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Chapter 10

Osteopontin is not crucial to protective immunity during murine tuberculosis

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Abstract

Upon *Mycobacterium (M.) tuberculosis* infection, the development of a strong Th1 mediated adaptive immune response is considered most important for the containment of the infection. Osteopontin (OPN) is a phosphorylated glycoprotein that is chemotactic for inflammatory cells and has been implicated in the induction of Th1 responses and granulomatous disease. We tested the hypothesis that OPN facilitates protective immunity during *M. tuberculosis* infection using wild-type (WT) and OPN knockout (KO) mice in a model of pulmonary tuberculosis. OPN expression was up-regulated in alveolar macrophages and lymphoid cells during *M. tuberculosis* infection. There were no significant differences in bacterial outgrowth, inflammation or recruitment of lymphocytes, macrophages and polymorphonuclear cells in the lungs after 2 and 5 weeks of infection. However, percentages of CD4⁺ and CD8⁺ T cells were reduced in the absence of OPN 5 weeks after infection. Cytokine concentrations appeared similar in lungs from both mouse strains, however, there was a trend towards decreased levels of IFN- γ in OPN KO mice 5 weeks after infection. Despite an unaltered immune response in the early phase of tuberculosis, OPN KO mice had a modest survival advantage. Of note, both pulmonary bacterial loads and lung inflammation were reduced in these mice 31 weeks after infection. These data suggest that OPN is not crucial to protective immunity upon *M. tuberculosis* infection and during the late phase of tuberculosis it may even be detrimental for the host.

Introduction

Mycobacterium (M.) tuberculosis and its associated disease tuberculosis is a serious threat to mankind. One-third of the world's population is infected with the tubercle bacillus and tuberculosis is responsible for two million deaths each year (1, 2). Upon infection with this pathogen healthy individuals develop a strong T-helper 1 (Th1) response which is able to contain the infection in granulomas and prevent active disease. However, *M. tuberculosis* bacilli are not eradicated from the lungs and remain a potential danger to the infected individual (2).

Osteopontin (OPN) is a phosphorylated glycoprotein, expressed by a broad range of tissues and cells, which is involved in a number of physiological and pathological processes. Originally considered a bone matrix protein, OPN is now known to regulate inflammation, tissue remodeling and cell survival (3-5). For example, OPN acts as a chemotactic factor for T-cells, macrophages and neutrophils and modulates the function and differentiation of these inflammatory cells (5-7). Importantly, ample evidence indicates that OPN can stimulate Th1 responses: interaction of OPN with one of its receptors, $\alpha_v\beta_3$ integrin, on mouse peritoneal macrophages induces interleukin (IL)-12, a cytokine that drives Th1 responses, whereas in the same cells interaction of OPN with another receptor, CD44, prevents lipopolysaccharide induced production of the Th2 cytokine IL-10 (8). Moreover, O'Regan et al. have shown that T-cell dependent IL-12 production by human peripheral blood mononuclear cells is enhanced by OPN, in part via its ability to regulate CD3-induced expression of interferon (IFN)- γ and CD40L by T-cells (9). Several *in vivo* studies have also supported the involvement of OPN during Th1 responses. For example, antigen-specific re-stimulation of draining lymph node cells from OPN knockout (KO) mice revealed enhanced levels of IL-10, but reduced levels of IL-12 and IFN- γ as compared to wild-type (WT) mice in several Th1 models, including experimental autoimmune encephalomyelitis, injection of polyvinyl pyrrolidone, and infection with herpes simplex virus-1 (8, 10). Furthermore, OPN appeared to be essential for elimination of the malaria parasite *Plasmodium chabaudi chabaudi*, since OPN KO mice showed lower levels of IL-12 and IFN- γ , and died from nonlethal infection in contrast to WT mice (11).

Considering its role in Th1 responses and cellular migration, it can be anticipated that OPN plays a role in granulomatous inflammation. Indeed, *Schistosoma mansoni* egg induced pulmonary granulomas contain OPN expressing macrophages, and in OPN KO mice granuloma size was reduced and macrophage content diminished (12). In addition, polyvinyl pyrrolidone injection was shown to induce an intense

granulomatous response in WT mice, whereas in OPN KO mice such a response could not be detected. Interestingly, T cell deficient mice displayed a granulomatous reaction only when OPN was coinjected with polyvinyl pyrrolidone, demonstrating that OPN can partially substitute for activated T-cells in this setting (8). A role for OPN during mycobacterial infection is supported by *in vitro* experiments showing that both human and murine alveolar macrophages enhance OPN mRNA expression upon infection with either *M. bovis* bacillus Calmette-Guérin (BCG) or *M. tuberculosis* (13). Furthermore, gene profile screening of *M. tuberculosis* resistant and sensitive macrophages infected with *M. bovis* BCG revealed that the OPN gene was highly expressed in resistant macrophages as compared to sensitive macrophages. Resistance to *M. tuberculosis* was significantly increased in sensitive macrophages during *M. bovis* BCG infection when macrophages were stimulated with OPN (14). *In vivo*, *M. bovis* BCG infection resulted in an increase in granuloma number and size in OPN KO mice, which was associated with a reduced clearance of *M. bovis* BCG, due to defective killing by OPN KO macrophages (15). Patients suffering from diverse granulomatous diseases including tuberculosis, silicosis and sarcoidosis displayed pulmonary OPN expression in association with granulomas (13, 16, 17). A possible protective role for OPN in mycobacterial infection was further supported by the observation that the extent of OPN protein expression in pathological lymph nodes from *M. bovis* BCG or *M. avium* infected patients was inversely correlated with disseminated infection and death (18). In addition, plasma OPN concentrations were found to be increased in pulmonary tuberculosis patients as compared to healthy controls, and to decline after successful anti-tuberculosis therapy (19, 20).

In spite of the abundant literature pointing to an important role for OPN in the immune response to tuberculosis, to the best of our knowledge the function of OPN during infection with *M. tuberculosis in vivo* has thus far not been directly investigated. Therefore, in the present study we sought to compare the course of the infection and the accompanying host response in WT and OPN KO mice after intrapulmonary delivery of viable virulent *M. tuberculosis*.

Materials & Methods

Mice

Pathogen-free 8 to 10 week old WT C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). OPN KO mice, backcrossed ten times to a C57BL/6 genetic background, were obtained from the Jackson Laboratories

(Bar Harbor, ME) and bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Experimental infection

A virulent laboratory strain of *M. tuberculosis* (Erdman) was grown in liquid Dubos medium containing 0.01% Tween-80. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed with sterile 0.9% NaCl. Tuberculosis was induced as described previously (21, 22). Briefly, mice were anesthetized by inhalation with isoflurane (Abott Laboratories LTD., Kent, United Kingdom) and infected intra-nasally with 150 live *M. tuberculosis* Erdman bacilli in 50 µl saline, as determined by viable counts on Middlebrook 7H11 agar plates. Groups of mice were sacrificed 1 day (n = 3 per strain) or 2 or 5 weeks (both n = 8 per strain) after infection; in addition, 15 mice per strain were followed for 31 weeks, after which the survivors were killed and their organs processed as described further. Lungs and liver were removed aseptically, weighed and homogenized in 5 volumes of sterile 0.9% NaCl. Ten-fold dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days incubation at 37°C. Numbers of CFU are provided per g of lungs.

Lung histology

Lungs were removed 2, 5 or 31 weeks after inoculation with *M. tuberculosis*, fixed in 10% buffered formaline for 24 h, and embedded in paraffin. Hematoxylin and eosin stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, oedema, granuloma formation and pleuritis by a pathologist blinded for groups. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of inflamed lung surface. For OPN staining the slides were deparaffinised and endogenous peroxidase activity was quenched by a solution of methanol/0.03% H₂O₂ (Merck, Darmstadt, Germany). After digestion with a 0.1 M sodium citrate solution, pH 6.0, non-specific binding was blocked with 10% normal swine serum (Dako, Glostrup, Denmark) and slides were then exposed to goat anti-OPN antibody (R&D Systems, Minneapolis, MN). After washes, slides

stained for OPN were incubated with a horseradish peroxidase-labeled swine anti-goat IgG antibody (Biosource International, Camarillo, CA) in the presence of 5% normal mouse serum (Sigma, St. Louis, MO). Slides were finally developed using 3,3'-diaminobenzidine-tetra-hydrochloride peroxidase substrate (Sigma) and slides were counterstained with methyl green. The sections were mounted in glycerine gelatine. As a negative control we used a WT lung (5 weeks after infection) without addition of the primary antibody.

Flow cytometry

Lung cell suspensions were obtained by crushing lungs through a 40- mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described previously (23, 24). Erythrocytes were lysed using ACK lysing buffer (Bio Whittaker, Verviers, Belgium); the remaining cells were washed twice with RPMI 1640 (Gibco, Life Technologies, Rockville, MD), and counted by using a hemocytometer. In addition, cells were brought to a concentration of 1×10^7 cells per mL of FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3 and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 minutes at 4°C using directly labeled antibodies against CD3 (CD3-phycoerythrin), CD4 (CD4-allophycocyanin), CD8 (CD8-peridinin chlorophyll protein) or GR-1 (GR-1-FITC). All antibodies were used in concentrations recommended by the manufacturer (BD Pharmingen, San Diego, CA). After staining, cells were fixed in 2% paraformaldehyde, and determined using flow cytometric analysis using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Percentages of polymorphonuclear cells (PMNs), macrophages and lymphocytes were determined using GR-1 expression (GR-1 high, intermediate and low, respectively) and T cell surface proteins were analyzed on CD3⁺ cells within the lymphocyte gate.

Assays

For cytokine measurements, organ homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl_2 , 2 mM CaCl_2 , 1% Triton X-100, 4-(2-aminoethyl)benzenesulfonyl fluoride (4 ug/ml), EDTA- Na_2 (50 ug/ml), pepstatin (10 ng/ml), and leupeptin (10 ng/ml; pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at $1,500 \times g$ at 4°C for 15 min, and supernatants were sterilized using a 0.22 mm filter (Corning Incorporated, Corning, NY) and stored at -20°C until assays were performed. Homogenates and supernatants IFN- γ , IL-4, IL-12p70, TNF- α , IL-10, IL-6, keratinocyte-derived cytokine (KC) and MIP-2 were

measured by ELISA using matched antibody pairs according to the manufacturer's instructions (R&D Systems).

Splenocyte stimulation

Single cell suspensions were obtained by crushing spleens through a 40 µm cell strainer as described previously.(25) Erythrocytes were lysed using ACK lysing buffer, the remaining cells were washed twice with RPMI 1640 supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic (Gibco). Cells were seeded in 96-well round bottom culture plates at a cell density of 1×10^6 cells per well in quadruplicate, and stimulated with 20 µg/ml tuberculin purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after 48 h incubation at 37°C in 5% CO₂, and cytokine levels were analyzed by ELISA.

Delayed-type hypersensitivity response to PPD

To measure delayed-type hypersensitivity (DTH) responses, we examined the swelling of footpads according to previously described methods (25). Briefly, WT and OPN KO mice (n = 8 mice per group) were immunized intra-dermally at the base of the tail with 0.1 mg of heat-killed *M. tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in 0.1 ml of mineral oil (Sigma-Aldrich). Thirteen days after immunization, mice were challenged with 20 µg PPD in saline into one of the hind footpads and in the other with saline alone. Measurements of footpad thickness were performed with a Mitutoyo model 7326 engineer's micrometer (Mitutoyo MTI Corporation, Aurora, IL) before, and 24 and 48 h after the PPD challenge. The increase in footpad thickness was calculated as the difference in swelling between 0- and 24- or 48-h measurements. Specific DTH reactivity was calculated as the difference between the swelling of the PPD-injected footpads and the swelling of the saline-injected footpads. 48 h after PPD challenge footpads were fixed in 10% buffered formaline for 48 h, decalcified in 15% EDTA for 4 weeks, embedded in paraffin, cut and stained with hematoxylin and eosin.

Statistical analysis

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

Results

Osteopontin expression during infection

To determine whether OPN is present in the lung during *M. tuberculosis* infection, OPN expression was determined in lungs from WT mice before, 2 and 5 weeks after infection by immunohistochemistry. Whereas in uninfected lungs no OPN immunoreactivity could be detected, at both 2 and 5 weeks after infection, alveolar macrophages, and to a lesser extent lymphoid cells, specifically expressed OPN (Figure 1).

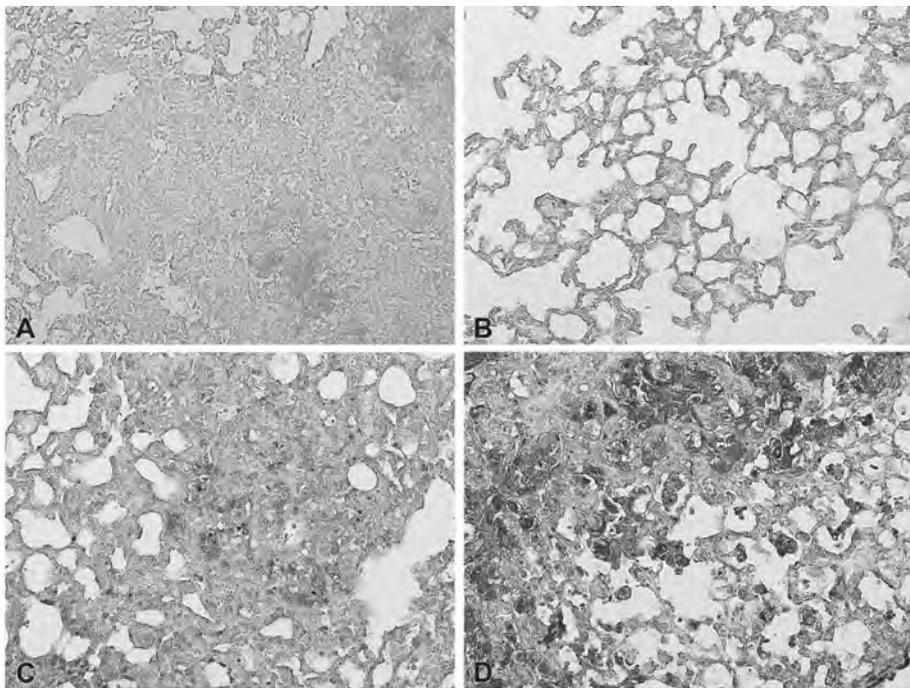


Figure 1: Increased osteopontin expression during *M. tuberculosis* infection. OPN expression in lungs from WT mice; (A) negative control, (B) before infection, (C) 2 weeks and (D) 5 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Whereas in uninfected lungs OPN could not be detected, tuberculosis was associated with increased pulmonary expression of OPN, especially in alveolar macrophages, and to a lesser extent in lymphoid cells. Original magnification 10x.

Osteopontin does not affect early mycobacterial growth and dissemination

To determine the role of OPN in antibacterial defense during tuberculosis, the outgrowth of *M. tuberculosis* was determined in lungs of WT and OPN KO mice 1 day, 2 and 5 weeks after infection. At all time points the numbers of CFU detected in lungs were similar in WT and OPN KO mice (Figure 2A). To study the dissemination of *M.*

tuberculosis, the mycobacterial load in liver was measured. Again, no differences in mycobacterial growth in liver were observed between WT and OPN KO mice 2 and 5 weeks after infection (Figure 2B).

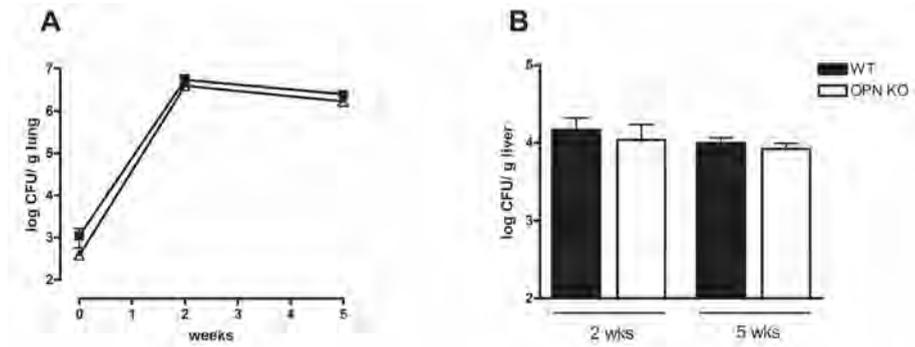


Figure 2: Osteopontin deficiency does not influence mycobacterial outgrowth in lungs and liver. Mycobacterial loads in lungs (A) and liver (B) from WT (closed symbols) and OPN KO (open symbols) mice 1 day, 2 and 5 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Data are mean \pm SEM of 3 (1 day) or 8 mice (2 and 5 weeks) per group.

Pulmonary inflammation

To investigate whether OPN deficiency was accompanied by altered lung inflammation, we performed histopathologic analyses of lung tissue slides prepared from WT and OPN KO mice 2 and 5 weeks after infection. At both time-points, both groups displayed granulomatous inflammation which increased in extent and severity from 2 to 5 weeks after infection in both mouse strains (Figure 3). Lung weights, the percentage of inflamed lung parenchyma and total lung inflammation scores did not differ between WT and OPN KO mice (Table I), although a trend towards a lower percentage of inflamed lung parenchyma in OPN KO was found 5 weeks after infection ($P = 0.1$).

Cellular composition of lung infiltrates

To obtain more insight into the cellular composition of the pulmonary infiltrates in WT and OPN KO mice, we prepared whole lung cell suspensions at 2 and 5 weeks after infection. At both time-points total leukocyte counts were similar in WT and OPN KO mice. To determine whether the cellular composition of the infiltrates was affected by OPN we counted the percentages of lymphocytes, macrophages and PMNs. There were no differences in cellular composition between WT and OPN KO

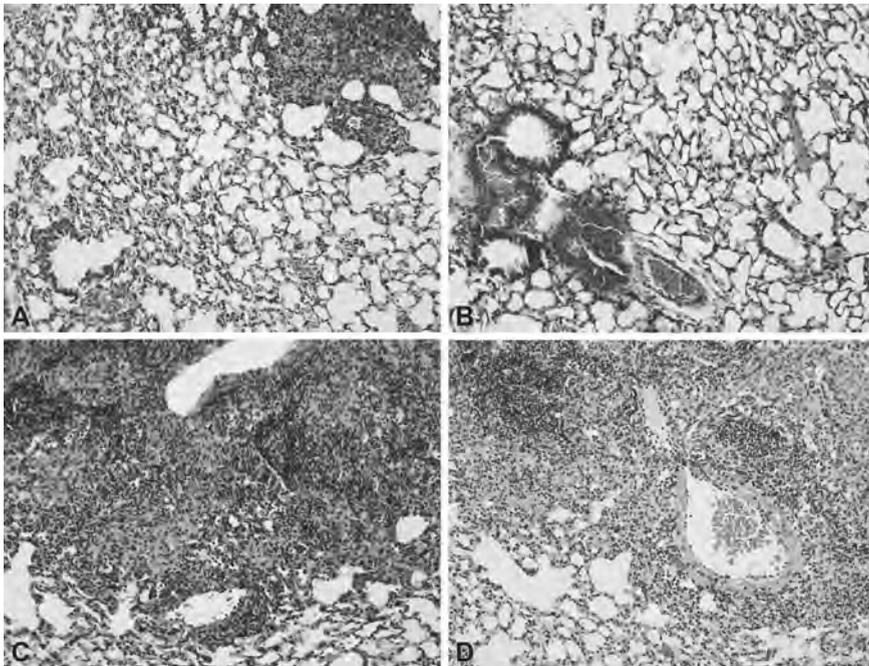


Figure 3: Lung histopathology. Representative lung histology of WT (A, C) and OPN KO (B, D) mice 2 (A, B) and 5 weeks (C, D) after intranasal infection with 150 CFU of *M. tuberculosis*. The lung sections are representative for 8 mice per group per time-point. H&E staining, original magnification 10x.

lung infiltrates at 2 (data not shown) and 5 weeks after infection (Table II). Since CD4⁺ and CD8⁺ T cells are important players during tuberculosis (26), we analyzed whole lung CD3⁺ lymphocytes with respect to expression of CD4 and CD8. This revealed that at 2 weeks after infection both mouse strains showed similar percentages of CD4⁺ T

Table I: OPN deficiency does not impact on lung inflammation.

	Lung weight (g)	Inflamed area (%)	Inflammation Score
2 wks			
WT	0.16 ± 0.01	4 ± 1	4.4 ± 0.9
OPN KO	0.16 ± 0.01	5 ± 2	6.0 ± 0.9
5 wks			
WT	0.29 ± 0.02	38 ± 4	10.8 ± 0.5
OPN KO	0.29 ± 0.02	29 ± 3	9.9 ± 0.7

Lung weights, % of inflamed lung parenchyma and histopathological changes (scored from H&E stained slides of left lungs) of WT and OPN KO mice 2 and 5 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Data are mean ± SEM of 8 mice per group.

cells. Of note, however, a trend towards decreased percentages of CD8⁺ T cells was found in OPN KO mice as compared to WT mice ($P = 0.06$, data not shown). At 5 weeks after infection, there was a trend towards reduced percentages of CD4⁺ T cells and significantly reduced percentages of CD8⁺ T cells in OPN KO mice as compared to WT ($P = 0.06$ and $P < 0.05$ respectively, Table II).

Table II: OPN KO mice demonstrate a diminished influx of CD8⁺ T cells into the lungs

	Leukocytes x 10 ⁵ / lung	Mφs %	PMNs %	Lymphocytes %	CD4 ⁺ %	CD8 ⁺ %
WT	3.7 ± 0.2	11.0 ± 0.7	4.4 ± 0.2	67.1 ± 1.2	61.6 ± 1.0	25.0 ± 0.7
OPN KO	4.1 ± 0.3	10.5 ± 0.8	5.0 ± 0.5	69.9 ± 1.6	52.2 ± 3.7	20.7 ± 1.1*

Total leukocyte counts (x10⁵/lung) and differential cell counts in lungs of WT and OPN KO mice 5 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Percentages of PMNs, macrophages and lymphocytes were determined on GR-1 expression (GR-1 high, intermediate and low, respectively) and T cell subsets (CD4⁺ and CD8⁺) are presented as the percentage positive cells in the CD3⁺ gate. Data are mean ± SEM of 8 mice per group. * $P < 0.05$ versus WT

Cytokine and chemokine response

Cytokines and chemokines play a pivotal role in the regulation of the immune response to tuberculosis (27, 28). Therefore we measured the concentrations of pro-inflammatory cytokines (IFN- γ , IL-12, TNF- α , IL-1 β , IL-6), anti-inflammatory cytokines (IL-4, IL-10) and chemokines (MIP-2, KC) in lung homogenates obtained 2 and 5 weeks after infection. The concentrations of all mediators were similar in lungs of WT and OPN KO mice at both time points. However, a trend towards decreased levels of the prototypic type 1 cytokine IFN- γ was found in OPN KO mice 5 weeks after infection as compared to WT mice ($P = 0.06$) (Table III).

Similar IFN- γ production upon ex vivo stimulation of splenocytes

Next we determined the capacity of splenocytes obtained from *M. tuberculosis* infected mice to respond to the recall antigen PPD. Splenocytes were harvested at 2 and 5 weeks after infection and stimulated with PPD for 48 hours, after which IFN- γ and IL-4 were measured in the supernatant. Antigen-specific IFN- γ production by splenocytes harvested from OPN KO and WT mice at either 2 or 5 weeks after infection was similar (Figure 4). IL-4 levels were undetectable in supernatants from both groups at both time-points.

Table III: Pulmonary cytokine and chemokine concentrations.

pg/mL	2 wks		5 wks	
	WT	OPN KO	WT	OPN KO
IFN- γ	1979 \pm 391	1566 \pm 269	2116 \pm 285	1314 \pm 107
IL-4	673 \pm 130	586 \pm 47	641 \pm 75	523 \pm 61
IL-12	225 \pm 72	179 \pm 42	85 \pm 12	83 \pm 5
TNF- α	1856 \pm 258	1861 \pm 221	5725 \pm 949	4876 \pm 482
IL-1 β	1427 \pm 257	1073 \pm 158	357 \pm 64	345 \pm 53
IL-10	1196 \pm 248	918 \pm 88	545 \pm 71	406 \pm 69
IL-6	511 \pm 68	490 \pm 55	1021 \pm 164	894 \pm 66
KC	1195 \pm 276	1012 \pm 180	1202 \pm 136	903 \pm 153
MIP-2	1275 \pm 232	1164 \pm 128	1853 \pm 255	1647 \pm 209

Cytokine and chemokine levels (in pg/mL) in lung homogenates of WT and OPN KO mice 2 and 5 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Data are mean \pm SEM of 8 mice per group.

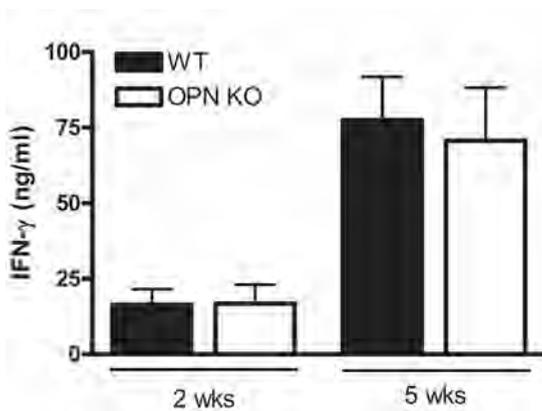


Figure 4: Similar IFN- γ production upon PPD re-stimulation. IFN- γ production after 48h PPD re-stimulation by WT (black bars) and OPN KO (white bars) splenocytes harvested 2 and 5 weeks after infection with 150 CFU of *M. tuberculosis*. Data are mean \pm SEM of 8 mice per group.

DTH reaction to *M. tuberculosis* antigen

The recruitment of leukocytes into inflamed areas is critical for the development of DTH responses. To investigate leukocyte recruitment during a DTH reaction against *M. tuberculosis*-specific antigens, mice were immunized and subsequently challenged in one footpad with PPD and with saline in the other. Both WT and OPN KO mice showed significant footpad swelling after the challenge. Both at 24 and

48 h after PPD challenge, the increase in footpad thickness was similar in WT and OPN KO mice (Figure 5A). Histological analysis confirmed that the DTH reaction to PPD was comparable in both mouse strains. Footpads from both groups showed a diffuse and dense infiltrate, and no differences were found with respect to severity of inflammation, edema and cellular composition (Figure 5B and C).

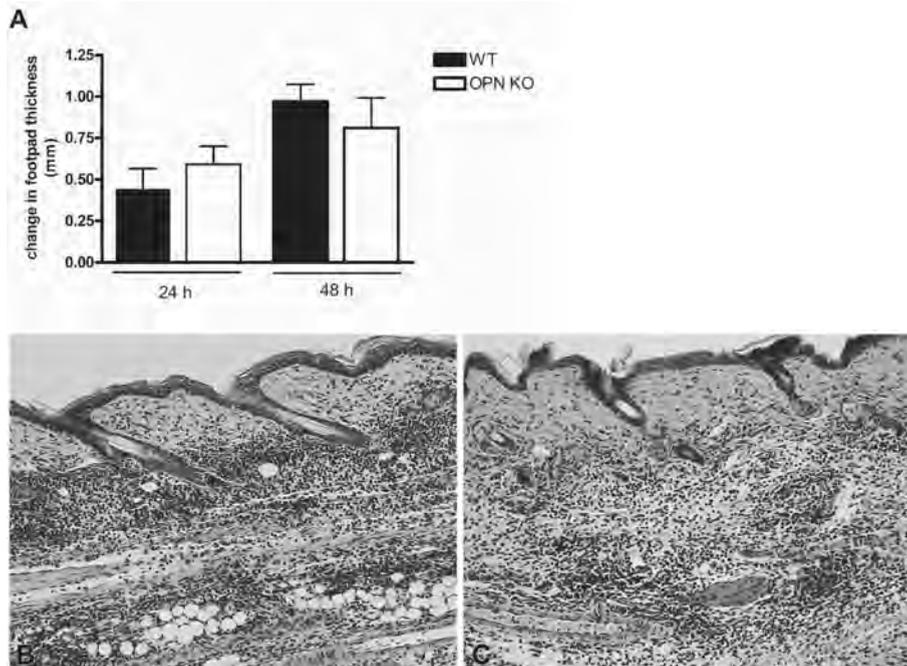


Figure 5: Similar DTH response to PPD challenge. (A) WT (black bars) and OPN KO (white bars) mice were immunized with heat-killed *M. tuberculosis* and challenged in one hind footpad with PPD and in the other saline. Footpad swelling was measured 0, 24 and 48 hours after challenge and calculated as described in the method section. Data are shown as mean \pm SEM of 8 mice per group. Representative view of the footpad of a WT (B) and OPN KO (C) mouse 48 hours after a DTH reaction. Footpads from both groups showed a diffuse and dense infiltrate, and no differences were found with respect to severity of inflammation, edema and cellular composition (H&E staining, original magnification 10x).

Osteopontin affects the late host response to *M. tuberculosis* infection

Having established that OPN deficiency did not have a major impact on the early immune response to *M. tuberculosis* infection, we next investigated the role of OPN in the long term response and outcome of tuberculosis infection. Therefore, WT and OPN KO mice were inoculated with *M. tuberculosis* and followed for 31 weeks. During this period OPN KO mice showed a modest but not significant survival

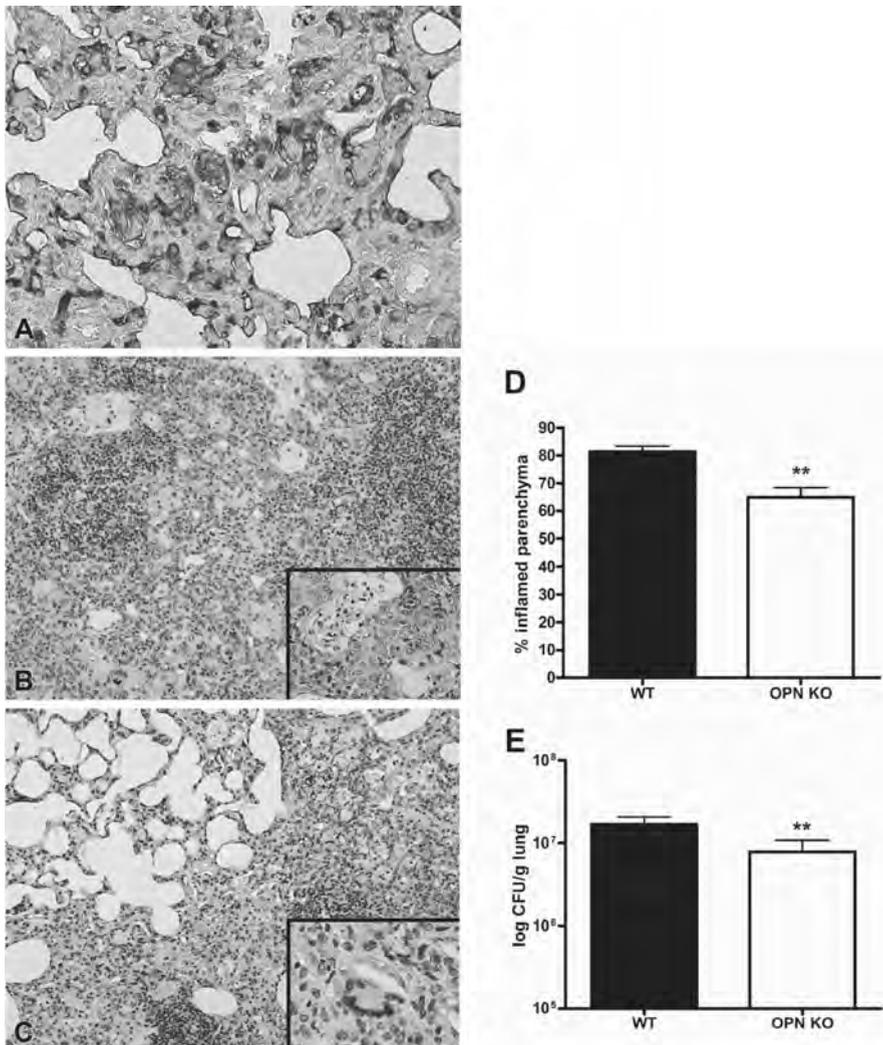


Figure 6: Osteopontin contributes to the chronic inflammatory response to *M. tuberculosis* infection. (A) OPN expression in lung from a representative WT mouse 31 weeks after infection with 150 CFU of *M. tuberculosis*. OPN expression was found in alveolar macrophages, and to a lesser extent in lymphoid cells. Representative lung histology of WT (B) and OPN KO (C) mice 31 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Lung tissue remodeling was found in WT lungs (B, insert) and multinucleated giant cells were found in OPN KO lungs (C, insert). The lung sections are representative for 11-14 mice per group. H&E staining, original magnification 10x and inserts 40x. Percentage of inflamed parenchyma (D) and mycobacterial growth (E) in lungs of WT (black bars) and OPN KO (white bars) mice 31 weeks after infection with 150 CFU of *M. tuberculosis*. Data are mean \pm SEM of 11-14 mice per group. ** $P < 0.01$ versus WT

advantage: after 31 weeks 4 out of 15 WT and 1 out of 15 OPN KO mice had died (data not shown). At the end of the experiment, the remaining mice were killed for

further analysis. In lungs from WT mice OPN expression could still be detected in alveolar macrophages and lymphoid cells (Figure 6A). Although, due to the relatively enhanced mortality in WT mice these data might be underestimated, OPN KO mice showed less lung inflammation as reflected by the percentage of inflamed lung tissue ($P < 0.01$, Figure 6B-D). Furthermore, in lung lesions of OPN KO mice multinucleated giant cells were found (insert Figure 6C), which were not present in the lungs of WT mice. On the other hand, WT mice displayed clear lung tissue remodeling (insert Figure 6B), which was not found in OPN KO lungs. In addition, OPN KO mice displayed reduced mycobacterial loads in their lungs at this late time point ($P < 0.01$, Figure 6E).

Discussion

The last decade numerous *in vitro* and *in vivo* studies proposed OPN as an important player in Th1 responses and granulomatous inflammation. In addition, patient studies revealed that OPN expression is associated with granulomas (13, 16, 17), and plasma OPN levels correlate with the extent of lung lesions and outcome during mycobacterial infection (18, 19). To the best of our knowledge, however, so far the functional role of OPN in *M. tuberculosis* infection has not been examined. We here demonstrate that OPN deficiency does not dramatically affect early responses during tuberculosis, whereas during more chronic infection pulmonary mycobacterial outgrowth and inflammation are reduced in OPN KO mice.

Our primary hypothesis was that OPN would play an important role in host defense against pulmonary tuberculosis. This hypothesis was based on several lines of evidence. Intracellular OPN has been found to be important for the function and differentiation of macrophages, the phagocytic cells that initially ingest tubercle bacilli that enter the lower airways (6). In addition, recombinant OPN inhibited nitric oxide production, a major antimicrobial mechanism, by RAW 264.7 cells and murine thioglycollate-elicited macrophages upon stimulation with lipopolysaccharide plus IFN- γ (29, 30). Moreover, several studies have implicated OPN in an adequate Th1 response to infection (8-11), which is also important for a protective immune response against *M. tuberculosis* (2). One previous study examined the role of OPN during infection with the closely related though non-virulent *M. bovis* BCG (15). In that study *M. bovis* BCG was administered via intraperitoneal injection, a clinically less relevant route. OPN KO mice demonstrated a reduced clearance of *M. bovis* BCG, which was accompanied by more extensive granulomatous inflammation in liver (15). In our experiments we used a virulent *M. tuberculosis* strain administered

via the airways and found no differences in mycobacterial outgrowth in lungs or liver in the first 5 weeks after infection. Moreover, in contrast to our hypothesis, we were not able to detect any significant differences in pulmonary type 1 cytokine concentrations; i.e. IL-12 concentrations did not differ at any time-point and only a trend was found towards reduced IFN- γ levels in OPN KO mice 5 weeks after infection. In addition, no differences were detected between WT and OPN KO mice with respect to pulmonary inflammation, the number and size of granulomas or the cellular composition of lung infiltrates. In this respect, it should be noted that some previous studies were unable to identify an essential role for OPN in Th1 responses. For example, contradicting reports on the role of OPN during *Listeria monocytogenes* infection have been published; whereas one study claimed OPN KO mice to be defective in their ability to clear *Listeria monocytogenes*, accompanied by reduced IFN- γ responses (8), another study showed that OPN KO mice mounted a normal immune response to this pathogen (31). Moreover, normal immune responses of OPN KO mice have been reported during vaccinia virus, influenza virus (31) and *Borrelia burgdorferi* infection (32, 33). In accordance with previous investigations (13, 34), we were unable to visualize OPN in lungs of uninfected mice. Lung inflammation has been found to result in OPN expression by alveolar macrophages, lymphocytes, and epithelial cells (34-37). In addition, *M. tuberculosis* infection has been shown to upregulate OPN gene expression in human monocytes *in vitro* (38). In line, we detected OPN expression by alveolar macrophages and lymphoid cells 2, 5 and 31 weeks after infection.

Recruitment of inflammatory cells to the site of infection is crucial for an adequate immune response. Excreted OPN acts as a chemo-attractant, which may result in more macrophages, neutrophils and T cells to migrate to the lung (5-7). Considering this, we were surprised to find no differences in the migration of either of these leukocyte subsets to the lungs of WT and OPN KO mice at 2 and 5 weeks after infection. Moreover, we did not find differences with regard to footpad swelling or cellular influx between WT and OPN KO mice in a DTH reaction with *M. tuberculosis*-specific antigens. An earlier study demonstrated significant inhibition of footpad swelling in a herpes simplex virus-1 specific DTH reaction in OPN KO and OPN neutralized mice (8). This suggests a stimulus specific rather than a general leukocyte migration effect of OPN.

Since both CD4⁺ and CD8⁺ T cells are important players in the immune response to *M. tuberculosis* infection (26), we analyzed whole lung CD3⁺ lymphocytes with

respect to CD4 and CD8 expression. Although total lymphocyte numbers appeared similar in both groups, we found a significantly reduced percentage of CD8⁺ T cells in OPN KO mice 5 weeks after infection and a similar trend was seen at 2 weeks after infection. The percentage of CD4⁺ T cells demonstrated a trend to be less in OPN KO mice only at 5 weeks after infection. The diminished numbers of CD8⁺ T cells in OPN KO mice are in agreement with a study showing that CD8⁺ T cell migration in a DTH reaction upon DNFB challenge was enhanced by OPN over-expression (39). We found WT and OPN KO splenocytes to produce equal IFN- γ concentrations upon PPD re-stimulation, demonstrating that the intrinsic capacity of OPN KO splenocytes for IFN- γ production is not altered; similar observations have been made in re-stimulated *M. bovis* BCG infected splenocytes (15). The slight reduction in CD4⁺ and CD8⁺ T cells in OPN KO lungs might be an explanation for the trend towards less IFN- γ in OPN KO lungs 5 weeks after infection.

Although OPN deficiency did not have a major impact on the early immune response to *M. tuberculosis* infection, the long term response to tuberculosis infection appeared to be affected by OPN deficiency. Indeed, whereas we did not find any significant differences in lung mycobacterial outgrowth in the early phase of tuberculosis, we did find significantly less bacteria in the lungs of OPN KO as compared to WT mice that survived the 31-week observation period. Moreover, at this time-point the percentage of inflamed lung parenchyma was significantly reduced in the absence of OPN. Of note, although these differences are modest, these data might be underestimated since more WT than OPN KO mice died during this observation period and one can speculate that animals that died were the ones with the highest bacterial loads and/or severest pulmonary inflammation. Since macrophages are the principal cells in which *M. tuberculosis* persists and the granulomatous inflammatory infiltrate in both mouse strains consisted particularly of foamy macrophages, the diminished numbers of mycobacteria in OPN KO lungs at this time point might be a consequence of less mycobacteria-containing macrophages. The fact that differences in pulmonary inflammation were found only in a late phase of tuberculosis might be explained by the fact that apart from regulating inflammation OPN is also known to mediate tissue remodelling (3, 5, 40). For example, recently it became clear that knockdown of OPN leads to rapid repair and reduced scarring in a murine wound healing model (41). Furthermore, OPN is required for transforming growth factor- β 1 induced myofibroblast differentiation *in vitro* (42), and OPN is up-regulated and acts pro-fibrotic in murine bleomycin-induced pulmonary fibrosis (34) and human

idiopathic pulmonary fibrosis (43). Moreover, remodelling processes and calcification of the vasculature and mature bone structures are modulated by OPN (3, 5).

In conclusion, our data suggest that, in contrast to our hypothesis, OPN is not crucial to a protective Th1 response during pulmonary tuberculosis. In contrast, the presence of OPN even may have a negative effect on the late phase of pulmonary tuberculosis as indicated by reduced pulmonary bacterial loads and less pulmonary inflammation in the absence of OPN.

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