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Nippostrongylus brasiliensis infection leads to the development of emphysema associated with the induction of alternatively activated macrophages

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Chronic obstructive pulmonary disease (COPD) is the 5th most prevalent disease worldwide leading to severe morbidity and mortality in developed countries. The disease is strongly associated with smoking, and can be characterized by progressive and irreversible deterioration in lung function and destruction of the lung parenchyma. We show here that infection with the hookworm *Nippostrongylus brasiliensis* results in deterioration in lung function, destruction of alveoli and long-term airways hyperresponsiveness, consistent with COPD and emphysema. *N. brasiliensis* infection leads to chronic low level hemorrhaging in the lung and the presence of hemosiderin-laden macrophages in the absence of an overt inflammatory infiltrate. Microarray analysis of gene expression in diseased lungs and quantitative RT-PCR analysis of purified macrophages revealed a state of prolonged tissue injury and the presence of alternatively activated macrophages producing MMP-12. Taken together, these data show that lung tissue damage caused by hookworm infection can result in the development of COPD and emphysema.

Key words: Chronic · Emphysema · Lung · Parasite · Th2

Introduction

Chronic obstructive pulmonary disease (COPD) is the 5th most prevalent disease worldwide [1]. Emphysema, a component of COPD, is characterized by the dilation of airspaces distal to the terminal bronchiole, due to a destruction and irreversible loss of alveolar septa resulting in airflow limitation [2]. The vast majority of individuals with emphysema have a history of smoking, however, only a small proportion of smokers develop emphysema [1]. Clearly, additional environmental and genetic factors have a substantial influence on disease development. Many mechanisms of disease development have been postulated, including the infiltration of inflammatory cells and an imbalance between proteases (such as elastase) and protease inhibitors. Indeed, analysis of lung lavage fluid from smokers and patients with emphysema has revealed an increased proportion of inflammatory cells such as macrophages and neutrophils, capable of secreting proteases [3, 4]. Data from experimental systems utilizing knockout mouse strains have implicated

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Abbreviations: **AHR:** airways hyperresponsiveness · **COPD:** chronic obstructive pulmonary disease · **HO-1:** heme-oxygenase-1 · **MetCh:** methacholine-chloride · **Nb:** *Nippostrongylus brasiliensis* · **NES:** Nb antigens · **PenH:** enhanced pause

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proteases including MMP-12 [5], and transgenic mice overexpressing cytokines such as IL-13 [6] and IFN-γ [7] have also been described to develop emphysema (in part through inducing expression of MMP). T cells have also been associated with emphysema, in particular $CD8^+$ T cells; however, their exact role in disease induction *versus* exacerbation is less clear. Similarly, eosinophils, typically associated with Th2 immune responses have been found in the lungs during exacerbations of COPD, but their role in disease development is unknown [3]. Whilst many of the effector mechanisms of emphysema have become known, it is still unclear how disease induction and progression are related.

Nippostrongylus brasiliensis (Nb) is a Helminth parasite against which a strong Th2 cell-mediated immune response develops, characterized by the presence of $CD4^+$ Th2 cells, eosinophils, mucus cell hyperplasia and IgE production by B cells [8, 9]. In contrast to typical allergic airway inflammation models, Nb larvae, similar to a number of human hookworm larvae [10], infect through the skin and migrate to the lung *via* the vasculature. After migrating through the vascular wall, the worms reach the large airspaces where they are coughed up and swallowed within 3–4 days [11]. Nb is not retained in the lung, nevertheless, a Th2 immune response characteristic of allergic airway inflammation, is sustained within the lung for an additional 14 days after the larvae have migrated out, possibly due to remaining allergens [12]. This infectious disease model has been widely used to unravel the key immunological factors that underlie Th2 immune responses. We recently made the surprising finding that even at very late time points after infection (>300 days), lungs from previously infected mice exhibited severe airways hyperresponsiveness. Histological analysis of the lungs showed significant structural changes to the airways had occurred resembling COPD and emphysema. The changes included a destruction of alveolar walls and consecutive enlargement of distal air spaces, resulting in a dramatic enlargement of lung size, as seen in some smoke-inhalation induced emphysema models as well as in human disease. Intriguingly, at late time points there was chronic active disease as evidenced by alveolar microhemorrhage, heme-oxygenase expression and hemosiderin deposition within macrophages. Quantitative real-time PCR and gene microarray analysis of lungs at late time points post infection also revealed the presence of alternatively activated macrophages and the up-regulation of the emphysema-related protease MMP-12. Taken together, these data show that lung tissue damage caused by hookworm infection can initiate a damage-repair process that results in the development of emphysema.

Results

Infection with *Nippostrongylus brasiliensis* induces chronic airway hyperresponsiveness

It has previously been established that the Th2 immune responses mounted against Nb in the lung typically lead to the development of airways hyperresponsiveness (AHR) [9]; however, the duration of such hypersensitivity is unclear. Thus, we infected mice with Nb and 14, 70, 100, or 300 (Fig. 1A–D) days later assessed airway hypersensitivity after methacholine challenge. As expected, on day 14 post infection, mice exhibited a severe dose-dependent increase in enhanced pause (PenH) upon methacholine challenge (Fig. 1A), which correlated with the peak of the inflammatory cell infiltrate into the airways (data not shown). At both 70 (Fig. 1B) and 100 (Fig. 1C) days post infection when the inflammatory cell infiltrate had essentially subsided, airway hypersensitivity remained high, indicating a chronic inflammatory state or irreversible damage following the initial response. By day 300 post infection mice exhibited severe difficulty in breathing upon exposure to low concentrations of methacholine (Fig. 1D) and were removed from the measurement chambers. Analysis of baseline PenH levels revealed a substantial change in the resting PenH of mice 70 days post infection, indicative of progressive lung damage (Fig. 1E). The airways hyperresponsiveness had Th2 characteristics as IL-13-deficient mice exhibited decreased AHR whilst IFN-γ-deficient mice exhibited exacerbated AHR (Fig. 1F).

Infection with *Nippostrongylus brasiliensis* leads to the development of pulmonary emphysema

We next sought to establish whether Nb infection affected the structure of the lung. Accordingly, lungs were inflated at fixed pressure with formalin and removed en bloc. The lungs of mice previously infected with Nb were dramatically larger than their uninfected age-matched counterparts (Fig. 2A). Histological analysis showed that mice previously infected with Nb had developed massive emphysema with dilation of distal airspaces due to loss of alveolar septa, which increased in severity at late time points post infection (Fig. 2B). Hemosiderin-laden macrophages within alveolar walls were enlarged and intraalveolar macrophages were increased in number. Inconspicuous focal peribronchial inflammatory infiltrates, consisting mainly of plasma cells or macrophages were present (data not shown) and bronchioles and bronchi showed goblet cell hyperplasia (Fig. 2C). Of note, emphysema similarly developed when the number of Nb injected subcutaneously was reduced to 250 Nb (data not shown). Mice on a C57BL/6

genetic background developed emphysema (data not shown), albeit less pronounced, indicating that BALB/c mice have some genetic predisposition to the development of this disease.

Exposure to Nb antigens leads to long-term airways hyperresponsiveness but not emphysema

We next assessed whether Nb antigens (NES) alone were sufficient to induce long-term AHR and emphysema, or whether infection with live Nb was required. Accordingly, we either infected mice with Nb or administered multiple rounds of NES intranasally and measured AHR on day 30. Notably, NES administration alone was sufficient to induce AHR (Fig. 3A), although no change in baseline PenH was detectable at any time point examined (data not shown). Histological analysis of NES-treated lungs revealed that no major structural changes had occurred (Fig. 3B–E), suggesting that indeed initial tissue damage during Nb infection was required to initiate emphysema development.

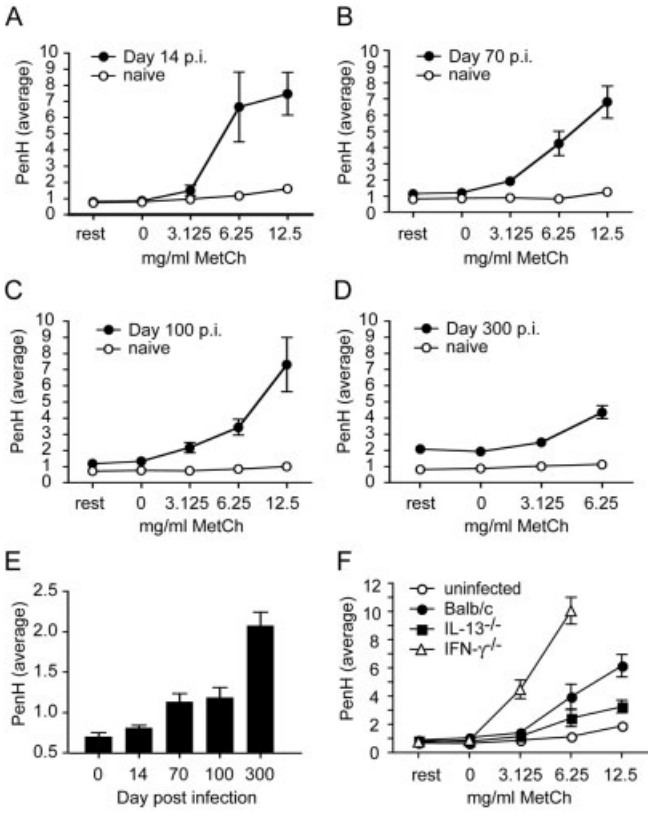

Figure 1. Infection with *Nippostrongylus brasiliensis* induces long-term airways hyperresponsiveness and lung dysfunction. Mice were infected with Nb and on days 14 (A), 70 (B), 100 (C) and 300 (D), airways hyperresponsiveness upon challenge with increasing concentrations of MetCh was assessed using a whole-body unrestrained plethysmograph. Filled circles represent individual infected mice; open circles represent naive age matched controls. (E) Baseline PenH measurements from experiments in (A–D). Average PenH on day 0 post infection represents the average resting PenH ± SD from all naive controls in (A–D). (F) Mice of the indicated genotypes were infected with Nb and on day 30 post infection airways hyperresponsiveness upon challenge with MetCh was assessed. Measurements of IFN-$\gamma^{-/-}$ mice were stopped at 6.25 mg/mL MetCh due to the severe response that developed.

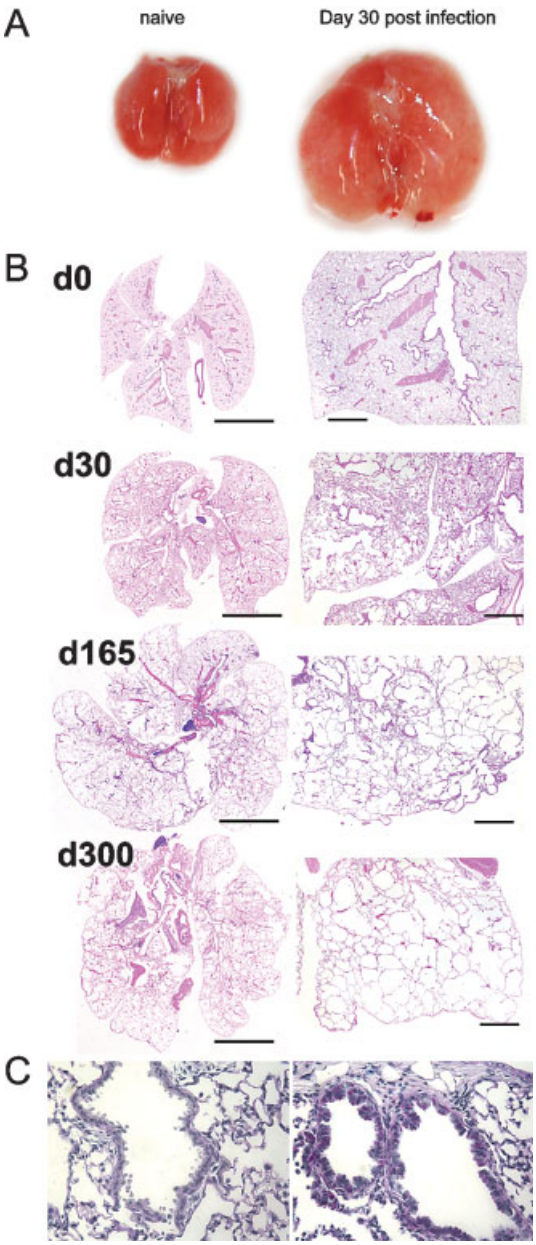

Figure 2. Infection with *Nippostrongylus brasiliensis* leads to the development of emphysema. Mice were infected with Nb, on d30 post-infection, and lungs were removed. (A) Photographs showing gross increase in lung size following Nb infection. (B) Representative microphotographs from H&E-stained lung sections at the indicated time points post infection. Scale bars are 5 and 1 mm on the left and right columns, respectively. (C) Goblet cell hyperplasia (40× magnification) as determined by Alcian Blue and PAS staining was evident on day 30 post infection (right panel) but not on day 0 (left panel).

Long-term hemorrhaging and alternative macrophage activation following infection with *Nippostrongylus brasiliensis*

The presence of hemosiderin-laden macrophages in the lungs was an indicator of recent phagocytosis of erythrocytes, and thus by proxy, hemorrhaging. We detected such macrophages at early time points post infection, soon after Nb had migrated through the tissue, and notably, hemosiderin-laden macrophages were still detected over a year after infection (Fig. 4A and data not shown), suggesting that Nb infection initiates a chronic damage process. Focal intraalveolar microhemorrhage was seen in diseased lungs. This microhemorrhage was associated with the detection of intracytoplasmic erythrocytes in alveolar wall and peribronchial macrophages (Fig. 4B), indicative that this microhemorrhage was not an artifact and had occurred before the mice were sacrificed. Indeed, heme-oxygenase-1 (HO-1) expression was detected in whole-lung tissue at multiple time points by quantitative real-time PCR (Fig. 4C) and immunohistochemical staining for HO-1 revealed its expression was primarily restricted to alveolar wall and peribronchial macrophages (Fig. 4D).

We next sorted macrophages from the lungs of previously infected mice, and found that the population of $Mac3^{hi}$ $CD11b^{hi}$ cells stained positive for hemosiderin (Fig. 4E). Quantitative real-time PCR analysis of these cells also revealed that the hemosiderin positive macrophages had an alternatively activated (AAM) phenotype as shown by the preferential induction of arginase as opposed to iNOS (Fig. 4F). Macrophages exhibiting this AAM phenotype were found in the lung later than 100 days post infection (data not shown). A number of differences in chemokine expression have been described between classical and alternatively activated macrophages [13–15]. To further characterize the $Mac3^{hi}$ $CD11b^{hi}$ hemosiderin-laden macrophages, we assessed CCL5, CXCL11 and CCL17 chemokine expression levels in sorted cells. In line with their putative AAM phenotype, the $Mac3^{hi}$ $CD11b^{hi}$ hemosiderin-laden macrophages preferentially expressed CCL17, whilst the $Mac3^{lo}$ $CD11b^{hi}$ cells expressed CCL5 and CXCL11 (Fig. 4G). We next assessed whether Nb-induced AAM produced the emphysema-related protease, MMP-12 and found substantial up-regulation of MMP-12 mRNA in purified macrophages at day 14 post infection (Fig. 4H). Analysis of MMP-12 expression in whole-lung samples revealed that it was rapidly up-regulated after infection, peaking around day 14 and remaining substantially above baseline levels for more than 150 days (Fig. 4I and data not shown).

Microarray analysis of *Nippostrongylus brasiliensis*-induced emphysematous lungs

To gain better insight into the extent to which Nb infection modified gene expression in these diseased lungs, we performed gene microarray analysis of diseased and normal lungs (Fig. 5). Filtering on genes with a statistically significant ($p < 0.05$) threefold differential expression revealed that 56 genes were distinctly expressed between the sample groups. Of these 56 genes, 13 belonged to the immunoglobulin heavy chain family (data not shown). In line with our quantitative RT-PCR analysis, a clear increase in alternatively activated macrophage genes was found in the diseased lungs including Retnla (*fizz1)* and *Chi3l3 (ym1)* and MMP-12 expression (Fig. 5A and B). The emphysematous tissue also presented a novel chemokine/chemokine receptor fingerprint showing significantly up-regulated expression of CCL9, CCL6, CXCL13,

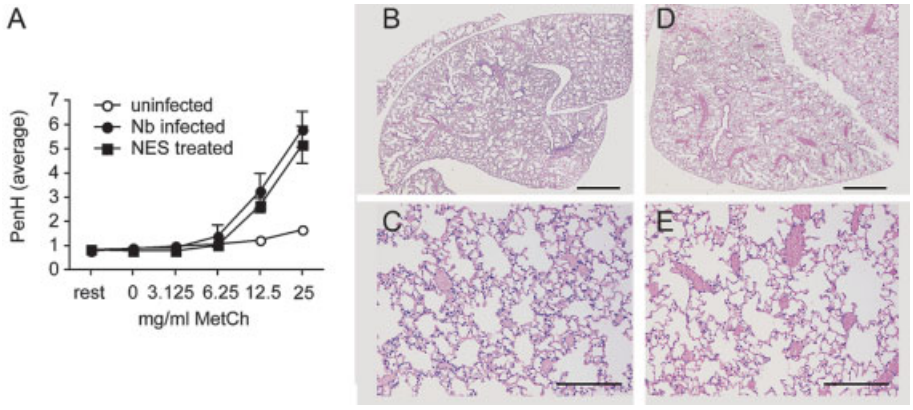

Figure 3. Exposure to *Nippostrongylus brasiliensis* antigens leads to long-term airways hyperresponsiveness but not emphysema. (A) Mice were infected with Nb or administered with NES on 4 consecutive days. On day 30 post infection airways hyperresponsiveness upon challenge with increasing concentrations of MetCh was assessed using a whole-body unrestrained plethysmograph. Representative microphotographs of (B, C) naive or (D, E) NES-treated lungs at day 30 post infection. Scale bars in (B, D) and (C, E) are 1 and 0.2 mm, respectively.

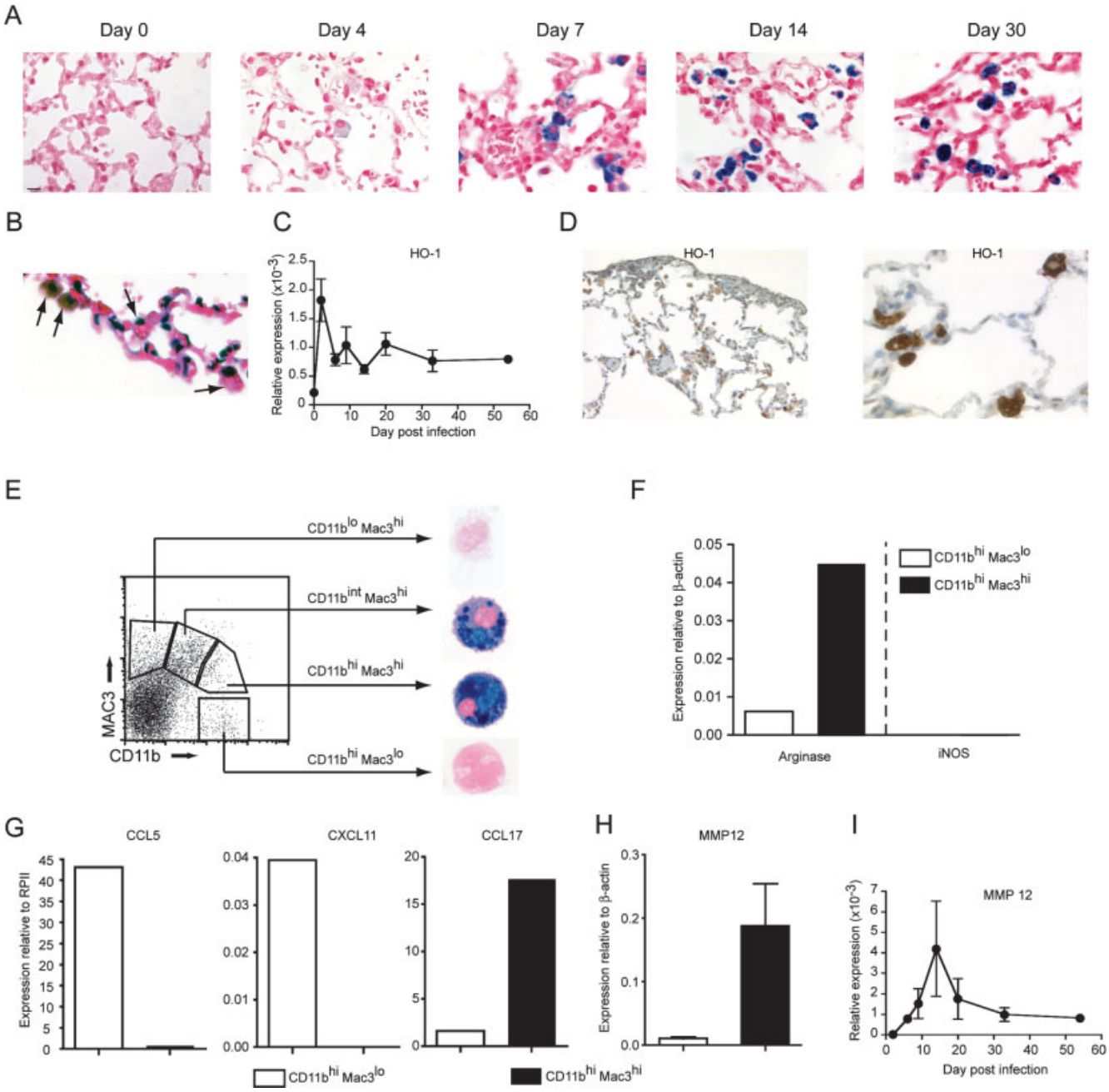

Figure 4. Hemosiderin-laden alternatively activated macrophages accumulate in the lungs after infection with *Nippostrongylus brasiliensis*. (A) Mice were infected with Nb and at the indicated time points lungs were removed, fixed and stained to allow identification of hemosiderin-laden macrophages. (B) Recently phagocytosed erythrocytes were evident in H&E-stained macrophages (indicated by arrows). (C) HO-1 expression relative to β-actin was determined at the indicated time points. (D) Protein expression on HO-1 in macrophages was confirmed by direct staining for HO-1 expression on lung tissue samples 40 days post infection. On day 14 post infection, macrophages from whole-lung homogenates were sorted based upon CD11b and Mac3 expression by flow cytometry. Sorted cells were (E) stained for hemosiderin; (F) assessed for expression of arginase and iNOS (G) CCL5, CXCL11 and CCL17 by quantitative RT-PCR. (H) MMP-12 production by sorted cells was determined by quantitative RT-PCR. (I) At the indicated time points post infection lungs were removed and whole tissue MMP-12 transcripts were measured by quantitative RT-PCR.

CXCR6, CCL8 and CXCL5 (Fig. 5C) – chemokines/receptor primarily associated with the recruitment of macrophages, B cells, and granulocytes.

MMP-12- and Th2-associated genes do not mediate *Nippostrongylus brasiliensis*-induced emphysema

Taken together, our results showed that infection with the Helminth Nb could initiate a process with a profound influence on lung function and structure. This process is associated with Th2 cytokines and the production of MMP-12. To ascertain whether these factors influenced the development of emphysema, we infected BALB/c, IL-4R-deficient, IL-5R-deficient, IL-13-deficient, IFN-γ-deficient and MMP-12-deficient mice with Nb (Fig. 6). Thirty days post infection, when emphysema is clearly detectable in BALB/c mice, lungs were inflated at fixed pressure with formalin and removed en bloc. As expected, wild-type BALB/c mice exhibited severe emphysema; however, this was the case with all of the knockout mice examined. Thus, in contrary to the development of AHR (Fig. 1), none of these factors played a major role in Nb-induced emphysema development.

Discussion

The gross characteristics of emphysema disease models share many similarities, despite distinct etiological mechanisms. The disease can be induced through smoke-inhalation [5, 16], genetic modification [6, 7], protease administration [17, 18] and now we show that infection with the hookworm, Nb, is also capable of starting the process that results in this chronic disease. Interestingly, Th2-mediated allergic airway inflammatory responses are sufficient to induce airways hyperresponsiveness, but alone are not sufficient to drive alveolar wall damage, which results in emphysema (Fig. 3). This observation suggests that Nb infection results in early tissue damage that activates an

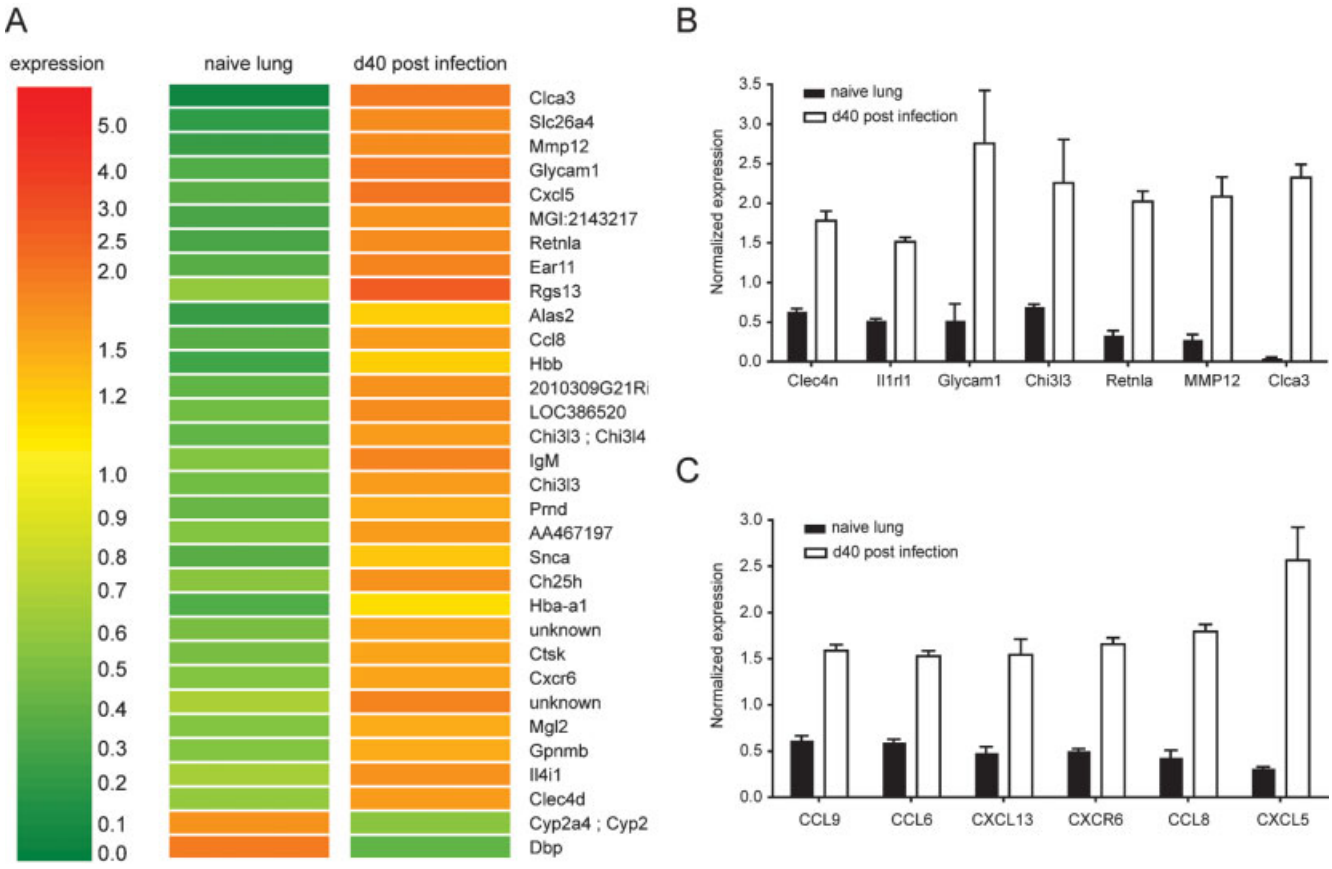

Figure 5. Gene microarray analysis of diseased lungs shows the accumulation of alternatively activated macrophage markers and sustained chemokine expression. Mice were infected with Nb, on day 40 post infection lungs were removed, and whole-tissue gene expression was assessed utilizing Affymetrix GeneChip microarray's. (A) Overview of genes significantly differentially expressed threefold between naive and diseased lungs (excluding immunoglobulin heavy chain genes). (B) T helper type 2 cell/macrophage normalized gene expression. (C) Chemokine and chemokine receptor normalized gene expression. Data are compiled from individual Affymetrix GeneChip microarray's of five lungs per group.

inflammatory cascade leading to long-term tissue damage that is associated with continued microhemorrhage and macrophage activation. Notably, this active process was seen in the absence of prominent lymphocytic inflammation, the cytokines IL-4, IL-5, IL-13, IFN-γ and the protease MMP-12. Moreover, treatment with anti-Helminth drugs excluded the possibility that chronic infection was responsible for the lung pathology (data not shown). Whilst each of these factors has been implicated in either Th2 responses, or the development of emphysema, individually they do not appear to be critical in this model; rather, physical damage by worm migration and an ensuing dysregulated repair mechanism appears to be a more plausible explanation.

The inflammatory response that developed during infection was acute, peaking around day 14 post infection, and subsiding by day 21 post infection, whilst the COPD/emphysema development appeared to slowly continue given that mice exhibited strikingly increased baseline breathing patterns at very late time points point infection (*i.e.* day 70+). These data are at odds to the conclusions drawn by Reece *et al.* [19], who reported that mechanical damage and hemorrhaging are rapidly resolved following Nb infection. Indeed, the early innate response and massive hemorrhaging that occurs immediately following Nb infection is largely resolved within 12 days, however, they did not examine later time points. Using a similar infection regime, our data at later time points (days 30–300 post infection) show that in fact the consequences of Nb infection to lung tissue are chronic and progressive. Microhemorrhage and both HO-1-positive macrophages and hemosiderin-laden macrophages were detectable in diseased lungs at very late time points post infection, indicating that the chronic destructive process was active long after the lung passage of the worms and in the absence of strong inflammation. It remains unclear whether these macrophages are actively involved in the tissue damage, or are merely indicative of ongoing damage. Alveolar microhemorrhage due to alveolar septal damage, followed by erythrophagocytosis by macrophages and possible macrophage activation may support this apparently self-perpetuating process resulting in progressive emphysema; however, further studies are needed to test this hypothesis.

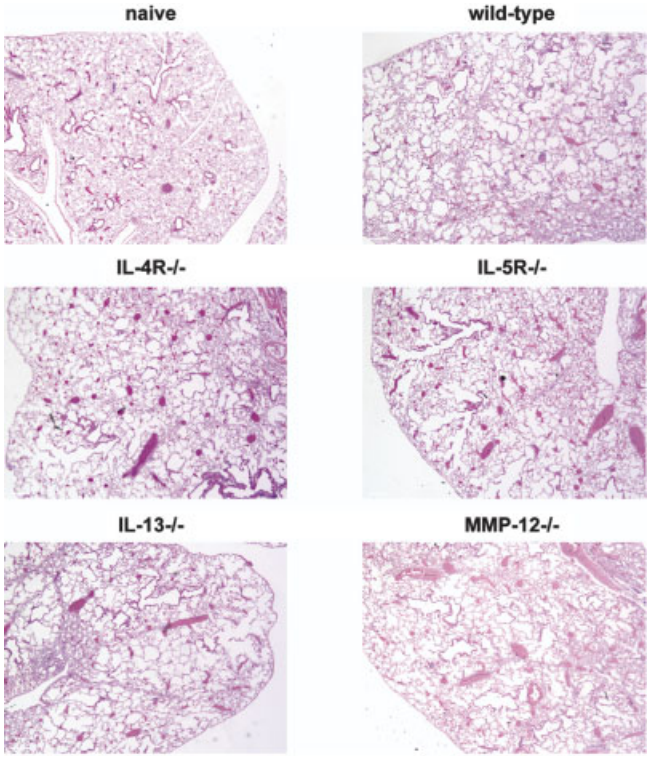

Figure 6. MMP-12 and Th2 associated genes do not cause emphysema development after infection with *Nippostrongylus brasiliensis*. Mice were infected with Nb and on day 40 post infection lungs were fixed under pressure and removed en bloc. Representative microphotographs from H&E-stained lung sections from mice on a BALB/c background of the indicated genotype (2.5× magnification).

One possibility is that antigen depots remain long-term in the lung, sustaining antigen-specific inflammatory infiltrates. Whilst plausible, this argument is weakened by the absence of a sizable lymphocytic inflammatory infiltrate at late time points after infection. The mechanism for the late phase disease progression remains to be elucidated, but may represent a self-perpetuating damage-repair cycle. Interestingly, such hemosiderin-laden macrophages are not present in the lungs of influenza-infected mice (data not shown), or during chronic allergen exposure despite strong inflammatory infiltrates. Moreover, prolonged administration of NES induces long-term AHR but does not lead to the development of emphysema, suggesting that late phase hemorrhage in lung tissue may flow on from the initial physical damage induced by worm migration through the lung tissue [12] (data not shown).

The hemosiderin-laden macrophages presented an alternatively activated phenotype, as shown by preferential expression of arginase over iNOS. Alternative macrophage activation is driven primarily through the presence of IL-4 although IL-13 and IL-21 have also been implicated in their differentiation [20, 21]. Infection by Nb induces a strong Th2 immune response and is known to activate alternative macrophages [19]; however, their long-term presence in the airways suggests that additional mechanisms flowing on from the original infection may sustain their renewal and differentiation. Notably, microarray analysis of lungs from smoke-induced emphysema [22] show a similar pattern of gene expression to that described here; specifically, the up-regulation of alternatively activated macrophage markers including FIZZ, YM1, and AMcase and the protease

MMP-12. It has been proposed that alternatively activated macrophages may play a role in tissue repair and dampening down responses, and thus their presence in these models of emphysema might be explained by the host-tissue repair response. Given the chronic nature of the tissue damage, it is tempting to speculate that such macrophages might in fact perpetuate the disease, although further studies are needed to test this possibility. Notably, plasma cells have also been reported to express markers typically associated with alternative macrophages; however, their role in tissue remodeling has yet to be revealed [23].

Filarial parasite infection can cause tropical pulmonary eosinophilia, which is associated with coughing/wheezing, asthma-like symptoms, and development of interstitial fibrosis in untreated cases [24]. However, such filarial parasite infections have not been linked to the development of emphysema, possibly because they do not migrate through the lung tissue causing damage, but rather are bound by specific antibodies and deposited in the pulmonary vasculature, becoming the focus of granulomas rich in eosinophils. Considering acute immune responses against non-migratory live parasites or the presence of immunogenic parasite-derived products in the lung is not sufficient to induce emphysema, it is likely that a critical factor for the induction of emphysema in our model is early tissue damage.

What are the factors that drive the development of emphysema? This model of Helminth-induced emphysema appears to be initiated after physical damage caused by Nb migration through the tissue. It is plausible that similar acute damage caused by other mechanical stresses may also lead to irreversible lung damage and potentially emphysema. Smoking, a leading cause of emphysema, may support ongoing lung damage, but alone appears insufficient to start a self-sustaining inflammatory cascade driving tissue damage, as stopping smoking is proposed to also limit disease progression. Furthermore, a relatively minor proportion of smokers go on to develop emphysema. Similarly, chronic bronchitis or acute viral/bacterial infections do not typically lead to the development of emphysema.

Taken together, the development of emphysema appears multifaceted: genetic predisposition and stress/physical damage provide a platform upon which subsequent inflammatory responses may lead to dysregulated repair mechanisms and chronic lung pathology.

Materials and methods

Mice and infection

BALB/c wild-type mice were obtained from Charles River (Germany) and maintained specific-pathogen free in BioSupport (Zurich) animal facility in isolated ventilated cages. Animals used in experiments were between 8 and 10 weeks of age. *N. brasiliensis* was maintained by passage through Lewis rats. Mice were infected subcutaneously with 550 live Nb L3 worms and sacrificed at the indicated time points. IL-13-deficient mice were kindly provided by Frank Brombacher with permission from A. McKenzie [25]. All animal experimental procedures were approved by the Zurich animal ethics committee.

Preparation and administration of NES

L3 infective *N. brasiliensis* larvae were isolated from fecal cultures, washed five times in sterile PBS then a further five times in an antibiotic cocktail (RPMI + 1/20 Penicillin Streptamycin + 1/100 Gentamycin (Gibco BRL, Invitrogen)). After the final wash, larvae were incubated for 60 min at room temperature in the antibiotic cocktail. Larvae were then incubated for 48 h at 37°C in RPMI 1640 (RPMI + 1/100 Penicillin Streptamycin + 1/100 Gentamycin) supplemented with 1% glucose. The supernatant was concentrated (Vivascience, Sartorius) and amount of protein NES determined by absorbance at 280 nm. NES were filter-sterilized and frozen in aliquots at –20°C until required. Mice were anesthetized and administered 5 μg of NES in a 50-μL volume intranasally on four consecutive days.

Measurement of airway responsiveness

At the indicated time point, mice were placed in individual unrestrained whole-body plethysmograph chambers (Buxco Electronics, Petersfield, UK). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methacholine-chloride (MetCh). This procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh (Aldrich Chemie, Steinheim, Germany) and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

Flow cytometry

Cells from lung tissue were washed with PBS/0.1% BSA and incubated with anti-CD32/CD16 mAb for 30 min at 4°C to block Fc binding. After another washing step, cells were stained with PE-labeled anti-MAC3 and APC-labeled anti-CD11b (BD PharMingen) for 15 min at 4°C. Subsequently, cells were washed with PBS/0.1% BSA and sorted using a FACSVantage SE (Becton Dickinson).

Histological preparation of tissue

Mice were sacrificed at the indicated time points, and 4% neutral buffered formalin was instilled *via* the trachea at a constant pressure of 20 cm of water. After fixation and paraffin embedding, lung tissues were processed for Alcian Blue and PAS, hematoxylin and eosin, or Prussian Blue staining for hemosiderin following standard histological techniques. HO-1 staining was performed with a polyclonal goat-anti heme-oxygenase-antiserum; 1:250 dilution; (Stressgen, Ann Arbor, MI). Stained sections were assessed using an Axioplan 2 microscope (Zeiss, Germany) Retiga EXi camera (Qimaging, UK) and Openlab software (Improvision, Coventry, UK).

Assessment of RNA levels

Whole lungs were perfused with PBS and homogenized. For cell sorting experiments, filtered lung homogenates were stained with CD11b-APC and MAC3-PE and sorted by flow cytometry. RNA was prepared with TRI-Reagent (Molecular Research Center) and treated with DNase (Invitrogen) to avoid genomic DNA contamination before RNA was reverse transcribed to cDNA (Invitrogen). Gene expression was quantified by real-time PCR (I-cycler, Bio-Rad) and samples were normalized to expression levels of the indicated house-keeping genes. Primers used were: RPIIf 5′-GCT TGG TTT AAT CCC CCT CA-3′; RPIIr 5′-CTT CAT TGC ACC TCA CAT CG-3′; β-actin rtF: 5′ CTT TTC ACG GTT GGC CTT AG 3′; β-actin rtR: 5′ CCC TGA AGT ACC CCA TTG AAC 3′; HO-1 rtF: 5′ TGC TCG AAT GAA CAC TCT GG 3′; HO-1 rtR: 5′ TCC TCT GTC AGC ATC ACC TG 3′; MMP12 rtF: 5′ TCC AAG TCT GGA GTG ATG TGA 3′; MMP12 rtR: 5′ TGT ACC ACC TTT GCC ATC AA 3′ ; CCL5 rtF; 5′ CGC ACC TGC CTC ACC ATA-3′; CCL5 rtR; 5′ CTG CAA GAT TGG AGC ACT TG 3′; CXCL11 rtF; 5′ GGA TGA AAG CCG TCA AAA TG 3′; CXCL11 rtR; 5′ CCA GGC ACC TTT GTC GTT TA 3′; CCL17 rtF; 5′ CAG GAA GTT GGT GAG CTG GT 3′; CCL17 rtR; 5′ TGG CCT TCT TCA CAT GTT TG 3′.

cDNA preparation

The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260 nm/280 nm ratio between 1.8-2.1 and a 28S/18S ratio within 1.5-2 were further processed. Total RNA samples (2 μg) were reverse-transcribed into double-stranded cDNA, *in vitro* transcribed in presence of biotin-labeled nucleotides using a IVT Labeling Kit (Affymetrix, P/N 900449, Santa Clara, CA), purified and quantified using BioRobot Gene Exp – cRNA Target Prep (Qiagen, Switzerland). The labeled cRNA quality was determined using Bioanalyzer 2100. Array hybridization biotin-labeled cRNA samples (15 μg) were fragmented randomly to 35–200 bp at 94°C in Fragmentation Buffer (Affymetrix, P/N 900371) and were mixed in 300 μL of Hybridization Mix (Affymetrix, P/N 900720) containing a Hybridization Controls and Control Oligonucleotide B2 (Affymetrix, P/N 900454), before hybridization to GeneChip® Mouse Genome 430 2.0 arrays for 16 h at 45°C. Arrays were then washed using an Affymetrix Fluidics Station 450 FS450_0001 protocol. An Affymetrix GeneChip Scanner 3000 (Affymetrix) was used to measure the fluorescent intensity emitted by the labeled target. Statistical analysis raw data processing was performed using the Affymetrix GCOS 1.4 software (Affymetrix). After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS5 algorithm [26]. To compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to target intensity (TGT value) of 500 as detailed in the statistical algorithms description document of Affymetrix (2002). Quality control measures were considered before performing the statistical analysis. These included adequate scaling factors (between 1 and 3 for all samples) and appropriate numbers of present calls calculated by application of a signed-rank call algorithm [27]. The efficiency of the labeling reaction and the hybridization performance was controlled with the following parameters: present calls and optimal 3′/5′ hybridization ratios (around 1) for the house-keeping genes (GAPDH and ACO7), for the poly A spike in controls and the prokaryotic control (BIOB, BIOC, CREX, BIODN).

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