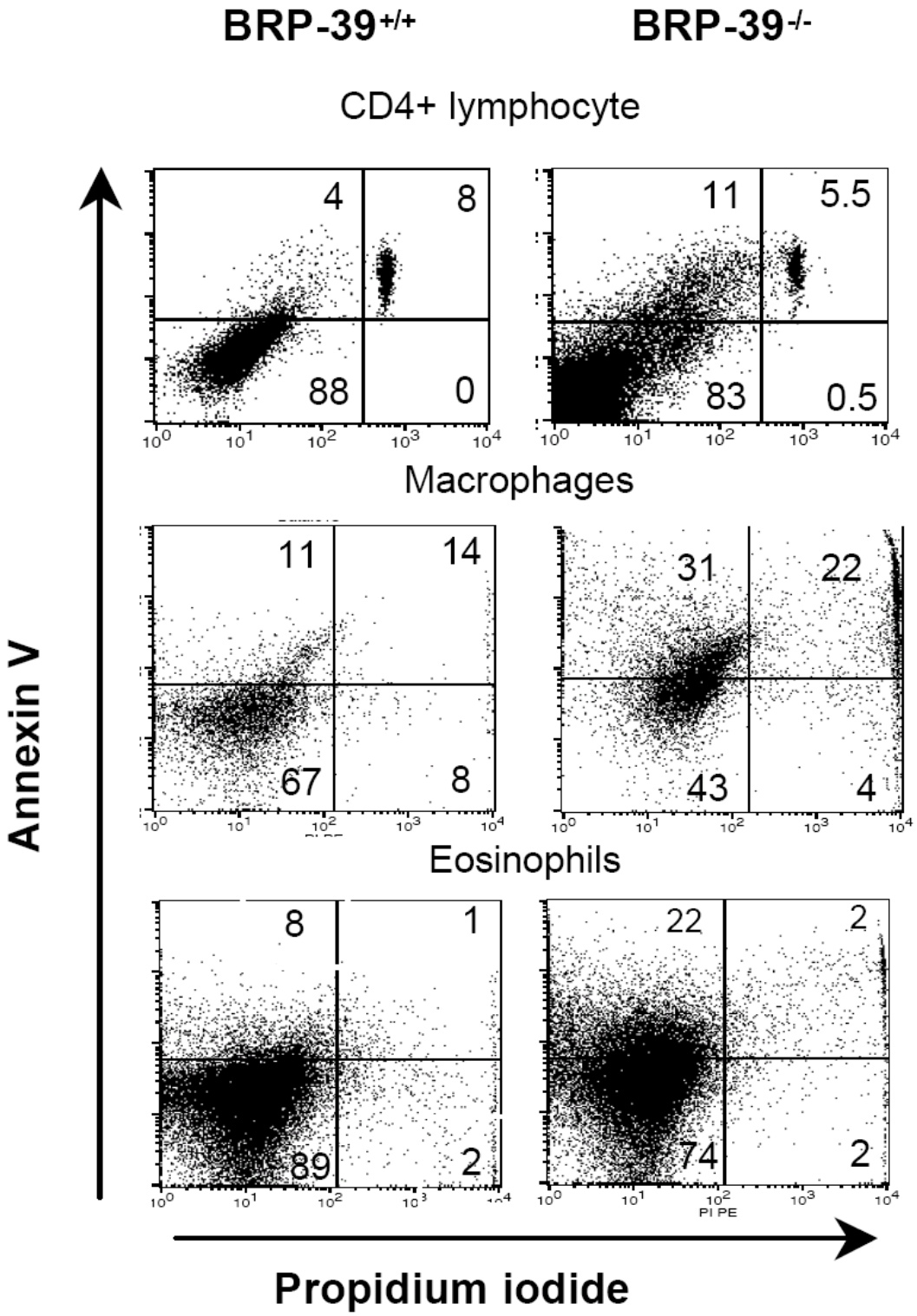
5.4 BRP-39 Regulation of Inflammatory Cell Apoptosis

In a further step, we compared the cell death responses, including apoptosis (programmed cell death) and necrosis, of inflammatory cells (macrophages, $CD4^+$ lymphocytes and eosinophils) in OVA sensitized and challenged wild-type and BRP-$39^{-/-}$ mice to evaluate whether BRP-39 might play a role in inflammatory cell apoptosis. These studies detected enhanced levels of annexin V staining of lung and BAL CD4 cells, macrophages and eosinophils in BRP-$39^{-/-}$ mice compared to wild-type mice (Figure 6A). This included cells with enhanced levels of expression of annexin V only (apoptosis) and cells that stained with annexin V and propidium iodide (PI) (late apoptosis / mixed apoptosis and necrosis) (Figure 6B). This *in vivo* cell death response(s) was associated with enhanced CD95 (Fas) surface expression, a key receptor involved in the induction of leukocyte apoptosis (Figure 6C). When viewed in combination, these studies demonstrate that BRP-39 is an important inhibitor of eosinophil, T cell and macrophage death receptor-mediated apoptosis *in vivo*.

A



B


BRP-39+/+
BRP-39-/-
CD4+ lymphocyte
4
8
88
0
11
5.5
83
0.5
Macrophages
11
14
67
8
31
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Eosinophils
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89
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22
2
74
2
Annexin V
PI PE
Propidium iodide

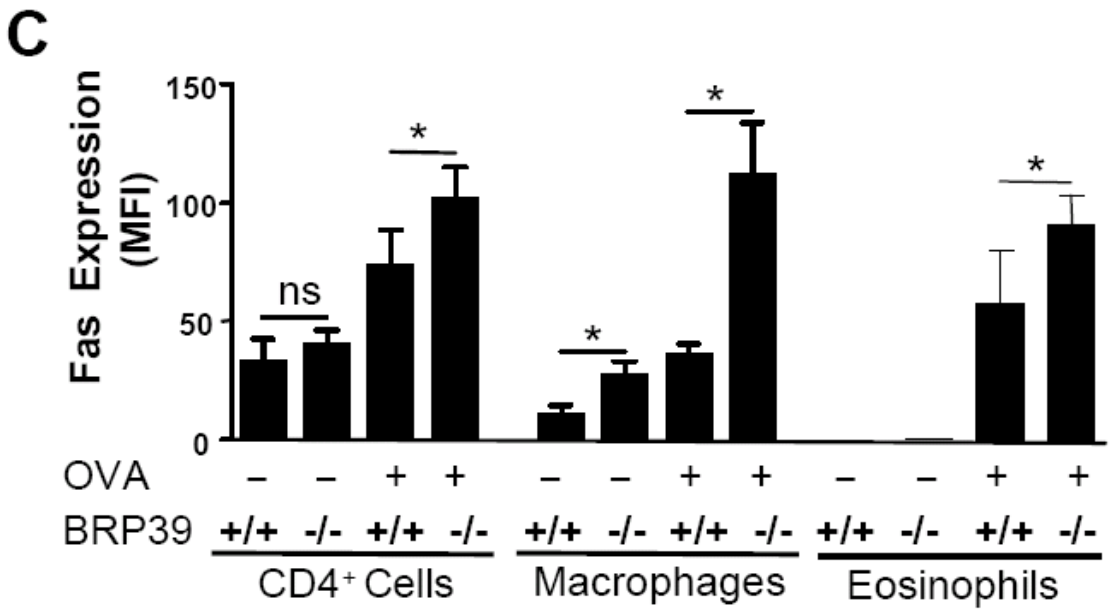


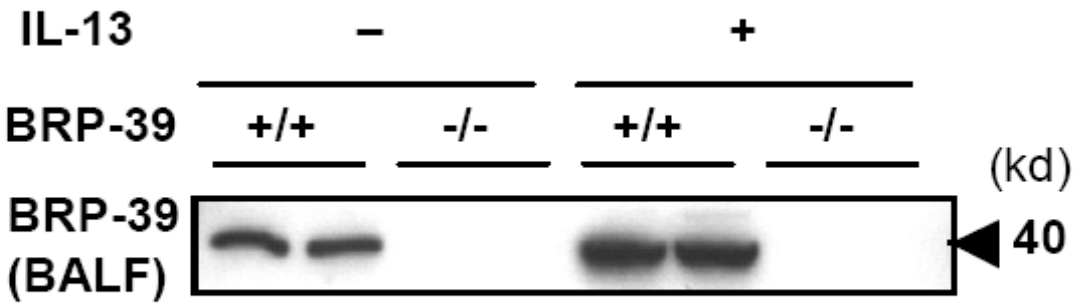
Figure 6. Role of BRP-39 in inflammatory cell apoptosis. Figure 6A: Apoptosis was analyzed by Annexin V surface staining on $CD4^+$ T cells, $F4/80^+$ alveolar macrophages and eosinophils in BAL of wild-type and BRP-39 knock-out mice. Where indicated, the OVA sensitization and challenge model was performed as described in detail in the methods section. Figure 6B depicts representative FACS plots of Annexin V and propidium iodide staining of $CD4^+$ T cells, $F4/80^+$ alveolar macrophages and eosinophils. Figure 6C shows Fas (CD95) surface expression on $CD4^+$ T cells, $F4/80^+$ alveolar macrophages and eosinophils in BALF. *$p<0.05$

5.5 BRP-39 and IL-13

IL-13 is the major effector cytokine at sites of Th2 inflammation and a powerful regulator of chitinase expression. [54] Thus, studies were undertaken to determine if IL-13 also regulates BRP-39 and the role of BRP-39 in the pathogenesis of IL-13-induced inflammation and tissue remodeling was defined. The levels of BRP-39 mRNA and protein were significantly higher in lungs from IL-13 transgenic (Tg) mice compared to the wild-type controls (Figure 7A). BRP-39 was most prominent in airway epithelial cells and alveolar macrophages (Figure 7B). In the latter experiments, IL-13 Tg mice with wild-type and null BRP-39 loci were generated and the tissue effects of IL-13 in these animals were compared. IL-13 overexpression in the transgene murine lung caused an increase of inflammatory cell ($CD4^+$ T cells and macrophage) apoptosis (Figure

7C). This induction of apoptosis was largely abrogated when BRP-39 was genetically knocked-out. These studies demonstrate that BRP-39 is induced by IL-13 and plays a critical role in IL-13-induced inflammatory cell apoptosis.

A.



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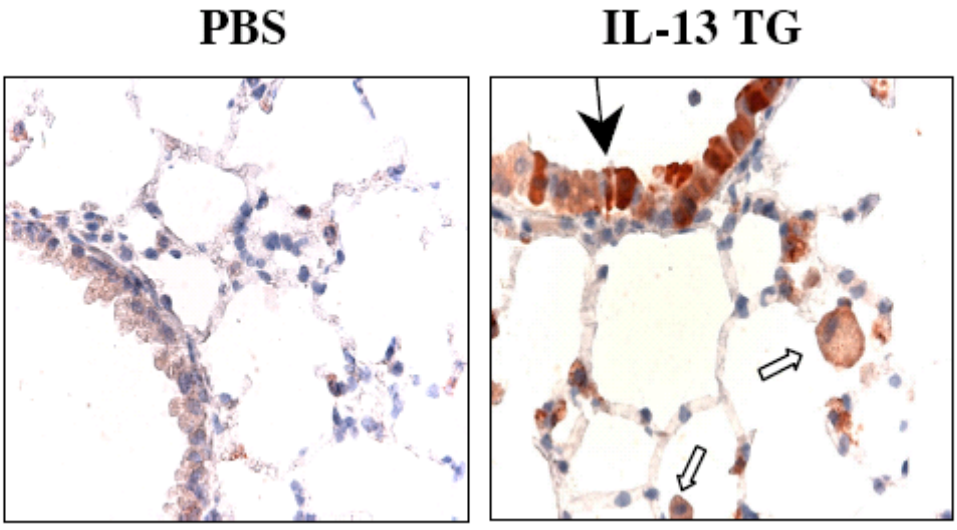

C.

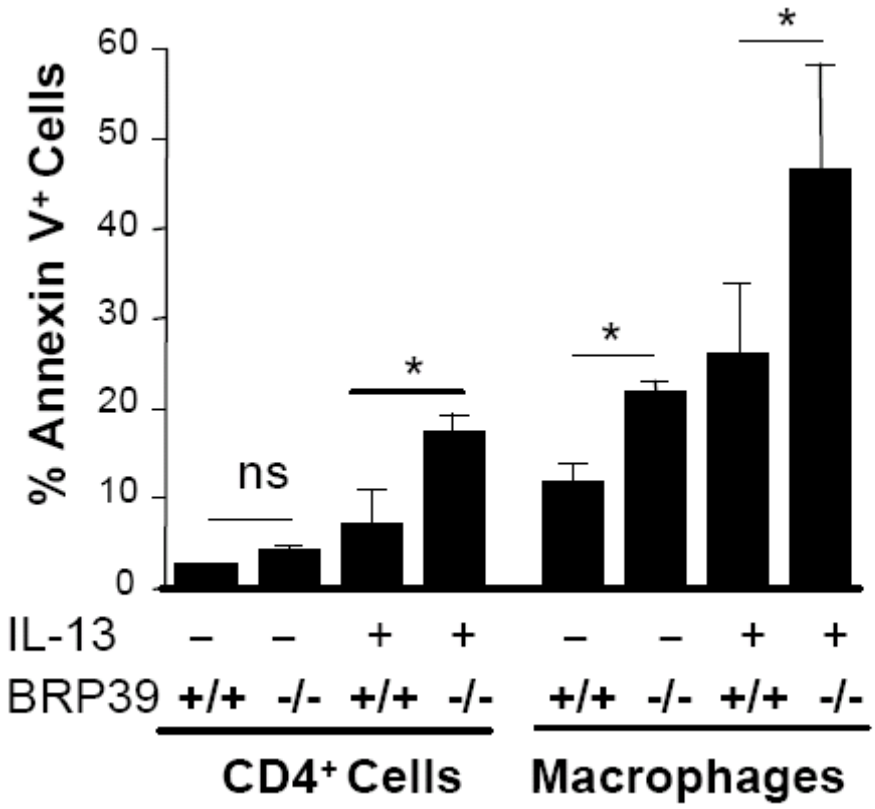

Figure 7. IL-13 induces BRP-39 expression and modulates IL-13-induced inflammatory cell apoptosis and airway remodeling. Figure A shows BRP-39 protein detection by Western blotting in BALF of wild-type and IL-13 transgenic overexpressing mice. Figure B shows BRP-39 staining using immunohistochemistry in lung tissue sections from wild-type and IL-13 transgenic overexpressing mice. The black arrow indicates BRP-39+ alveolar epithelial cells, the white arrows show BRP-39+ alveolar macrophages. Figure C shows Annexin V surface staining in $CD4^+$ T cells and $F4/80^+$ alveolar macrophages of BALF from wild-type and IL-13 tg mice. *$p<0.05$

6. DISCUSSION

These studies demonstrate for the first time a role for the chitinase-like protein BRP-39 in Th2 inflammation. Genetic knock-out studies and antibody blocking experiments substantiated the functional role of BRP-39 in the pathogenesis of Th2 inflammation *in vivo*. This effect was mediated by modulation of dendritic cells recruitment and activation, alternative macrophage activation and inflammatory cell apoptosis, possibly mediated via a Fas-dependent pathway. Together with data from human studies, these findings strongly suggest BRP-39 as novel pharmaceutical target in allergic asthma.

6.1 BRP-39

BRP-39 was initially discovered in mouse breast cancer cells [43]. Subsequently, a variety of differentially termed homologues were described, including human YKL-40, human HcGP-39, porcine 38 kDa heparin-binding glycoprotein (GP38K), bovine 39 kDa whey protein and drosophila Imaginal Disc Growth Factors [17,28,41]. In the breast, the expression of BRP-39 and bovine 39 kDa whey protein are increased during the extensive glandular remodeling that is seen in the involution phase after the cessation of lactation [43]. In drosophila, BRP-39-like molecules that lack chitinase activity act as growth factors. In porcine systems, GP38K induces the differentiation of cultured vascular smooth muscle cells [28]. Human YKL-40/HcGP-39 has been described to be produced by cultured chondrocytes and synovial cells, where it regulates cell proliferation and survival and has mitogenic effects on human skin and lung fibroblasts and synoviocytes [17,41]. This limited body of information suggests that BRP-39/YKL-40 might play a role in tissue remodelling and cell survival. Because the levels of HcGP-39/YKL-40 are elevated in a variety of human diseases, it is also been thought to contribute to pathologic remodeling responses such as lung diseases [10,23,26,38,41]. However, the biological roles of these BRP-39-like moieties in normal physiology and disease pathogenesis are poorly understood. In fact, our limited understanding of

the functions of these strongly conserved and therefore presumably biologically important moieties in mammals and man is believed to be one of the most pressing issues in chitinase/chitinase-like proteins biology [1]. Therefore, we performed studies using *in vitro* and *in vivo* experimental approaches, to define the functional role of BRP-39 in animal models of Th2 inflammation. These studies indicated an essential role for BRP-39 in dendritic cell accumulation and activation, alternative macrophage activation and inflammatory cell apoptosis/survival, mechanism that have a broad biological relevance also beyond allergic asthma. Further studies are, however, necessary to evaluate the functional significance of BRP-39 in other inflammatory disease conditions.

6.2 BRP-39 and Th2 inflammation

To address BRP-39 and Th2 inflammation, we generated and characterized BRP-39 knock-out mice. These studies add to our understanding of the biology of chitinase-like proteins by demonstrating for the first time, that BRP-39 plays a critical role in the pathogenesis of Th2 inflammation, Th2 cytokine production, dendritic cell accumulation and activation and IL-13-induced inflammation and remodeling. These studies also provide insights into the mechanisms that may underlie these effects by demonstrating that BRP-39 is an important regulator of $CD4^+$ T cell, macrophage and eosinophil apoptosis, CD95 (Fas) expression, dendritic cell accumulation and activation and alternative macrophage activation. BRP-39 seems to be involved in several steps of the allergic/Th2 immune response: accumulation and activation of antigen-presenting cells (dendritic cells), modulation of alveolar macrophages into alternatively activated (M2) macrophages and protecting recruited bronchoalveolar inflammatory cells ($CD4^+$ T cells, pro-inflammatory macrophage subsets and eosinophils) from apoptosis, thereby prolonging and enhancing the local airway inflammation. Our observation that genetic knock-out of BRP-39 increased the surface expression levels of the pro-apoptotic mediator / death receptor Fas (CD95) suggested an involvement of Fas-ligand/Fas-mediated apoptosis in BRP-39-induced tissue

responses. Based on this notion, BRP-39 might have an anti-apoptotic role *in vivo* and loss of BRP-39 triggers apoptosis by upregulation of Fas surface expression. Whether the effect on Fas is a direct or indirect consequence of lack of BRP-39 can not be differentiated based on our data. Further studies are required to understand the underlying pathways by which BRP-39 modulates inflammatory cell apoptosis/survival, downstream of Fas. Nevertheless, it is easy to conceive how elevated levels of tissue and/or circulating BRP-39 can inhibit inflammatory cell death and augment the accumulation of T cells, alternatively activated macrophages and dendritic cells, thereby regulating the intensity and natural history of Th2-dominated diseases such as asthma. These findings also provide a potential mechanism via which BRP-39 can contribute to the severity and activity of diseases, such as rheumatoid arthritis, diabetes, inflammatory bowel disease, pulmonary fibrosis, sarcoidosis, atherosclerosis and other chronic inflammatory diseases, in which T cells and macrophages are believed to play important pathogenic roles. Asthmatic airways dysfunction used to be considered largely in terms of the contraction of airway smooth muscle (bronchospasm). However, numerous studies have suggested a renewed appreciation of the essential role of inflammation in this disorder. The central roles of Th2 cells needs to be accented because they are now believed to be major contributors to the initiation and maintenance of airway inflammation, regulation of B cell and eosinophil function, and induction of mucus and stimulation of airway remodeling [1,4,13,15]. Previous studies from our laboratory demonstrated that the true chitinase acidic mammalian chitinase is induced by IL-13 and plays an important role in Th2 inflammation. They also demonstrated that acidic mammalian chitinase is expressed in an exaggerated fashion in human asthma [54]. The present studies add to our understanding of the relationships between 18 glycosyl hydrolase family members and Th2 inflammation by demonstrating that BRP-39 is induced during and plays a critical role in the pathogenesis of aeroallergen-induced Th2 responses. These observations suggest that a variety of chitinases and chitinase-like proteins contribute to the pathogenesis of Th2 inflammation. Because BRP-39 is not

a functional chitinase, they also suggest that their contributions in this setting may not be dependent on chitinase enzyme activity. Our demonstration that BRP-39 is a critical regulator of inflammatory cell apoptosis provides a chitinase-independent mechanism that may underlie this effect. This observation is in keeping with other studies that demonstrate the important roles that inflammatory cell (T cell, macrophage and eosinophil) apoptosis plays in the initiation, perpetuation and intensity of Th2 inflammatory responses [16,45]. Additional investigation will be required to determine wether acidic mammalian chitinase regulates inflammatory cell apoptosis and if acidic mammalian chitinase and BRP-39 regulate Th2 inflammation via identical mechanisms.

IL-13 is a product of a gene on chromosome 5 at q31 that is produced by stimulated $CD4^+$ Th2 cells [13,51]. Studies from our laboratory and others have demonstrated that it has a variety of effects that are relevant to asthma and Th2 inflammation including the ability to induce IgE production, eosinophil, lymphocyte and macrophage-rich inflammation, mucus metaplasia, airway remodeling and airways hyperresponsiveness (AHR) on methacholine challenge [14,51]. Exaggerated levels of IL-13 have also been seen in atopic and non-atopic asthma [21,22,30] and polymorphisms in the IL-13 promoter and coding region have been associated with asthma in study populations [19]. As a result of these observations, dysregulated IL-13 production is now felt to be a cornerstone in the pathogenesis of Th2 inflammation and remodeling. IL-13 also plays an important role in the pathogenesis of type II granulomatous responses and the fibrosis in schistosomiasis, idiopathic pulmonary fibrosis, scleroderma and radiation fibrosis [7-9,18,37,48,50]. In keeping with these important findings, a number of investigators have worked to define the mechanisms that IL-13 uses to generate its tissue effects. As a result, it is now known that among a myriad of involved proteins in particular chemokines and chemokine receptors, matrix metalloproteinases, TGF-β_1 and IL-11 contribute to the pathogenesis of pulmonary Th2 inflammation and remodeling [6,29,32,34,53]. The present studies demonstrate that BRP-39 is induced by and plays an important role in the

pathogenesis of IL-13 induced airway inflammation. They also demonstrate that, in the absence of BRP-39, exaggerated levels of T cell and macrophage apoptosis are seen in lungs from IL-13 Tg mice and that BRP-39 induces alternative macrophage activation. This is the first demonstration that a chitinase-like protein contributes to pathogenesis of a Th2 cytokine effector response and alternative macrophage activation. These studies also raise the possibility that the effects of BRP-39 in Th2 responses are mediated by its ability to induce the differentiation and augment the survival of alternatively activated macrophages. This is in keeping with the appreciation that these macrophages are a major source of fibrogenic TGF-β_1 in the IL-13-treated lung (Elias et al. unpublished observation). Further studies are required to understand the complex relationship between BRP-39, IL-13, alternative macrophage activation and TGF-β_1.

7. SUMMARY

In summary, these studies demonstrate, using genetic knock-out and antibody blocking experiments *in vivo*, that the chitinase-like protein BRP-39 is potently and selectively induced in macrophages and epithelial cells in lungs with Th2- and IL-13-induced inflammation, as murine model of human allergic asthma. These studies also demonstrate that BRP-39 plays a critical role in the pathogenesis of aeroallergen-induced Th2 inflammation and IL-13-induced inflammation and remodeling. Lastly, they demonstrate that BRP-39 mediates these effects, at least in part, by inhibiting inflammatory cell apoptosis, inhibiting Fas/CD95 surface expression, regulating myeloid dendritic cell accumulation and activation and inducing the alternative activation of local alveolar macrophages. These studies support BRP-39 as a therapeutic target against which interventions can be directed to control asthma or other Th2 or macrophage-mediated pathologies.

ZUSAMMENFASSUNG

Diese Studien zeigen, dass das Chitinase-ähnliche Protein BRP-39 eine entscheidende Rolle in der Pathogenese einer Th2 Entzündung in der Lunge spielt. Wir fanden, dass BRP-39 von Makrophagen und Epithelzellen in Lungen mit Th2 Inflammation und IL-13-induzierter Inflammation exprimiert wird. Unter Verwendung von genetischen BRP-39 *knock-out* Mäusen fanden wir, dass BRP-39 eine kritische Rolle in der Pathogenese der allergisch induzierten Th2 Inflammation und damit assoziierten pulmonalen Umbauprozessen spielt. BRP-39 vermittelt diesen Effekt über eine Hemmung der zellulären Apoptose, die Regulation von Fas/CD95 Oberflächenexpression, die Regulation der Rekrutierung und Aktivierung pulmonaler myeloider dendritischer Zellen und der Differenzierung lokaler Alveolarmakrophagen zu alternativ-aktivierten Makrophagen. Diese Studie unterstreicht die Rolle von BRP-39 als funktionelle Komponente und als therapeutisches Ziel bei Asthma bronchiale.

8. ATTACHMENT

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seit 02/2007	Nebentätigkeit als journalistische Mitarbeiterin des Thieme Verlages, Kurzreferate für die Zeitschrift Pneumologie
07/2007-02/2009	Forschungsauslandsaufenthalt an der Yale University, Section of Pulmonary and Critical Care Medicine, Department of Internal

Medicine, Leiter Prof. Jack A Elias, M.D., Chair of of Internal Medicine and Chief of the Section of Pulmonary and Critical Care Medicine, Projektbetreuung durch Dr. Dominik Hartl.

Forschungsprojekte an der Yale University:

- The effect of chitinases and chitinase-like proteins on leukocyte function in asthma
- The role of Toll-like receptors in lung disease

Stipendien:

2003/2004	Leistunsstipendium der medizinischen Universität Graz
2008	Auslandsstipendium der Gesellschaft für Pädiatrische Pneumologie
2008	Auslandsstipendium des Landes Steiermark
2008	Auslands-Kongress-Stipendium der Gesellschaft für Pädiatrische Pneumologie

Publikationen:

Hartl D, Latzin P, Hordijk P, Marcos V, Rudolph C, Woischnik M, Krauss-Etschmann S, **Koller B**, Reinhardt D, Roscher A, Roos D, Griese M. *Cleavage of CXCR1 on neutrophils disables bacterial killing in CF lung disease.* **Nature Medicine***;* 2007 Dec;13(12):1423-30. Impact factor 28.8

Koller B, Kappler M, Latzin P, Takyar S, Gaggar A, Kormann A, Lehmann N, Roos D, Griese M, Hartl D. *TLR expression on neutrophils at the pulmonary site of infection – TLR1/TLR2-mediated upregulation of TLR5 expression in cystic fibrosis lung disease*.
Journal of Immunology, 2008 Aug 15;181(4):2753-63. Impact factor 6.8

Koller B, Roos D, Sabroe I, Hartl D. *Toll-like receptors on neutrophils – functionality and involvement in chronic inflammatory diseases*. **J Leuk Biol** (in revision). Impact factor 4.1

Hartl D, **Koller B**, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, Griese M, Krauss-Etschmann S. *Quantitative and functional impairment of pulmonary $CD4^{+}CD25^{hi}$ regulatory T cells in pediatric asthma.* **Journal of Allergy and Clinical Immunology**, 2007 May;119(5):1258-66. Impact factor 7.7

D. Hartl, C. H. He, **B. Koller**, C. A. Da Silva, R. Homer, C. G. Lee, J. Elias. *In vivo characterization of acidic mammalian chitinase in murine lung disease models: Involvement of ADAM17/EGFR, apoptosis and chemokine production.* **J Biol Chem,** 2008. Impact factor 5.5 (in press)

Lee CG, Hartl D, **Koller B**, Chen NY, Matsuura H, da Silva CA, Humbles A, Kearley J, Coyle A, Chupp G, Reed J, Elias JA. *Role of breast regression protein 39 in Th2 and IL-13-induced tissue responses and leukocyte apoptosis*. **The Journal of Experimental Medicine** (in revision)