Wolbachia- and Onchocerca volvulus-Induced Keratitis (River Blindness) Is Dependent on Myeloid Differentiation Factor 88

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Endosymbiotic Wolbachia bacteria that infect the filarial nematode Onchocerca volvulus were previously found to have an essential role in the pathogenesis of river blindness. The current study demonstrates that corneal inflammation induced by Wolbachia or O. volvulus antigens containing Wolbachia is completely dependent on expression of myeloid differentiation factor 88.

The presence of intracytoplasmic bacteria in Onchocerca volvulus was first described in 1977 (13), and these bacteria were later identified as endosymbiotic Wolbachia (10). Pro-inflammatory responses in filarial-infected individuals and animals exposed to filaria have indicated that Wolbachia rather than the nematode is the cause of inflammation (3, 4, 12, 21). Wolbachia bacteria are essential for the pathogenesis of O. volvulus-induced keratitis, since (i) corneal inflammation was not induced by extracts derived from O. volvulus depleted of Wolbachia by doxycycline treatment of infected individuals and (ii) related filarial species containing Wolbachia induce keratitis but aposymbiotic species lacking Wolbachia do not (18). Both the systemic inflammatory response and the inflammatory response in the cornea were significantly reduced in C3H/HeJ mice (18, 21), which have a point mutation in the gene encoding Toll-like receptor 4 (TLR4) (16). Furthermore, the major surface protein of Wolbachia activates TLR2 and TLR4 (2). As TLR2 and TLR4 signal through the common myeloid differentiation factor 88 (MyD88) adaptor molecule, (19, 20, 22), we utilized MyD88−/− mice to examine the role of this intracellular signaling molecule in Wolbachia- and O.volvulus-induced keratitis.

Although our previous study demonstrated that Wolbachia has an essential role in O. volvulus-induced keratitis, we did not determine whether Wolbachia could induce keratitis in the absence of filarial antigens. Since insect Wolbachia from the Aa23 mosquito cell line activates macrophages (21), we determined whether this Wolbachia also induces keratitis. Isolated insect Wolbachia and a soluble O. volvulus antigen extract containing Wolbachia (OvAg) were quantified by PCR for the single-copy wsp gene as described previously (5, 14). Four microliters was injected into the corneal stroma of MyD88−/− mice and wild-type littermates, and the mice were sacrificed 18 h later at the peak of neutrophil infiltration (6). Eyes were embedded in paraffin, and 5-μm sections of the corneal stroma were immunostained with NIMP-R14, which is specific for neutrophils (6). As shown in Fig. 1A, Wolbachia induced a dose-dependent neutrophil infiltrate in the corneal stroma of immunocompetent mice. In marked contrast, no neutrophils were detected in the corneal stroma of MyD88−/− mice, even with the largest inoculum (Fig. 1A). Neutrophils were also absent in the corneas of MyD88−/− mice inoculated with O. volvulus extract compared with control mice (Fig. 1B). Figure 1C shows that the corneas of inoculated MyD88−/− mice were similar to saline controls.

The presence of neutrophils in the corneal stroma leads to loss of corneal transparency and can be measured by determining corneal haze by in vivo confocal microscopy (11). Wolbachia or O. volvulus extract containing Wolbachia was injected into the corneal stroma of control and MyD88−/− mice as described above, and corneal haze was calculated from stromal thickness and light intensity in combined images of the corneal stroma (11). Figure 2 shows that injection of Wolbachia-induced elevated levels of corneal haze in wild-type, immunocompetent mice compared with the haze in saline-inoculated mice. In contrast, there was no increase in corneal haze in Wolbachia-inoculated MyD88−/− mice compared with saline-inoculated corneas. Similar results were obtained for control and MyD88−/− mice inoculated with OvAg (Fig. 2B). Together with the impaired neutrophil recruitment to the corneas of MyD88−/− mice, these findings demonstrate that keratitis induced by Wolbachia and O. volvulus extracts is completely dependent on MyD88.

We previously demonstrated that neutrophil recruitment to the corneal stroma in immunized mice is dependent on CXCR2 and that intrastromal injection of O. volvulus stimulates CXC chemokine production by resident corneal cells (6). To determine if MyD88 is required for CXC chemokine production, O. volvulus antigen containing Wolbachia was injected into the corneal stroma of control and MyD88−/− mice. After 6 h, the peak time for CXC chemokine production and prior to neutrophil infiltration (6), corneas were dissected and sonicated, and the levels of chemokines were determined by an enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 3, the levels of both KC/CXCL1 and MIP-2/CXCL2 were signifi-

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cantly elevated in wild-type corneas inoculated with OvAg compared with the levels in saline-treated corneas (P < 0.05). However, neither chemokine was detected in MyD88−/− mice, indicating that production of these CXC chemokines by resident cells in the cornea was completely dependent on MyD88.

As neutrophils also produce CXC chemokines in response to filarial antigens containing Wolbachia (5), which likely exacerbates neutrophil infiltration into the cornea, we examined the role of MyD88 in neutrophil activation. Peritoneal neutrophils from control and MyD88−/− mice were isolated and incubated with isolated Wolbachia. Figure 4 shows that neutrophils incubated with Wolbachia produced KC/CXCL1, MIP-2/CXCL2, and tumor necrosis factor alpha. In contrast, neutrophils from MyD88−/− mice did not produce any of these cytokines in response to Wolbachia or lipopolysaccharide (LPS), although MyD88−/− neutrophils produced CXCL2/MIP-2 in response to phorbol myristate acetate, a non-TLR stimulus. Together, these data show that MyD88 has an essential role in Wolbachia-induced neutrophil activation and cytokine production.

In summary, the results of the current study show that Wolbachia- and O. volvulus-induced keratitis is completely ablated in MyD88−/− mice, demonstrating that the MyD88-dependent pathway has an essential role in this model of river blindness. Given that specific activation of TLR2, TLR4, and TLR9 in the mouse cornea requires MyD88 to induce corneal inflammation (11) and that neutrophils express TLRs and are activated by Wolbachia (5, 9), we propose that the response to Wolbachia is initiated by TLRs on resident cells in the corneal stroma. Secretion of CXC chemokines by these cells mediates neutrophil recruitment from the peripheral, limbal vessels into the avascular cornea and migration through the stromal matrix to the site of microfilaria degradation and release of Wolbachia (5). A second role for MyD88 in the inflammatory process is to initiate neutrophil activation and production of CXCL1/KC, CXCL2/MIP-2, and tumor necrosis factor alpha in response to Wolbachia, further mediating neutrophil recruitment. Neutrophil activation also leads to secretion of cytotoxic products, such as nitric oxide and oxygen radicals that disrupt the normal function of resident corneal cells, thereby leading to loss of corneal clarity.

Results of previous studies suggested that the inflammatory responses induced by Wolbachia and filaria extracts were mediated by LPS-like activity in Wolbachia (15, 18, 21); however, complete genome sequencing of Wolbachia from insects and Brugia malayi indicated that Wolbachia lacks the biosynthetic machinery for LPS (23; http://tools.neb.com/wolbachia). Although Wolbachia peptidoglycan and lipoproteins have not been tested yet, ligands for TLR2 and TLR4 are present in the Wolbachia surface protein, which is abundantly expressed in

FIG. 1. Neutrophils in the corneal stroma of wild-type and MyD88−/− mice after injection of Wolbachia or OvAg. Wolbachia was injected into the corneal stroma of MyD88−/− mice and wild-type littermates. After 18 h, mice were sacrificed, and corneas were stained for neutrophils using monoclonal antibody NIMP-R14. (A) Total number of neutrophils per 5-μm corneal section in control and MyD88−/− mice inoculated with 20,000, 10,000, or 2,500 bacteria. (B) Total number of neutrophils in control and MyD88−/− mice inoculated intrastromally with 4 μl OvAg in 4 μl. (C) Representative sections of wild-type and MyD88−/− mice inoculated with 4 μl Hanks balanced salt solution (HBSS) or with 20,000 Wolbachia bacteria. There were statistically significant differences between wild-type and MyD88−/− mice (P < 0.0001) for all Wolbachia concentrations and for OvAg. Data points represent individual corneas. epi., epithelium; endo., endothelium.
insect and filaria Wolbachia, is highly conserved among filarial species, and is recognized by filaria-infected individuals (1, 2, 17). Wolbachia surface protein from Dirofilaria immitis activates cells through TLR2 and TLR4 signaling in transfected fibroblasts, murine dendritic cells, and macrophages (2). As TLR2 and TLR4 signal through MyD88, these findings are consistent with our current findings and suggest that MyD88-independent pathways do not contribute to Wolbachia-mediated inflammatory responses.

In chronically infected individuals, adaptive immunity is established before significant microfilaria invasion of the corneal stroma; however, innate responses to Wolbachia in the cornea appear to be the triggering events for neutrophil infiltration and loss of corneal clarity, which is exacerbated in the presence of antibody and after recruitment of eosinophils (7, 8). Further studies of Wolbachia surface protein and other possible Wolbachia TLR ligands should reveal the contribution of the TLR pathways to the activation of innate and acquired immune responses to Wolbachia and filarial antigens associated with disease pathogenesis.
REFERENCES


