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NLRP3 inflammasome activation by mycobacterial ESAT-6 and dsRNA in intraocular tuberculosis

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ABSTRACT

The molecular basis of intraocular tuberculosis (TB) is not well understood. In this study, we investigated the role of two constituents of viable *Mycobacterium tuberculosis* – Early Secreted Antigenic Target-6 (ESAT-6), and mycobacterial RNA– in inflammasome activation in the retinal pigment epithelium (RPE) – a key site of inflammation in intraocular TB.

We found that ESAT-6 induced caspase-1 activation and inflammasome priming in mouse RPE cells, significantly more in wild-type than in *Tlr2/3/4/7/9*$^{-/-}$, *Myd88*$^{-/-}$ and *Nlrp3*$^{-/-}$ RPE cells. Sub-retinal ESAT-6 injection resulted in greater RPE degeneration in wild-type than *Nlrp3*$^{-/-}$ mice. In human ocular TB tissue sections, NLRP3 staining was noted in retina as well as RPE. Mycobacterial RNA, specifically its double stranded component, also induced caspase-1 activation, and the double stranded RNA was immunolocalized to human ocular TB sections. Our observations suggest that inflammasome activation in RPE by viable *M. tuberculosis* could potentially contribute to human intraocular TB.

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1. Introduction

Intraocular tuberculosis (TB) is a common cause of ocular inflammation in TB-endemic countries and is being increasingly reported from non-endemic countries [1,2]. Despite its widespread occurrence, several aspects of the disease including its molecular pathogenesis, remains unclear. The challenges in understanding pathogenesis of intraocular TB are primarily due to the paucibacillary nature of *Mycobacterium tuberculosis* infection in the eye [3]. In the current study, we have used mycobacterial products, associated with viable infection, to elucidate the molecular mechanisms of intraocular TB.

Microbes or microbial products generate innate immune response in the host through several types of pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and various nucleic acid receptors [4]. These PRRs not only sense the microbial product, but also determine the extent of immune response depending on the presence or absence of microbial viability and virulence factors [5].

M. tuberculosis has a wide range of virulence factors including lipids, cell envelope and secreted proteins, that could potentially activate inflammasomes [6]. Amongst these, the Early Secreted Antigenic Target-6 (ESAT-6), a 6kD protein, is secreted by live *M. tuberculosis* and has been demonstrated to be the most potent mycobacterial activator of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome [7]. ESAT-6 is also essential for colonization and granuloma formation *in vivo* [8]. Thus, ESAT-6 is not only a marker for mycobacterial viability, but also, a key virulence factor. However, it is unknown whether ESAT-6 activates NLRP3 in the eye, or whether NLRP3 is expressed in human intraocular TB specimens. Bacterial RNA also represents microbial viability to

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the innate immune system [5]. Once detected in the host cytosol, microbial RNA can activate NLRP3 inflammasome leading to caspase-1 dependent IL-1β production [9-11]. However, it is not known whether RNAs accumulate in human intraocular TB, or whether RNA from *M. tuberculosis* can activate the inflammasome.

In this study, we demonstrate NLRP3 inflammasome activation in human intraocular TB. We also show in *in vivo* and *in vitro* studies, that *M. tuberculosis* viability and virulence factors such as the secreted protein ESAT-6 and bacterial RNA (specifically double-stranded or dsRNA) can induce inflammasome activation in the retinal pigment epithelium (RPE). Finally, we demonstrate immunolocalization of dsRNA in human intraocular TB.

2. Methods

2.1. Immunostaining of human intraocular TB tissue sections

Paraffin-embedded tissue sections of human ocular tuberculosis obtained from archival material of enucleated globes and diagnosed by *IS6110* targeted real-time PCR or presence of acid-fast bacilli (n=5) or age-matched non-diseased controls (n=3) were first deparaffinized in xylene and decreasing concentrations of ethyl alcohol. The clinical diagnosis in the five human intraocular TB cases included panophthalmitis (n=2), panuveitis, choroiditis, and necrotising scleritis (all n=1). The sections were then depigmented with 0.25% potassium permanganate and 0.5% oxalic acid. Immunostaining was performed with mouse antibody against NLRP3 (1:100, Sigma Aldrich, St. Louis, MO) or dsRNA (1:1000, clone J2, English & Scientific Consulting, Szirák, Hungary) in later experiments, and the bound antibody detected with biotin-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA).

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Specificity of staining was ascertained by substituting isotype IgG for the primary antibody.

2.2. Cell culture

Human fetal and primary mouse RPE cells were isolated as previously described [18-19]. Mouse RPE cells were obtained from 6-8 week old C57BL/6J wild type (WT), *Nlrp3*$^{-/-}$, *TLR23479*$^{-/-}$ and *MyD88*$^{-/-}$ mice. All knock out mice used in our study were back-crossed with C57BL6/J and were free of the *Rd8* mutation. All cells were incubated at 37 ^{o}C at 5% CO_2. Mouse cells were maintained in Dulbecco modified Eagles medium (DMEM), supplemented with 20% fetal bovine serum (FBS) and penicillin-streptomycin, while 10% FBS supplementation was used for human RPE cells.

2.3. ESAT-6 treatment

ESAT-6 (BEI Resources, USA, catalogue #NR-14868), dissolved in Hanks BSS, as per manufacturer's instructions was used for treating RPE cell cultures. Cells with 80-90% confluence were treated for varying durations with 5μg of ESAT-6 per mL of culture medium in all experiments, except where other doses are mentioned. This dose was chosen based on reported use of 2.5μg dose of ESAT-6 for NLRP3 inflammasome activation in THP-1 macrophages [7].

2.4. Mycobacterial RNA extraction and treatment

M. tuberculosis H37Rv was grown in Middlebrook 7H9 supplemented with 0.5% glycerol and 10% BD Middlebrook ADC (Fisher Scientific, Hampton, NH) and 0.05% Tween-80 in rolling culture. *Exponential phase* cultures (OD_{600} between 0.5

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and 0.8) were harvested for RNA extraction using FastRNA Pro blue kit (MP Biomedicals, Santa Ana, CA) according to manufacturer's instructions. RNA extracts were treated with Turbo DNase (Thermo Fisher Scientific, Waltham, MA) until DNA free. The samples were analysed by Nanodrop-1000, for confirming quality as well as for quantification. The RNA was divided into aliquots of 10 μg each and stored at -80 ^{o}C.

10 μg of exponential phase *M. tuberculosis* RNA, either untreated or following various enzymatic treatments (Table 1) was transfected into human or mouse RPE cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A synthetically produced 300 nt long dsRNA, Alu RNA, that has been shown to cause NLRP3 dependent caspase-1 activation in the RPE (endogenously produced in age-related macular degeneration [14], was similarly transfected to be used as positive control. Enrichment of ssRNA from total RNA was performed using a ssRNA enrichment kit (Zymo, Irvine, CA).

2.5. Western blotting

After 24 hours incubation with either ESAT-6 or mycobacterial RNA, RPE cells were lysed with radioimmunoprecipitation assay (Sigma-Aldrich, St. Louis, MO) lysis buffer supplemented with protease inhibitor. Protein concentrations were measured with Bradford assay kit (Biorad, Hercules, CA) and equal amounts of protein samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels (Novex, Invitrogen, Carlsbad, CA). Proteins were then transferred to polyvinyledine difluoride membranes (Millipore, Burlington, MA), that were blocked for one hour with LI-COR buffer and then incubated overnight at 4 ^{o}C, with anti-caspase-1 antibody (1:1000, Invitrogen, Carlsbad, CA, diluted in LI-COR buffer as per manufacturer's

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instructions). The immunoreactive bands were identified on the Odessey CLx infrared imaging system (LI-COR, Lincoln, NE). Besides, ESAT6 treated RPE cells from WT, *Nlrp3*$^{-/-}$, *TLR23479*$^{-/-}$ and *MyD88*$^{-/-}$ mice were also tested for NLRP3 protein abundance (1:1000, Sigma-Aldrich, St. Louis, MO).

2.6. Real-time PCR

We tested inflammasome priming at various time points following ESAT-6 activation of mouse RPE cells. Total RNA was TRIzol extracted from RPE cells by following the manufacturer's instructions. Equal quantities of RNA were reverse transcribed using Quantitect kit (Qiagen, Germantown, MD) and the resultant cDNA was amplified in the 7900 HT Fast real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green detection system. Relative expressions of target genes were determined by the 2^{-DDCt} method using the 18S rRNA housekeeping gene. Oligonucleotide primers specific for mouse Nlrp3 (forward, 5′-ATGCTGCTTCGACATCTCCT-3′, and reverse, 5′-AACCAATGCGAGATCCTGAC-3′), mouse Il-18 (forward, 5′-GACAGCCTGTGTTCGAGGAT-3′, and reverse, 5′-TGGATCCATTTCCTCAAAGG-3′), and mouse 18S rRNA (forward, 5′-TTCGTATTGCGCCGCTAGA-3′, and reverse, 5′-CTTTCGCTCTGGTCCGTCTT-3′) were used.

2.7. Mice

All animal experiments were approved by institutional review committee and carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual

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Research. Wild type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Nlrp3*$^{-/-}$ mice have been previously described [9]. All knock out mice were backcrossed to C57BL6/J, and were confirmed to be free of *Rd8* mutation.

2.8. Sub-retinal injections of ESAT-6

Subretinal injections of ESAT-6 (1µg/1µL) were given to WT and *Nlrp3*$^{-/-}$ mice anesthetized with 100 mg/kg ketamine hydrochloride (Ft. Dodge Animal Health), with a 34G needle and Exmire microsyringe (Ito Corporation, Fuji, Japan). Pupils were dilated with 1% tropicamide drops (Alcon Laboratories, Fort Worth, TX).

2.9. Fundus photography and assessment of RPE degeneration

Fundus photographs of dilated mouse eyes were taken seven days after sub-retinal injection of ESAT-6, with TRC-50IX camera (Topcon, Tokyo, Japan) linked to a digital imaging system (Sony Corp., Tokyo, Japan). Following fundus photography, mice were sacrificed and whole mounts of RPE/ choroid were prepared that were fixed with 4% paraformaldehyde, and stained with rabbit polyclonal antibodies against mouse zonula occludens-1 (ZO-1, 1:100, Invitrogen, Carlsbad, CA), and visualized on immunofluorescence microscopy (SP-5, Leica, Exton, PA) with Alexa 594 (Invitrogen, Carlsbad, CA). Additional WT and *Nlrp3*$^{-/-}$ mice were euthanized, and retina and RPE were collected separately in RIPA buffer with proteinase inhibitor, and homogenized by sonication. Protein abundance was tested by western blot analysis, with antibodies against caspase-1 (1:1000, Invitrogen) and vinculin (1:1000, Sigma-Aldrich, St. Louis, MO)

2.10. *In vivo* imaging of caspase activation induced by ESAT-6

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Seventy-two hours after sub-retinal injections of ESAT-6, we gave intravitreal injections of a near-infrared fluorophore (NIRF, Ex: 790 Em: 800) conjugated pan-caspase probe (VAD-fmk). Fundus photos and near-infrared fluorescent imaging were acquired using Topcon TRC-50IX, 4 hours following the injection.

3. Results

3.1. ESAT-6 induces dose-dependent activation of caspase-1, and cytotoxicity in mouse RPE cells

ESAT-6 induces dose-dependent activation of caspase-1, and cytotoxicity in mouse RPE cells: We tested the effect of mycobacterial viability and virulence associated factors – ESAT-6 and bacterial RNA in inducing inflammasome activation in RPE cells *in vitro* and *in vivo*. We selected the RPE for our *in vitro* studies, as it is commonly involved in intraocular TB (multifocal serpiginoid choroiditis) and has been predicted to be the site for preferential localization of *M. tuberculosis* in the eye [12-13].

We found caspase-1 activation in mouse RPE cells after treatment with a range of ESAT-6 doses (Figure 1A). These results suggest that RPE cells are susceptible to innate immune activation by *M. tuberculosis* secreted protein ESAT-6. In addition, we examined inflammasome priming at various time points after ESAT-6 activation. We noted significantly increased *NLRP3* and *IL-18* mRNA abundance only after 24 hours of treatment (Figure 1B-C). These results suggest that RPE cells are able to mount an innate immune response to *M. tuberculosis* secreted protein ESAT-6.

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3.2. ESAT-6-induced caspase-1 activation is dependent on TLR signaling, NLRP3 and MyD88: We next investigated the upstream pathways for caspase-1 activation. Previous studies in mice and THP-1 cells have implicated NLRP3 activation as important inflammatory mediators activated by ESAT-6 [7]. Consistent with these previous findings, we found that ESAT-6-induced caspase-1 activation was dramatically reduced in *Tlr23479*$^{-/-}$, *Myd88*$^{-/-}$ and *Nlrp3*$^{-/-}$ RPE cells compared to wild-type RPE cells (Figure 1D).

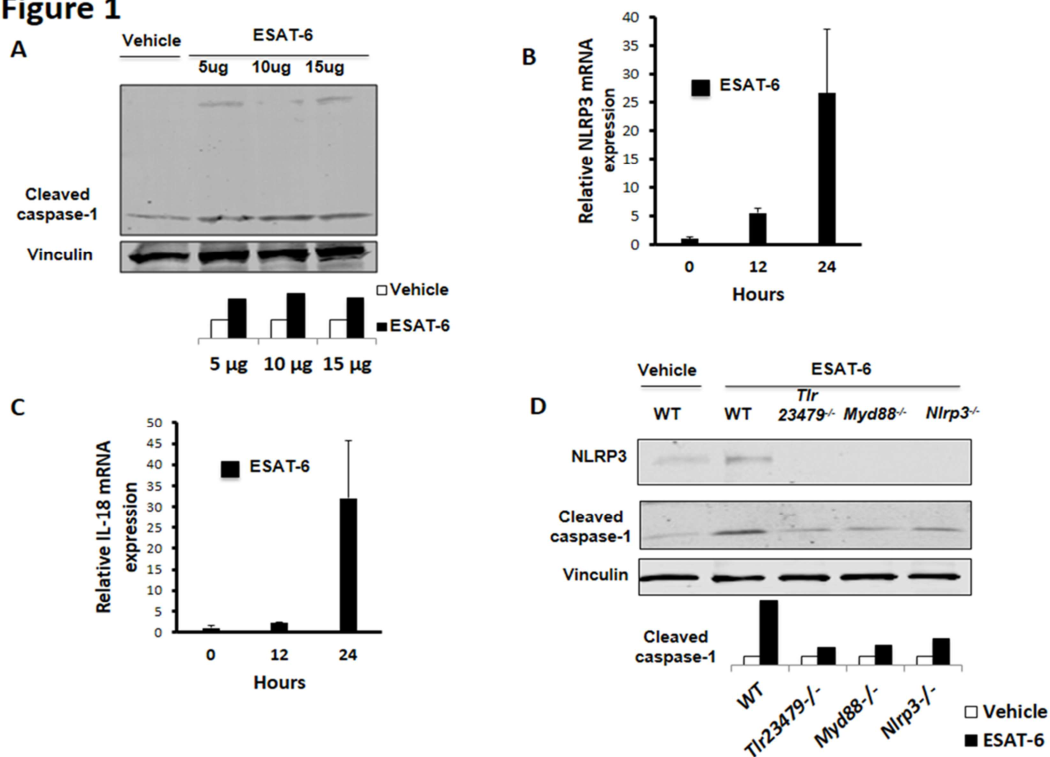

Figure 1. **ESAT-6 induces dose-dependent activation of caspase-1, and cytotoxicity in mouse retinal pigment epithelium (RPE) cells.** (A) Western blotting for active caspase-1 protein levels after treatment with 5, 10 and 15µg of ESAT-6. Priming of NLRP3 analyzed by mRNA abundance of NLRP3 (B) and IL-18 (C) after 0, 12 and 24 hours of treatment with 10µg of ESAT-6. (D) Absent NLRP3 protein, and reduced caspase-1 generation, after ESAT-6 treatment in *Tlr234*, *MyD88* and

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Nlrp3 deficient cells, as compared to wild type (WT) RPE cells, suggesting role of these mediators in NLRP3 inflammasome and caspase-1 activation.

3.3. Sub-retinal injection of ESAT-6 induces NLRP3 dependent RPE degeneration: The RPE is a common site of inflammation in some forms of intraocular TB such as multifocal serpiginoid choroiditis [12]. In mice, sub-retinal injection of ESAT-6, but not vehicle BSS, resulted in RPE degeneration around the injected area (Figure 2A). The extent of degeneration was confirmed on ZO-1-stained RPE flat mounts that revealed extensive distortion of cell morphology in ESAT-6 treated eyes compared with regular hexagonal cells in vehicle treated eyes (Figure 2B). Furthermore, *in vivo* imaging with a pan-caspase probe revealed increased intensity of the active probe in ESAT-6 treated eyes as compared to vehicle treated eyes (Figure 2C). Moreover, eyes from *Nlrp3*$^{-/-}$ mice were protected from ESAT-6-induced RPE degeneration compared to wild-type mice (Figure 2D). This is consistent with the finding (Figure 1F) that caspase-1 activation was dependent on NLRP3. Further indicating that ESAT-6 causes RPE degeneration via the NLRP3 inflammasome, protein extracted from both retina and RPE of ESAT-6 injected mice showed significantly higher activated caspase-1 (p20) in wild type mice as compared to *Nlrp3*$^{-/-}$ mice (Figure 2E).

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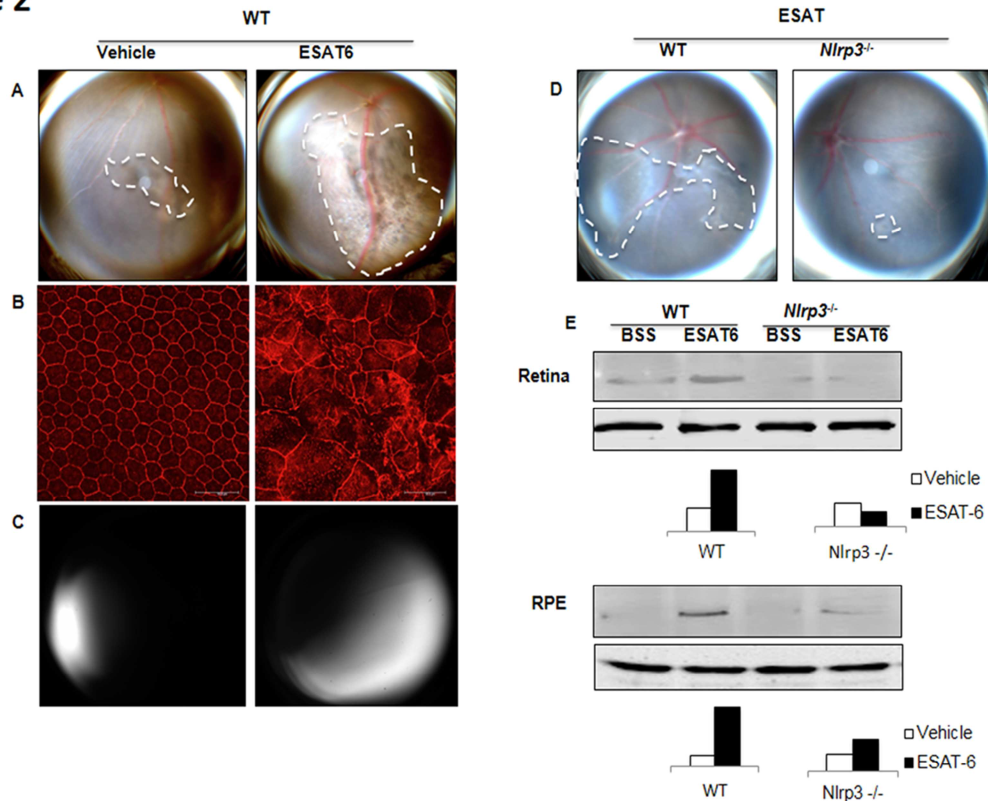

Figure 2. Sub-retinal injection of ESAT-6 induces NLRP3 dependent RPE degeneration. RPE degeneration after ESAT-6 injection as seen on (A) color fundus photograph. n=5 (B) zonula occludens-1 staining, n=4 and (C) bioimaging with pan-caspase probe. n=5. (D) Significantly reduced RPE degeneration in *Nlrp3* deficient mice, as compared to wild type (WT) mice. n=3 (E) Western blotting of pooled retina and RPE extracts showing dramatically reduced caspase-1 activation in Nlrp3 deficient mice

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3.4. Double-stranded RNA from *M. tuberculosis* induces NLRP3-dependent caspase-1 activation in RPE: We have previously demonstrated that accumulation of an endogenous RNA (*Alu* RNA) induces NLRP3 inflammasome activation and RPE degeneration in geographic atrophy – an

advanced form of age-related macular degeneration [14]. As such, we used *Alu* RNA as a positive control for caspase-1 activation. Transfection of *M. tuberculosis* total RNA also activated caspase-1 in primary human RPE cell cultures (Figure 3A). This finding is consistent with previous studies that demonstrated the immunological potential of bacterial RNA delivered into the cytosol of various types of eukaryotic cells [9-11].

To determine the characteristics of *M. tuberculosis* RNA that activate caspase-1, we treated *M. tuberculosis* total RNA with different ribonucleases (RNases) prior to incubation with RPE cell cultures (Table 1). While treatment with RNase I (which specifically degrades single-stranded RNA (ssRNA)) did not alter Caspase-1 activation, treatment with RNase III (which specifically degrades double-stranded RNA (dsRNA)) completely abrogated the response (Figure 3B). These data suggest that dsRNA from *M. tuberculosis* is responsible for caspase-1 activation. Further supporting this concept, ssRNA enriched from mycobacterial total RNA did not activate caspase-1 (Figure 3C). It is likely that caspase-1 activation by mycobacterial RNA in RPE cells is also mediated by NLRP3, as has been demonstrated by previous studies [9-11].

Table 1: Summary of various methods used to treat *Mycobacterium tuberculosis* RNA (10μg) prior to transfection into human retinal pigment epithelium

Treatment	Dose	Duration
RNase I (Life Technologies)	5U	30 minutes
RNase III (Life Technologies)	0.5U	1 hour

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ssRNA concentrator (Zymo Research)	As per manufacturer's instructions

We also detected dsRNA immunoreactivity in paraffin embedded tissue sections of patients with ocular tuberculosis by using the J2 antibody that recognizes long dsRNA [15]. dsRNA was detected in various cell types including the RPE in ocular tuberculosis tissue sections (4/4), but not in normal age-matched controls (refer to our earlier publication [14]) (Figure 3D). It is tempting to speculate that the J2-positive signal is mycobacterium-derived dsRNA; however, it is also possible that the observed dsRNA is host-derived, and is induced by bacterial infection or inflammation. Future work will be required to determine the source of dsRNA accumulation in ocular TB sections.

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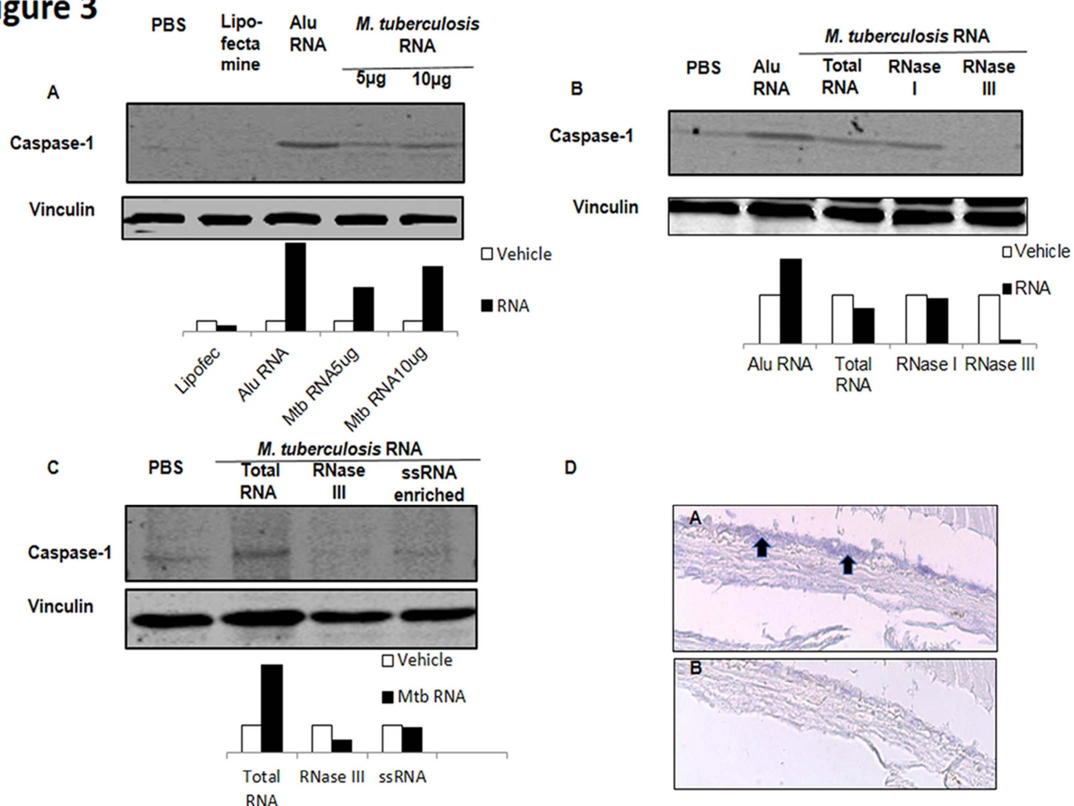

Figure 3: **Double-stranded RNA from exponential phase *Mycobacterium tuberculosis* (*Mtb*) H37Rv cultures activates caspase-1 in the retinal pigment epithelium (RPE)** (A) Immunoblot analysis of caspase-1 activation 24 hours after treatment with two different doses of *Mtb* RNA. Alu RNA (10 µg) was used as positive control while phosphate buffered saline (PBS) and lipofectamine were used as negative controls. (B) Caspase-1 activation in human RPE cells exposed to 10µg *Mtb* RNA previously treated with RNase I or RNase III (C) Caspase-1 activation in hRPE cells exposed to *Mtb* RNA previously treated with RNase III, or to single-stranded RNA-enriched *Mtb* RNA (D) Immunolocalization of double-stranded RNA in human intraocular TB (n=4) with J2 antibody in retinal pigment epithelium (purple, arrows), but not with isotype antibody.

3.5. **NLRP3 activation in human eyes with intraocular TB:** In further support of the role for NLRP3 in intraocular TB, we found immunoreactivity to NLRP3 in retina, RPE, choroid and iris from human intraocular TB tissue (Figure 4). These tissue sections had been obtained from enucleated eyes with bacteriologically confirmed TB. Increased abundance of NLRP3 protein in diseased tissues as compared to controls was suggestive of involvement of this pathway in intraocular TB. Interestingly, we found significant NLRP3 immunoreactivity in the photoreceptor layers of the retina. This needs to investigated in future studies as a mechanism of visual loss in intraocular TB and other forms of ocular inflammation.

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Figure 4

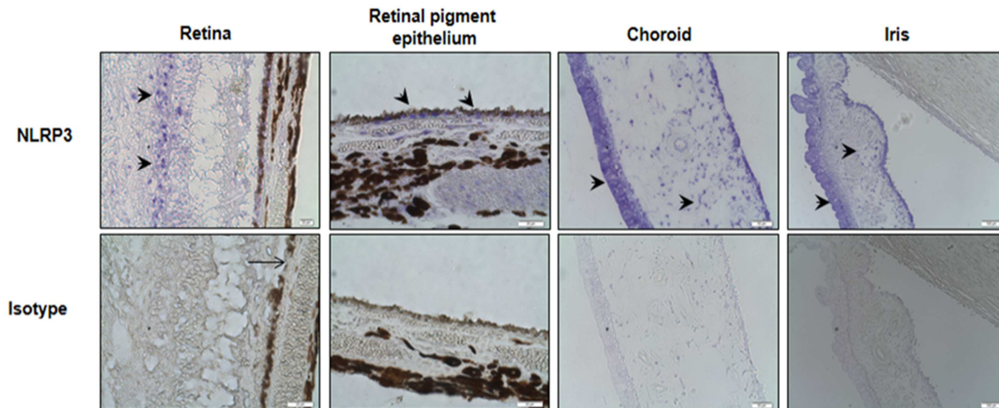

Figure 4. Immunolabeling of human intraocular TB, showing staining for NLRP3 (arrowheads, top panel) in retina, RPE, choroid (with detached RPE) and iris. n=4. No staining was seen with isotype IgG antibody. Focal area of necrosis in the RPE (arrow) is shown in the lower panel for RPE antibody.

4. Discussion

Inflammasome activation has been described previously in various forms of ocular inflammation [14,16]. Although *M. tuberculosis* is known to activate the NLRP3 inflammasome in systemic TB, the role of inflammasome activation in intraocular TB was not known. Our study demonstrates the involvement of the NLRP3 inflammasome in intraocular TB. We have demonstrated inflammasome activation by two markers of *M. tuberculosis* viability – a secreted protein, ESAT-6 and mycobacterial RNA. We found caspase-1 protein abundance and increased *NLRP3* expression after ESAT-6/ mycobacterial RNA treatment. In addition, caspase-1 activation was reduced in *Nlrp3*$^{-/-}$ cells. Further, we demonstrated RPE

degeneration and caspase-1 activation in RPE, following sub-retinal injection of ESAT-6. Both were significantly reduced in *Nlrp3*$^{-/-}$ mice. Finally, we provided direct evidence of NLRP3 by immunostaining in human ocular TB.

Primary involvement of RPE has been suggested in imaging studies in serpiginous-like choroiditis, a common form of intraocular TB. Histopathological study has demonstrated presence of *M. tuberculosis* within the RPE in an enucleated eye with panuveitis [13]. Most recently, RPE cells have been shown to phagocytose *M. tuberculosis* at rates similar to THP-1 macrophages [17]. Also, viable mycobacteria persisted longer in RPE cells than in THP-1 cells. Together, these observations suggest that RPE cells can be a potential reservoir for viable *M. tuberculosis* in the eye. Thus, inflammasome activation in the RPE by viable mycobacteria could be crucial for pathogenesis of intraocular TB.

A novel finding of our study is the relationship between intraocular inflammation and bacterial RNA. We found that RNase III sensitive RNA (double-stranded), but not RNase I-sensitive RNA (single-stranded) from *M. tuberculosis*, activated caspase-1 in RPE cells. Previous studies have documented similar differential responses of microbial RNA upon treatment with different RNases [9-11]. For example, Sander *et al.* found that *E. coli* RNA-induced IL-1β and lactate dehydrogenase production was abrogated in THP-1 macrophages after RNase I and III treatment, or with RNase III treatment alone, though they did not highlight the specific role of bacterial dsRNA in inflammasome activation [10]. We did not study the role of various bacterial RNA components (mRNA, rRNA and tRNA) individually, though previous reports show different responses to these RNA components in human and murine macrophages [11]. mRNA appears to be the only active component in murine macrophages, while all three components activate

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inflammasomes in human macrophages. We speculate that the differential response to double- or single-stranded RNA not only varies between different hosts but also between different cell types in the same host.

Our results would be stronger if replicated with live *M. tuberculosis*, and also compared with that from dead organisms. Additional studies with adoptive transfer of ESAT-6 specific T-cells, from immunized mice to healthy mice would help in ruling out the possibility of activated *M. tuberculosis* specific T-cells from non-ocular sites of infections migrating to the eye to cause uveitis. We plan to work on this interesting hypothesis in future studies. There were also incongruities between our experimental and human data, that we could not overcome in the current study. For example, we found clear evidence of RPE cell death *in vitro* and *in vivo*, while such was not evident in human tissue sections. This might be due to the extremely paucibacillary nature of human eye infections, and correspondingly low levels of mycobacterial proteins/ RNA. Similarly, mere demonstration of NRLP3 inflammasome in human tissues is not equivalent to its activation/oligomerization. These too need to be addressed in future studies.

In conclusion, our study links inflammasome activation in intraocular TB to mycobacterial viability. This association reiterates the importance of anti-mycobacterial therapy in the treatment of intraocular TB.

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AUTHORS CONTRIBUTIONS

Conceptualization: SB, BF, NK

Data curation: SB

Formal analysis: SB, BF, NK

Investigation: SB, BF, NK, KA, NR

Original draft: SB, BF, NK

Review and editing: SB, BF, NK, KA, NR

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Highlights

- Molecular mechanisms of ocular tuberculosis are unknown
- We investigated inflammasome activation by viable mycobacterial constituents in the eye
- ESAT-6 induced NLRP3-dependent caspase-1 activation in retinal pigment epithelium(RPE)
- Mycobacterial double-stranded RNA(dsRNA) also activated caspase-1 in RPE
- NLRP3 and dsRNA were immunolocalised in human ocular TB sections

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