Lack of Eosinophil Peroxidase or Major Basic Protein Impairs Defense against Murine Filarial Infection

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Eosinophils are a hallmark of allergic diseases and helminth infection, yet direct evidence for killing of helminth parasites by their toxic granule products exists only in vitro. We investigated the in vivo roles of the eosinophil granule proteins eosinophil peroxidase (EPO) and major basic protein 1 (MBP) during infection with the rodent filaria Litomosoides sigmodontis. Mice deficient for either EPO or MBP on the 129/SvJ background developed significantly higher worm burdens than wild-type mice. Furthermore, the data indicate that EPO or MBP is involved in modulating the immune response leading to altered cytokine production during infection. Thus, in the absence of MBP, mice showed increased interleukin-10 (IL-10) production after stimulation of macrophages from the thoracic cavity where the worms reside. In addition to elevated IL-10 levels, EPO−/− mice displayed strongly increased amounts of the Th2 cytokine IL-5 by CD4 T cells as well as a significantly higher eosinophilia. Interestingly, a reduced ability to produce IL-4 in the knockout strains could even be seen in noninfected mice, arguing for different innate propensities to react with a Th2 response in the absence of either EPO or MBP. In conclusion, both of the eosinophil granule products MBP and EPO are part of the defense mechanism against filarial parasites. These data suggest a hitherto unknown interaction between eosinophil granule proteins, defense against filarial nematodes, and cytokine responses of macrophages and CD4 T cells.

Eosinophils are a hallmark of allergic diseases (4, 16, 17, 33, 36) and helminth infection (22, 27). It is has been debated whether they are involved directly in the killing of worms or in orchestrating the immune response (13, 19, 31, 45).

Evidence for the importance of eosinophils in worm destruction comes from in vitro data: eosinophils operate via anti-body-dependent cytotoxicity directed against helminth parasites such as schistosomes (7) or filarial nematodes (10, 20). Data supporting eosinophil function in vivo is mainly indirect since the evidence refers to interference in interleukin-5 (IL-5)-dependent responses, which are known for recruitment of eosinophils to the site of infection (1, 25, 29) Thus, in nonper-missive models of filarial infection, blockade of IL-5-dependent pathways resulted in a higher number of transient worm stages (23, 26). In the permissive model of murine infection with Litomosoides sigmodontis, our group previously showed that IL-5 is a key cytokine for protection against invading L3 larvae, nodule formation, and control of microfilariae (2, 43). Furthermore, IL-5 is important for vaccine-induced protection against filarial nematodes in vivo (24, 28). Protection is associated with recruitment of eosinophils to the skin, which deposit matrix proteins to the incoming larvae. Eosinophil degranulation, but not recruitment, is antibody dependent, as shown in vaccination studies using B-cell-deficient mice. In these mice, recruited eosinophils did not degranulate and this was associated with reduced killing of infective L3 larvae and, consequently, the development of higher worm loads (30).

Degranulation (i.e., the release of abundant cationic proteins from morphologically distinct cytoplasmic granules) is an important function of eosinophils in the presence of antigen (15). The most prominent granule protein (25% of the total protein mass of the secondary granule) is a cationic heme-containing protein known as eosinophil peroxidase (EPO), which appears to be specific for the cell type eosinophils (9). The respiratory burst of activated eosinophils results in the release of EPO together with generation of the reduced oxygen component (14, 32). From in vitro data, it was concluded that the release of EPO could provide a potent defense mechanism against filarial parasites (21). A second protein is the major basic protein (MBP), which is also released when the eosino-phil degranulates. It is characterized as a small cationic protein of about 12 kDa and colocalizes with the characteristic electron-dense crystalline core of eosinophil secondary granules (35). MBP release is also believed to be critical for eosinophil-mediated activities and is responsible for many observed pathologies associated with allergic respiratory disease (11, 12, 18). However, mice deficient for either MBP or EPO did not display an altered course of disease in murine asthma, nor was lack of these proteins associated with a change in asthma-related pathologies (11, 12).

Using EPO and MBP knockout mice, our study shows that EPO, as well as MBP, is important for the control of filarial infection. Furthermore, deficiency in either EPO or MBP led to a perturbation of the cytokine response. Lack of either one
was associated with higher IL-10 production by macrophages, suggesting IL-10 as one factor leading to higher permissiveness of the infection. In addition, deficiency of EPO correlated with higher levels of IL-5 produced by CD4 T cells and a higher influx of eosinophils into the thoracic cavity compared to that in the wild type during filarial infection. In summary, these results provide evidence for MBP and EPO as important molecules for defense against filarial infection. Furthermore, they suggest that these molecules are involved in the regulation of immune responses.

MATERIALS AND METHODS

Animal maintenance and infection of mice with L. sigmodontis. Transgenic (129/SvE EPO+/− and 129/SvJ MBP+/−) (11, 12) and wild-type (129/SvJ and BALB/c, originally from Jackson Laboratories, Bar Harbor, Maine) mice were housed under specific-pathogen-free conditions in microisolator cages. Natural infections were performed as described previously (34). At least five mice were used for each group. BALB/c wild-type mice were used as a control for successful infection rate because of the semiresistant phenotype of 129/SvJ mice.

Parasite recovery. Adult worms were removed from the thoracic cavity and counted at day 28 postinfection (p.i.). The thoracic cavity was flushed with 1 ml of phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), and the worms were allowed to sediment. The microfilaria count was determined from 50 μl EDTA-treated peripheral blood or 200 μl of thoracic cavity flush after staining with Hinkelmann’s 0.5% [wt/vol] eosin Y, 0.5% [wt/vol] phenol, and 0.185% [vol/vol] formaldehyde in distilled water as described previously (3).

Determination of proportions of inflammatory cells in thoracic cavity fluid. Proportions of inflammatory, thoracic cavity cells were determined using cytospin preparations: 2 × 10⁶ thoracic cavity cells in PBS–1% FCS were centrifuged against a glass slide with absorptive filter paper using a Shandon cytocentrifuge. Cytospins were then stained using the Wright-Giemsa stain (Sigma, Munich, Germany). Neutrophils and eosinophils were differentially enumerated, and the total number was calculated.

Flow cytometric analysis. Staining of thoracic cavity cells was performed using the following antibodies (all from BD Biosciences, Heidelberg, Germany): rat anti-mouse CD4 (clone YTS 191.1 conjugated to fluorescein isothiocyanate [FITC]), rat anti-mouse CD8 (clone YTS 169.4 conjugated to phycoerythrin [PE]), rat anti-mouse CD45R (clone RA3-6B2–PE), rat anti-mouse MAC-1 (clone M1/70.15–FITC), rat anti-mouse DX5 (clone PK 136–FITC), and rat anti-CD3 (clone 145-2C11–PE). Cells were stained with a 1:100 dilution according to standard procedures and analyzed using a FACScan cytometer (BD Biosciences, Heidelberg, Germany).

B cells, CD4+ T cells, CD8 T cells, NK cells, and NK cells have been summarized as B cells because no significant differences were detectable between the different groups of mice.

Purification of CD4+ T cells by FACS. To isolate CD4+ T cells, thoracic cavity cells were labeled with rat anti-mouse CD4 (YTS 191.1–FITC) according to standard procedures. The cells were washed with PBS–1% bovine serum albumin and diluted in RPMI 1640 medium (supplemented with 50 μg/ml gentamicin, 2 mM glutamine, and 5% FCS). The samples were sorted using the fluorescence-activated cell sorter (FACS) DIVA using the DIVA software (BD Biosciences). The purified CD4+ T cells were then transferred into cell culture.

Cell culture. Splenocytes from uninfected mice were prepared and cultured at 37°C and 5% CO2 in 96-well microtiter plates. A total of 2 × 10⁶ cells per well were cultured in RPMI 1640 (PAA Laboratories, Parsching, Austria) supplemented with 5% fetal calf serum (PAA Laboratories), 2 mM L-glutamine (PAA Laboratories), and 50 μg/ml gentamicin sulfate (Cymbrex Bio Science, Walkersville, MD). The following stimuli were added: medium alone, L. sigmodontis antigen (whole adult worm extract, 10 μg per well), lipopolysaccharide (LPS) antigen from Escherichia coli (2 ng/well), anti-CD3 (1 μg/well), or concanavalin A (ConA; 0.5 μg/well). Supernatants were removed after 72 h for cytokine determination.

A total of 2 × 10⁶ thoracic cavity cells per well from L. sigmodontis-infected mice were stimulated with either medium, 10 μg/well adult worm antigen, or 2.5 μg/well concanavalin A. Supernatants were collected after 72 h.

Adherent macrophages were obtained by allowing thoracic cavity cells to adhere to petri dishes for 60 min at 37°C, following removal of nonadherent cells. Purity of the adherent macrophages was >95% as analyzed by cytospin (not shown).

RESULTS

EPO or MBP deficiency increases susceptibility to L. sigmodontis compared to wild-type mice. To analyze the influence of eosinophil peroxidase and major basic protein on parasite killing, we infected EPO−/− and MBP−/− mice as well as their corresponding wild-type controls (129/SvJ) with L. sigmodontis. Since this background is semiresistant to L. sigmodontis, we also included BALB/c mice as a positive control of susceptibility. Accordingly, at day 28 p.i., we observed a lower number of parasites in the thoracic cavity of 129/SvJ wild-type mice compared with BALB/c mice (Fig. 1a). In contrast, worm numbers in mice lacking the eosinophilic granule proteins EPO or MBP were increased compared with those in the 129/SvJ wild type and were similar to those in fully permisive BALB/c mice (Fig. 1a), suggesting a role for EPO and MBP in worm elimination in the first 28 days p.i. Analysis of the length from female adult worms at day 28 p.i. showed that the worms of both EPO- and MBP-deficient mice were significantly longer than those from the 129/SvJ strain (Fig. 1b), indicating better parasite development.

To further investigate the effect of EPO or MBP on full patenty, adult worm loads and production of microfilariae were analyzed during the late stage of infection. However, no live worms were detectable at 50 days p.i. in 129/SvJ wild-type, MBP−/−, and EPO−/− mice (data not shown). The elimination of the parasites before patency did not allow establishment of microfilariae in the blood, in contrast to fully susceptible BALB/c mice.

Lack of EPO increases migration of eosinophils into the thoracic cavity. To find out if a lack of eosinophilic granule proteins alters the recruitment of cells to the site of infection, the cell population that had migrated into the thoracic cavity during infection was analyzed by FACS and cytospin. Interestingly, at
day 28 p.i., in EPO−/− mice the number of eosinophils in the thoracic cavity was increased twofold compared to those in both MBP−/− and 129/SvJ wild-type mice (Fig. 2). Other inflammatory cells such as macrophages, lymphocytes, or neutrophils were not affected by the absence of EPO or MBP (Fig. 2). Less than 1% of eosinophils were found in uninfected mice (data shown). These data argue for EPO itself being able to orchestrate the immune response via the inhibition of eosinophil influx.

**EPO and MBP are correlated with modified cytokine responses of macrophages and CD4+ T cells.** Measurement of cytokines in the thoracic cavity lavage fluid of infected mice 28 days p.i. was performed. The overall concentrations of IL-10 were low, but significantly increased in EPO−/− mice compared with MBP−/− and 129/SvJ wild-type mice (Fig. 3). No significant differences were seen in the levels of IL-5, IL-4, and IFN-γ between any groups in the thoracic cavity lavage (data not shown).

To complement these data, we also investigated cytokine production by the cells that had accumulated at the site of infection. They were isolated from the thoracic cavity and stimulated with adult *L. sigmodontis* worm antigen. Thoracic cavity cells from EPO−/− mice and BALB/c mice produced significantly more of both IL-5 and IL-10 compared with MBP−/− or wild-type mice. MBP−/− cells produced more IL-10 than wild-type cells (Fig. 4a). IL-5 secretion of MBP−/− thoracic cavity cells did not differ from that of infected wild-type mice. IFN-γ was not detectable in all groups of mice (data not shown). In contrast, IL-4 concentrations were significantly higher in 129/SvJ wild-type mice compared to both EPO−/− and MBP-deficient strains (Fig. 4a).

To determine the source of the cytokines, macrophages (Fig. 4b) and CD4+ T cells (Fig. 4c) were purified and their cytokine production was measured. Adherent macrophages of both deficient strains showed significantly higher IL-10 levels in response to adult worm antigen compared with the wild type. The largest amounts of IL-10 were observed in macrophages of EPO−/− mice (Fig. 4b).

This was in contrast to sorted CD4 T cells from these mice after antigenic stimulus. IL-10 production of EPO−/− CD4+ cells did not differ from that of either wild-type or MBP−/− mice. Overall, IL-10 concentrations were rather low (Fig. 4c). Interestingly, EPO−/− CD4 T cells produced two- to threefold more IL-5 in comparison to wild-type and MBP−/− cells. In
FIG. 3. Cytokine levels in the thoracic cavity fluid at day 28 p.i. All groups of mice secrete equivalently high levels of IL-5. IL-10 is not detectable (ND) in 129/SvJ and MBP−/− mice, while EPO−/− mice and BALB/c wild-type cells produce significantly more IL-10. IL-4 and IFN-γ are not detectable in the thoracic cavity fluid from all mouse strains (data not shown). Each group contains five animals. The data are from one representative experiment out of two. Asterisks indicate significant differences (P < 0.05).

DISCUSSION

The increased recruitment of eosinophils is a phenomenon of helminth and allergic diseases (4, 16, 17, 22, 27, 33, 36). Despite this, relatively few studies can assign a clear in vivo function to eosinophils. In vitro, destruction of helminth larvae has been observed in several settings and eosinophils were shown to participate in an antibody-dependent manner (6, 8, 25). The direct involvement of various eosinophil-derived granule proteins against microfilariae in vitro was observed at a relatively high concentration (21). The role of eosinophils, however, as well as eosinophil granule proteins, has not been fully elucidated (22). Depletion of IL-5 from fully susceptible BALB/c mice infected with Litomosoides sigmodontis results in a prolonged survival of adult worms (2) and elevated microfilaria; these effects were more pronounced in IL-5-deficient mice (44). Conversely, IL-5-overexpressing mice on a BALB/c background have a higher number of eosinophils and significantly reduced life span of adult worms (29). Genetic deficiency in IL-5 also prevents vaccine-induced protective immunity in both susceptible BALB/c as well as resistant C57BL/6 mice (24). However, since IL-5 in addition to recruitment of eosinophils also affects B-cell maturation (38) and B cells are required for protective immunity following immunization, these two effects could not be dissected before. This became possible by the use of eotaxin-1-deficient mice in experimental injection of Brugia malayi microfilaria, where the resulting lack of eosinophil recruitment into the injection site, i.e., the peritoneal cavity, was associated with longer microfilaria survival (40). These studies, however, did not determine whether the essential function of eosinophils was due to the production of cytokines or the production of granule-associated factors.

The availability of MBP- and EPO-deficient mice has now provided a suitable approach to investigate the role of these granule proteins in vivo. Studies with O. volvulus, a nonpermissive filarial parasite in mice, did not detect a difference between EPO−/− and wild-type mice in L3 to L4 molting capacity (1). In experimental Brugia pahangi infection, also nonpermissive in mice, neither deficiency of EPO nor MBP led to an alteration of parasite loads, whereas administration of an eosinophil-depleting anti-CCR3 antibody resulted in higher parasite numbers, suggesting that eosinophils, but not EPO or MBP, are important for parasite control in this model (37).

L. sigmodontis infection shows a graded pattern of resistance and susceptibility across different mouse strains. Certain strains, such as BALB/c, are highly permissive, and infection regularly develops into patency with circulating microfilariae. Other strains are semiresistant, such as C3H, FVB, and 129/SvJ, in which a considerable number of adult worms develop, while resistant mouse strains such as C57BL/6 only have a small proportion of incoming larvae developing into adult worms, and these are regularly infertile. In BALB/c mice, there is a protective effect of IL-5 both in primary infections as well as after vaccination. IL-5 deficiency in resistant C57BL/6 mice, however, contrasts with that of susceptible BALB/c mice as it does not control parasite recovery during primary infection, but does play an important role in protective immunity follow-
ing immunization (24). Taken together, these results suggest that eosinophils play a role in the killing of filarial parasites, but there are multiple mechanisms in play.

Our study demonstrates that on a semiresistant background, 129/SvJ, the lack of EPO or MBP leads to higher worm loads, suggesting that both granule proteins are important for the control of development of infective larvae into adult worms. Our results do not exclude that on a more resistant back-

FIG. 4. (a) Production of IL-4, IL-5, and IL-10 by thoracic cavity cells. Cells were recovered 28 days p.i., and cytokines were measured after stimulation with *L. sigmodontis* (LS) adult worm antigen, concanavalin A, or medium only as control. The data show that antigen-specific IL-5 and IL-10 production of cells from EPO−/− mice and BALB/c wild-type mice was significantly higher than that in the other mouse strains. Furthermore, cells of MBP−/− mice showed a higher capacity of IL-10 production than 129/SvJ controls, while IL-5 production was equivalent to that in 129/SvJ wild-type mice. Thoracic cavity cells from 129/SvJ wild-type mice secreted more IL-4 after antigen-specific stimulation compared to MBP−/− and EPO−/−. Cells incubated with medium or concanavalin A show no significant differences between the groups. Each group contained five animals. The data are from one representative experiment out of two. Bars show the mean ± standard deviation. Asterisks represent a significant difference (*P* < 0.05). ND, not detected. (b) Cytokine production by adherent macrophages from thoracic cavity cells. Stimulation of macrophages with adult worm antigen shows that MBP−/− and EPO−/− macrophages produce significantly higher IL-10 levels than 129/SvJ wild-type mice. EPO−/− macrophages secrete more IL-10 when stimulated with LPS. Each group contains five animals. The data are from one representative experiment out of two. Bars show the mean ± standard deviation. Asterisks represent significant differences (*P* < 0.05). ND, not detected. (c) Cytokine production by purified CD4+ T cells separated by FACS and stimulated with adult worm antigen or concanavalin A. CD4+ T cells of 129/SvJ wild-type mice show a significantly higher production of IL-4 than those of both knockout strains. IL-5 levels are significantly higher in EPO−/− CD4+ T cells compared to the other mouse strains. Each group contains five animals. The data are from one representative experiment out of two. Bars show the mean ± standard deviation. Asterisks represent significant differences (*P* < 0.05). ND, not detected.
ground, such as C57BL/6, the effect of background genetics or alternate effector mechanisms may be dominant over the contribution of EPO or MBP. This may explain why a recent study failed to show a role for EPO or MBP in C57BL/6 mice infected with *Brugia pahangi* (37). Whether the deficiency of either granule protein on a fully permissive background such as BALB/c would also lead to a higher worm load is also an interesting question that could help determine whether the protective role for IL-5 is mediated through eosinophils or a separate effector mechanism.

One question that arises is whether genetic deficiency of one granule protein leads to inhibited expression of the other. In this situation, it may be that only one of the two granule proteins is essential and that the phenotype seen in both mouse strains is due to the loss of one granule protein. Analysis of EPO activity in MBP-deficient mice and, conversely, testing whether there were MBP-positive eosinophils in EPO-deficient mice demonstrated that disruption of one gene did not affect the expression of the other.

Thus, EPO and MBP appear to have distinct roles in protective immunity, with both being essential for killing of *L. sigmodontis* parasites.

Considering the in vitro data that deposition of granule proteins is associated with attack and ultimately degeneration of worm larvae, it may be argued that the major effect of granule proteins is that of direct toxicity to the helminth. Alternatively, or in addition, the higher degree of susceptibility in knockout mice compared to the wild-type strain could be due to altered cytokine responses. In this regard, macrophages were shown here as one source of increased IL-10 in knockout mice (39). Since it is known that IL-10 is associated with higher permissivity both in resistant C57BL/6 (41) as well as in semi-resistant FVB mice (Specht et al., unpublished data), the higher propensity of macrophages to produce IL-10 during *L. sigmodontis* infection (Fig. 4b), may be one important factor in the higher worm load observed.

Interestingly, deficiency of EPO but not of MBP was associated with increased production of IL-5 by both unseparated thoracic cavity cells and purified CD4+ T cells. While higher secretion of IL-5 by thoracic cavity cells could also have been the result of the increased proportion of eosinophils observed...
in this knockout strain, this hypothesis could be excluded by the finding that similar levels of secreted IL-5 were observed when purified CD4⁺ T cells were used. In the converse argument, since the data suggest that IL-5 is mainly T-cell derived, the finding that similar levels of secreted IL-5 were observed in this knockout strain, this hypothesis could be excluded by

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susceptibility. Comparison of levels of cytokine production by IL-4 levels (since they produce IL-4) (5) or whether they regulate the IL-4 production by other cellular sources.

is whether the cytokine patterns in the knockout strains are more similar to the cytokine patterns in the classically susceptible BALB/c mice, indicating a common mechanism of susceptibility. Comparison of levels of cytokine production by throracic cavity cells of BALB/c mice, however, revealed that the BALB/c cytokine pattern bears both characteristics of the more resistant 129/SvJ wild-type strain (regarding IL-4 production) and those of the more permissive knockout strains (regarding adult worm antigen-induced IL-5 and IL-10 production). Thus, it seems that each mouse strain has its own characteristic cytokine pattern that does not directly relate to resistance or susceptibility, making it difficult to compare cytokines between different genetic backgrounds. Therefore, interpretations on the biological significance of cytokine alterations that are associated with knockout strains should always be made keeping within the respective genetic background.

In conclusion, this study shows that it is possible to demonstrate a function for eosinophil granular proteins EPO and MBP in vivo for the control of filariae, a finding that complements several in vitro studies demonstrating that these proteins, mostly MBP, can damage filarial parasites. The inability of heterologous filarial species such as Onchocerca volvulus to develop in mice or backcrossing to a more resistant mouse strain such as C57BL/6 may have precluded similar findings in other studies. Eosinophil granular proteins may not only be effective by direct attack on worms, but also may be involved in regulation of cytokine responses that are known to lead to different degrees of permissiveness. Our data underscore the important role of eosinophils in murine filarial infection both in their cytotoxic activity and as important regulators of the immune response (31).

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REFERENCES


