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Phenotyping of mice with genetic defects relevant to allergic diseases: Proteinase 3/neutrophil elastase double-knockout mice

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1 INTRODUCTION

Exacerbations of obstructive airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) may be associated with an increased number of neutrophilic granulocytes in the airways. Although airway eosinophilic inflammation is recognized as an important feature of chronic stable asthma, recent evidence supports an important role for neutrophils in acute severe asthma (Busse and Sedgwick 1992; Sur, Crotty et al. 1993; Fahy, Kim et al. 1995; Lamblin, Gosset et al. 1998).

Understanding the type of cellular inflammation in acute severe asthma has important implications not only for the treatment of acute severe asthma but also for its prevention.

There are at least three possible physiologic roles for the neutrophil in acute severe asthma. First, neutrophil proteases, especially neutrophil elastase, are important mucin secretagogues for goblet cells and submucosal gland cells (Kim, Nassiri et al. 1989; Sommerhoff, Nadel et al. 1990). Thus, neutrophils may be important mediators of the prominent mucous hypersecretion seen in acute severe asthma. Second, neutrophil products may be important mediators of epithelial cell activation and heightened vascular permeability in acute severe asthma (Persson 1986; Amitani, Wilson et al. 1991). Third, neutrophil proteases can activate eosinophils (Liu, Lazarus et al. 1999).

1.1 Neutrophils

Neutrophils, which are also known as polymorphonuclear leukocytes (PMN), are a major effector cell of innate immunity (Nathan 2006).

They were discovered by Ilya Metchnikoff, who inserted rose thorns into starfish larvae and then described the infiltrating phagocytic cells as macrophages and microphagocytes. The latter cell type was then called neutrophil granulocyte by Paul Ehrlich, who had already encompassed the functional significance of neutrophils in the inflammatory process. About one hundred years ago, in 1908, the Nobel Prize in Medicine was awarded jointly to Metchnikoff and Ehrlich for their work that, as many scientists claim today, founded the basis for modern immunology (Kaufmann 2008).

Neutrophils play a crucial role in the first-line defence against invading bacteria, fungi and protozoa (Faurschou and Borregaard 2003). The neutrophil-mediated inflammatory response can be regarded as a multi-step process involving the initial adhesion of circulating neutrophils to activated vascular endothelium, the subsequent extravasation and migration of neutrophils towards inflammatory foci, and the ultimate in situ elimination of foreign microorganisms through phagocytosis, generation of reactive oxygen metabolites, and release of microbicidal substances. Most of the steps in this process are dependent on the mobilisation of cytoplasmic granules and secretory vesicles.

The various subsets of granules contained within the neutrophil constitute an important reservoir not only of antimicrobial proteins, proteases, and components of the respiratory burst oxidase, but also of a wide range of membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation (Dang, Cross et al. 2001).

It is the controlled mobilisation of these cytoplasmic organelles that permits transformation of the neutrophil from a passively circulating cell to a potent effector cell of innate immunity. Additionally, the regulated exocytosis of granules enables the neutrophil to deliver its arsenal of potentially cytotoxic granule proteins in a targeted manner, thus preventing widespread damage to host tissue in most situations (Faurschou and Borregaard 2003; Herrero-Turrion, Calafat et al. 2008).

The development of neutrophils in the bone marrow takes about two weeks; during this period, they undergo proliferation and differentiation (Zhang, Zhang et al. 1997). During maturation, they pass through six morphological stages: myeloblast, promyeloblast, myelocyte, metamyelocyte, non-segmented (band) neutrophil, segmented neutrophil. The segmented neutrophil is a fully functionally active cell. It contains cytoplasmic granules (primary or azurophil and secondary or specific) and a lobulated chromatin-dense nucleus with no nucleolus. The bone marrow of a normal healthy adult produces more than 10^{11} neutrophils per day and more than 10^{12} per day in settings of acute inflammation. Upon release from the bone marrow to the circulation the cells are in a non-activated state and have a half-life of only 4 to 10 h before marginating and entering tissue pools, where they survive for 1 to 2 days. Cells of the circulating and marginated pools can exchange with each other. Senescent neutrophils are thought to undergo apoptosis (programmed cell death) prior to removal by macrophages (Lum, Bren et al. 2005). The viability is significantly shorter in individuals suffering from infectious or acute inflammatory diseases when the tissue requirement for newly recruited neutrophils increases considerably.

1.2 Neutrophil Granules

Neutrophil granules are formed sequentially during myeloid cell differentiation (Faurschou and Borregaard 2003). Formation of granules (granulopoiesis) is initiated in early promyelocytes, when immature transport vesicles bud off from the Golgi and fuse (Bainton and Farquhar 1966; Bainton, Ullyot et al. 1971). The resultant early-appearing granules were originally defined by their high content of myeloperoxidase (MPO) and consequently named “peroxidase-positive granules”, but they are also referred to as “azurophil granules” due to their affinity for the basic dye azure A (Spicer and Hardin 1969), or simply designated “primary granules”. The production of MPO ceases at the promyelocyte/myelocyte transition. Accordingly, granules formed at later stages of myelopoiesis are peroxidase negative. Peroxidase-negative granules can be subdivided into specific (secondary) and gelatinase (tertiary) granules, based on their time of appearance and content of granule matrix proteins. Specific granules are formed in myelocytes and metamyelocytes and have a high content of lactoferrin and a low content of gelatinase, while gelatinase granules form in band cells and segmented neutrophils and are low in lactoferrin but high in gelatinase (Bainton, Ullyot et al. 1971; Kjeldsen, Bainton et al. 1993; Borregaard, Sehested et al. 1995) Secretory vesicles, like granules, are regulated exocytic vesicles that appear in segmented neurophils (Borregaard, Miller et al. 1987; Cowland and Borregaard 1999). The fact that these vesicles contain plasma proteins suggests that secretory vesicles form by endocytosis (Borregaard, Kjeldsen et al. 1992). All granule subsets share common structural features such as a phospholipid bilayer membrane and an intragranular matrix containing proteins destined for exocytosis or delivery to the phagosome. However, substantial differences exist between the various granule subsets with regard to protein content. Several studies

have shown that proteins synthesised at a given stage of myeloid cell development typically localise to the same granules (Borregaard, Sehested et al. 1995; Sorensen, Arnljots et al. 1997; Arnljots, Sorensen et al. 1998). Furthermore, if the biosynthetic window of a specific granule protein is experimentally changed from the myelocytic to the promyelocytic stage, it will localise to azurophil granules [12]. Together, these findings have given rise and support to the targeting-by timing hypothesis, which states that targeting of proteins into distinct granule subtypes is determined simply by the time of their biosynthesis (Borregaard, Sehested et al. 1995). The extraordinary heterogeneity of neutrophil granules is thus explained by a fine-tuned and highly individually regulated expression of the granule protein genes, mediated by combinations of myeloid transcription factors that, like the granule proteins, are present at specific stages of neutrophil development (Cowland and Borregaard 1999; Bjerregaard, Jurlander et al. 2003). Not all granule proteins are targeted to granules with equal efficiency, and the degree of targeting of a given protein may, furthermore, depend on the stage of neutrophil development at which the protein is formed (Arnljots, Sorensen et al. 1998). The mechanisms responsible for sorting between the regulated and constitutive exocytic pathways in neutrophils are unknown (Borregaard and Cowland 1997).

1.2.1 Neutrophil Serine Proteases

Neutrophils contain and can secrete a variety of potent proteases, including neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3), which are stored in the cytoplasmic, azurophilic granules. Serine proteases are a class of proteases that have a serine residue in the active site of the enzyme. The enzymatic

mechanism of serine proteases is based on the *catalytic triad* consisting of three amino acids in the active center, in which the hydroxyl group of the serine starts the reaction cascade by exerting a nucleophilic attack on the carbonyl carbon of the scissile peptide bond. This finally leads to the cleavage of the substrate polypeptide chain. The specificity of the proteases mainly depends on the amino acid residues neighboring the active center, since these residues govern the recognition and proper alignment of the substrate prior to the hydrolysis of the peptide bond.

PR3 and NE are two closely related enzymes with overlapping and potentially redundant substrate specificities, which are different from that of CG. All three neutrophil serine proteases (NSPs) are implicated in anti-microbial defense by degrading engulfed microorganisms inside phagolysosomes of neutrophils (Belaaouaj, McCarthy et al. 1998; Belaaouaj, Kim et al. 2000; Reeves, Lu et al. 2002; Weinrauch, Drujan et al. 2002; Segal 2005).

The importance of neutrophil proteases lies in their abundance, their effectiveness at reasonably high (i.e. extracellular) pH values and their broad specificity. Among many other functions ascribed to these enzymes, PR3 and NE were also suggested to play a fundamental role in granulocyte development in the bone marrow (Bories, Raynal et al. 1989; Skold, Rosberg et al. 1999; El Ouriaghli, Fujiwara et al. 2003).

While the vast majority of the enzymes is stored intracellularly, minor quantities of PR3 and NE are externalized early during neutrophil activation and remain bound to the cell surface, where they are protected against protease inhibitors (Owen, Campbell et al. 1995; Campbell, Campbell et al. 2000).

These membrane presented proteases were suggested to act as “path clearers” for neutrophil migration by degrading components of the extracellular matrix (Shapiro 2002). This notion has been addressed in a number of studies, which yielded

conflicting results (Carden and Korthuis 1996; Hirche, Atkinson et al. 2004; Young, Thompson et al. 2004). Thus, the role of PR3 and NE in leukocyte extravasation and interstitial migration still remains controversial.

Surprisingly, all three NSPs present in azurophilic granules have been reported as targets of autoantibodies in certain pathological situations. While the occurrence of autoantibodies against NE and CG appears to be a concomitant phenomenon of diseases such as cocaine-induced midline lesions (Wiesner, Russell et al. 2004) and ulcerative colitis (Halbwachs-Mecarelli, Nusbaum et al. 1992), respectively, the development of anti-PR3 autoantibodies represents a characteristic feature for a systemic, autoimmune vasculitis called Wegener´s granulomatosis (Jenne, Tschopp et al. 1990). Interestingly, myeloperoxidase, which is stored together with the NSPs in the azurophilic granula, is the principal autoantigen of a related autotimmune vasculitis called microscopic polyangiitis.

The potential role of neutrophil proteases in the pulmonary tissue destruction of emphysema is well known and is argued to result from disruption of a protease-antiprotease balance (Snider 1989). The contribution of proteolytic effects to the pathogenesis of asthma is much less clear, whether the enzymes come from netrophils or any other inflammatory cell.

Extracellular NE was detected by immunohistochemistry in two of four asthmatic airways in a study carried out by Fujisawa whereas eosinophil proteins were detected in all four airways (Fujisawa, Kephart et al. 1990). The same concerns about localization and levels and detection approaches, apply as much to neutrophil proteases as the neutrophil themselves. However, airway hyperreactivity in an animal model (sheep) was blocked by elastase inhibitors (O'Riordan, Otero et al.

1997). Consequently the presence of these enzymes in asthma, and even more their possible contribution, must remain enigmatic.

1.2.1.1 Neutrophil Elastase

NE is a potent serine proteinase with a primary translational product of 267 amino acids and variable glycosylation, migrating with a molecular mass of 28 to 31 kd (Sinha, Watorek et al. 1987). Mature NE, a highly cationic glycoprotein, is stored in an active form in primary granules at high concentration (~ 4 µg/10^6 cells), making it a major component of neutrophils (Liou and Campbell 1995). NE is synthesized in the promyelocytic stage of myeloid development and stored in large quantities in its active form in neutrophil azurophil granules (Takahashi, Nukiwa et al. 1988). Catalytic activity is conferred by the His-Asp-Ser triad which forms a charge relay system, resulting in a powerful nucleophile capable of attacking peptide bond substrates. NE has potent catalytic activity against a broad array of extracellular matrix substrates, including the highly resistant elastin that imparts structural stability to the lung (Shapiro, Goldstein et al. 2003). Neutrophil elastase is a potent secretagogue for both airway epithelial cells (Breuer, Christensen et al. 1987) and submucosal gland cells (Nadel 1991).

Human NE has been localized to chromosome 19 and mouse NE to chromosome 10 (Jenne 1994). The gene for mouse NE is composed of 5 exons and 4 introns, similar to the human (Belaaouaj, Walsh et al. 1997).

NE is responsible for the blistering in bullous pemphigoid, an autoimmune skin diseas characterized by subepidermal blisters and autoantibodies against hemidesmosome-associated proteins (Liu, Shapiro et al. 2000).

Also, most patients with neutropenia also have NE mutations, results suggesting that mutations of the gene encoding NE cause both cyclic and severe congenital neutropenia (Dale, Person et al. 2000).

1.2.1.2 Proteinase 3

PR3 is an -26,000-dalton neutral serine proteinase produced during myeloid differentiation and stored in the azurophil granule of polymorphonuclear leukocytes (Baggiolini, Bretz et al. 1978; Sturrock, Franklin et al. 1992). These granules are specialized lysosomes that also contain other proteinases and antimicrobial agents. Their contents may be released outside of the cell as part of the host defence mechanism. PR3 is major constituent of the azurophil granules, present in amounts comparable to those of human leukocyte elastase (Gabay, Scott et al. 1989).

The PR3 gene contains five exons and four introns, which is similar to several other mammalian serine proteinases. PR3 gene is on human chromosome 19 and on mouse chromosome 10 (Sturrock, Franklin et al. 1992; Belaaouaj, Moog-Lutz et al. 1999).

PR3 has been associated with a number of human diseases. It is the target antigen of the cytoplasmic pattern of antineutrophil cytoplasmic autoantibodies (c-ANCA) detected in the circulation of Wegener's granulomatosis (WG) patients (Ludemann, Utecht et al. 1990; Korkmaz, Kuhl et al. 2008).

These circulating anti-PR3 antibodies increase prior to vascular inflammation in WG patients, and monitoring c-ANCA levels is an important part of both diagnosis and treatment of Wegener's disease (Jennette and Falk 1991). The cause of PR3 autoantibody formation is unknown. PR3 also is involved in growth and

differentiation of leukaemia cells (Bories, Raynal et al. 1989), and animal models have indicated a role for PR3 in emphysema (Kao, Wehner et al. 1988).

1.2.1.3 Cathepsin G

CG is a neutral serine protease that is expressed and synthesized at the promyelocyte stage of development and is packaged in the azurophil granules. CG has been proposed to play a role in neutrophil responses against a variety of bacteria (MacIvor, Shapiro et al. 1999).

CG has a number of potential substrates and activities that are difficult to classify, including the conversion of angiotensin I to angiotensin II (Klickstein, Kaempfer et al. 1982), the activation and damage of cultured airway epithelial cells (Nahori, Renesto et al. 1992), the stimulation of secretion by airway gland serous cells (Sommerhoff, Nadel et al. 1990), the induction of transendothelial albumin flux (MacIvor, Shapiro et al. 1999), and the processing of NF-kB in vitro (MacIvor, Shapiro et al. 1999). It is not yet clear that any of these activities represent physiologic roles of this enzyme. Finally, CG has been proposed to play an important role in tissue remodeling at sites of wounding or tissue injury (Sabri, Alcott et al. 2003; Rafiq, Hanscom et al. 2008).

1.3 Asthma

1.3.1 Definition of Asthma

The guideline of Global Strategy for Asthma Management and Prevention by Global Initiative for Asthma (GINA) (NHLBI 2002) states that asthma is chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (NHLBI 2002). The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli (Bousquet, Jeffery et al. 2000).

Asthma is not a simple condition but a heterogeneous collection of clinical phenotypes (Harris, Nystad et al. 1998). It comprises a spectrum of diseases ranging from paroxysms of coughing, wheezing and dyspnoea occurring periodically and with symptom-free periods to severe persistent asthma where symptoms are continuously present (Anderson and Morrison 1998). Allergic asthma is characterized by infiltration of bronchial mucosa with eosinophils and T helper (Th) 2-Type cells, circulating specific immunoglobulin (Ig) E antibodies and positive skin test reactions to common aeroallergens, together with hyperresponssiveness, defined as an increased sensitivity to bronchoconstrictors such as histamine or cholinergic agonists (Humbert, Menz et al. 1999; Johansson, Hourihane et al. 2001; Renauld 2001). In contrast non-allergic asthmatics are negative in skin test, have no

allergy history and the serum total IgE concentration is in the normal range (Humbert, Menz et al. 1999).

1.3.2 Pathogenesis of Asthma

The course of allergic asthma involves the development of allergen-specific response to inhalants or other allergens, a process normally starting during childhood and in subset of individuals resulting in the development of Th2-polarized immunological memory which increase the risk of allergic respiratory disease (Prescott, Macaubas et al. 1999). In asthma pulmonary inflammation is characterized by mucosal edema, epithelial damage, increased neuronal responsiveness, bronchoalveolar eosinophilia, increased mucus secretion, and decreased mucociliary clearance (Fahy, Corry et al. 2000). The inflammatory infiltrate in both allergic and non-allergic asthma consist of mast cells, eosinophils, macrophages, lymphocytes, neutrophils and plasma cells (Anderson and Morrison 1998; Hamid, Tulic et al. 2003). Inflammatory cytokine production in airway wall in the absence of inflammatory stimulation is relatively low. However in asthmatics, eosinophils and other inflammatory cells as well as structural cells (airway epithelial cells, fibroblasts, endothelial cells and smooth muscle cells) produce a wide range of cytokines and mediators, most probably as part of tissue's response to chronic inflammation (Barnes 1998; Smart and Kemp 2002).

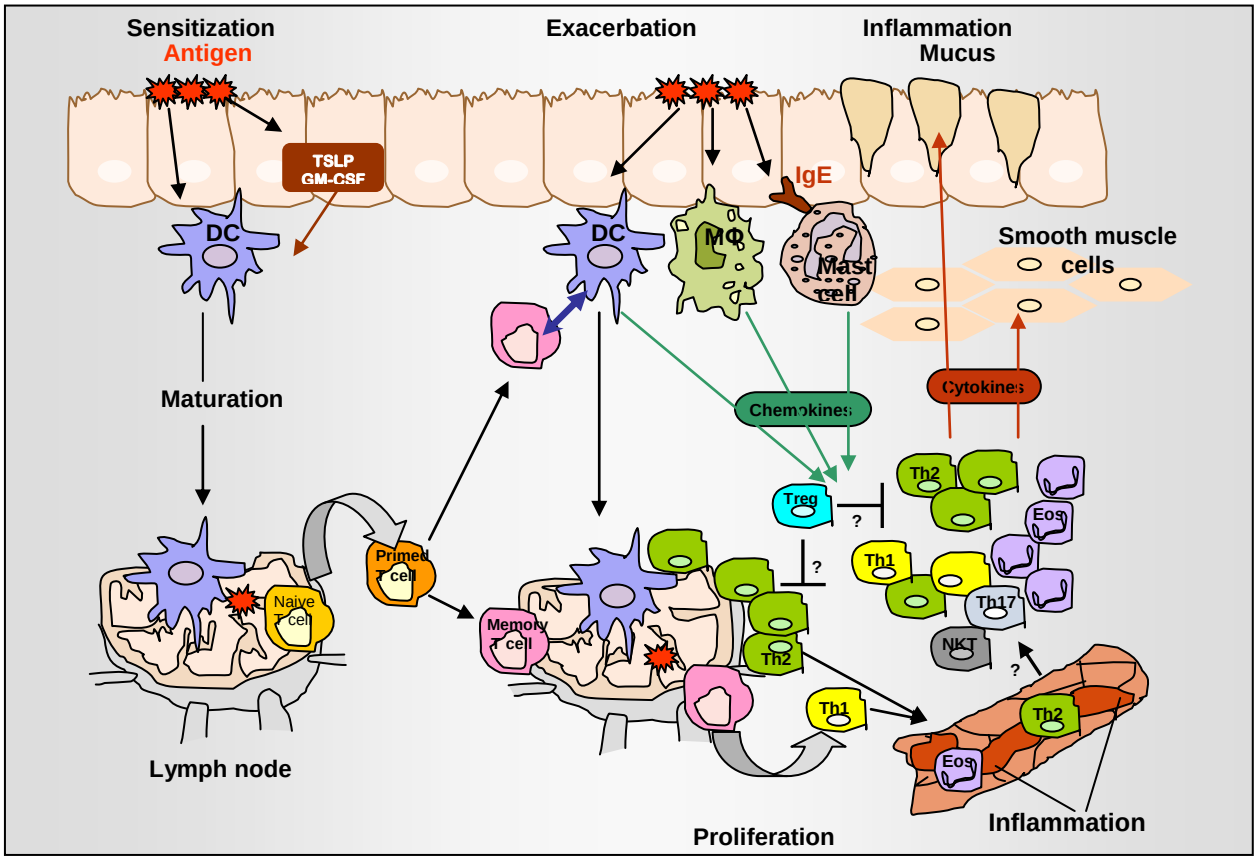


Figure 1. Asthma pathogenesis. (*a*) Sensitization to an allergen results from uptake by airway dendritic cells (DCs), maturation of the DCs, migration to lymphoid tissue, and antigen presentation to T cells. Maturation of the DCs requires secondary stimuli generated from epithelial cells following antigen exposure. These primed T cells then reenter the lung, where they provide surveillance for the allergen. (*b*) Exacerbation of allergic airway inflammation occurs when there is reexposure to allergen and uptake by airway DCs with presentation of antigen to airway-associated T cells as well as T cells in lymphoid tissue. T cells in the lymph node then proliferate and home to the lung, where they amplify the airway inflammation. (*c*) The full allergic airway phenotype results from cytokine production from T cells as well as from inflammatory mediators released from recruited eosinophils and other cells in the lung. This results in mucus hypersecretion, smooth muscle cell hyperreactivity, and airway remodeling with chronic inflammation. (PMN, polymorphonuclear cell; Eos, eosinophil; M, macrophage; Treg, T regulatory cell; NKT, natural killer cell) (Medoff, Thomas et al. 2008).

1.3.3 Neutrophils in Asthma

It is now generally agreed that inflammatory cell responses likely play a major pathogenic role in asthma. The role of neutrophils in asthma is less clear than of

eosinophils (Wark, Gibson et al. 2000). Most of the emphasis has been on the effects of locally accumulated eosinophils while neutrophils have been considered to be either not significantly increased or not pathogenically important. However, recently investigations have suggested that neutrophils are playing a greater pathogenic role than previously considered. Comparing bronchial lavage from eight mechanically-ventilated patients with status asthmaticus, other asthmatics and other mechanically-ventilated patients, increased levels of neutrophils and NE as well as eosinophils in the status asthmaticus lavages has been shown, pointing to a possible role for neutrophils as well as eosinophils (Lamblin, Gosset et al. 1998).

High levels of NE in the sputum of patients with asthma and chronic bronchitis have also been found, raising the possibility that neutrophil effects might play a role in airway remodeling in asthma as well as in chronic bronchitis (Vignola, Bonanno et al. 1998).

Neutrophils appear early and prominently in sites of developing late phase IgE-mediated reactions following allergen challenge (Zweiman, Moskovitz et al. 1998). However, the significance of this accumulation is unclear since biopsy and lavage studies in chronic mild to moderate asthma have not shown impressively increased numbers of neutrophils or deposition of neutrophil elastase (Adcock, Ford et al. 2008). Neutrophil prominence in the airways of fatal asthma suggests that neutrophils play a pathogenic role in asthma, at least in its more severe presentations (O'Byrne P and Postma 1999).

Bronchial asthma even in its mild forms, is characterized by local infiltration and activation of inflammatory and immunoeffector cells, including T lymphocytes, macrophages, eosinophils and mast cells (Busse and Sedgwick 1992; Fahy, Kim et

al. 1995; Frangova, Sacco et al. 1996). The overall hypothesis is that, at least in allergic asthma, the eosinophil accumulation is controlled by T-cell and mast cell products, and that eosinophil-mediated damage results in increased bronchial hyperresponsiveness (Busse and Sedgwick 1992; Sur, Crotty et al. 1993). In addition to eosinophils, bronchial or bronchoalveolar neutrophils have been described by different investigators in asthmatic patients but the pathogenetic role of neutrophils in asthma is still poorly understood (Kim, Nassiri et al. 1989; Sommerhoff, Nadel et al. 1990).

1.3.4 Animal Models of Allergic Asthma

The use of animals for research and teaching has now become an issue of interest in the world. New techniques and methods to produce knockout, knockin, and transgenic animals provide researchers with ways to more efficiently study human diseases and the therapeutics that hold promise for those diseases (Kertz 1996).

The use of genetically engineered animal models to explore the function of genes and for the selection of appropriate drug targets holds great promise in speeding the development of valuable therapies. These models provide effective ways to test new drug compounds, as well as aid in our understanding of specific disease processes. Decisions in favour of one or the other animal model are often guided by logistical and technical issues like cost factors, required housing conditions and animal handling, duration of generation times, or number of offspring. On the other side, it is important to choose experimental models where results can be successfully extrapolated to human physiology and diseases. Depending on the field of research, different organisms turned out to be appropriate. The principal functional and

pathogenic characteristics of allergic asthma have been observed in a number of animal models. The following species have been used: mouse, [13] rat, [14] guinea pig, [15] rabbit, [16] ferret, [17] dog, [18] cat, [19] sheep, [20] pig, [21] some primates, [22] and even horse [23]. Some of these species develop allergic asthma spontaneously, while in others it is experimentally induced. Of the latter cases, in addition to the mouse, the most commonly used experimental models have been the guinea pig, the sheep, and the monkey. The model of guinea pig sensitized to ovalbumin is characterized, as in human asthma, by an early acute phase and a later chronic phase following contact with the allergen, the existence of eosinophilic lung inflammation, and by bronchial hyperreactivity [15, 24]. This was the most frequently used model up until the first models were described in mouse, and has been largely used to evaluate the therapeutic interest of various molecules [15]. However, it does not offer the genetic versatility of the mouse, nor does it offer as many species-specific reagents for the identification of molecular and cellular changes associated with pathogenesis [25]. The sheep model of asthma is also characterized by an early acute phase followed by a later chronic phase, both accompanied by bronchial hyperreactivity [26]. In addition, from an anatomical and functional perspective, the lungs of the sheep under normal conditions are very similar to those of humans [20, 27, and 28]. Although initially animals were studied that were spontaneously sensitized to *Ascaris suum*, [32] there now exists an induced model, in which the animals are sensitized to dust mites, that reproduces practically all of the clinical and pathogenic characteristics of human asthma [22]. In spite of the scientific interest, the cost of working with sheep and primates, the length of the experiments, the difficulty of the manipulations, the lack of reagents developed for the evaluation of molecular expression, and the stricter control of their use in the

laboratory compared with other species means that these models are rarely used due to their poor scientific exploitability. For reasons that are fundamentally scientific, but also economic and based on ease of use (housing, manipulation, availability of species-specific reagents), the animal that is nowadays universally used as a model of allergic asthma is the mouse (Torres, Picado et al. 2005). Compared with other species, another advantage of the mouse is its ability to be manipulated genetically (transgenics and knockouts) and immunologically (mice with spontaneous or induced immunodeficiencies) and also the comparative analysis between mouse and man revealed genomic sequence homology of approximately 40%, even up to 90% within the protein-coding regions, indicating that the obvious differences between both species are mainly not based on the DNA sequenced level, but on species-specific regulation of gene expression, different splice variants or protein modification after transcription (Waterston, Lindblad-Toh et al. 2002).

Although the best approach to investigate asthma processes, and to identify crucial pathways and potential novel targets for drug therapy, is to perform studies in human asthmatics, there are constraints that can be avoided when using animal models. First, ethical issues limit the study of allergen sensitization in humans. Second, there are few clinical indications for bronchoalveolar lavage (BAL) and lung biopsy in asthmatics, thus restricting access to lesions and reducing our capacity to study disease pathogenesis. Third, testing novel treatments in humans is difficult due to the tough regulations that need to be met before a drug is accepted for clinical phase trials (Epstein 2004). In 1994, the first mouse models resembling allergic asthma were published (Brusselle, Kips et al. 1994; Gavett, Chen et al. 1994; Kung, Jones et al. 1994; Lukacs, Strieter et al. 1994) and have since resulted in significant strides in our understanding of the pathophysiology of allergic disease. Because asthma is a

complex multifactorial disease, it is unlikely that a single animal model of asthma that replicates all of the morphological and functional features of the chronic human disease will ever be developed. However, animals to model specific features of the disease can be used and much of the current understanding of disease processes in asthma, and in particular the response to allergens, comes from studies in laboratory animals such as guinea pigs, rats and mice. The mouse is the most widely used species, mainly because of the availability of transgenic animals and because of the wide array of specific reagents that are available for analysis of the cellular and mediator response (Nials and Uddin 2008).

1.3.5 Mouse Phenotyping

Model organisms, especially the mouse, have proven to be important tools to learn more about gene functions. There are many mouse mutant lines (MMLs) available, but a major part of them lacks complete phenotypic characterization. Due to pleiotropic effects, one and the same gene may have different functions in different organ systems. Thus, mouse mutant lines have to be systematically and comprehensively phenotyped in a standardized way to exploit the knowledge, which otherwise might remain uncovered. In order to overcome the bottleneck of standardized, comprehensive phenotyping in mouse genetics, several research centers around the world have been established like the German Mouse Clinic, Munich, Germany (http://www.mouseclinic.de).

The German Mouse Clinic (GMC) contributes the phenotyping of MMLs as an open access platform for the scientific community. In collaboration with different teams, which consists of experts from various fields of mouse physiology and pathology,

examinations in the following areas can be performed: allergy, behavior, clinical chemistry, bone and cartilage, energy metabolism, eye development and vision, host-pathogen interactions, cardiovascular analyses, steroid metabolism, immunology, lung function, molecular phenotyping, neurology, nociception, and pathology (Gailus-Durner, Fuchs et al. 2005).

The goal of the Allergy screen within the German Mouse Clinic is to search for IgE mutants in order to establish mouse models for allergic diseases and to find new strategies for antiallergic therapy. The increased production of IgE in response to common environmental antigens is the hallmark of atopic diseases in man (Hamelmann, Tadeda et al. 1999). Mouse mutants with phenotypic alterations in IgE production represent a valuable tool to study and characterize the molecular mechanisms of IgE-mediated allergic hypersensitivity (Zhang, Lamm et al. 1997).

PR3/NE $^{-/-}$ mice and their control littermates in 129/SvEv background were also screened in primary screening in German Mouse Clinic.

2 AIM OF THE WORK

There is increasing evidence that neutrophils may play a role in acute severe asthma (Jatakanon, Uasuf et al. 1999). Neutrophils contain, release, synthesize and secrete materials that have the potential to injure the cells and structural elements of the airways. Although up to now in numerous studies airway eosinophilic inflammation is recognized as an important feature of chronic stable asthma, recent evidences support an important role for neutrophils in acute severe asthma (O'Donnell and Frew 2002). Understanding the role of neutrophils and its proteases has important implication not only for the treatment of asthma but also for its prevention (Kawabata, Hagio et al. 2002).

In this study the role of neutrophil serine proteases in an animal model of asthma was investigated. The primary phenotyping of Proteinase 3 (PR3)/ Neutrophil elastase (NE) deficient mice was performed in GMC.

Then a more detailed allergic phenotyping characterization for PR3/NE double knockout mice was performed in both, 129/SvEv and BALB/c background.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

The reagents, buffers and instruments used in the experimental procedures of this thesis are shown in tables 1-6.

Reagent	Company
Albumin from Chicken Egg White Grade V	Sigma-Aldrich, USA
Sheep Anti-Mouse-IgE (monoclonal)	The Binding site, England
Purified Mouse IgE (Clone IgE-3)	Pharmingen, USA
Biotin-Conjugated Rat Anti-Mouse IgE (monoclonal)	Pharmingen, USA
Biotin-Conjugated Rat Anti-Mouse IgG2a	Pharmingen, USA
Biotin-Conjugated Rat Anti-Mouse IgG1	Pharmingen, USA
Anti-Chicken Egg Ovalbumin Clone OVA 14	Sigma-Aldrich, USA
Block-Buffer Albumin Bovine, Fraction V	Sigma-Aldrich, USA
Streptavidin-Peroxidase	Calbiochem, USA
TMB (Tetramethylbenzidin)	Fluka, Switzerland
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich, USA
Carbonate-Bicarbonate Buffer Capsules	Sigma-Aldrich, USA
Citric Acid Monohydrate	Sigma-Aldrich, USA

Trisma-Base	Sigma-Aldrich, USA
Microtiter-Plate NUNC-Maxisorp U-Form	NUNC GmbH, Germany
Polyoxyethylene-Sorbit Monolaurate (Tween-20)	Sigma-Aldrich, USA
H_2O_2	
Stop-Solution, 2 M Sulphoric Acid	Merck, Germany
HCl	Roth, Germany
Diethanolamin	Sigma, Germany
Tris-hydrochloride (Tris-Hcl)	Roth, Germany

Table 1. Reagents for ELISA and FACS analysis

Buffer	Composition
FACS Staining Buffer	1x PBS 0.5%(w/v) BSA 0.02% (w/v) NaN_3
Coating Buffer	1x PBS 0.02%(w/v) NaN_3
Blocking Buffer	1x PBS 0.5% (w/v) Tween20 1% (w/v) BSA 0.02%(w/v) NaN_3
Wash Buffer	1x PBS 0.5% (w/v) Tween20 0.02%(w/v) NaN_3
Substrate Buffer	1x Aqua double distillated 0.1% (w/v) Diethanolamin 0.2% (w/v) $Mgcl_2$ 0.02%(w/v) NaN_3
Lysis Buffer	0.3M Tris 0.17M NH_4Cl

Table 2. Buffers for ELISA and FACS analysis

All buffers used for FACS staining were filtered using a Stericup 0.22 µm vacuum filtering system, in order to avoid blocking of the FACS analyser.

Reagent	Company
Ketamin 10%	Essex Pharma GmbH, Germany
Rompun	Bayer HealthCare, Germany
Amoniumchlorid (NH_4Cl)	Sigma, Germany
Ether	Merck, Germany
Phosphate Buffer Salt Solution (PBS)	BiochromAG, Germany

Table 3. Reagents for Bronchoalveolar Lavage (BAL)

Reagent	Company
Diff-Quik set	Medion Diagnostics, Germany
Giemsa	Merck, Germany
Entellan	Merck, Germany
Formaldehyde	Sigma, Germany

Table 4. Reagents for Histology

Reagent	Company/Composition
100 bp Size Marker	Invitrogen, Germany
DNeasy Tissue Kit	Qiagen, Germany
Gel Loading Solution	Sigma-Aldrich, USA
Agarose Gel	Sigma-Aldrich, USA
HotStarTaq Master Mix Kit	Qiagen, Germany
Electrophoresis Buffer Tris-Acetate-EDTA (TAE)	242 g Tris 57.1 ml Acetic acid 100 ml 0.5 M EDTA, pH 8.0 Add H2O to 1 litre

Table 5. Reagents for PCR

Instrument	Company
Microtiter-Plate washer Wellwash AC	Thermo Electron Corporation, USA
Microtiter-Plate Reader, Multiskan Ascent	Labsystems, USA
Microtiter-Plate Photometer, MRX	Dynatech Laboratories, Denkendorf
Microtiter-Plate Shaker, Micromix	DPC Biermann GmbH, Bad Nauheim
Cytocentrifuge, Cytospin 3, Shandon	Thermo Electron Corporation, USA
PCR System	Applied Biosystem, USA
Gel Documentation System	Bio-Rad, USA
PH Meter, Inolab	VWR International GmbH, Germany
Electronic Balance, PB303 DeltaRange	Mettler Toledo GmbH, Germany
Leight Microscope, DMLB	Leica, USA
Microlitre Centrifuge, Biofuge	DJB Labcare, England
Centrifuge, Megafuge	DJB Labcare, England
Incubator, Hareaeus	VWR International GmbH, Germany
Sterilizer	Thermo Electron Corporation, USA
Plate Shacker	Titramax 101, Heidolph Instruments, Schwabach, Germany
Mixer, REAX 2	Heidolph Instruments, Germany
Multicolor Flow Cytometry	FACS Calibur, Becton Dickinson, Mountain View, CA
FlowJo software	TreeStar Inc, USA
Vacuum Filtering System	Milipore Corporation, Bedford, USA
PARI-Boy Nebulizer	PARI, Starnberg, Germany
Luminex 100	Bio-Rad, USA
Whole-body Plethysmography System	Buxco Electronics, Sharon, CT, USA

Table 6. Instruments

3.1.2 Animals

Simultaneous deficiency of the PR3/NE gene cluster in 129/SvEv mice was generated by homologous recombination in embryonic stem cells by Dr. Dieter Jenne at the Max-Planck-Institute for Neurobiology, Dept. of Neuroimmunology (Pfister, Ollert et al. 2004). PR3/NE heterozygous offspring from 129/SvEv background were crossed into another different genetic background (BALB/c) to generate an inbred and outcross line. Genotyping of offspring was performed by Polymerase Chain Reaction (PCR).

Prtn3 and Ela2 are both mapped to murine chromosome 10 within a 3 kb region (Jenne, Tschopp et al. 1990) . Because of this close proximity and the functional redundancy regarding co-expression and substrate specificity of the two genes, a double-knock-out approach was chosen.

The animals have normal lifespan and normal reproduction. They were housed in pathogen-free animal facility of Helmholtz Zentrum München (Neuherberg, Germany) with food and water *ad libitum*. All the experiments were conducted under federal guidelines for the use and care of the laboratory animals and were approved by the local government (Regierung von Oberbayern) and the Animal Care and Use Committee of the Helmholtz Zentrum München.

3.2 Methods

3.2.1 Gene Characterization of Proteinase 3/Neutrophil Elastase Deficient and Control Mice

To distinguish PR3/NE $^{-/-}$ mice from WT animals, genomic DNA was analysed.

Genotyping of offspring was performed by PCR using primers DJ186 (5' CTCGGCCTTATGTGGCATCC; exon 2 PR3) and DJ187 (5' CCGGCATAGGAAGGTGACCA-3'; exon 4 PR3 reverse) for the wild-type allele and the primer pair DJ218 (5'-AGCTCCCATGCTGTGTTTC-3'; Intron 1 PR3) and DJ104 (5'-TCGGTCTTTGGGATGGGTAAG-3', exon 5 NE reverse) for the recombinant allele.

3.2.2 Polymerase Chain Reaction (PCR)

The PCR was performed using a thermocycler (Applied Biosystem). All PCR reactions were started with a pre-incubation step termed “Hot Start”, which denatures the template DNA at 94-100°C so that the primers can anneal after cooling. The second step, otherwise referred to as “touchdown”, allows the oligonucleotide primers to anneal to the denatured template by lowering the temperature to 37-65°C depending on the annealing temperature of the primers.

The reaction proceeds with the extension, or elongation of the primers at 72°C.

The duration of the extension steps can be increased if longer templates are being amplified. Usually, the elongation time of the final cycle is longer (up to 10 minutes) to ensure that all product molecules are fully extended. Steps 1-3 constitute one cycle of the PCR. The whole PCR reaction is usually carried out in 25-35 cycles.

Higher cycle numbers may result in an increase of unwanted artifacts, while no increase in the desired product is achieved.

DNA was diluted 1:5 in H_2Odd. Each DNA sample was amplified two times, once for Knock-out Loci and then for WT Loci. This approach allowed the clear identification of homozygote and heterozygote mice. All reactions were subjected to 35 cycles of amplification, using Taq-DNA-Polymerase and buffers of the company Qiagen, according to manufacturer's data.

Amplification of WT-Allel

Denaturierung: 94°C 1min

Annealing: Primer DJ104, DJ218, 58°C 1min

Elongation: 72°C 1min 30s

Amplification of Knockout-Allel

Denaturierung: 94°C 1min

Annealing: Primer DJ186, DJ187, 60°C 1min

Elongation: 72°C 1min 45s

3.2.3 Gel Electrophoresis

Agarose gel electrophoresis enables the user to monitor PCR procedure. The percentage of agarose in the gel varies. In this work, 0.5% agarose was used.

Ethidium bromide (EtBr) (end concentration: 1 µg/ml) was included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. The gels were then submerged in electrophoresis buffer (TAE) in a horizontal electrophoresis apparatus. After the samples were mixed with gel loading dye and loaded into the

sample wells, the electrophoresis was initiated by applying 100 mV for 30-45 minutes at RT. Size marker was co-electrophoresed with DNA samples for fragment size determination. After electrophoresis, the gel was placed on a UV light box and the fluorescent ethidium bromide-stained DNA pictured using the Gel Documentation System, Bio-Rad, USA.

3.2.4 Sensitization

The genetic background has a striking and selective impact on induction of allergic pulmonary asthma. As the reliability of the sensitization protocol has a crucial role in successful phenotyping of mouse models, and as there are numerous protocols for different mouse strains, we tried to establish a suitable sensitization protocol for our mouse models in which we could analyse the mice for allergic parameters.

We compared different concentration and frequency application of antigen in induction of allergic responses in both 129/SvEv and BALB/c background.

Although many different sensitisation and challenge protocols have been used, the basic model is consistent. Sensitisation protocols usually require multiple systemic administration of the allergen in the presence of an adjuvant.

Ovalbumin (OVA) derived from chicken egg is a frequently used allergen and the most commonly used model antigen that induces a robust, allergic pulmonary inflammation in laboratory rodents (Kips, Anderson et al. 2003).

Adjuvants such as alum [aluminium hydroxide ($AlOH_3$)] are known to promote the development of the Th2 phenotype by the immune system when it is exposed to an antigen (Brewer, Conacher et al. 1999). Adjuvant-free protocols have also been

described (Blyth, Pedrick et al. 1996), but these usually require a greater number of exposures to achieve sustainable sensitisation.

The 129/SvEv and BALB/c mice in our study were sensitized by an intraperitoneal injection of OVA and alum dissolved in PBS on the first day. The animals received a booster injection of alum-OVA mixture on day 14. The mice were exposed to aerosolized OVA (1%) dissolved in saline in different time points (Figure 2 and Figure 3).

24 h after the last OVA aerosol challenge the mice were sacrificed and blood collection and bronchoalveolar lavage (BAL) was performed.

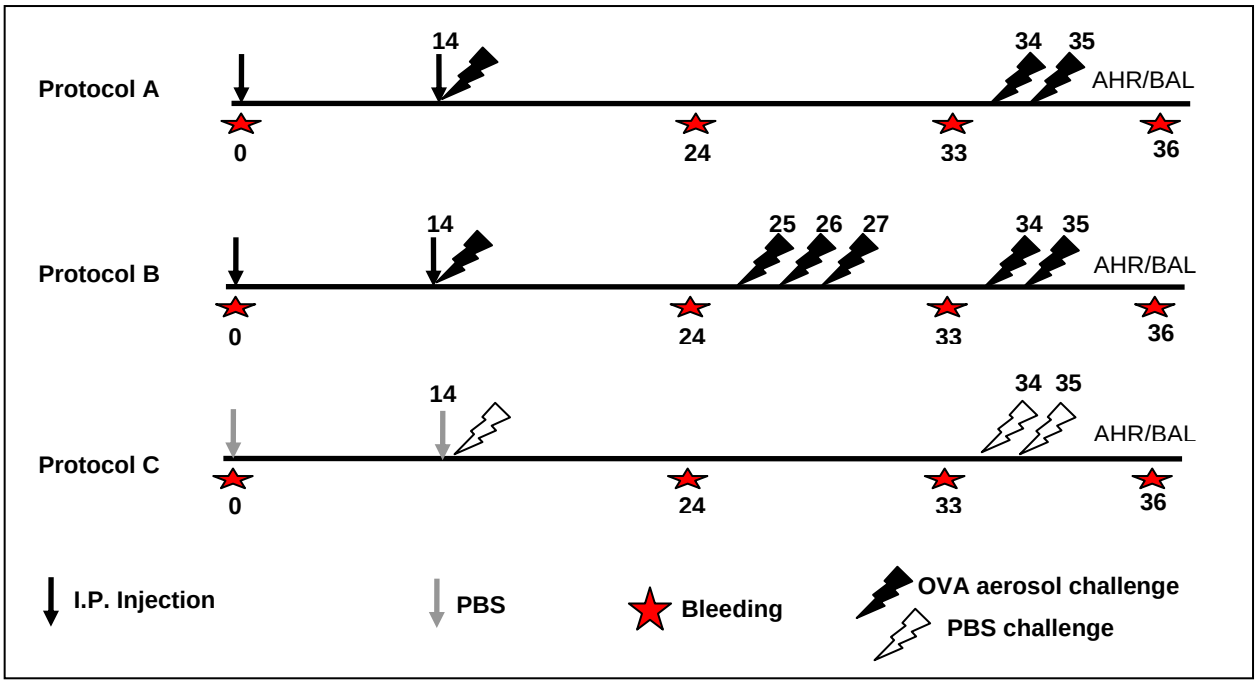


Figure 2. Experimental protocols in 129/SvEv animals. Mice in the experimental protocols A and B were given OVA complexed with alum and in protocol C PBS alone, by the intraperitoneal route (I.P.) on the days indicated. The animals in protocols A and B received OVA aerosol exposure (1%) and in protocol C, PBS aerosol exposure on the days mentioned. Days of blood sampling are indicated as well as AHR/BAL.

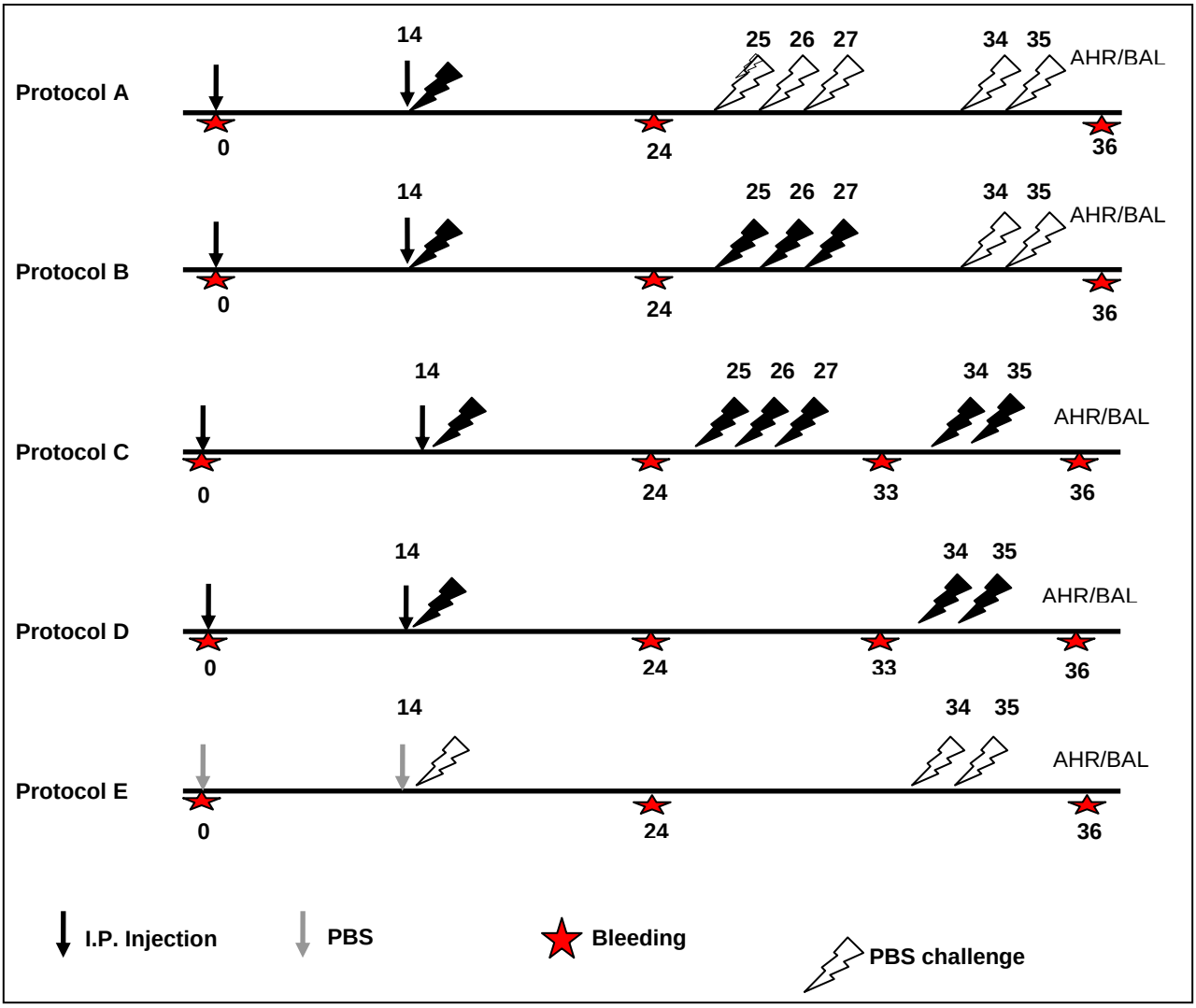


Figure 3. Experimental protocols in BALB/c animals. Mice in the experimental protocols A, B, C and D were given two doses of ovalbumin complexed with alum and in protocol E, PBS alone, by the intraperitoneal route (I.P.) on the days indicated. The animals in protocols A, B, C and D received OVA aerosol exposure (1%) and in protocol E, PBS aerosol exposure on the days mentioned. Days of blood sampling are indicated as well as AHR/BAL.

Based on studies on different sensitization protocols on 129/SvEv and BALB/c background in our group, we used the following protocols to achieve a clear outbreak of the disease.

8 weeks old 129/SvEv mice were sensitized by intraperitoneal injection of 10 µg OVA and BALB/c mice with 20 µg OVA, both emulsified in 2 mg of alum on days 0 and 14 in 200 µl PBS. Mice were challenged on days 14, 34 and 35 by inhalation exposure to aerosols of OVA (1% OVA in saline for 30 min using Pari-Boy nebulizer)

(Figure 4 and Figure 5). One day after the last OVA-aerosol challenge, the animals were sacrificed by cervical dislocation. Control animals were injected with equal volumes of PBS and were exposed to PBS-aerosol.

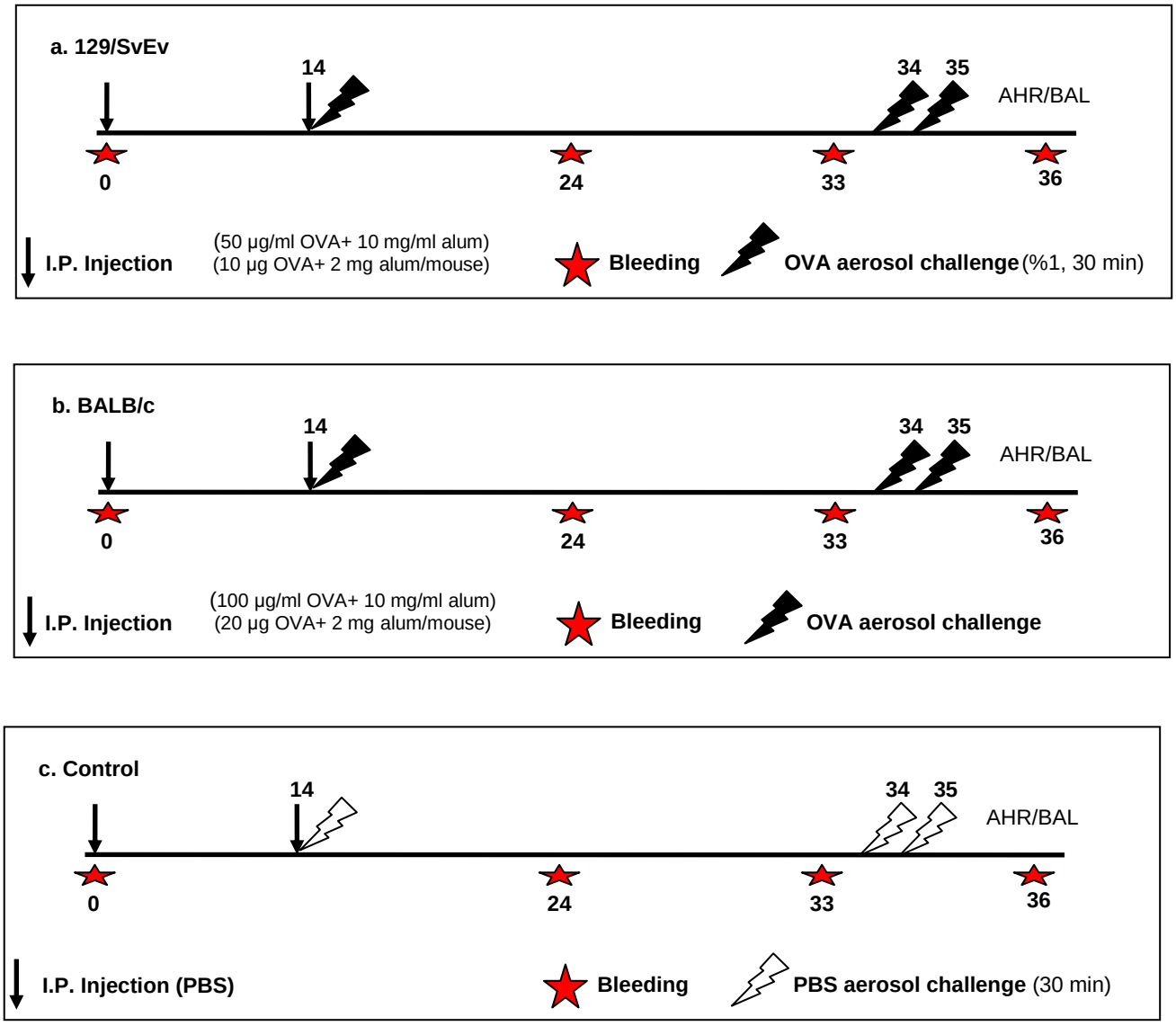


Figure 4. Scheme of sensitization protocols. Briefly, a and b. (OVA sensitized animals) Two I.P. injections of OVA-alum (10 µg) (20 µg) in 129/SvEv and BALB/c mice, respectively on day 0 and 14. OVA aerosol challenge at days 14, 34 and 35 (1% OVA, 30 min). c. (Controls) two I.P. injections of PBS and PBS aerosol challenge on the same days as the first protocol.

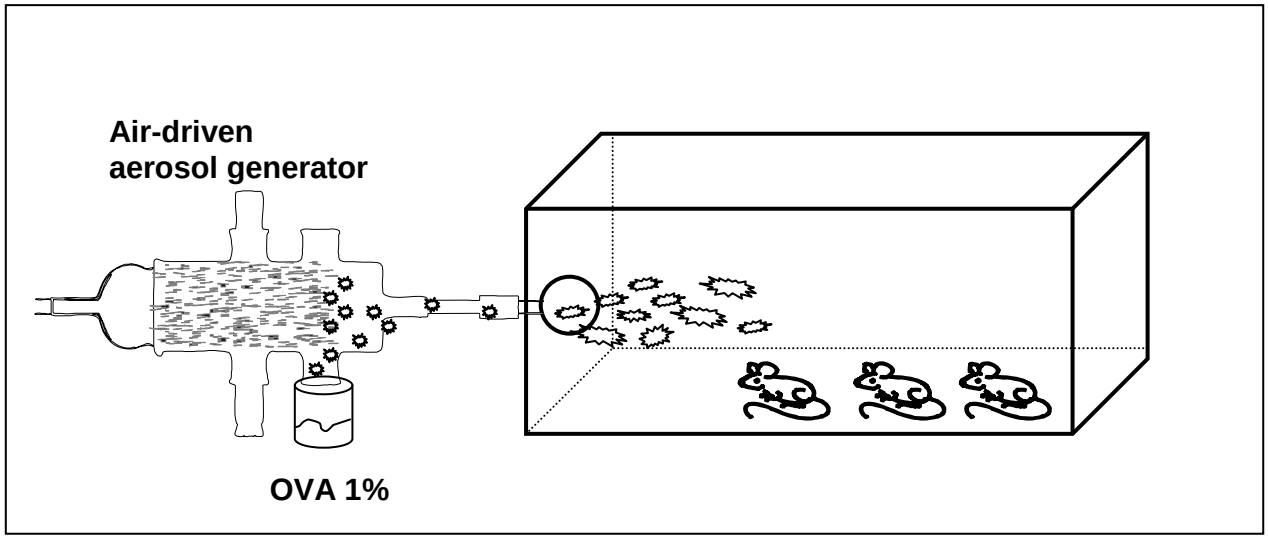


Fig 5. OVA aerosol challenge. The mice were exposed to 1% aerosolized OVA dissolved in PBS for 30 min.

3.2.5 Bronchoalveolar Lavage

The bronchoalveolar lavage (BAL) is a widely used technique developed in order to evaluate inflammatory and immune processes in the human lung. This technique has been also employed in screening different animal models of lung disease and has been shown to be very useful for evaluating allergic phenotypes. The BAL allows the quantification and differentiation of both the cellular infiltration in the airways and various inflammatory markers in the cell-free Bronchoalveolar lavage fluid (BALF), i.e. the status of the allergic airway disease. TH1/TH2 phenotype in the BALF can be easily evaluated using commercially available bioassays for cytokine detection. Furthermore, an increase in protein concentration, caused by an enhanced permeability of the bronchoalveolar-capillary barrier, can be easily measured in BALF (Coomassie Protein assay). Lastly, lung histology can be helpful to evaluate the perivascular and peribronchiolar cellular infiltrate, which in the allergic phenotype is characterized mainly by eosinophils and lymphocytes, in

addition to epithelial mucus-cell hyperplasia and eventually the structural changes seen in airway remodeling.

- After euthaniziation and exsanguinations, the mouse's chest is opened and the trachea is cannulated.
- BAL is performed five times with 1 ml PBS instilled in the lungs and harvested gently.
- Lavage fluid is collected, centrifuged at 600 rpm for 10 min, supernatant is stored at -20˚C for further evaluation.
- Cells are resuspended in 1 ml PBS. After quantification of total cell viability and yield, differential count of BAL cells is made on slides prepared by centrifugation of samples at 600 rpm for 10 min. These slides are fixed and stained with Diff-Quick and a total of 300 cells in each sample are counted by microscopy. Macrophages, neutrophils, lymphocytes and eosinophils are enumerated (Figure 6).

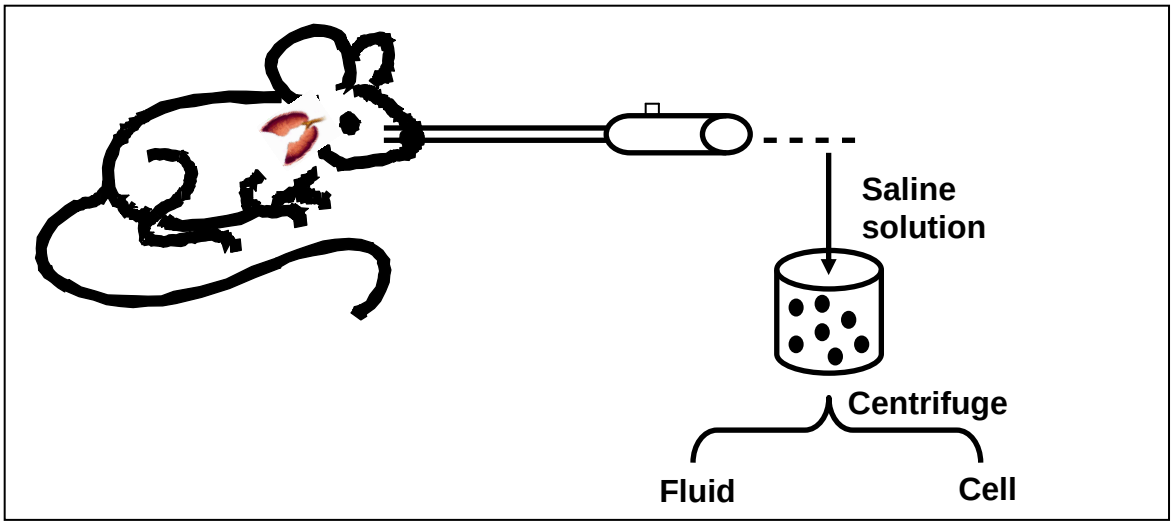


Figure 6. Bronchoalveolar lavage. BAL fluid was collected by rinsing 1 ml of PBS through the trachea into the lungs and recovering it. Cells in the lavage fluid were resuspended and a small aliquot was counted using a hemocytometer.

3.2.6 Bodyplethysmography

The Airway hyperresponsiveness (AHR) of freely moving conscious mice to aerosolized methacholine (MCh) was measured just before BAL by whole-body plethysmography (Buxco) (Figure 7). Readings were obtained at baseline and after exposure to aerosolized saline or MCh (10-40 mg/ml). Results are expressed as percentage increase of the enhanced pause (Penh) following challenge with each concentration of MCh, where baseline Penh (after saline challenge) was expressed as 100%.

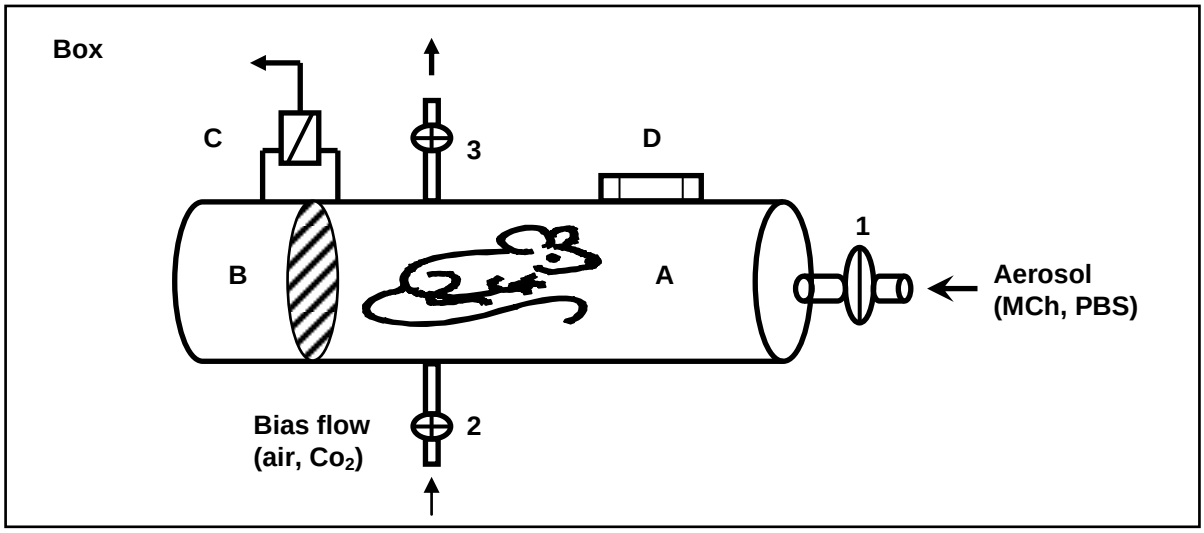


Figure 7. Schematic diagram of the whole-body plethysmograph. (A) Main chamber containing the mouse. (B) Reference chamber. (C) Pressure transducer connected to analyzer. (D) Pneumotachograph. (1) Main inlet for aerosol closed by valve. (2) Inlet for bias flow with four-way stopcock; (3) Outlet for aerosol with four-way stopcock.

3.2.7 Coomassie Protein Assay

The protein concentration was determined by using a coomassie protein assay reagent kit (Pierce), which is a colorimetric method for total protein quantification. When coomassie dye bindes the protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant colour change from brown to blue (Figure 8).

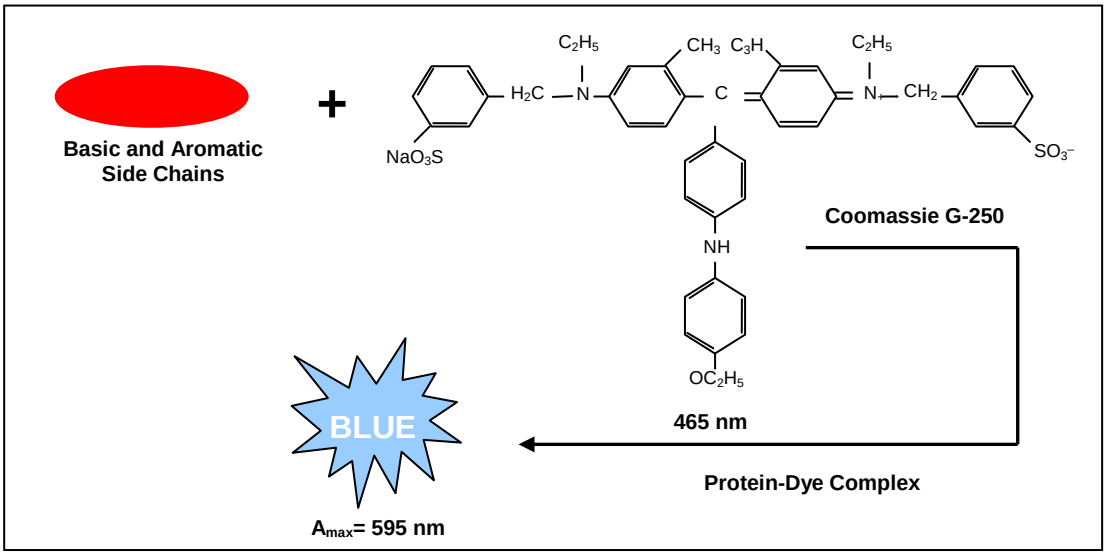


Figure 8. Reaction schematic for the Coomassie Protein Assay

Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions, which are assayed alongside the unknown samples.

3.2.8 ELISA Procedure

An isotype-specific sandwich ELISA technique with a lower detection limit of 1 ng/ml was intended for the quantitative determination of total IgE, OVA specific IgE, IgG1 and IgG2a in plasma. Briefly, microtiter plates were coated with 10 µg/ml of IgG fraction of sheep anti-mouse IgE (The Binding Site) to detect total IgE, or with 1 mg/ml OVA to detect OVA specific IgE, IgG1 and IgG2a in carbonate-bicarbonate buffer (Sigma). After incubation plates were washed with Tris buffer (pH 7.4) (Sigma) and blocked with 3% (w/v) bovine serum albumin (Sigma) at room temperature. Diluted plasma samples and standard were added to the plates. After overnight incubation biotinylated rat anti-mouse IgE (clone R35-118, Pharmingen), rat anti-mouse IgG1 (clone A85-1, Pharmingen) and rat anti-mouse IgG2a (clone R19-15, Pharmingen) were used following by incubation with peroxidase-labeled streptavidin (Calbiochem) and tetramethylbenzidine (TMB) (Fluka). Plates were read in a standard microplate reader at a wavelength of 450 nm. A standard curve for each assay was obtained using biotin-conjugated IgE, and results were expressed as ng/ml of specific IgE of plasma relative to the standard curve.

3.2.9 Flow Cytometric Analysis of Bronchoalveolar Lavage Fluid

To measure the cellular composition of the lungs following allergen challenge, we did in addition to Diff- Quick, BAL flow cytometry. 8, 24 and 48 hours after the last aerosol challenge, mice were sacrificed by cervical dislocation. BAL was performed with 3 ml of $Ca2^{+-}$ and $Mg2^{+-}$ free PBS supplemented with 0.1 mM EDTA. For erythrocyte lysis, cell pellets were resuspended in 200 µl NH4CL-Tris (RT) and incubated for 10 min at RT on a plate shaker (van Rijt, Prins et al. 2002). Half of the

cells were used for cytospins and the remaining BALF cells were stained in a 96-well U-bottom plate.

Plates were centrifuged (530 RCF, 3 min, 10°C) and supernatants were discarded by vigorous tipping over. Cells were centrifuged as described above, and pellets were checked for proper lysis. If the lysis was not complete, this step was repeated. Then the cells were washed in 200 µl FACS buffer for 30 seconds on the plate shaker. After subsequent centrifugation and discarding the supernatant, unspecific binding of the staining Abs via Fc-receptors was blocked by pre-incubation with 50 µl Fc-block Ab solution (anti CD16/CD32).

In addition, life/death discrimination was initiated by adding ethidium monoazide (EMA) (1:1000 diluted in FACS buffer), which binds stable to DNA of dead cells after photo-cross linking through light exposure (40 W of approximately 50 cm distance) for 20 min (O'Brien and Bolton 1995).

Cells were subsequently washed by addition of 150 µl FACS buffer per well, centrifuged and the pellets were resuspended in 50 µl antibody staining mixes (as outlined in Table 7).

The following main cell populations were analyzed: B cells ($CD19^+$ clone 1D3), B1 B cells ($CD19^+$CD5+, clone 53-7.3), T cells ($CD3^+$, clone 145-2C11), $CD4^+$ T cells (clone RM4-5), $CD8^+$ T cells (CD8α, clone 53-6.7), γ/δ T cells (clone GL3) and granulocytes (Gr-1^+, clone RB6-8C5). We also analyzed additional subpopulations based on the following surface antigens: B cells (B220 clone RA3-6B2), macrophages (CD11b clone M1/70), lymphocytes (CD103 clone 2E7), T regulatory cells (CD25 clone PC61), leukocytes (CD62L clone MEL-14) and granulocytes (Ly-6G/Ly-6C clone RB6-8C5).

After 20 min incubation on ice in the dark, cells were washed twice with 150 µl

FACS buffer and then fixed in 100 µl 1% Paraformaldehyde solution (PFA) for 30 min on ice in the dark. Finally, cells were pelleted, washed twice in 200 µl FACS buffer, resuspended in 100 µl PBS, and stored at 4°C in the dark.

Data were acquired on a FACS Calibur (Becton Dickinson, San Diego, USA) and were analyzed using FlowJo software (TreeStar Inc, USA). All samples were acquired until a total number of 25,000 cells were reached.

Antibody	Clone	Cells	Supplier
CCR3	83101	Eosinophils	R&D systems
B220	RA3-6B2	B cells	BD PharMingen
CD3	17A3	T cells	BD Pharmingen
CD4	H129.19	T cells	BD PharMingen
CD8α	53-6.7	T cells	BD PharMingen
CD11b	M1/70.15.11.5HL	Macrophages	BD Pharmingen
CD19	1D3	B cells	CALTAG
CD25	PC61.5	T regulatory cells	BD PharMingen
CD62L	MEL-14	Leukocytes	BD PharMingen
CD103	2E7	Lymphocytes	BD PharMingen
Gr-1	RB6-8C5	Granulocytes	BD PharMingen
Ly-6G/Ly-6C	RB6-8C5	Granulocyte	BD PharMingen
TCRγδ	GL3	T cells	BD PharMingen

Table 7. List of antibodies used in FACS staining. CD3 and CD11b Abs were conjugated to Cy5 fluorescence dye, using the CyTM5 mAb Labelling Kit from Amersham Biosciences according to the manufacture's recommendations.

3.2.10 Peritoneal Inflammation and Lavage

Alum is used as a potent adjuvant to induce T helper type 2 (Th2)-mediated inflammation in murine asthma models (Eisenbarth, Colegio et al. 2008). Interaperitoneal injection of alum is frequently used in mice to induce cellular Th2 immunity and humoral immunity to adsorbed protein Ags (Kool, Petrilli et al. 2008).

Here cell accumulation and cytokine production were assessed in the peritoneal cavity of mice following antigen challenge. Leukocyte migration into the peritoneal cavity was induced by I.P. administration of 1 ml OVA and alum. Control mice received PBS. After 8 h, animals were killed by cervical displacement, the peritoneal cavity was opened and washed with 3 ml of Hanks' balanced salt solution (HBSS) containing heparin (10 U/ml); approximately 90% of the initial volume was recovered. Total cell count present in the peritoneal lavage fluid (PLF) was determined in a haemocytometer. Differential cell counts were performed after cytocentrifugation and staining with May-Grünwald-Giemsa under oil immersion (×1000). At least 300 cells were counted and results were expressed as the number of cells per millilitre of PLF. For cytometry analysis cells were collected 8 h after OVA challenge, washed twice in PBS containing 3% fetal calf serum (FCS) and 0.1% sodium azide and subsequently stained, according to the standard method. Stained cells were resuspended in PBS containing 3% FCS and 0.1% sodium azide. Cell samples were analysed on a FACScan Flow Cytometer in the same way as those from BAL.

3.2.11 Bioplex Cytokine Assay

The levels of different cytokines in the blood plasma were measured after OVA sensitization. In principle, the Bio-Plex (Bio-Rad, Munich, Germany) works similar to a standard sandwich ELISA, in which the analyte is first captured by a surface-coupled mAb. In the next step, the amount of captured substance is determined by a second, fluorescence-conjugated mAb, and analysis is performed by flow cytometry. Discrimination of the measured parameters is obtained by different bead sizes and/or colour (Figure 9). The big advantage of this bead-based assay over ELISA is that it allows simultaneous detection of several substances/cytokines in the same sample. Here the Bio-Plex Mouse Cytokine Th1/Th2 Panel, 8-Plex, 1x 96-well GM-CSF Panel with the Bio-Plex Suspension Array System (Bio-Rad) for IFN-gamma, IL-2, IL-4, IL-5, IL-10, TNF-alpha, IL-12 (p70) (Bio-Rad, Munich, Germany) was used. The 8-Plex Assay was acquired on a Luminex 100.

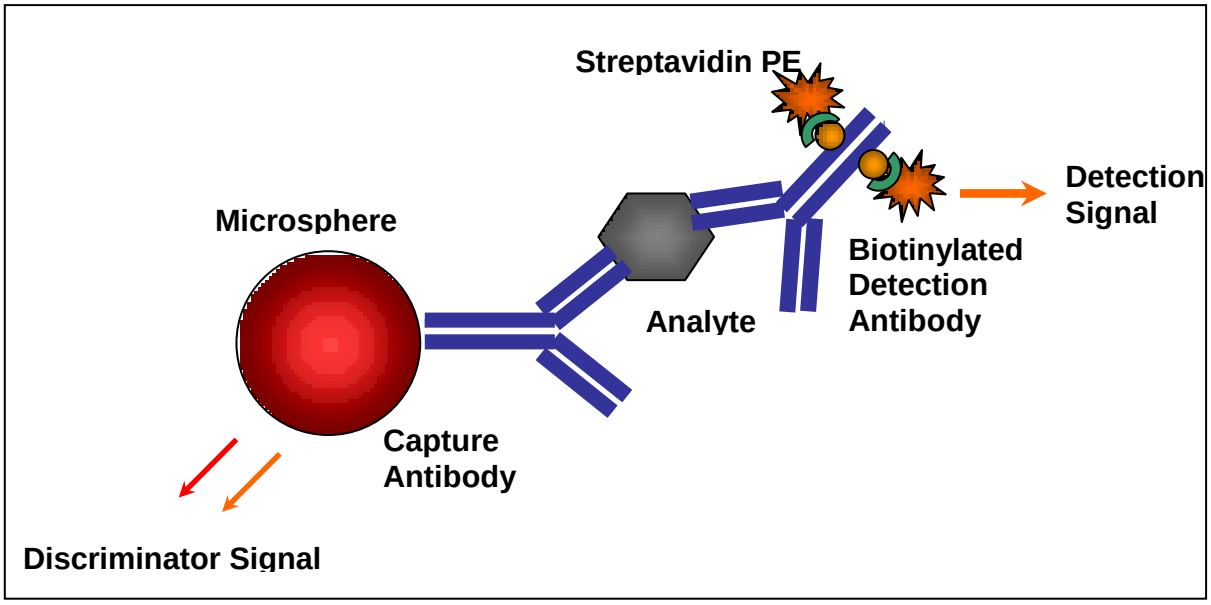


Figure 9. Bioplex cytokine assay

3.2.12 Histology

Lungs obtained from separate animals are instilled with 4% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy are stained with standard hematoxylin and eosin (HE). Stained sections were analyzed by light microscopy with the indicated magnifications.

3.2.13 Statistics

Values of all measurements are expressed as the mean ± standard error of means (S.E.M). Student's t-test was used to determine the levels of difference between all groups. Statistical analysis was performed with Microsoft Excel for Windows Office XP. A P-value of less than 0.05 was considered statistically significant.

4 RESULTS

4.1 Characterization of Proteinase 3/Neutrophil Elastase Double Deficient Mice

The PR3/NE double-deficient mouse line was established by targeted gene disruption in embryonic stem cells as shown in Figure 10.

Previous characterization experiments proved the positive recombination of the PR3/NE locus by Southern blotting of embryonic stem cell clones (Fröhlich, Ph.D. thesis): PR3/NE depleted mice showed no expression of mRNA for PR3 and NE in bone marrow cells as assessed by RT-PCR.

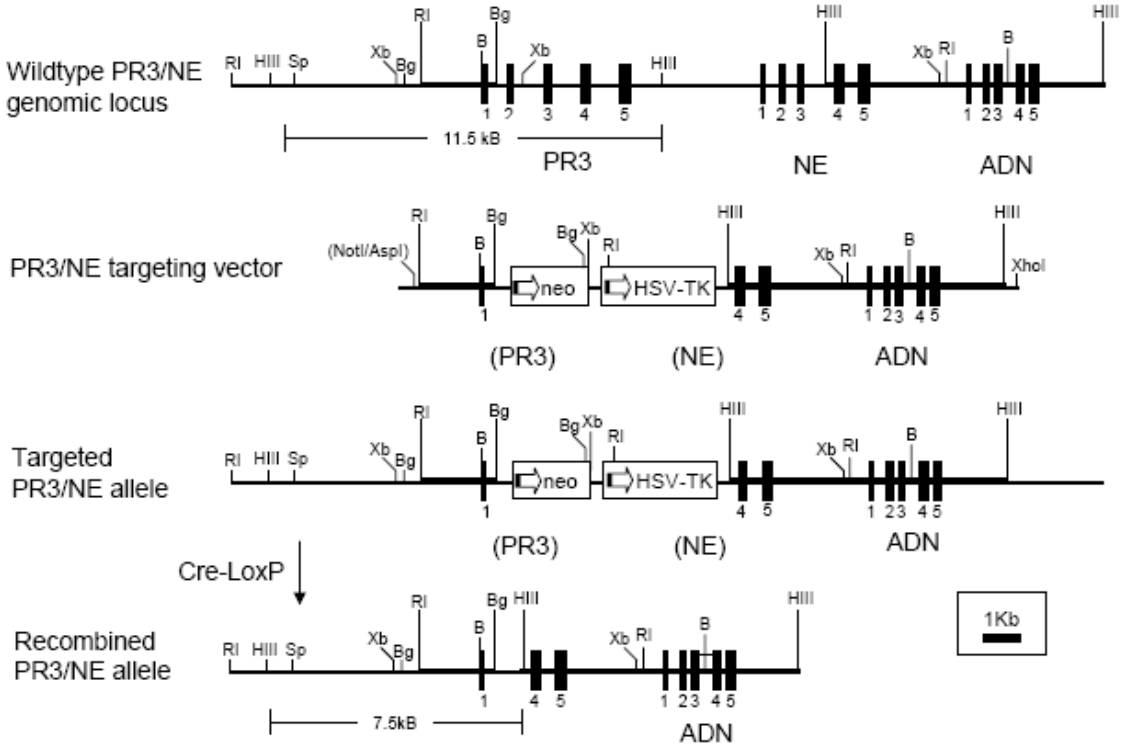


Figure 10. Generation of PR3/NE knockout mice. Schematic presentation of the wildtype PR3/NE locus, gene-targeting vector, targeted allele and the correctly recombined PR3/NE knockout allele. Gene-targeting results in deletion of exons 2-5 of the PR3 gene as well as exons 1-3 of the NE gene leaving the neighboured ADN gene unchanged.

Figure adapted from Kai Kessenbrock, Ph.D thesis (2009), LMU München, Department of Biology

4.2 Neutrophil Shape and Number in Proteinase 3/Neutrophil Elastase Deficient and Control Mice

In order to characterize the phenotype of the mutant allele in another inbred strain, we outcrossed the heterozygous PR3/NE knockouts with 129/SvEv background to BALB/c inbred mice. This resulted in the regular offspring of wild-type, heterozygous and homozygous genotypes of this gene according to the Mendelian ratio.

The investigation of PR3/NE deficient mice with BALB/c background didn't reveal profound changes comparing to their control littermates, so after preliminary measurements, we didn't plan further experiments for these mice.

Despite the absence of two abundant serine proteases and in contrast to expectations from previous reports (Bories, Raynal et al. 1989; Skold, Rosberg et al. 1999; El Ouriaghli, Fujiwara et al. 2003), we found unchanged neutrophil morphology (Figure 11) and regular neutrophil populations in the peripheral blood of the mutant 129/SvEv mice using flow cytometry to determine the differentiation markers CD11b and Gr-1 (Hestdal, Ruscetti et al. 1991; Fleming, Fleming et al. 1993) (Figure 12).

Hence, the proteases are not crucially involved in granulopoiesis and ablating PR3 and NE in the germ line represents a valid approach to assess their biological significance in vivo.

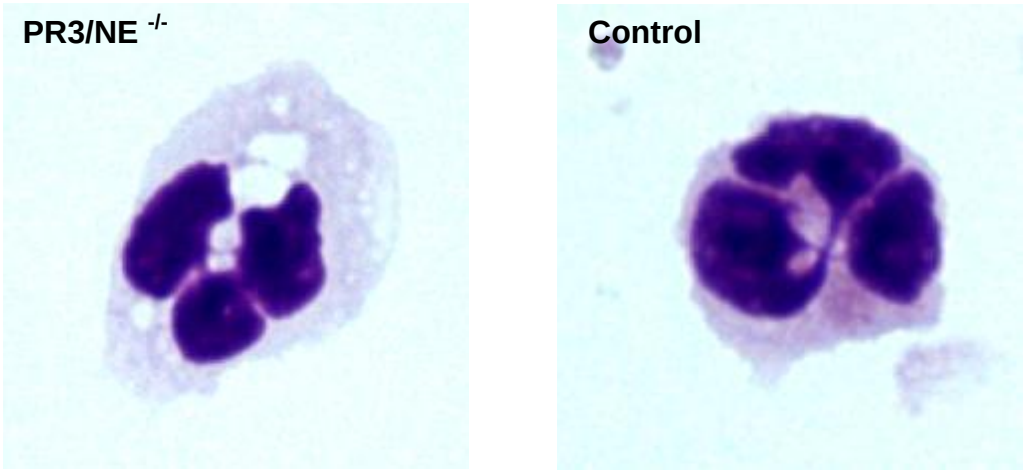


Figure 11. Microscopic analysis of neutrophils in 129/SvEv mice. Analysis of Giemsa-stained blood smears revealed normal granulocyte morphology in PR3/NE $^{-/-}$ mice displaying polymorphic nucleus (dark blue) identical to control neutrophils.

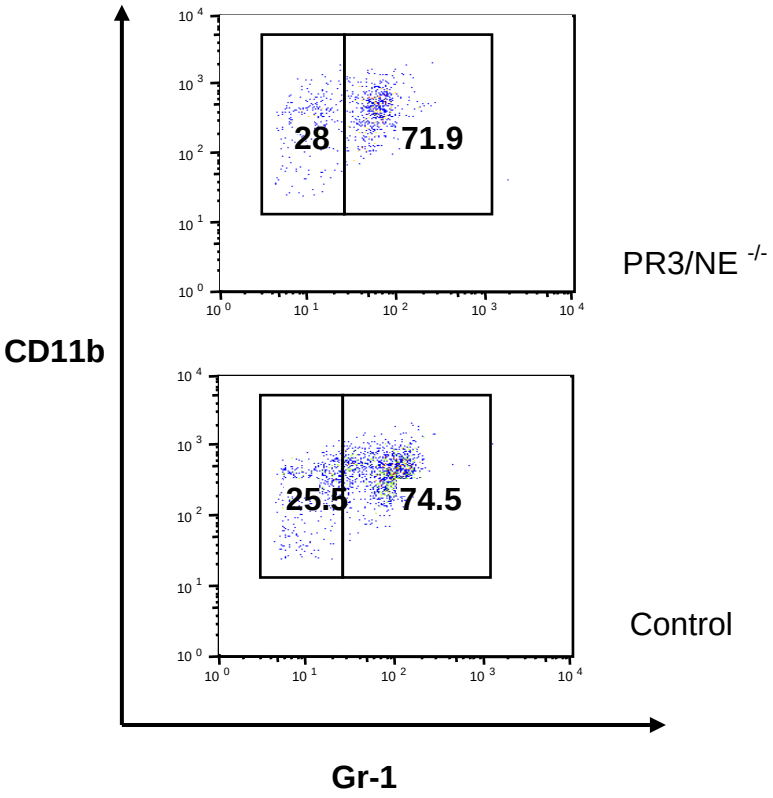


Figure 12. Representative dot plots of PR3/NE $^{-/-}$ and control littermates in 129/SvEv mice. Comparing the population of neutrophils in peripheral blood of PR3/NE $^{-/-}$ to their controls using CD11b and Gr-1 markers.

4.3 Effect of Different OVA Sensitization Protocols on Induction of Specific Ig Production in Proteinase 3/Neutrophil Elastase Deficient Mice

We studied different sensitization protocols on 129/SvEv and BALB/c background to find the most efficient protocol in modulating the physiologic, inflammatory and immunologic features characteristic of allergen-induced airway disease.

We evaluated the efficiency of the protocols frequently used in the literature. Total IgE, OVA-specific plasma IgG1 and IgG2a levels, the number of total cells after OVA aerosol challenge and percentage of different cells in BALF were measured to compare humoral immune responses in different sensitization protocols in both murine backgrounds (Figure 13, Figure 14, Figure 15 and Figure 16).

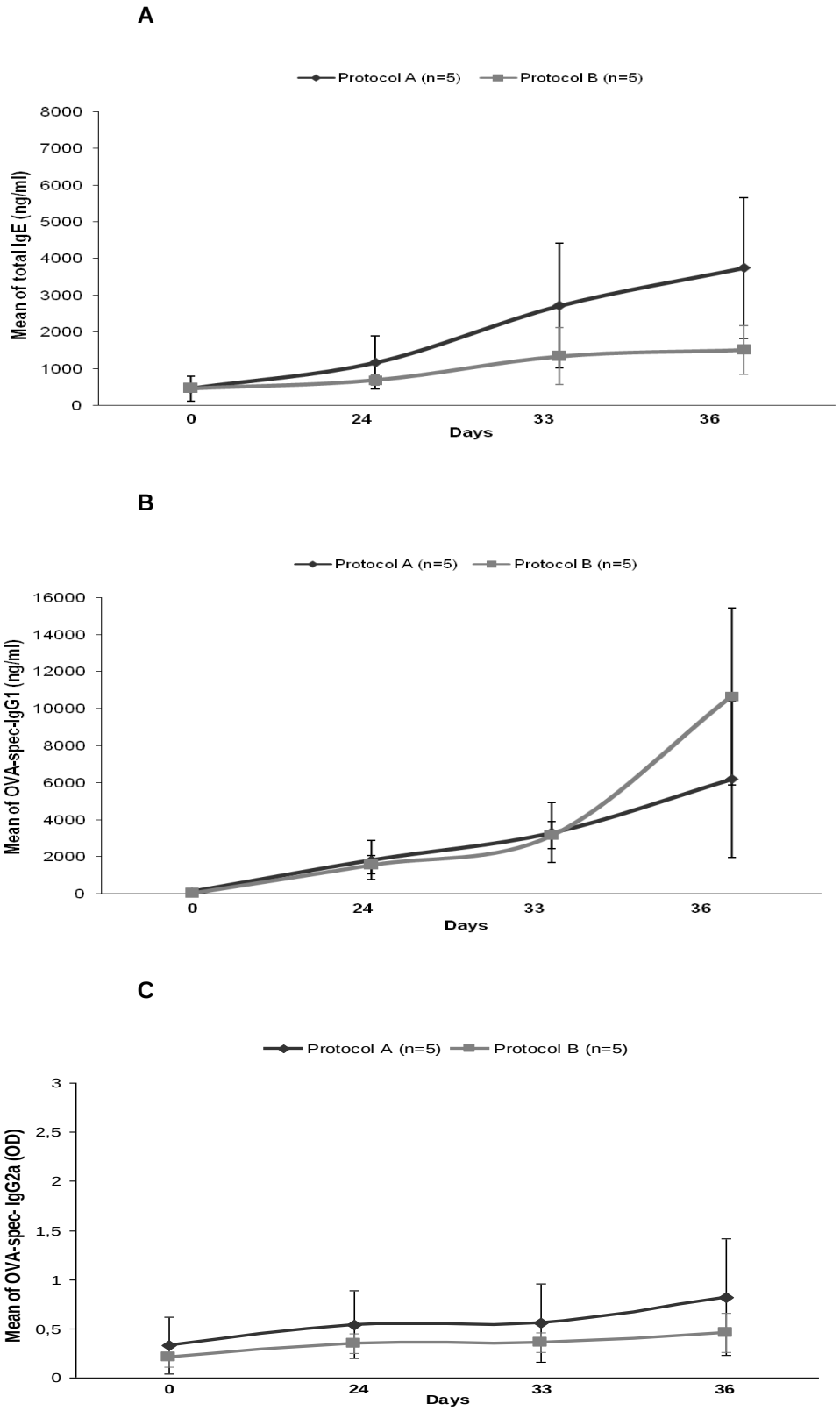


Figure 13. Time course of (A) Total IgE, (B) OVA-specific IgG1 and (C) OVA-specific IgG2a in 129/SvEv mice using 2 different sensitization protocols. Mice were sensitized with OVA-alum mixture on days 0 and 14 and challenged on different days. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 2). Total IgE, OVA-specific IgG1 and OVA-specific IgG2a were measured by ELISA. Error bars indicate the standard error of mean antibody titres.

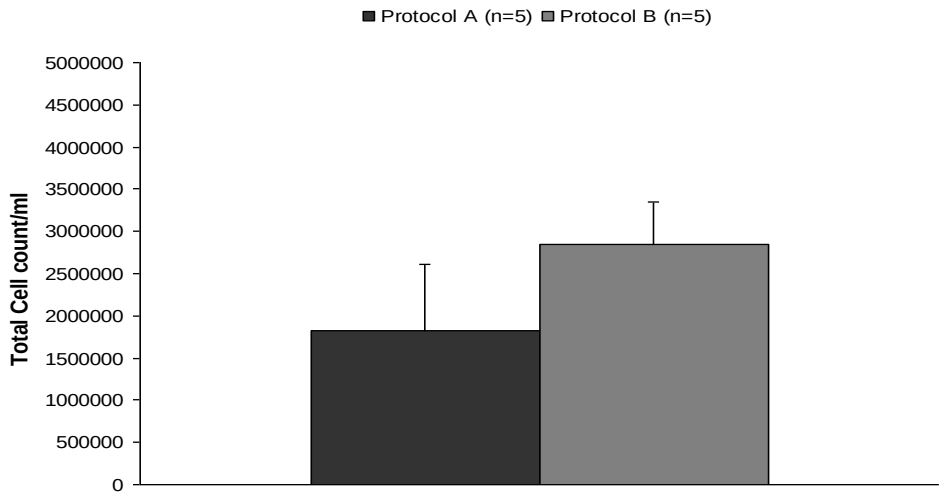


Figure 14. Total cells in 129/SvEv mice using 2 different sensitization protocols. Mice were sensitized with OVA-alum mixture on days 0 and 14 and challenged on different days. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 2). The number of total cells was measured on day 36. Error bars indicate the standard error of mean antibody titres.

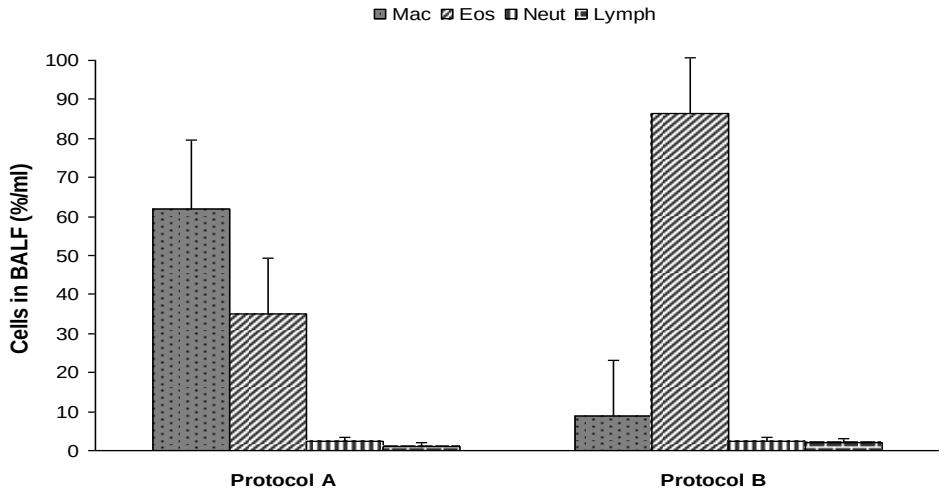


Figure 15. Effect of different OVA sensitization and challenge protocols on cell infiltration into the lungs of 129/SvEv mice. Mice were sensitized with OVA-alum mixture and challenged as already described. Cells were investigated 24 h after challenge (Figure 2). Results are represented as mean ± SEM.

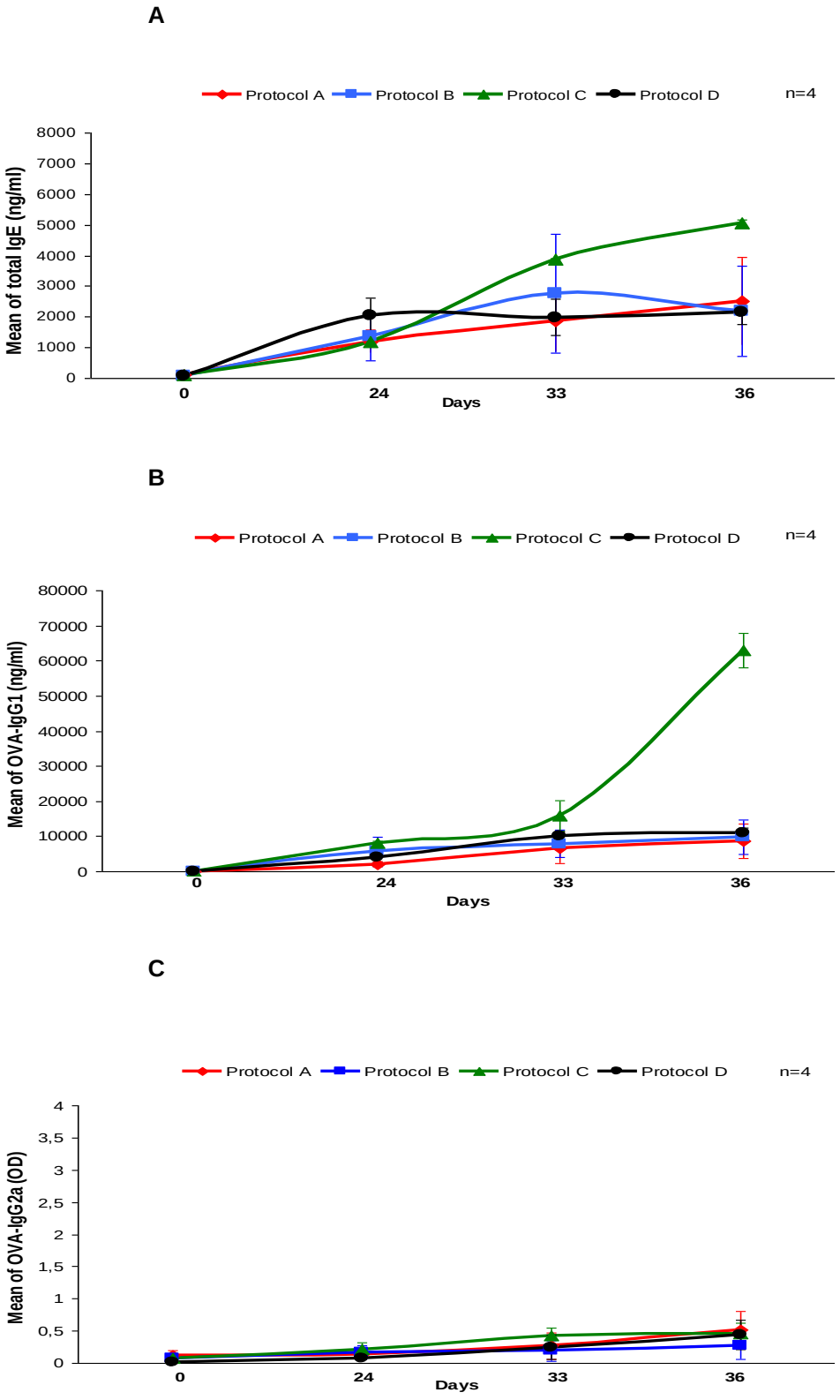


Figure 16. Time course of (A) Total IgE, (B) OVA-specific IgG1 and (C) OVA-specific IgG2a in BALB/c mice using 4 different sensitization protocols. Mice were sensitized with OVA-alum mixture on days 0 and 14 and challenged on different days. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 3). Total IgE, OVA-specific IgG1 and OVA-specific IgG2a were measured by ELISA. Error bars indicate the standard error of mean antibody titres.

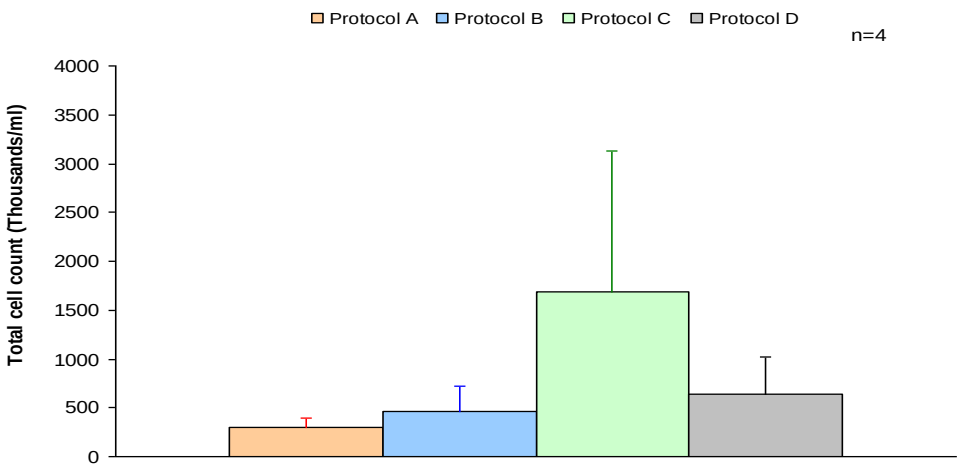


Figure 17. Total cells in BALB/c mice using 4 different sensitization protocols. Mice were sensitized with OVA-alum mixture on days 0 and 14 and challenged on different days. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 3). The number of total cells was measured on day 36. Error bars indicate the standard error of mean antibody titres.

Although prolonged allergen exposure in both murine backgrounds induced more cell infiltration and more eosinophils in lungs, it didn't result in significant differences in antibody production.

Taken together, the intraperitoneal injections of 50 µg/ml OVA with 10 mg/ml alum in 129/SvEv mice and 100 µg/ml OVA with 10 mg/ml alum in BALB/c mice together with two final OVA aerosol challenges proved to be the most efficient sensitization method of inducing an asthmatic reaction in our mouse models (Figure 5).

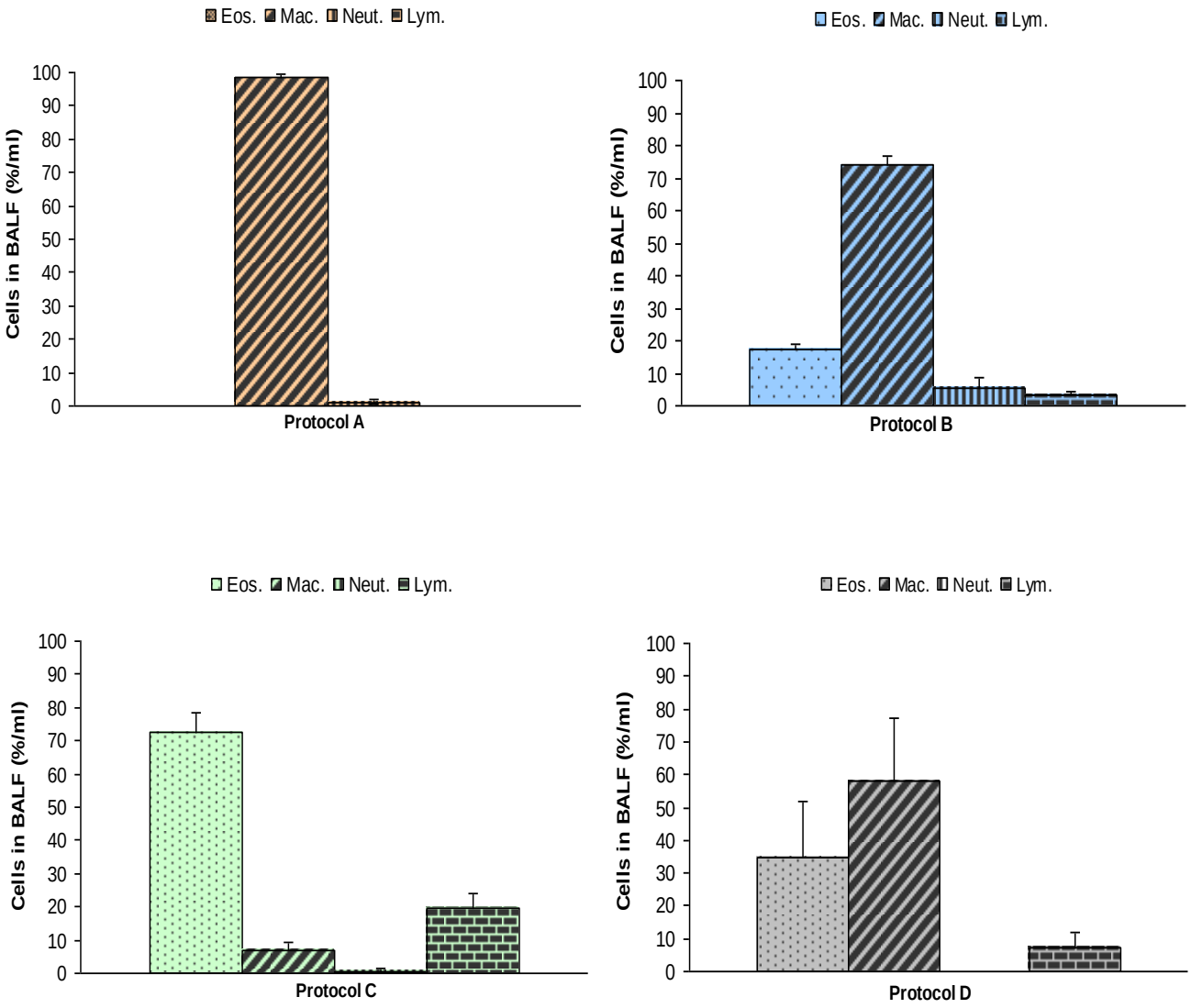


Figure 18. Effect of 4 different OVA sensitization and challenge protocols on cell infiltration into the airways of BALB/c mice. Mice were sensitized with OVA-alum mixture and challenged as already described (Figure 3). Cells were investigated 24 h after challenge. Results are represented as mean ± SEM.

We performed all the measurements for both murine backgrounds 24 h after the last challenge. As there were no significant differences comparing OVA-sensitized PR3/NE deficient and control mice, we performed further investigations on 129/SvEv background, 8, 24 and 48 hours after the last challenge.

To investigate the kinetics of the cell influx into the airways, BAL fluid from 129/SvEv animals was obtained also at different time points following OVA aerosol challenge.

4.4 Total and OVA-specific Plasma Immunoglobulin Levels in Proteinase 3/Neutrophil Elastase Deficient and Control Animals

Total IgE and OVA-specific plasma IgG1 and IgG2a levels were measured by ELISA technique to compare humoral immune responses between PR3/NE $^{-/-}$ mice and their control littermates in 129/SvEv and BALB/c background (Figure 19 and Figure 20).

The immunization with OVA and alum resulted in increased circulating levels of total IgE and OVA specific IgG1 and IgG2a in both PR3/NE $^{-/-}$ and control animals. There were no significant differences comparing PR3/NE $^{-/-}$ and control animals.

In 129/SvEv animals, PR3/NE $^{-/-}$ mice sensitized with OVA induced much the same levels of IgE and OVA-specific IgG2a as control animals. Although the levels of OVA-specific IgG1 was higher in knockout mice, statistical analysis showed no significant differences comparing to control mice.

Similarly, concerning the IgE, OVA-spesific IgG1 and IgG2a levels, there was no significant difference comparing PR3/NE $^{-/-}$ mice to controls in BALB/c background.

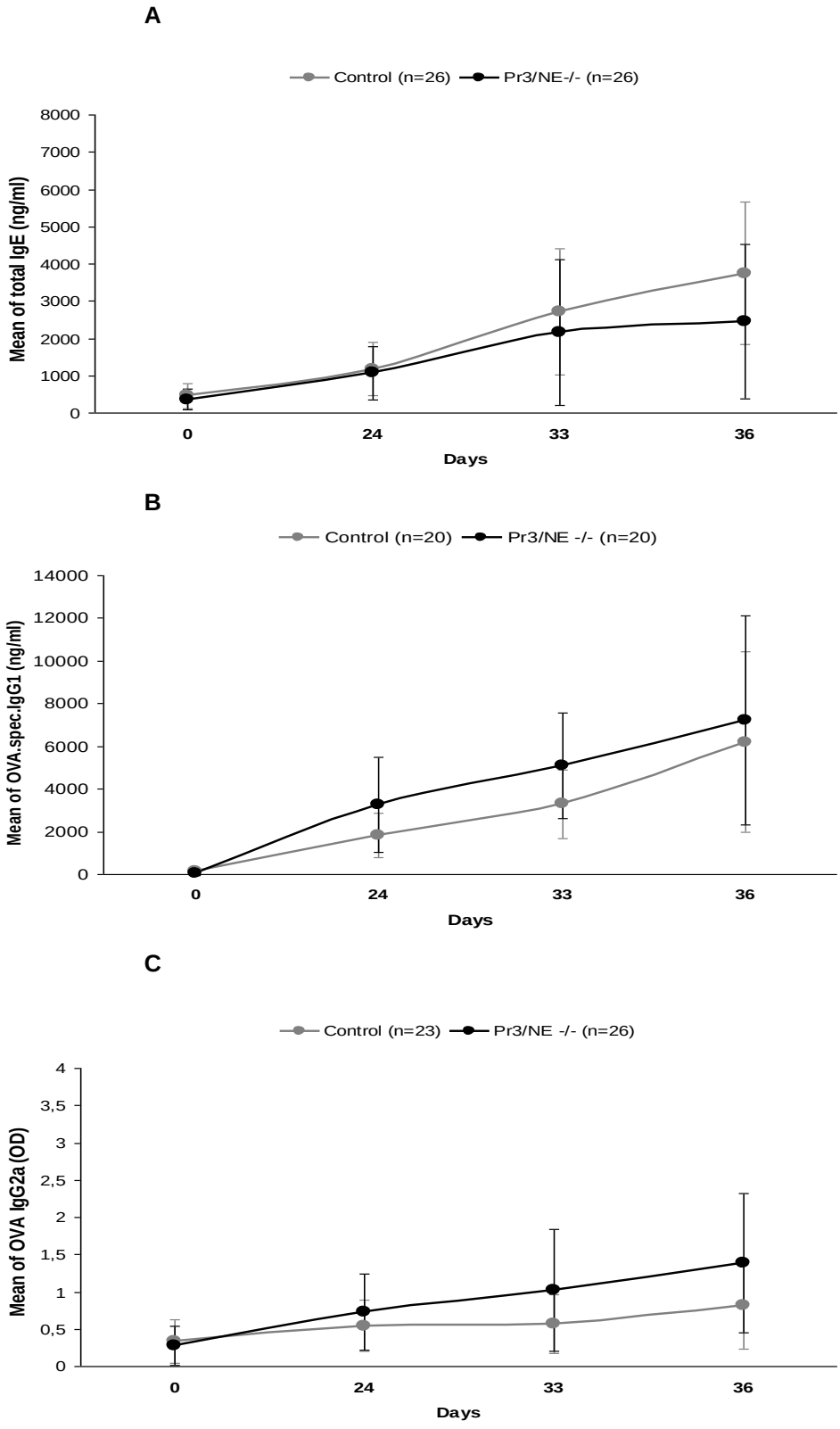


Figure 19. Time course of (A) Total IgE, (B) OVA-specific IgG1 and (C) IgG2a in PR3/NE $^{-/-}$ and control mice sensitized with OVA in 129/SvEv mice. Mice were sensitized with OVA/alum mixture on days 0 and 14 and challenged on days 14, 35 and 36. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 4). Total IgE, OVA-specific IgG1 and IgG2a were measured by ELISA. Error bars indicate the standard error of mean antibody titres.

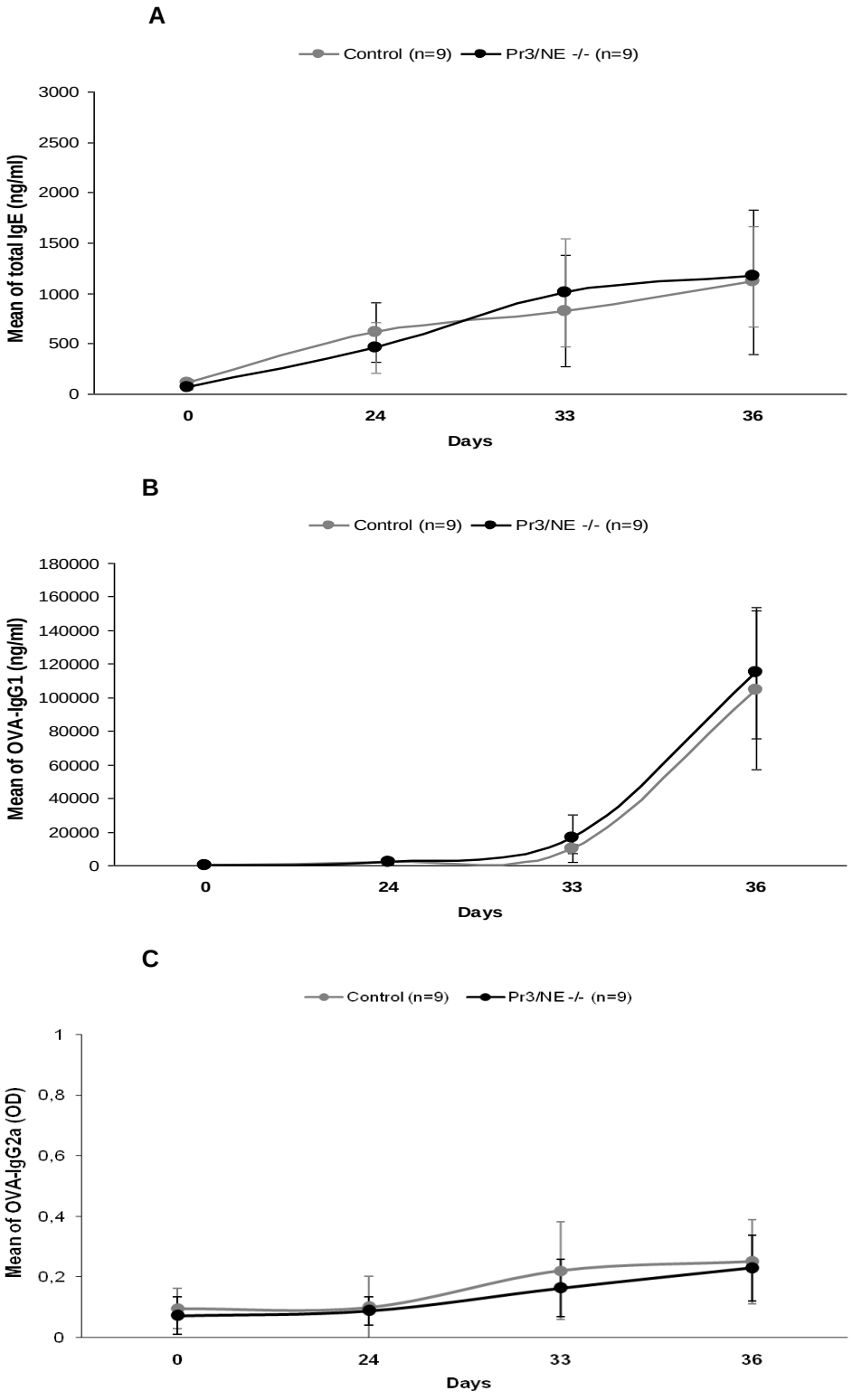


Figure 20. Time course of (A) Total IgE, (B) OVA-specific IgG1 and (C) IgG2a in PR3/NE $^{-/-}$ and control mice sensitized with OVA in BALB/c background. Mice were sensitized with OVA/alum mixture on days 0 and 14 and challenged on days 14, 35 and 36. Plasma samples were collected on days 0, 24, 33 and 36 (Figure 4). Total IgE, OVA-specific IgG1 and IgG2a were measured by ELISA. Error bars indicate the standard error of mean antibody titres.

4.5 Total Cell Number in Bronchoalveolar Lavage in Proteinase 3/Neutrophil Elastase Deficient and Control Mice

Aerosol OVA challenge in sensitized mice elicits an airway inflammatory response that can be detected in both BAL and peribronchial areas. Figure 21 shows the changes in total number of BAL cells in PR3/NE $^{-/-}$ mice and control littermates in 129/SvEv and BALB/c mice and Figure 22 shows the same results at 8, 24, and 48 h after aerosol challenge in 129/SvEv animals. Pronounced increases in the number of total cells were observed in sensitized mice of knockout and control group after challenge.

The inflammatory response was maximal at 48 h after challenge in 129/SvEv which was significantly different comparing PR3/NE $^{-/-}$ and control animals. Namely, the BAL cells of PR3/NE $^{-/-}$ mice in 129/SvEv background showed significantly greater changes after challenge.

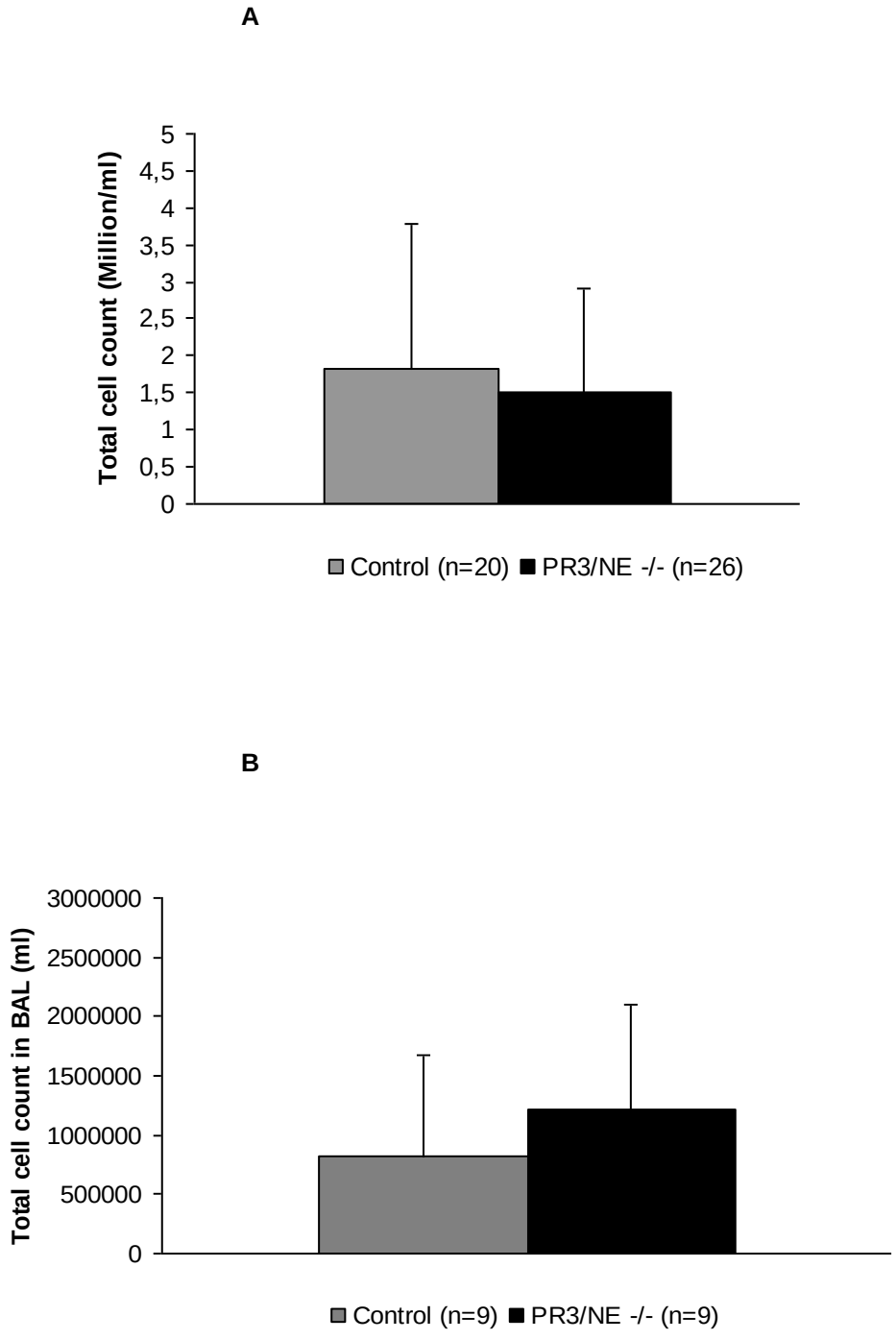


Figure 21. Total number of cells recovered from BAL in (A) 129/SvEv and (B) BALB/c mice. Bronchoalveolar lavage fluid samples from PR3/NE $^{-/-}$ and control mice were collected 24 h after challenge (Figure 4). Results are represented as mean ± SEM and are representative of four different experiments for 129/SvEv and two experiments for BALB/c animals. Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

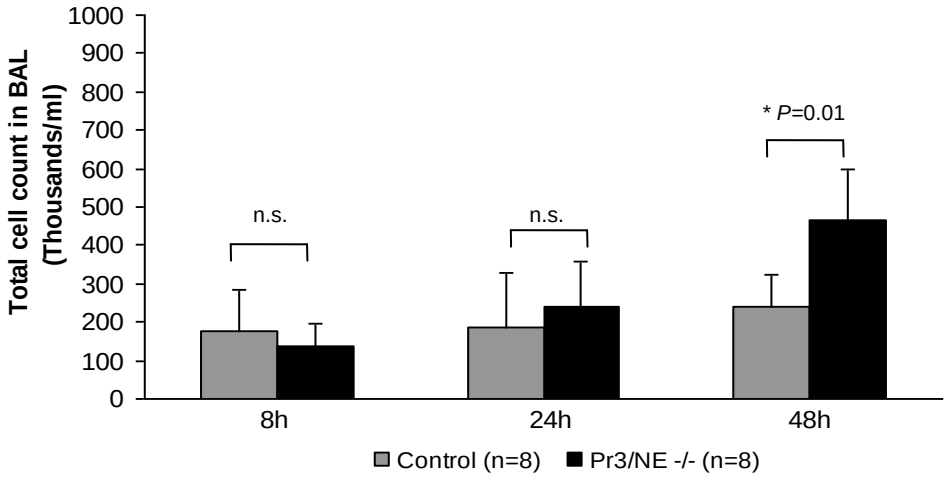


Figure 22. **Time course of total number of cells infiltrated into the lungs after OVA challenge in PR3/NE$^{-/-}$ and control littermates in 129/SvEv mice.** BALF samples from animals were collected 8, 24 and 48 h after OVA challenge (Figure 4). Results are represented as mean ± SEM (n=8 mice per group) and are representative of two different experiments. Statistical significance was analyzed using a Student's *t*-test. * *P*<0.05

PR3/NE $^{-/-}$ and control mice were sensitized intraperitoneally with alum-precipitated OVA and challenged with aerosolized OVA to compare the ability of late asthmatic responses to induce accumulation of cells in the lungs. The number of eosinophils in the BALF gradually increased in both background with time and reached a peak, 48 h after the last aerosol challenge in 129/SvEv animals (Figure 23 and Figure 25). The number of neutrophils in 129/SvEv mice reached a small peak 8 h after the last challenge with no apparent difference between knockout and control animals (Figure 23). To make it more clear we also compared absolute number of neutrophils from knockout and control animals at different time points after challenge (Figure 24).

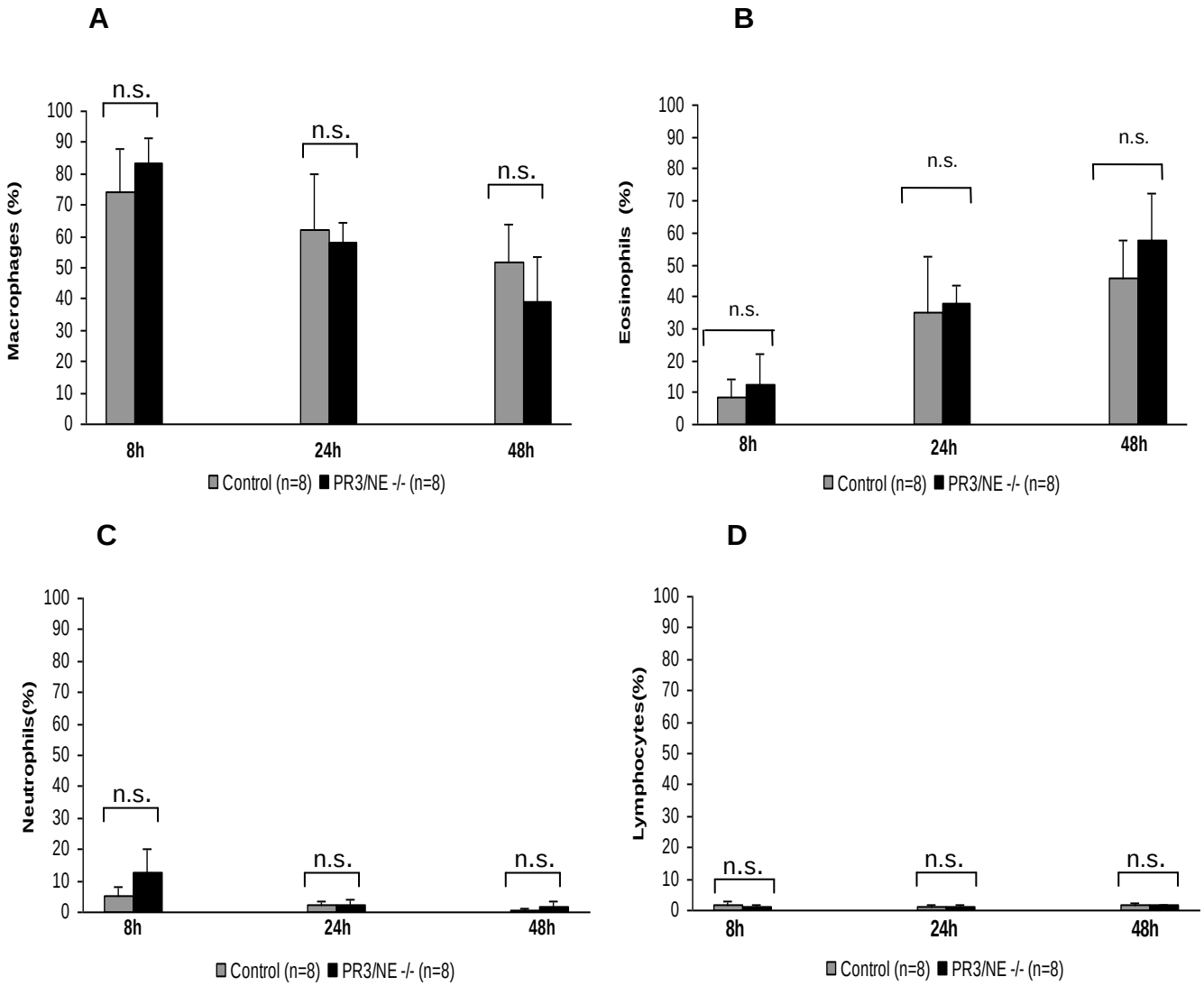


Figure 23. Effect of OVA sensitization and challenge on (A) macrophages, (B) eosinophils, (C) neutrophils and (D) lymphocytes infiltration into the airways of PR3/NE $^{-/-}$ and controls in 129/SvEv mice. Mice were sensitized with OVA-alum mixture and challenged as described (Figure 4). BALF samples from 129/SvEv mice were collected 8, 24 and 48 h after the last challenge. Results are represented as mean ± SEM and are representative of two different experiments. Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

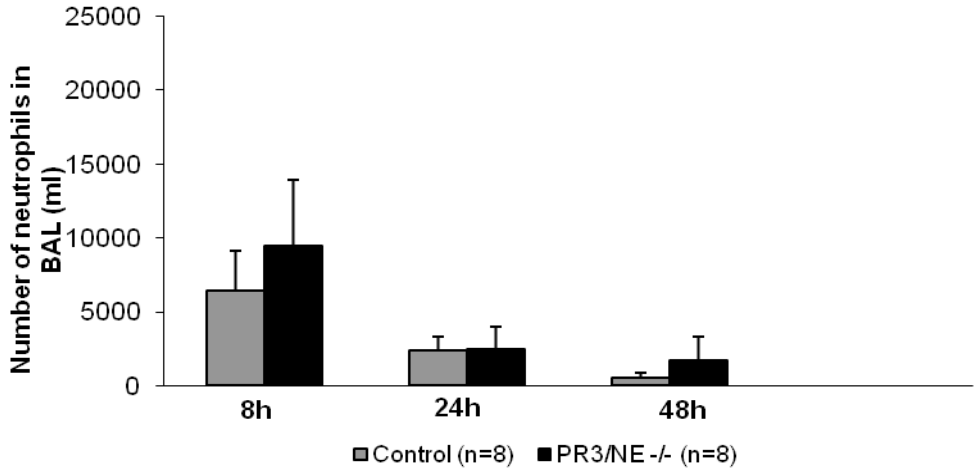


Figure 24. Absolute number of neutrophils in BAL of PR3/NE $^{-/-}$ and controls in 129/SvEv mice. BAL samples from animals were collected 8, 24 and 48 h after OVA challenge (Figure 4). Results are represented as mean ± SEM (n=8 mice per group) and are representative of two different experiments. Statistical significance was analyzed using a Student's *t*-test. * *P*<0.05

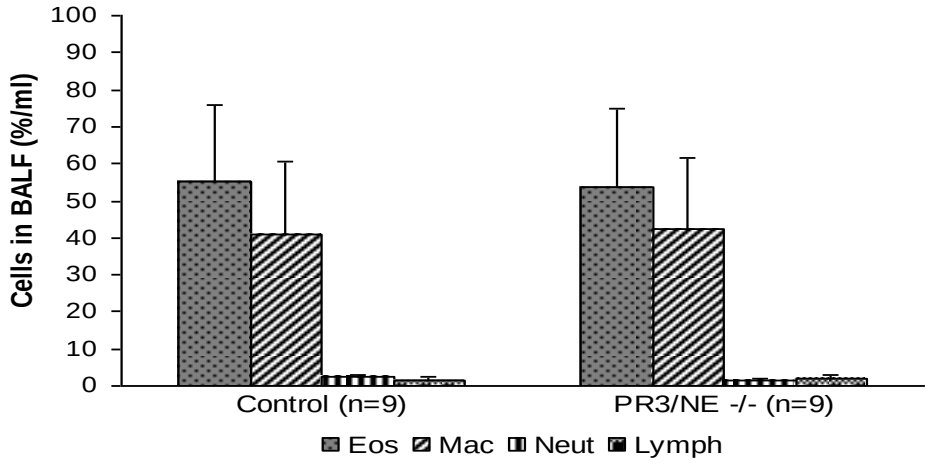


Figure 25. Effect of OVA sensitization and challenge on cell infiltration into the airways of PR3/NE $^{-/-}$ and controls in BALB/c mice. Mice were sensitized with OVA-alum mixture and challenged as described (Figure 4). Cells from BALB/c background were investigated 24 h after challenge. Results are represented as mean ± SEM and are representative of two different experiments. Statistical significance was analyzed using a Student's *t*-test. * *P*<0.05

BAL analysis showed sequentially: first a neutrophilic influx at 8 h after aerosol challenge, followed by a massive eosinophilic cell infiltration concomitant with a small lymphocyte cell number decrease at 24 and 48 h after challenge. BAL eosinophils were present at the first time point studied (8 h), peaked 48 h after the last challenge. BAL lymphocytes were present at all the time-points studied (Figure 26).

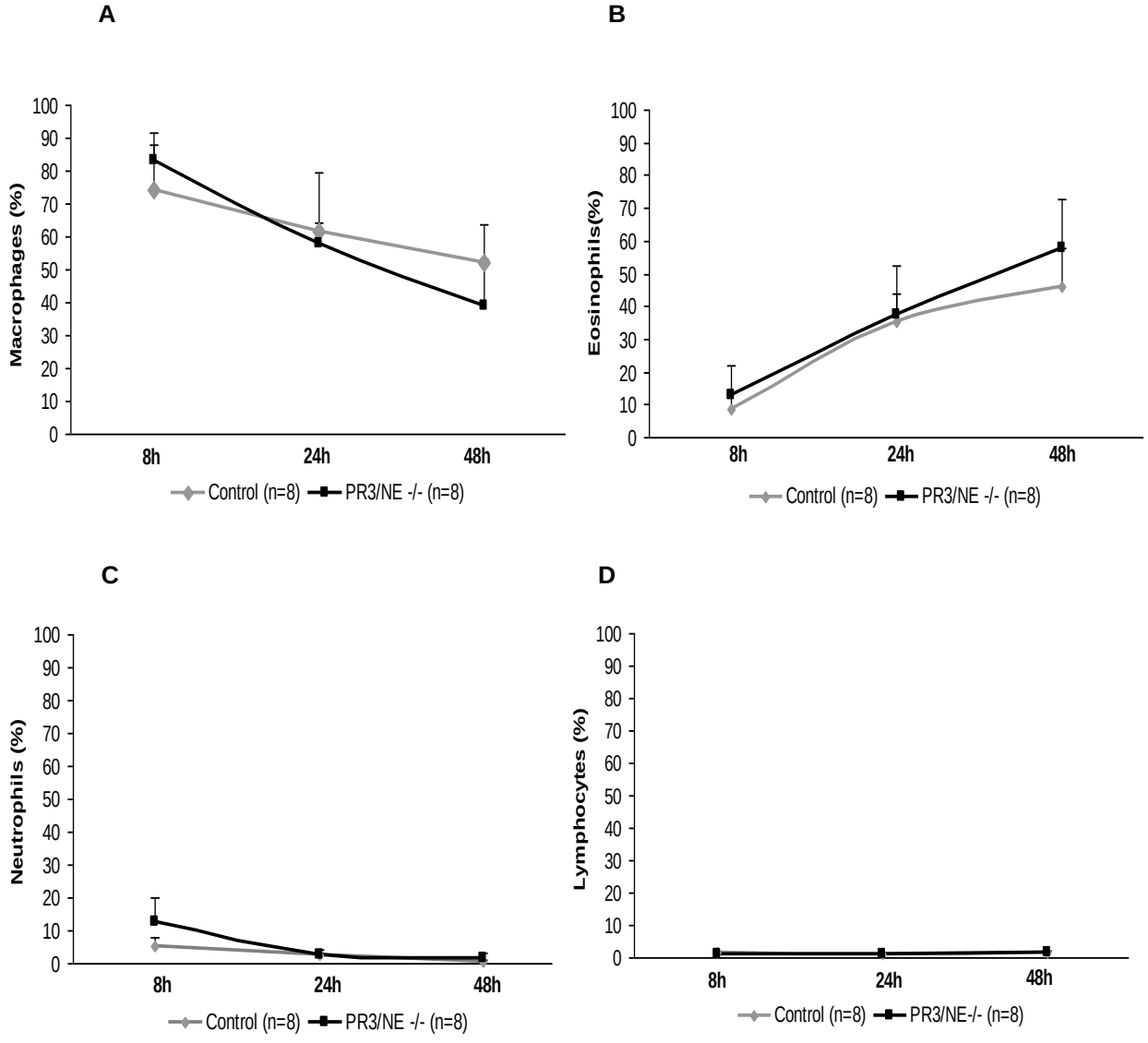


Figure 26. Time course of (A) macrophages, (B) eosinophils, (C) neutrophils and (D) lymphocytes infiltration into the lungs of PR3/NE $^{-/-}$ and control mice in 129/SvEv background. Results are represented as mean ± SEM (n=8 mice per group) and are representative of two different experiments. Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

4.6 Histology

24 h after the last challenge and after performing the BAL, the lungs were removed and excised. OVA aerosol exposure in both PR3/NE $^{-/-}$ and control mice had the same effects on lung histopathology (Figure 27).

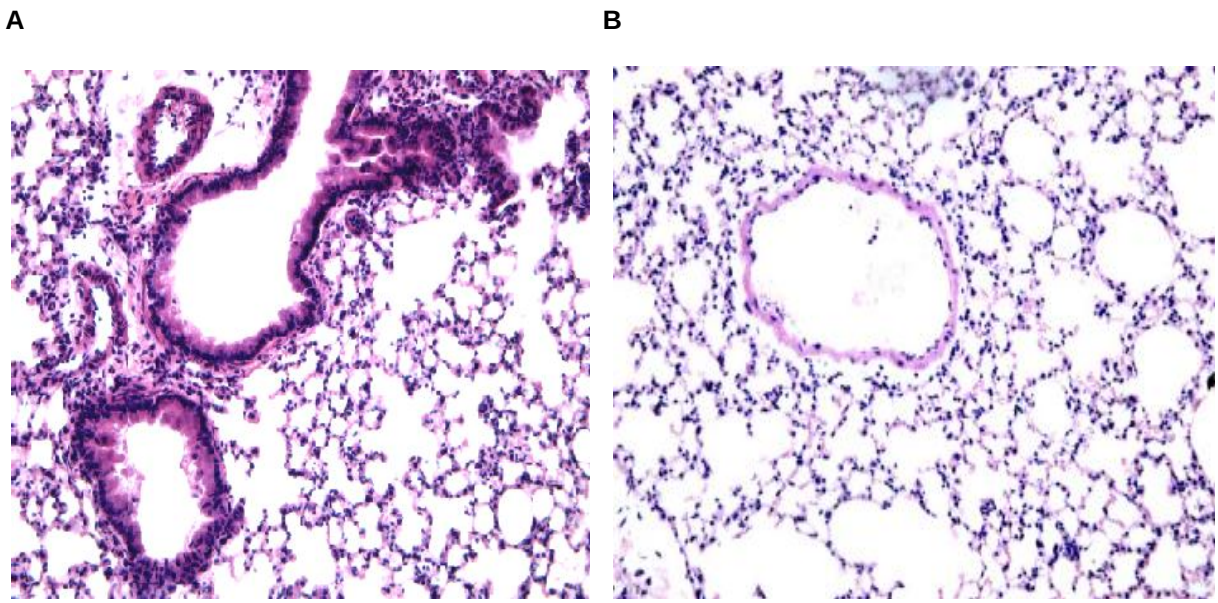


Figure 27. Histological analyses of lung tissue sections stained with standard HE technique in (A) sensitized 129/SvEv mic and (B) untreated mice. (original magnification x 200)

In paraffin-embedded lung sections, moderate inflammation was observed in all groups, and no differences in PR3/NE $^{-/-}$ and control mice were observed.

4.7 Total Protein Concentration in Bronchoalveolar Lavage in Proteinase 3/Neutrophil Elastase Deficient and Control Mice

Protein levels in BAL fluid were determined for BALB/c mice, 24 h after the last challenge and for 129/SvEV animals 8, 24 and 48 h after the last challenge using a Coomassie protein assay kit (Figure 28). There was no significant difference in total protein levels comparing PR3/NE $^{-/-}$ to control mice at different time points after challenge in both backgrounds.

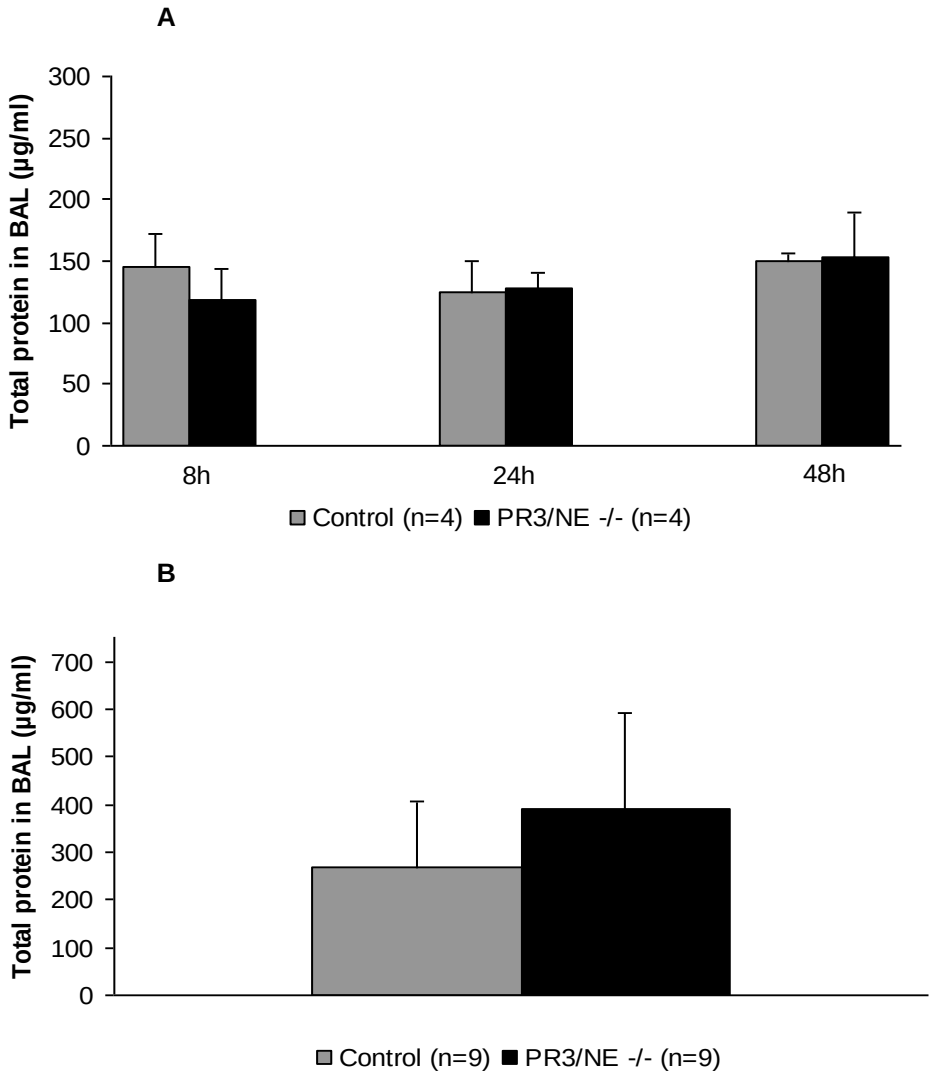


Figure 28. Total protein levels in BAL in (A) 129/SvEv and (B) BALB/c background. Using a coomassie protein assay reagent kit, the concentration of total protein was measured for 129/SvEv mice, 8, 24 and 48 h and for BALB/c mice, 24 h after the last challenge. Results are represented as mean ± SEM. Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

4.8 Airway Hyperresponsiveness

AHR was assessed as a percent increase of Penh in response to increasing the dose of MCh.

To measure MCh responsiveness, mice were exposed for 5 min to PBS, followed by increasing dosages (10–40 mg/ml) of aerosolized MCh (freshly prepared in PBS); Penh was monitored for each dosage. Results were expressed for PBS and methacholine as the percentage of baseline Penh values before PBS exposure.

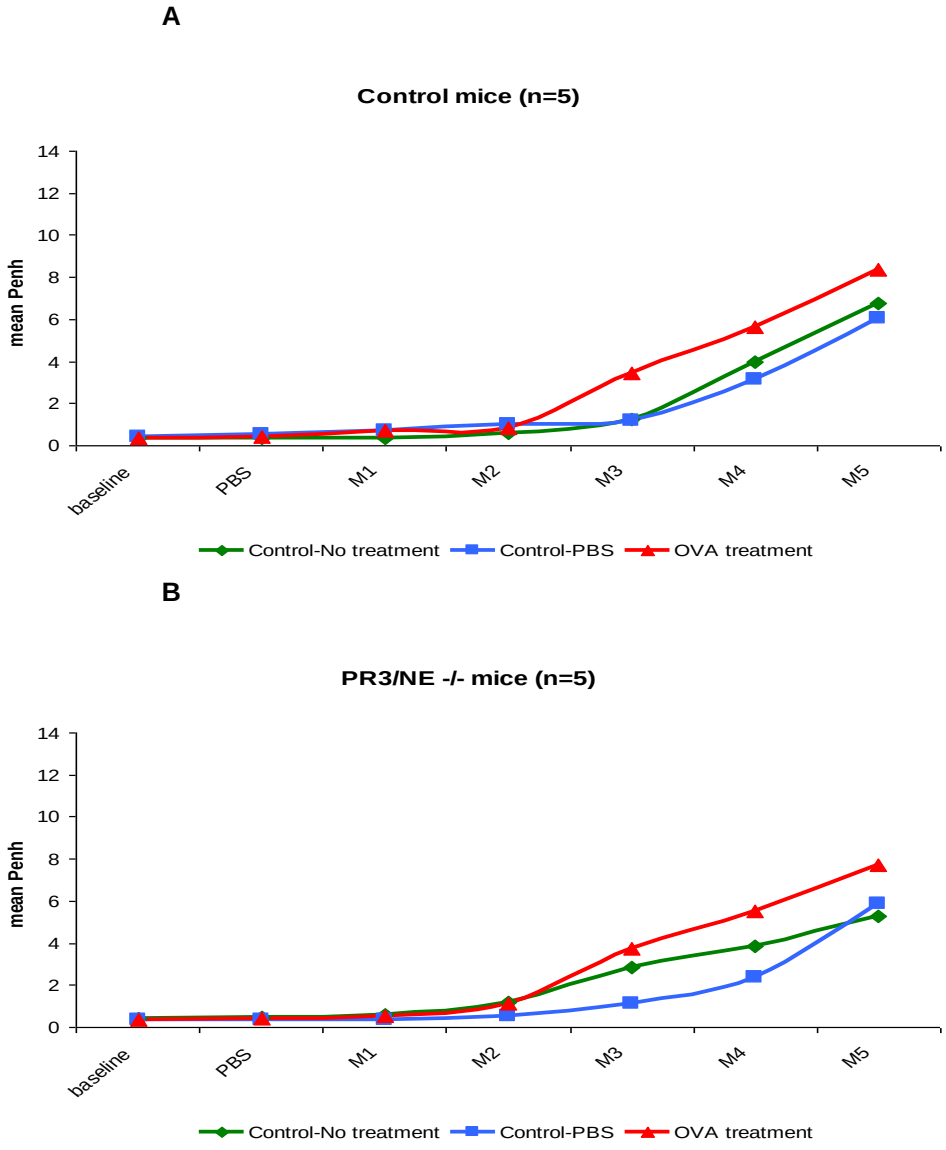


Figure 29. Effect on AHR in 129/SvEv background. Airway hyperresponsiveness (represented by Penh) was measured by non-invasive body plethysmography 24 h after ovalbumin challenge in (A) untreated mice (Control-No treatment), (B) sensitized (OVA treatment) and PBS mice.

AHR following MCh provocation was measured in sensitized and control and PBS mice, 24 h after OVA challenge (Figure 29).

The doses of MCh aerosol applied were the following: 10 mg/ml MCh nebulized for 1, 2, or 4 min (M1–M3), and 40 mg/ml MCH for 2 min (M4, M5). A data recording interval of 3 minutes was introduced after each MCh level. The mean of the Penh values determined in the 2nd and 3rd minute was used for quantifying AHR. The PenH determined before and after MCh exposure was applied as an index of AHR.

In OVA-sensitized animals, lung function tests performed 24 h after OVA challenge showed an increase in Penh after increasing MCh concentrations. There was no difference in baseline lung function, measured after PBS challenge, between control and PR3/NE $^{-/-}$ mice.

A whole-body plethysmography system (Buxco Electronics, Sharon, CT) was used to assess both spontaneous breathing patterns and AHR in unrestrained animals.

4.9 Analysis of Cellular Composition of Bronchoalveolar Lavage Fluid Using Flow Cytometry

In addition to Diff-Quick, to measure the cellular composition of the lungs following allergen challenge, we performed FACS staining of cells from BAL.

Immunofluorescence staining of BAL cells was carried out using directly conjugated monoclonal antibodies.

A

	Control mice			
Parameter[%]	**PBS**	**8h after challenge**	**24h after challenge**	**48h after challenge**
CCR3$^+$	16,2±6,1	17,6±2,1	36,9±7,7	62,7±19
CD3$^+$	1,6±1,5	4,8±2,1	6,2±3,4	8±3,5
CD19$^+$	2,03±1,5	3,1±0,7	2,2±0,6	2,1±0,8
CD19$^+$CD5$^+$	42,9±16,3	5,7±1,9	11,3±4,5	15,7±2,8
CD4$^+$	1,18±1,1	4,5±0,4	5,3±1,8	9,4±3,8
CD4$^+$CD25$^+$	16,03±1,6	12,5±2,2	31,5±6,9	16,9±8,8
CD4$^+$CD62Lhi	29,2±21,3	8,8±4,7	14,8±1,2	11,2±7,6
CD4$^+$CD62Llo	70,7±21,3	90,2±5,2	84,5±10,4	88,5±7,5
CD8a$^+$	0,5±0,3	0,28±0,04	0,52±0,5	1,02±0,3
CD8$^+$CD103$^+$	43,6±16,6	12,6±1,1	21,4±0,7	25,7±2,4
CD8a$^+$CD62Lh	54,3±19,6	51,2±7,8	32,2±18,9	37,1±12,05
CD8a$^+$CD62Llo	45,6±19,6	34,5±7,3	65,2±17,8	61,2±10,5
CD11b$^+$	8,8±2,8	9,4±3,3	12,6±7,5	28±1,1
CD11b$^+$CCR3$^+$	27,6±17,5	52,7±7,5	87,1±6,4	94,7±5,6
CD11b$^+$Ly6C$^+$	61,8±7,6	59,6±11,5	62,5±7,9	40,7±7,1
Gr1$^+$	11,68±6,9	28,75±7,2	29,5±4,4	31,5±8,8

B

	PR3/NE $^{-/-}$			
Parameter[%]	**PBS**	**8h after challenge**	**24h after challenge**	**48h after challenge**
CCR3$^+$	17±8,5	18,96±076	44±6,6	47,2±22,2
CD3$^+$	2,6±0,5	7,4±3,1	6,8±3,1	11,5±4,5
CD19$^+$	3±1,7	10,08±0,8	3,2±1,3	1,8±0,7
CD19$^+$CD5$^+$	40,2±13,3	9,7±1,8	9,1±5,1	12,05±9,5
CD4$^+$	1,9±0,5	5,1±2,9	5,9±2,8	5,9±3,2
CD4$^+$CD25$^+$	23,3±8,9	17,5±6,2	28,3±1,1	22,6±12,4
CD4$^+$CD62Lhi	31,4±18,7	5,7±2,5	13,6±2,1	11,3±8,4
CD4$^+$CD62Llo	68,5±18,7	93,9±2,05	86,1±12,05	88,4±8,2
CD8a$^+$	0,66±0,4	0,62±0,2	0,65±0,2	0,6±0,2
CD8$^+$CD103$^+$	38±12,2	9,6±2,8	20,9±4,4	25,04±1,4
CD8a$^+$CD62Lh	52±14,9	53,1±7,3	27,3±12,6	35,4±15,7
CD8a$^+$CD62Llo	47,9±14,9	42,1±8,6	69,6±10,8	62,4±14,3
CD11b$^+$	6,2±7,2	6,66±1,8	9,4±3,7	33,7±11,6
CD11b$^+$CCR3$^+$	31,9±14,1	50±10,8	83,6±2,2	94,9±5,5
CD11b$^+$Ly6C$^+$	65,3±5,6	64,6±4,6	63,6±3,8	45,2±7,8
Gr1$^+$	10,66±2,2	36,6±9,1	36,8±3,1	44,1±4,7

Table 8. Analysis of immunological parameters in BALF in (A) control and (B) PR3/NE $^{-/-}$ 129/SvEv mice. BAL cells from 129/SvEv mice in different time points after the last challenge and from PBS mice were stained with conjugated monoclonal antibodies. There were no statistically significant differences comparing cells from PR3/NE mice and their controls. Results are presented as mean±SEM. Statistical significance was analyzed using a Student *t*-test. * $P<0.05$

We analyzed different main cell populations in bronchoalveolar lavage fluid: B cells ($CD19^+$), B1 B cells ($CD19^+CD5^+$), T cells ($CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells, granulocytes ($Gr\text{-}1^+$), and eosinophils ($CCR3^+$).

We also analyzed additional subpopulations based on the following surface antigens: B cells (B220 clone RA3-6B2), macrophages (CD11b clone M1/70), lymphocytes (CD103 clone 2E7), T regulatory cells (CD25 clone PC61), leukocytes (CD62L clone MEL-14) and granulocytes (Ly-6G/Ly-6C clone RB6-8C5) (Table 8).

Data were acquired on a FACS Calibur (Becton Dickinson, San Diego, USA) and were analyzed using FlowJo software (TreeStar Inc, USA). All samples were acquired until a total number of 25,000 cells was reached.

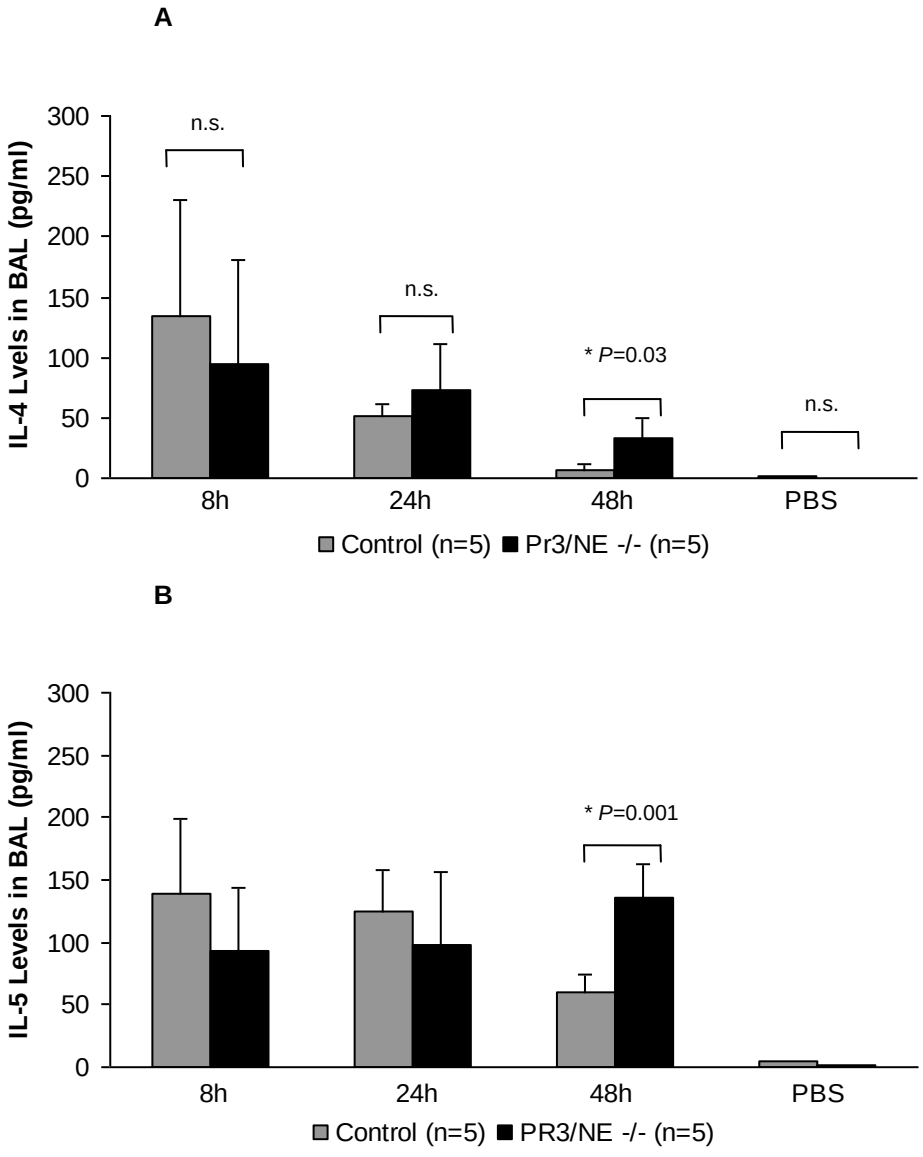
In order to study the eosinophilic inflammation of the lung, the $CCR3^+$ cells in BAL were studied. OVA aerosol challenge caused a strong eosinophilic inflammation of the lung of OVA sensitised mice and this was reflected in the bronchoalveolar lavage fluid of both PR3/NE $^{-/-}$ and control mice. The number of $Gr1^+$ and $CD11b^+$ cells in the lungs was also increased after OVA aerosol challenge.

There was a slight increase of T cells ($CD3^+$ cells) after OVA aerosol challenge in both PR3/NE $^{-/-}$ and control mice.

We didn’t see any major changes in other cell populations. Altogether, there were no statistical differences between the PR3/NE $^{-/-}$ and control mice concerning different cell populations in different time points after challenge.

4.10 Cytokine Levels in Bronchoalveolar Lavage Fluid

As ELISA techniques are time consuming and need relatively high amounts of plasma because each parameter has to be determined separately, measurements of cytokine subclass levels were transferred to bead array systems, allowing the simultaneous detection of different analytes in the same plasma sample.



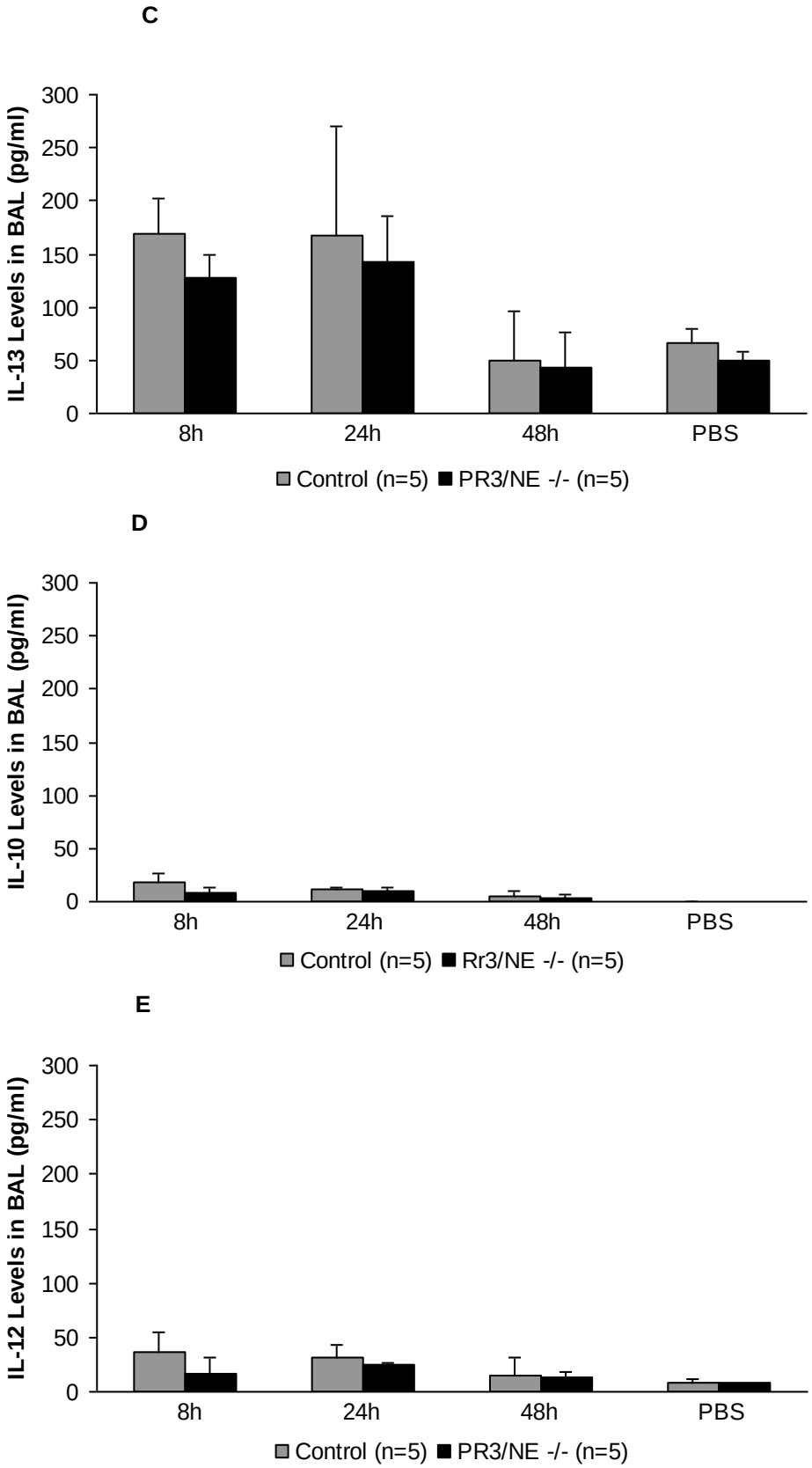


Figure 30. Cytokine levels in BAL in 129/SvEv mice. (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-10 and (E) IL-12 levels, generated in the airway tissues of OVA-sensitized PR3/NE $^{-/-}$ and control mice challenged with aerosolized OVA. Results are represented as mean ± SEM (n=5 mice per group). Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

To characterize the cytokine pattern in BALF, the levels of IL-4, IL-5, IL-13, IL12 and IL-10 were measured in different time points after challenge by multi bead array technique.

Compared with PBS challenge, OVA challenge caused a significant increase in the BALF concentrations of IL-4, IL-5 and IL-13 in both control and PR3/NE $^{-/-}$ mice (Figure 30).

There was no difference between PR3/NE $^{-/-}$ and control animals with respect to IL-13, IL-10 and IL-12, but 48 h after the last challenge, there were significantly higher levels of IL-4 and IL-5 comparing to controls.

4.11 Peritoneal Lavage

The I.P. injection of OVA and alum to PR3/NE $^{-/-}$ and control mice induced a marked increase in the number of total cells after 8 h. The increase of the total cells in peritoneal cavity was not significantly different comparing two groups of animals.

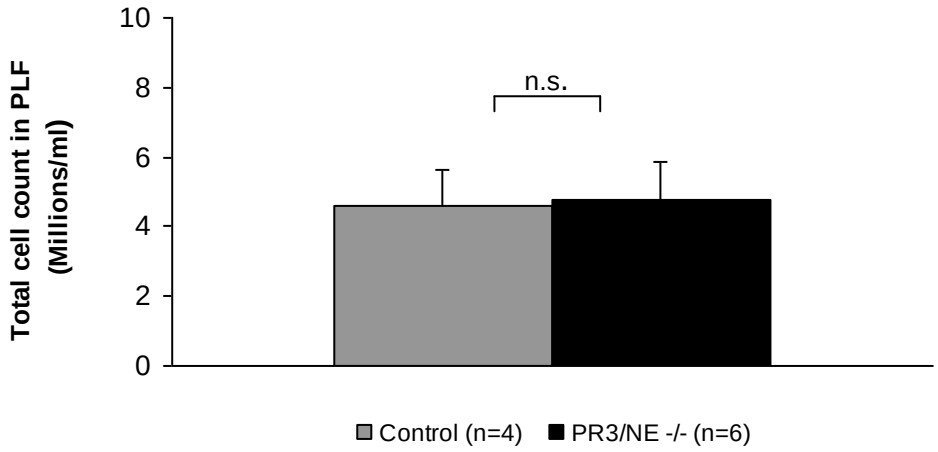


Figure 31. Cell accumulation in the peritoneal cavity in 129/SvEv mice after challenge. Cell accumulation in the peritoneal lavage fluid of OVA injected PR3/NE $^{-/-}$ and control mice. Results are represented as mean ± SEM. Statistical significance was analyzed using a Student's *t*-test. * $P<0.05$

Differential cell counts were performed after cytocentrifugation and staining with May–Grünwald–Giemsa under oil immersion. At least 300 cells were counted and results were expressed as the percentage of cells per millilitre of PLF. Differential cell counts showed significant increases in neutrophils (Figure 32) 8 h after I.P. challenge with OVA and alum. Although no differences were apparent in total cell numbers in the PLF from PR3/NE $^{-/-}$ and control mice 8 h after OVA-challenge (Figure 31), we measured the cytokine levels of IL-5, IL-4, IL-13 and IFN-gamma in PLF (Figure 33).

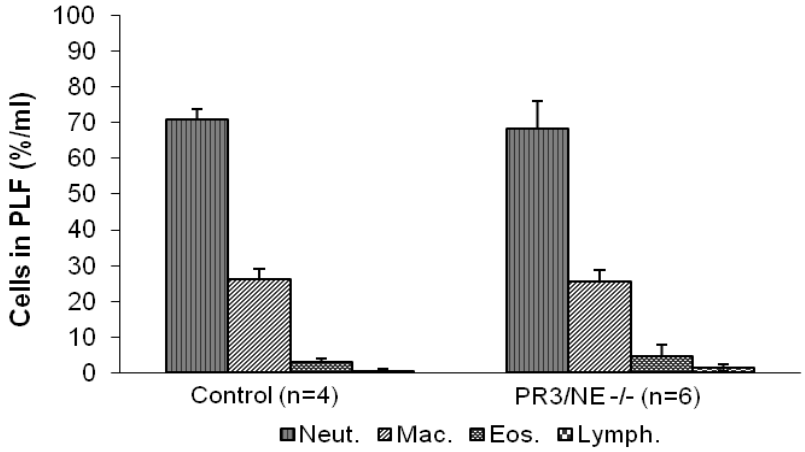


Figure 32. Analysis of cells in the peritoneal lavage fluid from 129/SvEv mice. Cells accumulated in the peritoneal lavage fluid of OVA injected PR3/NE $^{-/-}$ and control mice were investigated 8 h after challenge. Results are represented as mean ± SEM. Statistical significance was analyzed using a Student's *t*-test. * *P*<0.05

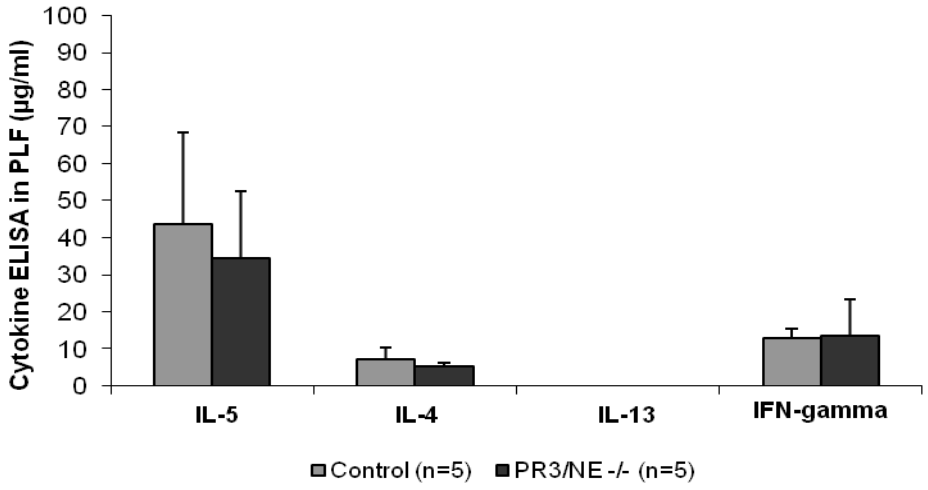


Figure 33. Cytokine levels in PLF in 129/SvEv mice. Cytokines generated in the peritoneal lavage fluid of OVA injected PR3/NE $^{-/-}$ and control mice. Results are represented as mean ± SEM (n=5 mice per group). Statistical significance was analyzed using a Student's *t*-test. * *P*<0.05

Concerning PLF cytokine levels, we couldn't find any significant differences comparing PR3/NE $^{-/-}$ and control mice.

To determine the presence and the phenotype of cells in the peritoneal cavity of OVA-challenged mice, cells were stained and analyzed by flow cytometry in the same way as those from BAL.

	PR3/NE $^{-/-}$		**Control**	
Parameter	OVA sensitized	PBS	OVA sensitized	PBS
$CD19^+$ (%)	7,03±2,3	27,3±4,5	8,1±7,02	24,4±1,2
$CD19^+CD5^+$ (%)	1,84±0,1	33,1±8,6	2,08±1,09	33,1±3,7
$Gr1^+$ (%)	75,6±5,2	1,6±0,6	59,3±19,6	2,3±0,3
$CD3^+$ (%)	1,7±0,5	3,05±1,1	1,2±0,7	4,8±2,4
$CD4^+$ (%)	2,5±0,9	11,0±3,1	4,6±4,7	15,9±3,1
$CD4^+CD25^+$ (%)	20,8±9,6	31,1±12	29,4±2,3	26,1±11,7
$\gamma/\delta^{CD3^+}TCR^+$ (%)	31,5±13,5	70±10,5	44,5±17	51,2±10,2
$CD8a^+$ (%)	0,67±0,3	2,1±0,3	1±0,7	3,8±1,6

Table 9. Analysis of immunological parameters in PLF in 129/svEv mice. Cellular composition of PLF cells of OVA challenged PR3/NE $^{-/-}$ mice and their controls, compared with PBS challenged mice. There were no statistically significant differences comparing cells from PR3/NE mice and their controls. Results are presented as mean±SEM. Statistical significance was analyzed using a Student *t*-test. * $P<0.05$

Analysis of PLF cells showed significant increases in $Gr1^+$ cells (Table 9) after I.P. challenge with OVA and alum in sensitized mice of both groups but there was no statistically significant difference comparing PR3/NE $^{-/-}$ and their control littermates. There was also decrease numbers of $CD3^+$, $CD4^+$ and $CD19^+$ cells after OVA

challenge in both sensitized PR3/NE $^{-/-}$ and their control littermates but no significant difference comparing knockout mice to their controls.

Comparing PR3/NE $^{-/-}$ mice to their control littermates concerning cellular composition of peritoneal lavage cells, there was no significant difference.

4.12 Primary Screening Results of Proteinase 3/Neutrophil Elastase Deficient Mice in German Mouse Clinic

Mouse mutants entering the GMC are examined in a primary screen according to the following standard workflow (Figure 34) (Gailus-Durner, Fuchs et al. 2005).

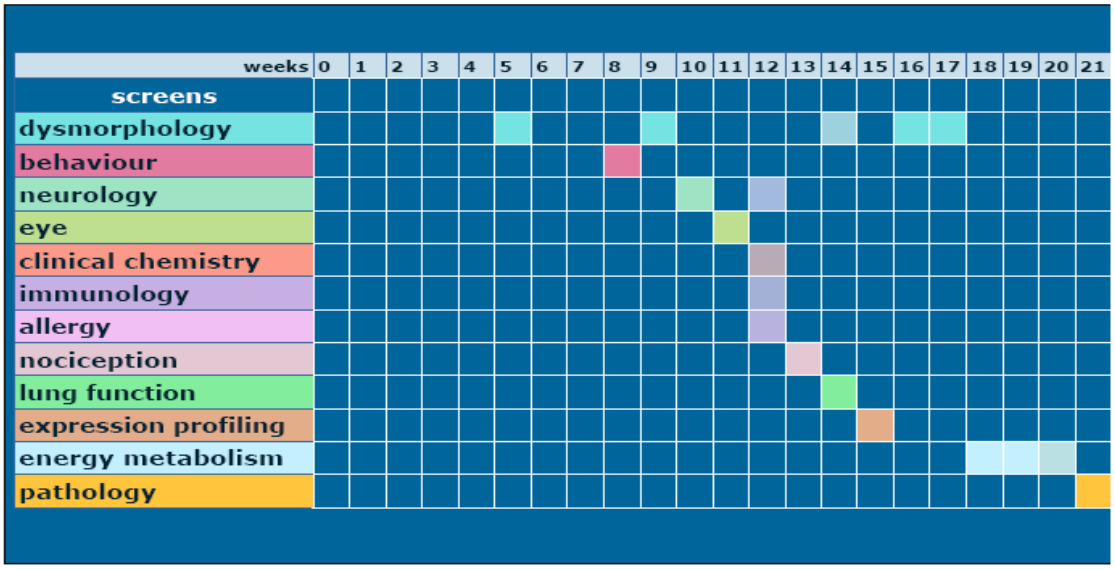


Figure 34. GMC standard workflow.

After the mice arrive at the GMC, they are acclimatized in the new environment for one week. The males then start in the Behavior Screen. There they stay for three weeks. Directly after the Behavior Tests, the anatomical inspection of the Dysmorphology Screen is performed. In the next week, the Neurology Screen is applied. One week later the mice go through the tests of the Eye Screen.

When the mice were 12 weeks old, blood is taken, and samples are distributed to the blood-based screens for Clinical Chemistry, Immunology, Allergy and the Lactate test. One week later, the animals are tested in the Nociceptive Screen. Two weeks after testing of the first blood sample, a second sample is taken to confirm outliers, and to supply the Dysmorpholgy Screen with material for determination of blood-based bone-related parameters. In parallel, 10 mutant animals (5 males / 5 females) and 10 controls (5 males / 5 females) leave the animal facility for the Lung Function Analysis, which for technical reasons is located elsewhere. These animals are, for hygienic reasons, not allowed to re-enter the German Mouse Clinic. The females go directly to Pathology. The males are used to freeze organs for future expression profiling on demand (remaining organs from those animals are analyzed by the Pathology). All other animals go through the bone and cartilage tests of the Dysmorphology Screen, and then stay three weeks in the Metabolic Screen. After completion of the primary screen, all animals end up in the Pathology.

In the primary screen 50 PR3/NE $^{-/-}$ mice (20 mutant mice and 30 littermate controls) have been analyzed in the German Mouse Clinic:

Behaviour Screen

Only female mutant mice showed a prominent behavioural phenotype. Female mutant mice showed a reduction in social affinity and an increase in anxiety-related behaviours. They completely avoided the board and exhibited pronounced thigmotaxis. There were no consistent genotype effects in male mice.

Dysmorphology Screen

Progressive deafness in male mutant mice was detected, which should be investigated in more detail.

Neurology Screen

Female mutant mice indicated a lower locomotor activity and a slower spontaneous behaviour as compared to female littermate control mice. Male mutant mice were impaired in the visual placing test and displayed altered vocalization behaviour.

Clinical Chemistry Screen

Several clinical-chemical parameters displayed altered levels in mutant mice: the plasma concentration of potassium, uric acid, cholesterol, triglyceride, sodium, and chloride were significantly different.

Immunology Screen

Under standard screen conditions, mutant mice showed significant differences comparing to controls. These results were especially pronounced for Gr-1^+ cells, which were decreased in knockout mice.

Metabolic Screen

Genotype-specific differences were rather moderate. Although of the same body weight, control males consumed more food and have higher values in energy uptake and metabolized energy - even when calculated per unit body weight. Food restriction did not provoke any physiological adaptation, which could be seen in a

decrease of caloric value of feces, in a dramatic decrease of feces production or in an increase of food assimilation coefficient.

In other screens no genotype-specific differences could be found.

5 DISCUSSION

In the present study the role of neutrophil serine proteases, proteinase 3 and neutrophil elastase in a mouse model of allergic asthma has been investigated.

Asthma is a chronic inflammatory disorder of the airways affecting between 5 to 10% of the world's population. Allergic asthma is characterized by infiltration of the bronchial mucosa with eosinophils and T helper (Th) 2-Type cells, circulating specific antibodies and positive skin test reactions to common aeroallergens, together with hyperresponsiveness, defined as an increased sensitivity to bronchoconstrictors such as histamine or cholinergic agonists (Figure1) (Humbert, Menz et al. 1999; Johansson, Hourihane et al. 2001; Renauld 2001; Medoff, Thomas et al. 2008).

Although there are clear evidences demonstrating distinct infiltration and presence of neutrophils in asthma (Cowburn, Condliffe et al. 2008; King and Moores 2008), an understanding of neutrophil function is of considerable importance to respiratory researchers. Neutrophils belong to the body's first line of cellular defense and respond quickly to tissue injury and invading microorganisms (Nathan 2006). The important role of neutrophils in acute severe asthma has been already demonstrated in several studies (Busse and Sedgwick 1992; Fahy, Kim et al. 1995; O'Byrne P and Postma 1999). Accumulation and activation of neutrophils in the airways has been reported in severe refractory asthma in adults (Wenzel, Szefler et al. 1997; Jatakanon, Uasuf et al. 1999) as well as acute asthma exacerbation in young children (Norzila, Fakes et al. 2000; Yoshihara, Yamada et al. 2006). Neutrophils may aggravate airway inflammation in asthma where eosinophils are presumably the major effector cells (Gleich 2000).

The granule-associated elastase homologues neutrophil elastase and proteinase 3 are involved in immune defense reactions of neutrophils (Jenne 1994). To investigate the role and function of neutrophil serine proteases in asthma we analyzed PR3/NE $^{-/-}$ mice in this study.

Prior to analysis of PR3/NE $^{-/-}$ mice, we necessarily investigated and compared different mouse models of asthma. Although mouse models rarely completely reproduce all the features of the respective human disease, wild-type mice after sensitization and respiratory tract challenges with antigen, develop a phenotype that closely resembles allergic asthma characterized by eosinophilic lung inflammation, airway hyperresponsiveness (AHR), increased IgE, mucus hypersecretion and eventually airway remodelling. Identifying mutant mouse lines with elevated total IgE or enhanced allergen-specific IgE sensitization and allergic airway inflammation after allergen exposure and challenge is likely to provide important advances with regard to the pathophysiology, diagnosis, and the preventive as well as therapeutic treatment of IgE-mediated allergic diseases. In this study, a variety of different allergen challenge models have been developed in mice and a number of sensitisation and challenge protocols have been employed. As genetic background has a striking and selective impact on mouse models of asthma (Zhang, Lamm et al. 1997), we used different OVA sensitization protocols for 129/SvEv and BALB/c backgrounds. We found that a combination of intraperitoneal injections of OVA with alum together with OVA aerosol challenge with concentrations which are already mentioned in Figure 4 are the most efficient sensitization methods of inducing an asthmatic reaction in our mouse models.

Sensitization of 129/SvEv mice using the selected protocol (Figure 4) causes infiltration of inflammatory cells, which can be detected in broncoalveolar fluid at a rate of about 40% eosinophils (Figure 23). We could also measure elevated levels of

total IgE in these mice, which was significantly up-regulated compared to non-sensitied mice (Figure 19). Therefore we applied this protocol as an optimized sensitization protocol for investigation of 129/SvEv mice in the GMC allergy screening platform.

For BALB/c mice we chose also a protocol which had the most efficient results of inducing an asthmatic reaction in our mouse model (Figure 4). Through this protocol we could measure a significant increase of about 60% eosinophils infiltration into the BAL (Figure 25). This protocol has been also demonstrated as a standard optimized protocol for the BALB/c mouse strain in the GMC allergy screen.

To get a better understanding of the role of neutrophils in asthma, we focused on PR3/NE $^{-/-}$ double knockout mice on previously mentioned backgrounds.

In our study, the sensitized PR3/NE $^{-/-}$ mice in both backgrounds which were challenged with allergen, have shown more or less the same cellular as well as humoral allergic reaction compared to a control group. In PR3/NE $^{-/-}$ mice and their control littermates in 129/SvEv background, 24 h after the last OVA challenge, 40% eosinophils and about 3% neutrophils were infiltrated into the bronchial lumen. 48 h after the last OVA aerosol challenge we observed more eosinophils and less neutrophils in these animals (Figure 23).

In BALB/c background, sensitized mice showed about 60% eosinophil and 3% neutrophil infiltration in BAL. There was also no significant difference in PR3/NE $^{-/-}$ double knockout mice as compared to control mice in this background.

Our cellular analysis showes a short increase of neutrophils in sensitized mice after 8 h that subside to the primary status after 48 h, whereas eosinophils remain highly increased 48 h after the aerosol challenge (Taube, Dakhama et al. 2003; Kung, Jones et al. 1994) (Figure 25).

In the recent past, increased numbers of neutrophils in the airways have been found at different stages of the disease (Martin, Cicutto et al. 1991; Tanizaki, Kitani et al. 1993; Fahy, Kim et al. 1995; Wenzel, Szefler et al. 1997). Following allergen challenge in patients with allergic asthma, neutrophils are the first inflammatory cells to accumulate within the airways (Koh, Dupuis et al. 1993; Nocker, Out et al. 1999; Kelly, Busse et al. 2000). In murine models, an increased number of neutrophils following an allergen challenge has also been described (Taube, Dakhama et al. 2003). It implicates neutrophilic airway inflammation not only as a feature of severe asthma, where it has been well documented (Sur, Crotty et al. 1993; Fahy, Kim et al. 1995; Wenzel, Szefler et al. 1997; Ordonez, Shaughnessy et al. 2000), but also as the basis of a distinct inflammatory phenotype which may be present either alone or in conjunction with eosinophilic inflammation. High levels of NE found in the sputum of patients with asthma raises the possibility that neutrophils may play a role in airway remodelling in asthma (Vignola, Bonanno et al. 1998) and in addition to eosinophils, bronchial neutrophils have been described in asthmatic patients (Kim, Nassiri et al. 1989; Sommerhoff, Nadel et al. 1990). Therefore, in this study it was hypothesized that impaired or functionally defected neutrophils may affect eosinophil infiltration in BAL.

To assess cytokine production of sensitized PR3/NE $^{-/-}$ mice after allergen challenge, a broad panel of cytokines have been analyzed. We could measure a significant increase of IL-4 and IL-5 concentration in BAL in control and in PR3/NE $^{-/-}$ mice 48 h after the last challenge (Figure 30). It's well known that Th2-type cytokines, e.g., IL-4 and IL-5 play important roles in eosinophil infiltration into the lungs (Daser, Meissner et al. 1995). IL-4 evokes transendothelial migration of eosinophils (Schleimer, Sterbinsky et al. 1992) whereas IL-5 strongly promotes the maturation, adhesion and

activation of eosinophils (Lopez, Sanderson et al. 1988; Yamaguchi, Hayashi et al. 1988).

While there was no significant increase of IL-2, IL-13 and IL-10 in challenged mice, PR3/NE $^{-/-}$ mice showed three times more IL-4 production and about two times higher concentration of IL-5 in BAL 48 h after challenge (Figure 30) .

The cellular analysis of BAL showed an increased trend of CCR3^{+} cells in bronchial lumen 48 h after challenge in both PR3/NE $^{-/-}$ and control mice (Table 8). This finding was in line with the increased number of eosinophils in BAL.

Beside different cellular markers of granulocytes (CCR3, CD11b and Gr1), we also closely investigated T-cells in our mouse model of asthma. Although CD3^{+} cells were significantly increased in BAL even 24 h after aerosol challenge, there was no significant difference between the control and PR3/NE $^{-/-}$ mice (Table 8).

A recent study of type III allergic reactions demonstrated a strongly diminished immune complex–mediated (IC-mediated) neutrophil infiltration *in vivo* as well as reduced activation of isolated neutrophils by ICs *in vitro* (Kessenbrock, Frohlich et al. 2008). In this study we focused on type I allergic reactions and concluded that in mice with neutrophils lacking to express sufficient levels of neutrophil elastase and proteinase 3, normal attending of neutrophils in bronchial lumen after aerosol challenge can be followed by recruitment /infiltration of the normal levels of eosinophils.

Our primary hypothesis that impaired neutrophils may cause significant changes in eosinophil infiltration in BAL was surprisingly not confirmed. We observed marginal or minimal impact of neutrophil proteases in chronic allergic inflammation of airways.

This observation raises the hypothesis that neutrophil serine proteases may exert a delayed cellular clearance in the inflammation site. Currently there is no explanation

for this discrepant finding. Furthermore, an understanding of the fundamental mechanisms regulating neutrophil and eosinophil inflitration to the sites of inflammation may provide a guide to novel and beneficial therapies for the treatment of allergic diseases.

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9 ABBREVIATIONS

Ab	Antibody
ADN	Properdin factor D; gene symbol
Ag	Antigen
AHR	Airway hyperresponsiveness
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CD	Cluster of differentiation
CG	Cathepsin G
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
GMC	German Mouse Clinic
IFN	Interferon
IL	Interleukin
I.P.	Interaperitoneal
KO	Gene knockout
MCh	Methacholine
MPO	Myeloperoxidase
NE	Neutrophil elastase
OVA	Ovalbumin
Penh	Enahanced pause
PFA	Paraformaldehyde
PR3	Proteinase 3
PMN	Polymorphonuclear
PLF	Peritoneal lavage fluid
TCR	T cell receptor
TNF	Tumor necrosis factor
WT	Wild-type

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12 Summary

Understanding the type of cellular inflammation in acute severe asthma has important implications not only for the treatment of disease but also for its prevention. Although there is clear evidence demonstrating distinct infiltration and presence of neutrophils in asthma, their specific function for the disease is not fully elucidated.

The granule-associated elastase homologues neutrophil elastase and proteinase 3 are involved in immune defense reactions of neutrophils.

The use of genetically engineered animal models to explore the function of genes and for the selection of appropriate drug targets holds great promise in accelerating the development of valuable therapies. Model organisms, especially the mouse, have proven to be important tools to learn more about gene functions.

Understanding the role of neutrophils and its proteases has important implication not only for the treatment of asthma but also for its prevention.

In this study the role of neutrophil proteases in an animal model of asthma was investigated. We mainly focused on type I allergic reactions. Intrestingly, we found that after aerosol challenge of mice lacking the neutrophil proteases, Elastase and proteinase 3, normal attending of neutrophils in bronchial lumen can be followed by recruitment /infiltration of the normal levels of eosinophils.

Thus, surprisingly, our primary hypothesis that impaired neutrophils may cause significant changes in eosinophils infiltration in bronchoalveolar lavage was not confirmed. This observation raises the hypothesis that neutrophil serine proteases may exert more subtle effects such a delayed cellular clearance in the inflammation site.

13 Curriculum Vitae

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BOOK CHAPTER CONTRIBUTION

- Standards of Mouse Model Phenotyping. Edited by Martin Hrabé de Angelis, Pierre Chambon, and Steve Brown © 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim ISBN: 3-527-31031-2

- Gene Knockout Protocols: Second Edition. Ralf Kühn, Wolfgang Wurst (eds.), vol. 530ª 2009 Humana Press, a part of Springer Science+Business Media DOI 10.1007/978-1-59745-471-1_25 Springerprotocols.com

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Javaheri A, Behrendt H, Jakob T and Ollert M. The German Mouse Clinic: Allergy Screen. **EUMORPHIA 3rd Annual Meeting – Barcelona/Spain February 2006**

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