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The NLRP3 inflammasome is active but not essential in endotoxin-induced uveitis

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Abstract

Objective—The inflammasome complex involving caspase-1 and nucleotide-binding domain, leucine-rich repeat containing protein (NLRP)3, also known as NALP3 or cryopyrin is important for host responses to microbial pathogens and several autoinflammatory diseases. We investigated the extent to which NLRP3 and caspase-1 control ocular interleukin (IL)-1β production and severity of uveitis (intraocular inflammatory disease) in an established, acute inflammatory uveitis model, endotoxin-induced uveitis (EIU).

Methods—Expression of NLRP3, its adaptor molecule ASC, also known as PYCARD (PYD and CARD domain containing), and caspase-1 were examined by immunoblotting. IL-1β production was measured by enzyme-linked immunosorbent assay (ELISA). Using knockout mice, roles for caspase-1 and NLRP3 were examined in uveitis induced by intraocular injection of *Escherichia coli* lipopolysaccharide (LPS).

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Results—NLRP3, ASC, and caspase-1 proteins are constitutively expressed in eye tissue. During EIU, IL-1β protein production increases; this requires the presence of both caspase-1 and NLRP3. However, severity of EIU is not altered by deficiency in either caspase-1 or NLRP3, as assessed by both intravital microscopy and histology.

Conclusions—These data identify the importance of the NLRP3 inflammasome for IL-1β production in the eye, yet indicate that its participation in EIU is nonessential.

Keywords

NLRP3; Caspase-1; Inflammasome; Uveitis; Mice; Lipopolysaccharide

Introduction

The inflammasome is a multiprotein complex containing the protease, caspase-1, that cleaves IL-1β and IL-18 into their secreted and biologically active forms. The importance of the inflammasome in host responses to various microbial pathogens is increasingly being realized. Elucidation of molecular underpinnings of the inflammasome has also revolutionized our understanding of the involvement of IL-1β in autoinflammatory diseases. It is known that caspase-1 activity is tightly regulated in a signal-dependent manner involving key interactions with the adaptor molecule, ASC, also known as PYCARD (PYD and CARD domain containing) and a subgroup of the nucleotide-binding domain, leucine-rich repeat containing (NLR) proteins that make up different inflammasome complexes [1-3]. Studies of gene-deficient mice and cells have defined roles for the nucleotide-binding domain, leucine-rich repeat containing protein (NLRP)3 inflamma-some in responses to a wide range of microbial pathogens as well as endogenous danger-associated signals [1, 3]. However, few studies have investigated the extent to which the NLRP3 inflammasome controls IL-1β production within the eye or its contribution to ocular inflammation.

The importance of the NLRP3 inflammasome is underscored by the discovery that its mutant forms are responsible for a set of rare autoinflammatory diseases known as the cryopyrin-associated periodic syndromes (CAPS) [4, 5]. The CAPS encompass a spectrum of three diseases: familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and chronic infantile neurological, cutaneous, and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID). All three diseases involve episodic fevers that coincide with varying degrees of inflammation involving multiple organs, including skin, joints, and sometimes eyes. Insights into the mechanisms of the NLRP3 inflammasome have led to remarkable success in treatment of these syndromes with anakinra (recombinant IL-1 receptor antagonist, IL-1RA) [6]. Interestingly, in-flammasome regulators such as pyrin or proline-serine threonine phosphatase-interacting protein 1 (PSTPIP1) as well as other NLR family members including nucleotide-binding oligomerization domain (NOD)1 and NOD2 are associated with rare human autoinflammatory diseases related to CAPS that also involve inflammation of joints, skin, or eyes [4, 6], and IL-1β is an implicated pathogenic factor in more common diseases involving joints and eyes such as ankylosing spondylitis and Behçet's disease [6-13].

Uveitis, or intraocular inflammatory disease, is a leading cause of blindness worldwide. Despite the concurrence of uveitis with systemic autoinflammatory disorders involving IL-1β, little is understood regarding the inflammasome pathway in intraocular inflammation. The messenger RNA (mRNA) transcripts of inflammasome components (NLRP3, ASC, and caspase-1) are known to be present in murine eye tissues [14], indicating their potential involvement in uveitis, yet their functional contribution remains to be determined. The eye's susceptibility to intraocular injection of LPS and the ensuing uveitis in mice have been well

documented by our group and others. This model is historically referred to as endotoxin-induced uveitis (EIU) [15]. Here, we tested the hypothesis that deficiency in NLRP3 inflammasome signaling would abrogate ocular inflammation in the EIU model. We evaluated protein expression of the inflammasome components (NLRP3, ASC, and caspase-1) in uveitic eye tissue. Using mice deficient in caspase-1 or NLRP3, we examined how IL-1β production was altered in their absence and whether severity of uveitis was impacted. We found that caspase-1 was activated and that IL-1β production was dependent on both caspase-1 and NLRP3. Somewhat surprisingly, however, EIU occurred independently of NLRP3 and caspase-1.

Materials and methods

Mice

NLRP3 knockout (KO) mice (on C57BL/6 background) were provided by Genentech (San Francisco, CA). Cas-pase-1 KO mice along with all congenic controls were purchased from Jackson Laboratory (Bar Harbor, ME). All animal experiments complied with the ethical and animal experiment regulations of the Association of Assessment and Accreditation of Laboratory Animal Care International and our Institutional Animal Care and Use Committee. All animals had access to water and food ad libitum.

Induction of endotoxin-induced uveitis

For intraocular LPS injections, anesthetized (1.7% isoflurane in oxygen) mice were administered a 2 μl intravitreal (i.v.t.) injection of 250 ng LPS in one eye and of saline in the contralateral eye while observing the needle with a dissecting microscope to avoid injuring the lens.

Intravital microscopy

Intraocular inflammation of the iris vasculature and tissue in mice anesthetized with 1.7% isoflurane was video recorded as previously described [16]. At time of imaging, mice were i.p. injected with 35 mg/kg rhodamine 6G (Sigma-Aldrich) for visualization of leukocytes. Digital videos (10 s each) of three independent regions of the iris were captured with a black and white video camera (Kappa Scientific, Gleichen, Germany) on an epifluorescence microscope (modified Orthoplan; Leica, Wetzlar, Ger-many) at 200x. The diameter and length of each vessel segment and the iris tissue as well as the numbers of rolling, adherent, and infiltrating leukocytes were determined offline using ImageJ analysis software as previously reported [16, 17].

Histopathology

At the indicated times, mice were sacrificed, and the eyes were enucleated, fixed in 10% neutral buffered formalin, and embedded in paraffin. Seven-micrometer tissue sections were stained with hematoxylin and eosin (H&E), and the severity of inflammatory changes of the eye (i.e., infiltration of leukocytes into the aqueous humor of the anterior segment and vitreous body of the posterior segment) were quantified by a masked observer.

Immunoblotting

Enucleated eyes were homogenized in lysis buffer containing protease inhibitors as previously described [18], and protein concentrations were determined by bicinchoninic acid (BCA) assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel (Bio-Rad) and transferred onto polyvinylidene difluoride membrane (Millipore). Primary antibodies to caspase-1 (Abcam), ASC (Millipore), NLRP3 (Epitomics), and β-actin (clone AC-15; Sigma Chemical, St.

Louis, MO) were used. The secondary antibodies were goat anti-rabbit immunoglobulin G (IgG) with IRDye 680 and goat anti-mouse IgG with IRDye 800CW (LI-COR, Lincoln, NE), and their near-infrared fluorescence signals were detected with a LI-COR Odyssey scanner and software.

ELISA

IL-1β production in eye tissue homogenates (protein isolated and quantified as described above) was determined by sandwich ELISA with paired antibodies (R&D Systems) as per manufacturer's instructions.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical evaluation of differences between experimental groups was determined by analysis of variance (ANOVA) and Student's *t* test (GraphPad Prism). Statistical significance was accepted at $p < 0.05$.

Results

Production of IL-1β in the eye requires caspase-1 activation

IL-1β production occurs through two distinct phases: an initial priming signal triggers mRNA expression and subsequent synthesis of the 31-kDa pro-IL-1β protein, and a second signal activates caspase-1 within the NLRP3 inflam-masome to cleave pro-IL-1β into its functional, secreted 17-kDa form [19, 20]. We found that the 45-kDa precursor form of caspase-1 is constitutively expressed in eye tissue and that the level of the cleaved and activated form is increased in response to LPS (Fig. 1a). At 5 h post LPS, the ratio of full-length to cleaved caspase-1 was ~2, suggesting relatively low levels of caspase-1 activation (Fig. 1b). We next tested the requirement for caspase-1 in IL-1β production. We found that IL-1β levels in eye tissue homogenates increase during EIU and that deficiency in caspase-1 abrogates IL-1β production (Fig. 1c). These data confirm the need for caspase-1 for production of IL-1β in EIU.

Severity of EIU is not altered by caspase-1 deficiency

If IL-1β has a substantial role in EIU, then caspase-1 KO mice should have a diminished response to i.v.t. LPS. To test this, we used intravital videomicroscopy, which enables us to visualize leukocyte trafficking within the iris in vivo and quantify cellular responses based on distinct behaviors such as leukocyte rolling and adherence to the endothelium within the iris microvasculature or cells that infiltrate the iris tissue [16]. Our previous reports on EIU have found the peak intravascular response at 5 h following LPS injection. We therefore examined the caspase-1 dependency of leu-kocytic responses at this time. We found that deficiency in caspase-1 did not significantly alter the cellular trafficking responses, as the number of rolling, adhering, or infiltrating leukocytes within the iris were similar to that of the WT responses (Fig. 2a). By 24 h following LPS injection, the cellular inflammatory response within the iris vasculature is in its resolution phase. We did not observe any significant differences between WT and caspase-1 KO mice at this point in time (data not shown).

One of the hallmarks of EIU is increased fibrin deposition and leukocytic infiltration into the aqueous humor of the anterior segment (Fig. 2c). Accordingly, we counted the number of leukocytes within the aqueous humor (Fig. 2b). We found that caspase-1 KO mice retained their responsiveness to LPS and did not exhibit any significant reduction in infiltrating cell numbers compared with WT controls. At this time, minimal inflammation was observed in the posterior segment, and caspase-1 deficiency did not alter this response (Fig. 2b). No other pathological differences, such as structural changes within defined tissues (iris, cornea,

retina), vasculitis, or hemorrhaging, were noted between WT and caspase-1 KO mice. These findings do not support a critical role for caspase-1 in EIU.

NLRP3 and its adaptor, ASC, are expressed in eye tissue and are responsible for IL-1β production

At least five NLR proteins can form inflammasome complexes with caspase-1, and in some cases NLRP3 has been reported to activate caspase-1-independent pathways [21, 22]. We therefore examined the extent to which NLRP3 contributes to IL-1β production within the eye and uveitis. Immunoblotting demonstrated constitutive NLRP3 protein expression in the eye that was not altered by LPS exposure (Fig. 3a). The caspase activation and recruitment domain (CARD)-containing adaptor molecule, ASC, participates in the NLRP3 inflammasome scaffold by recruiting caspase-1. We found that ASC is expressed in control, i.v.t. saline-injected eyes, and its expression is slightly increased in response to LPS (Fig. 3a). We evaluated the necessity for NLRP3 in IL-1β production in EIU (Fig. 3b) and determined that, akin to caspase-1 KO mice, NLRP3 KO mice have markedly impaired IL-1β production. These results support an essential role for the NLRP3 inflammasome in IL-1β production in EIU.

Mice with NLRP3 deficiency do not exhibit altered EIU

Using NLRP3 KO mice, we evaluated the function of NLRP3 in EIU. Because NLRP3 KO mice are on a C57BL/6 background, we were unable to perform intravital microscopy because the iris pigmentation impedes visualization of the fluorescent cells. Standard histological assessment, however, revealed that NLRP3 KO mice exhibited similar responsiveness to EIU as WT controls, and no significant difference in cellular infiltration within the aqueous humor or vitreous body was observed (Fig. 4). Akin to the caspase-1 KO mice, we did not observe any pathological aberrancy in the NLRP3 KO mice.

Discussion

Uveitis can arise from infection but is also a manifestation of many autoinflammatory diseases. IL-1 is one of the most studied cytokines in infection and inflammatory disease, yet very little work has investigated the extent to which the inflammasome contributes to uveitis. The data presented here demonstrate the presence of three NLRP3 inflammasome constituents within eye tissue and the capacity of caspase-1 and NLRP3 to regulate IL-1β production during EIU.

These studies show that deficiency in NLRP3 or cas-pase-1 did not significantly alter the severity of EIU. IL-1β can play an important role in leukocyte recruitment by its ability to trigger endothelial cells to produce chemokines, e.g., monocyte chemotactic protein-1 (MCP-1) and IL-8, and adhesion molecules, e.g., intercellular adhesion molecule-1 (ICAM-1), that are known to be critical for EIU [23-25]. Our finding that abrogated IL-1β production in the caspase-1 KO or NLRP3 KO mice did not alter LPS-triggered leukocyte recruitment was somewhat unexpected. KO and WT mice exhibited similar cellular trafficking responses within the iris, which coincided with increased leukocyte accumulation in the aqueous humor. Akin to the traditional model of EIU in WT mice, neutrophils appear to represent the predominant cell type. The lack of a role for IL-1β in uveitis is, however, consistent with prior reports investigating uveitis induced by LPS [26], as well as NOD1- or NOD2-dependent models of uveitis [18, 27]. NOD2 and NOD1 are NLRs associated with the autoin-flammatory diseases, Blau syndrome and sarcoidosis, wherein uveitis and arthritis are dominant clinical manifestations. In experimental forms of uveitis, caspase-1 was required for IL-1β production in the eye, yet IL-1 signaling was not essential for development of uveitis. These experimental data are consistent with clinical reports of the

ineffectiveness of anakinra in treating uveitis in patients with Blau syndrome [28, 29]. The apparent lack of a pathologic role for IL-1β in acute inflammatory uveitis driven by innate immune signals might reflect functional redundancy with a yet to be determined cytokine. These findings do not rule out the potential of the inflammasome in other experimental uveitis models such as experimental autoimmune uveitis (EAU), wherein IL-1 has been implicated [30, 31]. It is interesting to conjecture whether involvement of a T cell component as in EAU or the chronicity of uveitis may alter the inflammatory pathways involved in the pathogenesis of experimental uveitis.

There is precedent for caspase-1 mediating inflamma-tory responses to LPS. Indeed, resistance of caspase-1 deficient mice to endotoxin shock induced by LPS has long been known [32-34]. This leads us to consider how the ocular environment may alter the extent to which the caspase-1 inflammasome is required for inflammatory responses. The eye has an immunosuppressive milieu believed to protect its nonregenerating tissues that can suffer irreversible destruction in the face of inflammation, hence its immune-privileged state. The increased production of IL-1β in a caspase-1- and NLRP3-dependent mechanism indicates adequate mRNA synthesis, which has been previously reported [26, 35, 36], as well as inflam-masome activity. This would lead us to believe that intraocular injection of LPS might result in endogenous release of adenosine triphosphate (ATP) sufficient for activation of the inflammasome, as has been reported to occur in human monocytes [37, 38].

It is interesting to consider that the activities of secreted IL-1β are impeded in some way as an underlying mechanism of the immune-privileged state within the eye. The immunosuppressive milieu that exists within the eye [for example, increased presence of anti-inflammatory factors such as transforming growth factor-beta (TGF-β), neuropeptides, or IL-1Ra] may enhance the eye's resistance to certain inflammatory cytokines such as IL-1β. Future investigation into how the actions of IL-1β may be controlled within the eye will be important for our understanding of uveitis. We have recently examined the extent to which IL-1R contributes to EIU. Consistent with our findings here, deletion of IL-1R had no effect on EIU severity. However, IL-1Ra had a profound impact on controlling the severity of EIU as the uveitis was markedly exacerbated in mice deficient for IL-1Ra (Planck, Woods, Clowers, Nicklin, Rosenbaum, and Rosenzweig, submitted). Thus, there may be a threshold below which IL-1β activity is successfully blocked by IL-1Ra and above which IL-1β potentiates uveitis. This may be especially relevant to diseases characterized by high systemic IL-1β levels. Anakinra successfully ameliorated uveitis in a patient with the CAPS disease, NOMID (also referred to as CINCA) [39]. Emerging data support a beneficial effect for anti-IL-1 therapy in uveitis in patients with Behçet's disease as well [40]. Thus, dysregulation of IL-1 signaling has the potential to impact uveitis, and the extent to which targeting IL-1β therapeutically is beneficial likely varies depending on the pathogenesis of a particular disease.

The importance of IL-1β processing by the inflamma-some is being increasingly realized in a wide spectrum of host defense responses and diseases, yet its role in uveitis is poorly understood. The present study demonstrates the presence of inflammasome components ASC, caspase-1, and NLRP3 in murine eye tissue. Caspase-1 is activated during EIU, and both caspase-1 and NLRP3 are required for the ensuing IL-1β production. Somewhat surprisingly, absence of NLRP3 or caspase-1 did not profoundly alter EIU. These data indicate that the NLRP3 inflammasome is dispensable in EIU.

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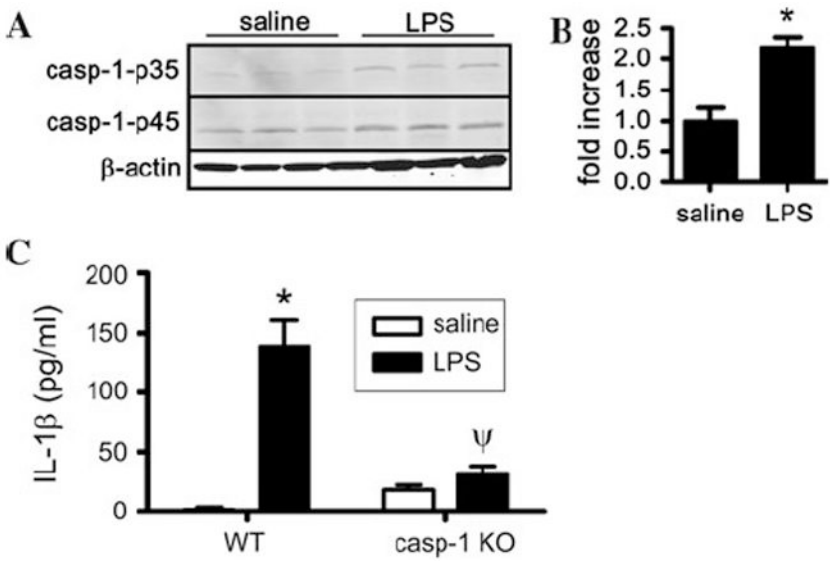

Fig. 1.

Production of IL-1β in the eye requires caspase-1. Regulation of IL-1β production and caspase-1 (casp-1) expression within eye tissue were examined 5 h following LPS or saline injection. **a** Expression of caspase-1 and its cleaved form were examined by immunoblotting, and **b** the relative intensity of cleaved caspase-1 compared with its pro-form is depicted graphically. Images are representative of three individual mice/treatment group (a total of six individual mice/treatment group were examined). **c** IL-1β production in eye tissue was measured by ELISA in wild-type (WT) and caspase- 1 KO mice injected with LPS or saline. $^{*}p < 0.05$ comparison between LPS and saline within a genotype, $^{\Psi}p < 0.05$ comparison between LPS-treated WT and LPS-treated caspase-1 KO mice (n = 8–10 mice/treatment group/genotype)

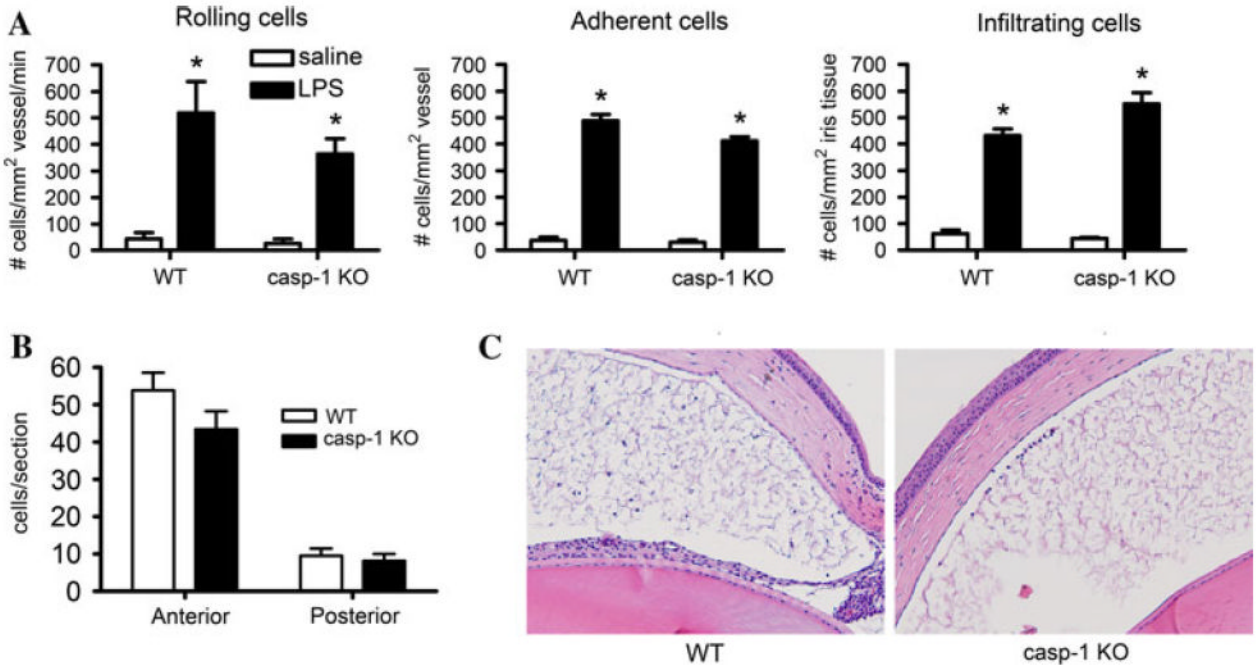

Fig. 2.

Severity of EIU is not altered by caspase-1 deficiency. Caspase-1 KO mice or WT congenic controls were challenged with LPS, and ocular inflammatory responses were measured at 5 h following injection. **a** Intravital microscopy demonstrates the number of rolling and adhering leukocytes within the iris vasculature along with the number of infiltrating cells within the iris tissue. *$p < 0.05$ comparison between LPS and saline within a genotype. **b** Histological assessment of uveitis was performed, and the infiltrating cells present within the aqueous humor of the anterior segment and vitreous body of the posterior eye segment were quantified. **c** Representative images of the anterior segment of eyes of LPS-challenged caspase-1 KO and congenic control mouse. There was no significant difference between genotypes (n = 10–12 mice/treatment group/genotype)

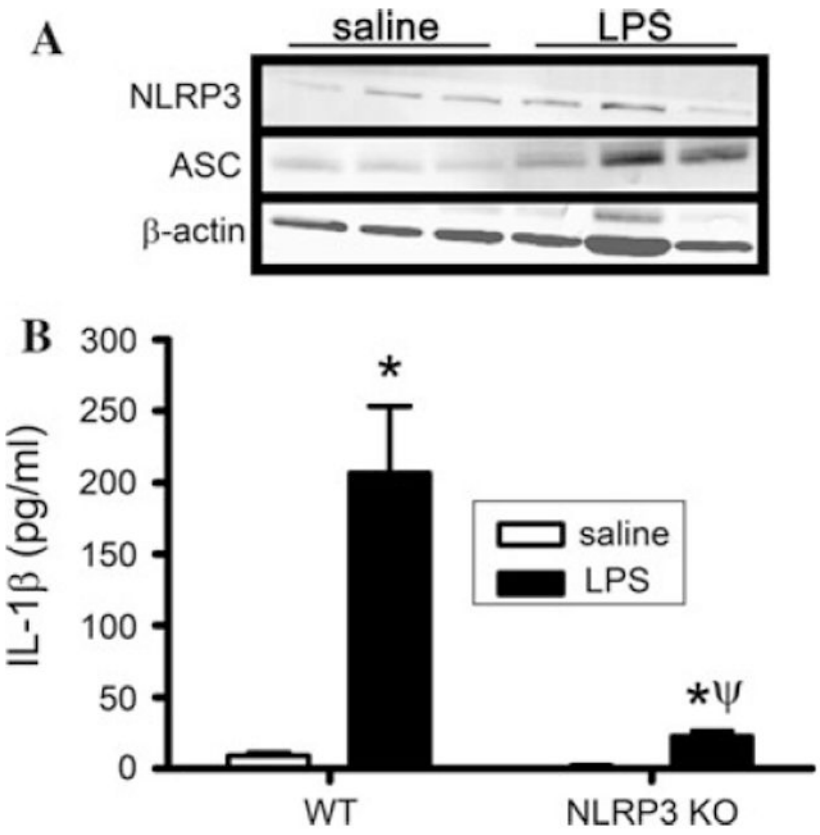

Fig. 3.

NLRP3 and its adaptor, ASC, are expressed in eye tissue and are responsible for IL-1β production. **a** Relative expression levels of NLRP3 and ASC in eye homogenates at 5 h post LPS injection were assessed by immunoblotting. Images are representative of three individual mice/treatment group (a total of six individual mice/treatment group were examined). **b** IL-1β production was measured by ELISA in WT and NLRP3 KO mice injected with LPS or saline. *$p < 0.05$ comparison between LPS and saline within a genotype, $^{\psi}p < 0.05$ comparison between LPS-treated WT and LPS-treated NLRP3 KO mice ($n = 8$ mice/treatment group/genotype)

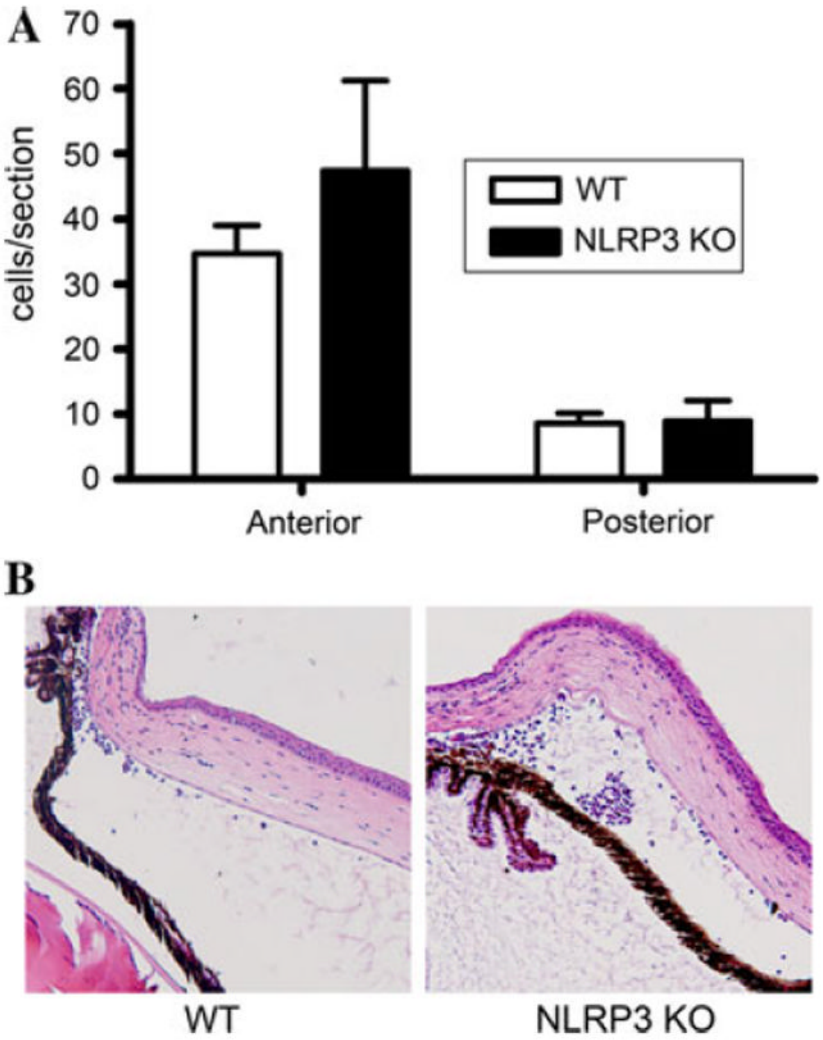

Fig. 4.

Mice with NLRP3 deficiency do not exhibit altered EIU. NLRP3 KO mice or WT congenic controls were challenged with LPS, and ocular inflammation was assessed histologically 5 h later. **a** Quantification of the number of infiltrating cells present in the aqueous humor of the anterior eye segment or the vitreous body of the posterior eye segment. **b** Representative images of the anterior chamber of the eyes of LPS-challenged NLRP3 KO and a congenic, control mouse. There was no significant difference between genotypes (n = 10–12 mice/ treatment group/genotype)