Supplementary information.

Nitric oxide controls tuberculosis immunopathology by inhibiting NLRP3 inflammasome-dependent IL-1β processing

Bibhuti B. Mishra, Vijay A. K. Rathinam, Gregory W. Martens, Amanda J. Martinot, Hardy Kornfeld, Katherine A. Fitzgerald, and Christopher M. Sassetti

1Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA
2Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA
3Division of Pulmonary, Allergy and Critical Care, Department of Medicine, University of Massachusetts Medical School, Worcester, MA
4Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA
5Howard Hughes Medical Institute, Chevy Chase, MD
Supplementary figure 1. IL-1β production from *Mtb*-infected macrophages requires the NLRP3 inflammasome and the bacterial ESX1 secretion system. Pretreatment with IFN-γ is required for IL-1β suppression. a. BMDMs obtained from mice lacking various NLRs were primed with LPS and infected with *Mtb* H37Rv. 24hrs following infection, IL-1β and TNF were measured in the culture supernatants by ELISA. *P* values were calculated by students *t*-test (two tailed). b. BMDMs derived from C57BL/6 mice were primed with LPS and infected with various strains of Mtb as indicated. IL-1β and TNF were measured in the supernatant. *P* values were calculated by students *t*-test (two tailed). ∆ESX1 contains the “RD1” deletion removing a large portion of the esx1 locus. The ∆rv3616 strain lacks a distally encoded component required for function of the system. ∆rv3616 :: rv3616 is a complemented version of this strain. c. IFN-γ was either omitted (Pam₃CSK₄->Mtb), added to macrophages 24 hours before infection (IFN-γ+ Pam₃CSK₄->Mtb), or 16 hours after infection (Pam₃CSK₄->Mtb->IFN-γ). Secretion of IL-1β into the culture supernatant was quantified by ELISA. *P* values were calculated two way ANOVA with Bonferroni’s multiple comparison post test. Throughout the figure, white bars indicate IL-1β and black bars indicate TNF.
Supplementary figure 2. IFN-γ pretreatment does not alter bacterial or macrophage viability.  

**a.** Macrophage-associated bacteria were enumerated by plating immediately after infection and 24 hours later. CFU: colony forming unit. **b.** Macrophage viability was assessed by quantifying intracellular ATP concentration using a luciferase-based assay\(^1\). Relative ATP concentration is represented by “light units”.

Supplementary figure 3. NO donors produce a physiologically relevant dose of this compound.  

**a.** NO production was estimated by measuring nitrite in the supernatant of \(Mtb\)-infected C57BL/6 and \(iNos^{-/-}\) BMDMs, using the Griess reagent. Supernatants are the same as those assayed for IL-1\(\beta\) in Fig. 6a. **b.** BMDMs were treated with SNAP or GSNO and nitrites were quantified as in panel “a”. Supernatants are the same as those assayed for IL-1\(\beta\) in Fig. 6d.
Supplementary figure 4. cGMP does not alter IL-1β release. BMDMs were treated as indicated. “8-Br-cGMP” indicates treatment with this cell permeable analog of cGMP². This compound was added 12 hrs before Mtb infection and maintained in the media throughout. Supernatants were assayed for IL-1β 16hrs after Mtb infection by ELISA.
Supplementary figure 5. Caspase-1 autoprocessing is not inhibited by nitric oxide.
The indicated NLRP3- or AIM2-inflammasome components were transfected into 293T cells along with 25ng of caspase-1 expression plasmid (first six lanes). In parallel, 250ng of caspase-1 expression plasmid was transfected alone to cause overexpression-induced caspase-1 autoprocessing ("10x Casp1"). Cells were treated with or without SNAP (125-250µM) and caspase-1 maturation into its active form was monitored by immunoblotting. To account for the different levels of caspase-1 expression, the blots were exposed for different times (as indicated). SNAP treatment suppressed NLRP3-dependent caspase-1 maturation, consistent with the inhibition of IL-1β processing shown in Figure 7a. However, this treatment had no effect on either AIM2-dependent caspase-1 maturation or the autoprocessing of caspase-1.
Supplementary figure 6. Adaptive immunity controls inflammatory pathology in tuberculosis. A protective Th1 response (left) will produce IFN-γ and promote the induction of iNOS from both resident and recruited macrophages\(^3,4\). This response has two distinct effects. NO can inhibit bacterial replication by poisoning the respiratory chain and intermediary metabolism of the pathogen\(^5,6\). In addition, we show that NO suppresses the production of active IL-1 by inhibiting the assembly and activity of the NLRP3 inflammasome. This suppression prevents the persistent production of IL-1 and the subsequent granulocytic inflammation promoted by this cytokine. Both activities of NO are required to produce the balanced anti-bacterial and anti-inflammatory state that is necessary to contain the infection. In contrast, weak or inappropriately polarized T cell responses (right) are unable to control bacterial replication, and the resulting high bacterial burden triggers robust activation of the NLRP3 inflammasome. As a consequence, the persistent production of IL-1 causes sustained neutrophil recruitment, tissue damage, and disease\(^7,8\). These two scenarios could represent different individuals with altered immune function or different lesions in an individual that undergo distinct fates during progression of disease.
Supplementary references.