NOD2, the Gene Responsible for Familial Granulomatous Uveitis, in a Mouse Model of Uveitis

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PURPOSE. NOD2 plays an important role in the recognition of intracellular bacteria through its ability to sense the components of bacterial peptidoglycan (PGN), namely muramyl dipeptide (MDP) and muramyl tripeptide (MTP). Specific mutations in the human NOD2 gene cause Blau syndrome, an autosomal dominant form of uveitis, arthritis, and dermatitis. As a first step toward understanding the role of NOD2 in the pathogenesis of uveitis, the authors developed a mouse model of MDP-dependent uveitis.

METHODS. BALB/c mice and mice deficient in L-selectin or NOD2 received intravitreal injection of MDP, MTP, or PGN. The intravascular response within the iris and cellular infiltration was quantified by intravital microscopy and histologic analysis.

RESULTS. MDP induced an acute, ocular inflammatory response, wherein rolling and adhering leukocytes within the vasculature were significantly increased within 6 hours after MDP treatment. A minor increase in cellular infiltration occurred at 12 hours after MDP treatment. The adhesion molecule L-selectin participated in MDP-induced vascular inflammation because L-selectin knockout mice showed a significant decrease in the number of rolling cells. Importantly, NOD2 plays an essential role in ocular inflammation induced by MDP, as indicated by the fact that uveitis did not develop in Nod2 knockout mice in response to MDP. Nod2 knockout mice also showed abolished ocular inflammation in response to MTP but not to PGN treatment.

CONCLUSIONS. These findings demonstrate a novel mouse model of uveitis, wherein NOD2 plays an essential role in inflammation induced by the minimal components of PGN. Thus, innate immune responses mediated by NOD2 may participate in the development of uveitis in response to bacterial products.


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Innate immunity plays a fundamental role in host defense through its ability to rapidly respond to invading pathogens. The capacity of the innate immune system to initiate inflammatory responses is attributed to the presence of germ line-encoded receptors referred to as pattern recognition receptors (PRRs). Such PRRs are known to sense pathogen-associated molecular patterns (PAMPs), or invariant molecular structures present in pathogens, and endogenous danger signals (so-called DAMPs). Although the toll-like receptor (TLR) family has represented the major constituent of the PRRs, it is now apparent that the NOD-like receptor (NLR) family also plays an important role in the detection of bacterial products. The role of members of the NLR family in the eye has been relatively uncharacterized. However, it is likely that they may participate in the basic immune mechanisms involved in ocular inflammation, as has been previously demonstrated for TLRs such as TLR4 in endotoxin-induced uveitis.

The discovery of a single nucleotide change that results in the development of uveitis is a seminal finding that clearly implicates the gene, NOD2, whose contribution to ocular inflammation must be clarified. NOD2 belongs to the NLR family and so shares conserved structural domains, including an N-terminal caspase recruitment domain (CARD), a central nucleotide-binding oligomerization domain (NOD), and C-terminal leucine-rich repeats (LRRs). NOD2 has been shown to sense muramyl dipeptide (MDP) and muramyl tripeptide (MTP), which are naturally occurring breakdown products of the bacterial cell wall component peptidoglycan (PGN). The capacity of NOD2 to sense MDP is dependent on its LRR domains. In response to MDP, NOD2 forms a multiprotein complex containing RICK/RIP2, which promotes the IKK complex to activate the transcription factor NF-kB. Signaling pathways other than NF-kB that may be activated by NOD2 are not well understood, although apoptosis, the MAP kinase pathway, and the inflammasome have all been implicated. NOD2 also appears to play a role in controlling different bacterial infections, including Listeria monocytogenes and Salmonella typhimurium.

The importance of NOD2 in regulating inflammatory responses is further underscored by the discovery that mutated forms of NOD2 are responsible for Blau syndrome. Blau syndrome (also referred to as Jabs syndrome) was first identified as an autosomal dominant disease characterized by multiorgan granulomatous inflammation of the eye, skin, and joint. The uveitis is usually bilateral, causes chorioretinal scarring, and can result in severe visual loss. It was subsequently discovered that patients with Blau syndrome had missense changes within the NOD domain affecting specific residues 334 and 469 and other coding region mutations, thereby definitively identifying NOD2 as the gene responsible for Blau syndrome. Intriguingly, additional polymorphisms in NOD2 have been associated with increased susceptibility to Crohn disease, a granulomatous inflammation of the bowel that can also involve the uvea and joints. Thus, this provides us with the unique opportunity to examine how the function of a particular gene such as NOD2, which is involved in innate immunity,
may participate in the initiation of uveitis. Indeed it was recently demonstrated that Nod2 is expressed in mouse eye tissue and within human vascular endothelial cells from the iris, choroid, or retina, little is known regarding the in vivo function of NOD2 within the eye.

As a first step toward understanding the pathogenesis of eye inflammation in Blau syndrome, we sought to investigate the role of NOD2 in a mouse model of uveitis. We tested the hypothesis that activation of NOD2 by MDP results in an ocular inflammatory response in mice. Given that the hallmark of ocular inflammation is the activation and recruitment of leukocytes through the vasculature of the eye, we used an established technique of intravital microscopy to visualize such a response to MDP. We demonstrated that an intravitreal injection of MDP elicited a transient increase in the number of rolling and adhering leukocytes within the iris microvasculature and a minor cellular infiltrate within the iris tissue. The process by which leukocytes interact with the vasculature and infiltrate inflamed tissue has been described and occurs in a stepwise fashion that involves specific adhesion molecules. We took advantage of intravital microscopy to visualize the functional role of L-selectin in MDP-induced inflammation and showed that deficiency in L-selectin significantly reduced the number of rolling cells in response to MDP. Importantly, such cellular inflammatory responses were mediated by NOD2 because we found that MDP-induced cellular inflammation was abolished in Nod2 knockout mice. NOD2 was also required for the ocular inflammatory response induced by MTP, another breakdown product of PGN. In contrast, uveitis induced by the administration of intact PGN, which is a TLR2 agonist, did not cause we found that MDP-induced cellular inflammation was abolished in Nod2 knockout mice. NOD2 was also required for the ocular inflammatory response induced by MTP, another breakdown product of PGN. In contrast, uveitis induced by the administration of intact PGN, which is a TLR2 agonist, did not require NOD2. To our knowledge, these are the first findings to demonstrate a functional role for NOD2 in ocular inflammation in mice. We believe these studies provide the foundation for future evaluation of NOD2-mediated ocular effects.

**METHODS**

**Reagents**

Synthetic stereoisoforms (L and D) of MDP and synthetic MTP were purchased from Bachem (Torrance, CA) and dissolved in sterile saline. MDP tested below the lower limit of detection of endotoxin activity by Staphylococcus aureus was purchased from Fluka (Buchs, Switzerland). For MDP treatment, mice were given intravitreal injections (2 μL volume) using a Hamilton syringe with a 30½-gauge needle.

**Mice**

Age-matched (8–10 weeks old) female BALB/c mice, tyrosinase knock-out (albino) C57BL/6, L-selectin knockout mice, and their appropriate strain controls (nonobese diabetic background) were obtained from Jackson Laboratories (Bar Harbor, ME). Nod2 knockout mice were kindly provided by Richard Flavell of Yale University, which we then backcrossed 10 generations onto a BALB/c background. Mice were housed in a facility approved by the Association of Assessment and Accreditation of Laboratory Animal Care International. Procedures were carried out according to National Institutes of Health, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and Oregon Health & Science University Institutional Animal Care and Use policies.

**Intravitral Microscopy**

The leukocyte response within the vasculature and extravascular tissue of the iris was assessed by intravitreal microscopy according to a previously established method. Briefly, at the time of imaging, animals were injected with 35 mg/kg rhodamine 6G (Sigma, St. Louis, MO) and anesthetized with 1.7% isoflurane. Digital images of the iris vasculature were captured with a black-and-white video camera (Kappa Scientific, Gleichen, Germany) on an epifluorescence intravitral microscope (modified Orthoplan; Leica, Wetzlar, Germany) in three independent regions. Diameter and length of each vessel segment or iris tissue and leukocyte phenotype (i.e., rolling, adhering, infiltrating) were quantified off-line with Image J analysis software, previously described.

**Histology**

Mouse eyes were prepared for histologic assessment as previously described. Briefly, whole eyes were dissected, fixed, and embedded in paraffin for sectioning. Seven-micrometer tissue sections were stained with hematoxylin and eosin. An observer masked to treatment groups counted the number of leukocytes within the anterior chamber of seven sections for each eye (approximately every tenth slide of a whole eye sectioned completely). The mean number of leukocytes per section was then calculated for each mouse eye.

**Statistical Analysis**

Data are represented as mean ± SEM. Mean differences between treatment and genotype were analyzed using two-way and one-way analysis of variance with Bonferroni test or t-test post hoc analyses. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**MDP Induces an Ocular Inflammatory Response in Mice**

In this study, we sought to investigate whether NOD2 participates in uveitis resulting from innate immune responses in mice. Some previous studies in rabbit models of uveitis showed that systemic treatment with PGN complexes or MDP elicited an acute, ocular inflammation. Since then, the discovery of TLRs and, more recently, NLRs has provided new insights into the mechanisms by which bacterial derived products (PAMPs) induce inflammation. Here we took advantage of the availability of chemically synthesized MDP, devoid of contamination of other bacterial products, to examine whether MDP could induce uveitis in mice. Because earlier studies involving MDP had been performed in rabbits, we started with a dose–response study, wherein mice were treated with intravitreal injection of MDP (dose range, 5–100 μg), and inflammation was assessed by intravitral microscopy 6 hours later. Although we did observe a significant effect of systemically administered 100 μg MDP on rolling (320 ± 113 vs. saline, 10 ± 10 cells/mm² vessel/min) and adhering (70 ± 24 vs. saline, 8 ± 4 cells/mm² vessel; data not shown) leukocytes within the iris vasculature, we determined that an intravitreal injection of 100 μg MDP was most effective in consistently eliciting ocular inflammation. Shown as Supplementary Figures online at http://www iovs.org/cgi/content/full/49/4/1518/DC1 are images taken of mice treated with intravitreal injections of saline or 100 μg MDP; their intravascular inflammatory responses were imaged 6 hours later.

To determine the duration of the intravascular inflammatory response induced by MDP, mice were treated with intravitreal injections of 100 μg MDP or saline. Given that it had been reported that the biological activities of MDP are influenced by the stereo-configuration of MDP, wherein the second amino acid (D-Glx) is replaced with L-Glx enantiomer (L-MDP), we also investigated whether the chemically synthesized Lisoform of MDP (100 μg) could induce an ocular inflammatory response to the same extent as MDP. Cellular inflammation was then assessed by intravitral microscopy at different time intervals after treatment (Fig. 1). We found that MDP-induced in-
distinct processes of leukocyte rolling, sticking, and extravasal adhesion molecules have been identified that mediate the eye. In the mouse endotoxin–induced uveitis model, these processes have been visualized in vivo in several systems, including the mouse endotoxin–induced uveitis model, indicating that MDP elicits an inflammatory response to MDP within the iris vasculature. We did not observe any significant effect of the L-MDP within the same duration of time after treatment (Fig. 1), thereby indicating that the inflammatory capacity of MDP is dependent on the stereoisomeric configuration. Consistent with this conclusion, we did not observe a decrease in the amount of MDP-induced intravascular inflammation in the presence of threefold concentration of the L-MDP (data not shown). We have also found that the potential of MDP to elicit ocular inflammation occurs in the C57BL/6 mouse strain in addition to the BALB/c strain because albino C57BL/6 mice (tyrosinase knockout) also showed a significant increase in the number of rolling and adhering cells in response to MDP (data not shown). These data define the specific dose and timeframe of a novel model of MDP-induced uveitis in mice.

**L-Selectin Participates in Leukocyte Rolling within the Vasculature in Response to MDP**

Leukocyte diapedesis has been well described and is dependent on an array of adhesion molecules expressed on the leukocytes and the endothelium. Specific adhesion molecules mediate such interactions in a stepwise progression (i.e., rolling, adherence, and extravasation), and each of these steps has been visualized in vivo in several systems, including the eye. In the mouse endotoxin-induced uveitis model, several adhesion molecules have been identified that mediate the distinct processes of leukocyte rolling, sticking, and extravasation.

We took advantage of the ability of intravitreal microcopy to assess specific leukocyte-endothelium interactions to test the functional role for L-selectin in MDP-induced inflammation.

Mice that lacked expression of L-selectin received intravitreal injections of MDP, and the intravascular inflammatory response within the eye was assessed by intravitreal microscopy 6 hours later (Fig. 2). As expected, the wild-type (WT) control mice treated with MDP showed significant increases in the number of rolling leukocytes; this was significantly reduced in the L-selectin knockout mice. There was an obvious trend, though it was not statistically significant, toward fewer adhering cells in response to MDP in the L-selectin knockout mice. This is consistent with the mechanisms involved in rolling preceding those involved in adherence and further suggests that MDP upregulates other adhesion molecules responsible for such downstream events. These data demonstrate a critical role for L-selectin in mediating the initiation of the intravascular inflammatory response to MDP.

**NOD2 Is Essential for MDP-Induced Uveitis in Mice**

We went on to test directly whether the ocular inflammatory response to MDP was altered in Nod2 knockout mice. Nod2 knockout mice or their congenic controls received intravitreal injections of MDP, and the intravascular inflammatory response was assessed by intravitreal microscopy 6 hours later. As expected, the WT mice showed significantly increased numbers of rolling and adhering leukocytes in response to MDP. In contrast, the Nod2 knockout mice did not exhibit an increase in rolling or adhering cells in response to MDP (Fig. 3). Nod2 knockout mice also failed to show a decreased intravascular inflammation or cellular infiltration in the iris tissue as assessed by intravitreal microscopy 12 hours after treatment (data not shown). This was consistent with a lack of leukocyte presence within the aqueous of the anterior chamber of the eye 12 hours after treatment (data not shown) and is the first direct evidence for a model in which uveitis is dependent on NOD2.
metabolites that include MDP and MTP, PGN itself is also
NOD2 in response to PGN. Although PGN is degraded into
response is dependent on NOD2. We then tested the role for
show an abolished response to MTP, indicating that this re-
within the eye of WT mice. In contrast, 
increase in the number of rolling and adhering leukocytes
response was assessed by intravital microscopy 6 hours after
treatment (Fig. 4). We found that MTP elicits a significant
ocular inflammation in mice. Because this had not been previ-
ously demonstrated, the dose and time of the vascular inflam-
matory response induced by an intravitreal treatment of either
PGN or MTP were optimized, as assessed by intravital micros-
permade to be crucial players in some inflammatory eye diseases. The
heterogeneity of uveitis suggests that multiple pathways
can contribute to its pathogenesis. Microbial triggers are consid-
ered to be crucial players in some inflammatory eye diseases. Therefore, the innate immune recognition system and its pat-
ter recognition receptors (PRRs) within the eye and their interactions with their microbial PAMPS may play important roles in the underlying mechanisms involved in uveitis. The discovery of a single nucleotide change that virtually guaran-
tees the development of uveitis as a component of Blau syn-
drome is a seminal finding that clearly identifies a gene, NOD2, whose contribution to ocular inflammation must be clarified.

**DISCUSSION**

Uveitis, or intraocular inflammation, is an immune-mediated
disease that occurs as recurrent episodes of inflammation or as
chronic inflammation. The complications of uveitis can include
cataracts, glaucoma and retinal detachment, and it is one of the
leading causes of blindness. Although the characteristics of
uveitis can be confined to the eye, it can be associated with
systemic immune-mediated disease such as ankylosing spondy-
litis, juvenile idiopathic arthritis, reactive arthritis, Behçet syn-
drome, inflammatory bowel disease, and Blau syndrome. The
gene discovery of a single nucleotide change that virtually guaran-
tees the development of uveitis as a component of Blau syn-
drome is a seminal finding that clearly identifies a gene, NOD2, whose contribution to ocular inflammation must be clarified.

**NOD2 Essential for Ocular Inflammation Induced by the Muramic Acid MTP but Not by PGN**

In vitro studies demonstrated that NOD2-expressing cell lines responded to PGN and that NOD2 requires intact muramyl peptides with two MDP or three MTP amino acids. These observations prompted us to further define the functional role of NOD2 in vivo within the eye. We first assessed the ability of synthesized MTP (wherein the third amino acid is a lysine residue) or purified PGN of *Staphylococcus aureus* to induce ocular inflammation in mice. Because this had not been previously demonstrated, the dose and time of the vascular inflammatory response induced by an intravitreal treatment of either PGN or MTP were optimized, as assessed by intravital microscopy (data not shown). We then went on to investigate the possible requirements for NOD2 in MTP- or PGN-induced ocular inflammation. *Nod2* knockout mice were treated with intravitreal injections of MTP, and the ocular inflammatory response was assessed by intravital microscopy 6 hours after treatment (Fig. 4). We found that MTP elicits a significant increase in the number of rolling and adhering leukocytes within the eye of WT mice. In contrast, *Nod2* knockout mice show an abolished response to MTP, indicating that this response is dependent on NOD2. We then tested the role for NOD2 in response to PGN. Although PGN is degraded into metabolites that include MDP and MTP, PGN itself is also recognized by TLR2. Mice were treated with intravitreal injections of PGN, and inflammation was assessed by intravital microscopy 6 hours later (Fig. 5). We found that though PGN induced a significant ocular inflammatory response, this response was not entirely dependent on NOD2 because the number of rolling cells was only partially reduced and no difference was detected in the number adhering cells. This finding suggests that NOD2 may contribute in part to PGN responses such as leukocyte rolling. Thus, NOD2 is required for ocular inflammation induced specifically by the minimal peptide fragments of PGN, and it demonstrates that *Nod2* knockout mice are fully capable of mounting an intraocular inflammatory response to other stimuli of innate immune responses.
Here, we report the novel finding that NOD2 functions in ocular inflammation in mice. We show that activation of NOD2 by MDP elicits a transient increase in rolling and adhering leukocytes within the vasculature of the eye and a mild infiltration within the iris tissue. The capacity of MDP to induce ocular inflammation in mice was dependent on the stereoisomeric configuration of the isoglutamine moiety because the L-isomer did not induce significant ocular inflammation. This is consistent with an earlier study in vitro that showed stimulation of NOD2-transfected cell lines with L-MDP did not activate a NF-κB luciferase reporter. We demonstrated that the adhesion molecule L-selectin participates in the initiation of the inflammatory cascades or adhesion molecules in the eye, this mouse model should help to clarify specific ocular activities of NOD2.

In addition to its role in host defense, the importance of NOD2 in regulating inflammation is further emphasized by the fact that mutations in NOD2 cause Blau syndrome, an autosomal dominant, multiorgan, granulomatous inflammation of the eye, skin, and joints. Distinct polymorphisms of NOD2 have also been strongly linked to the onset of Crohn disease, an inflammatory disorder of the intestine and that can involve the uvea. Mutations in other NLR family members also cause autoinflammatory diseases, many of which are associated with uveitis. Notably, the three common residues affected by the mutations in NOD2 are also strongly linked to the onset of Crohn disease.

Treatment with bacterial cell wall components such as peptidoglycan complexes or MDP has been shown to elicit acute anterior uveitis in rabbits. In the present study, a direct function of NOD2 was shown for the first time to be essential for ocular inflammation induced by an intravitreal treatment of MDP or MTP in mice. Although intravitreal and systemic administration of MDP in the rabbit resulted in ocular inflammation, we found that intravitreal injection of MDP in mice was more effective than systemic treatment. PGN-induced uveitis did not completely require NOD2 because Nod2 knockout mice were still capable of increased rolling and sticking leukocytes within the iris vasculature. This finding is consistent with the observation that TLR2 mediates the recognition of PGN and that NOD2 is capable of being activated independently of TLRs. Our results also emphasize that it is unlikely that MDP or MTP is capable of inducing ocular inflammation independently of NOD2 (e.g., by TLRs) because MDP- and MTP-induced ocular inflammations were completely abolished in the Nod2 knockout mice. Given the few studies of the functional consequences of NOD2 activation on inflammatory cascades or adhesion molecules in the eye, this mouse model should help to clarify specific ocular activities of NOD2.

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NOD2 Essential in Murine MDP–Induced Uveitis


References


