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Human Gene Variants Linked to Enhanced NLRP3 Activity Limit Intramacrophage Growth of *Mycobacterium tuberculosis*

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Activation of the NLRP3 inflammasome and subsequent generation of interleukin 1 β is initiated in macrophages upon recognition of several stimuli. In the present work, we show that gain-of-function gene variants of inflammasome components known to predispose individuals to inflammatory disorders have a host-protective role during infection with *Mycobacterium tuberculosis*. By isolation of macrophages from patients and healthy blood donors with genetic variants in *NLRP3* and *CARD8* and subsequent infection of the cells with virulent *M. tuberculosis*, we show that these gene variants, combined, are associated with increased control of bacterial growth in human macrophages.

Keywords. NLRP3; CARD8; inflammasome; IL-1 β ; human macrophage; C10X; Q705K; tuberculosis; mycobacteria.

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a global health threat. However, most people who are exposed to *M. tuberculosis* never develop active disease, suggesting that individual host factors influence the susceptibility to the bacterium. *M. tuberculosis* resides in human macrophages, where it

activates several receptors, including NLRP3 [1]. NLRP3 activation leads to recruitment of the adaptor ASC, which, together with NLRP3, forms the NLRP3 inflammasome. This inflammasome in turn activates caspase-1, cleaving pro-interleukin 1 β (IL-1 β) to active IL-1 β [2]. IL-1 β secretion is further regulated by the adaptor protein CARD8 via interaction with caspase-1 and/or by inhibiting NF κ B-mediated proIL-1 β synthesis [3]. The mechanisms behind IL-1 β release remain elusive, but production of this cytokine plays a pivotal role in the control of tuberculosis [4]. On the other hand, upregulated IL-1 β production, as observed in individuals carrying gain-of-function variants of the *NLRP3* gene, predisposes for inflammatory disorders [5]. We have previously described 2 such variants in the *NLRP3* gene linked to inflammatory disorders: a unique M299V mutation in 2 siblings [6] and a combination of a common Q705K polymorphism in *NLRP3* and a C10X polymorphism in *CARD8* in a patient [7]. The C10X polymorphism introduces an early stop codon in *CARD8*, resulting in decreased expression levels of CARD8 [8]. These polymorphisms are common, with allele frequencies of 6.5% (Q705K) and 34% (C10X) in a Swedish cohort and approximately 4% for both polymorphisms combined [7]. Here, we studied whether gene variants in inflammasome genes, besides predisposing for inflammatory disorders, could have a protective role during intracellular infection with *M. tuberculosis*. We show that macrophages from individuals carrying a gain-of-function variant in the *NLRP3* gene (ie, M299V or Q705K) in combination with C10X in the *CARD8* gene are superior in controlling *M. tuberculosis* growth.

MATERIALS AND METHODS

A detailed description is presented in the [Supplementary Materials](#).

Patients and Blood Donors

The first study subject (patient 1, a male with the Q705K and C10X polymorphisms) has a history of inflammatory disease, as previously described [7]. The second subject (patient 2, a male with the M299V and C10X polymorphisms) and the third subject (patient 3, a female with the M299V polymorphism) are siblings with previous symptoms of inflammation, as described in more detail elsewhere [6]. Human monocyte-derived macrophages (hMDMs) from sex-matched individuals carrying wild-type *NLRP3* and *CARD8* served as controls. Whole-blood specimens from healthy donors was collected at the blood bank at Linköping University Hospital.

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Preparation of Cells

hMDMs from whole-blood specimens (from healthy blood donors, patients, or healthy controls) were prepared as described elsewhere [9].

Preparation of Bacteria and Experimental Infection

The *M. tuberculosis* strains (luciferase-expressing H37Rv-lux, H37Rv-GFP, and H37Ra-GFP) were grown in Middlebrook 7H9 broth (Difco) supplemented with albumin-dextrose-catalase (Becton Dickinson) and Tween-80.

Evaluation of Bacterial Growth

The number of bacteria in infected samples was evaluated by luminescence, in which the luminescence correlates to the number of colony-forming units, as described in the [Supplementary Materials](#).

Evaluation of CD63 Colocalization or LysoTracker Staining

For CD63 staining, fixed cells were stained with anti-CD63 antibody (Sanquin) and Alexa 594-conjugated goat anti-mouse antibody (Invitrogen). For LysoTracker (Invitrogen) staining, LysoTracker Red DND-99 was added during the last 2 hours of incubation. The H37Ra-GFP used has earlier been shown to inhibit phagolysosomal fusion to the same extent as H37Rv [10].

Genotyping Assay

DNA was isolated from whole blood, using the Maxwell Blood DNA Purification Kit (Promega). The Q705K polymorphism of *NLRP3* (rs35829419) and the C10X polymorphism of *CARD8* (rs2043211) were genotyped using the Taqman SNP Genotyping assay (Applied Biosystems).

Statistical Analyses

The distribution of bacterial growth data in macrophages from 43 healthy donors included in the study was tested with the D'Agostino and Pearson test. On the basis of results of that test, the Student's *t* test (for comparison between 2 groups) and 1-way analysis of variance with the Tukey post hoc test (for comparison between multiple groups) were performed using Graph Pad Prism 5. Data are means \pm standard error of the mean. Statistical significance is defined as a *P* value of $<.05$.

Study Approval

Written informed consent was obtained from all subjects, and the study was approved by the regional ethics committee in Linköping (M177-07). Peripheral blood mononuclear cells were isolated from blood specimens obtained from these subjects and from anonymous, healthy blood donors in accordance with the guidelines of the local ethics committee and the declaration of Helsinki.

RESULTS

As a part of previous studies of patients with genetic variants in inflammasome-related genes linked to increased IL-1 β production [6, 7], we tested the ability of hMDMs from these patients to handle *M. tuberculosis* infection. Cells were infected with H37Rv-lux, and bacterial growth was evaluated after 48 hours (day 2) and compared to findings for healthy, matched controls. hMDMs from patient 1 (who had the Q705K and C10X polymorphisms) and patient 2 (who had the M299V and C10X polymorphisms) displayed increased control of *M. tuberculosis*, compared with those from wild-type control subjects, whereas hMDMs from patient 3 (who had the M299V polymorphism only) showed decreased growth control (Figure 1A). On the basis of these observations, we hypothesized that combined genetic variants in *NLRP3* and *CARD8* conferred the elevation of IL-1 β levels and increased the *M. tuberculosis*-restricting capacity of the cells. An increased IL-1 β level during mycobacterial infection has also been shown to enhance phagolysosomal fusion [11]. To investigate whether phagosomal maturation was altered in hMDMs carrying genetic variants of *NLRP3*, patient-derived macrophages were infected with GFP-expressing H37Rv, and the percentage of CD63-positive phagosomes was analyzed. hMDMs from patient 2 (who had *NLRP3* M299V and *CARD8* C10X) showed an increase in the percentage of CD63-positive phagosomes as compared to healthy donors and patient 3 (who had *NLRP3* M299V and wild-type *CARD8*; Figure 1B).

Previous studies have shown that *NLRP3* Q705K in combination with *CARD8* C10X results in constitutive secretion of IL-1 β (20–200 pg/mL) in nonstimulated cells [5, 7]. The enhanced growth control and phagolysosomal fusion seen in patients in the present study could be because their hMDMs are being differentiated in a more proinflammatory milieu *in vivo* and/or are preactivated. To mimic this scenario, hMDMs were differentiated *ex vivo* in the presence of IL-1 β for 7 days before cells were infected and CD63-positive phagosomes or acidified phagosomes were evaluated. However, neither CD63 translocation (Supplementary Figure 1A) nor acidification (Supplementary Figure 1B) was affected by differentiation in the presence of IL-1 β . Furthermore, differentiation in the presence of IL-1 β not only failed to stimulate increased phagosomal functionality, but also did not affect growth control (Supplementary Figure 1C). Increasing the IL-1 β concentration (250 pg/mL, 2.5 ng/mL, and 25 ng/mL) during differentiation did not affect the outcome (data not shown), nor did preactivation of cells with IL-1 β shortly before infection (Supplementary Figure 1D). Recently, a case report showed that the IL-1 receptor antagonist anakinra caused reactivation of latent tuberculosis [4]. To mimic latent tuberculosis, hMDMs were infected with a lower multiplicity of infection of 1 before either generation of IL-1 β or signaling by IL-1 β was blocked, and bacterial growth was

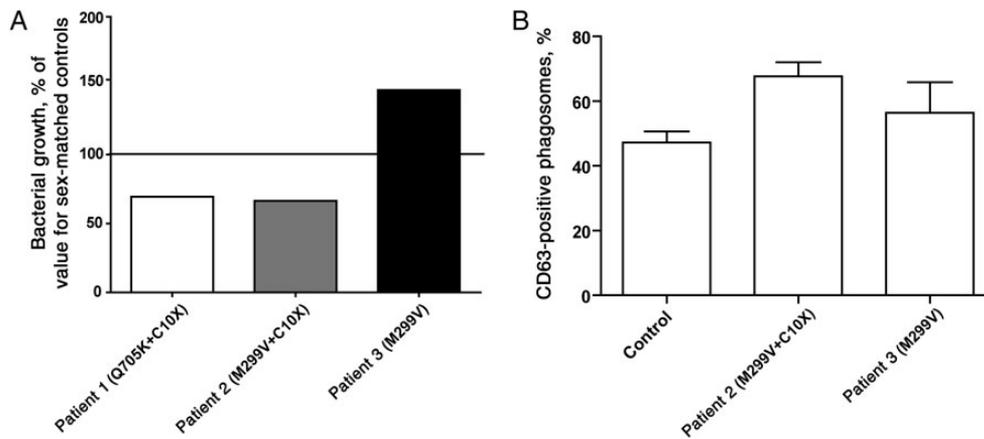


Figure 1. Macrophages from patients with inflammatory disease display increased growth restriction of H37Rv and increased phagosomal fusion. Human monocyte-derived macrophages (hMDMs) from patients carrying different genetic variants in *NLRP3* and/or *CARD8* were infected with H37Rv at multiplicity of infection of 10. *A*, Luminescence was evaluated as a measure of phagocytosis on day 0. At 48 hours (ie, on day 2), luminescence was evaluated again, and the fold-change in growth was calculated as the day 2 value divided by the day 0 value. The figure shows the total fold-change from both the lysate and supernatant fraction, where the fold-change of H37Rv in cells from individuals with the respective gene variant are compared to that in cells from sex-matched control individuals with no polymorphisms. *B*, hMDMs from patient 2 and patient 3 were seeded on glass coverslips and stained for CD63 at 48 hours after infection. The percentage of CD63-positive phagosomes was evaluated by confocal microscopy in a blinded fashion ($n = 2$). Cells from healthy blood donors were included as controls ($n = 18$).

analyzed. Although only low amounts of IL-1 β are seen during low-burden infection in hMDMs, blocking of IL-1 β signaling by the addition of anakinra significantly increased the growth of *M. tuberculosis* (Supplementary Figure 1E), whereas no effect could be seen during inhibition of caspase-1 by YVAD (Supplementary Figure 1F).

Because it was not possible to generate hMDMs resembling the cells from the patients by adding exogenous IL-1 β during incubation ex vivo, we decided to take advantage of the fact that *NLRP3* Q705K and *CARD8* C10X are both relatively common polymorphisms. To this end, blood specimens from 43 anonymous blood donors were used to isolate hMDMs,

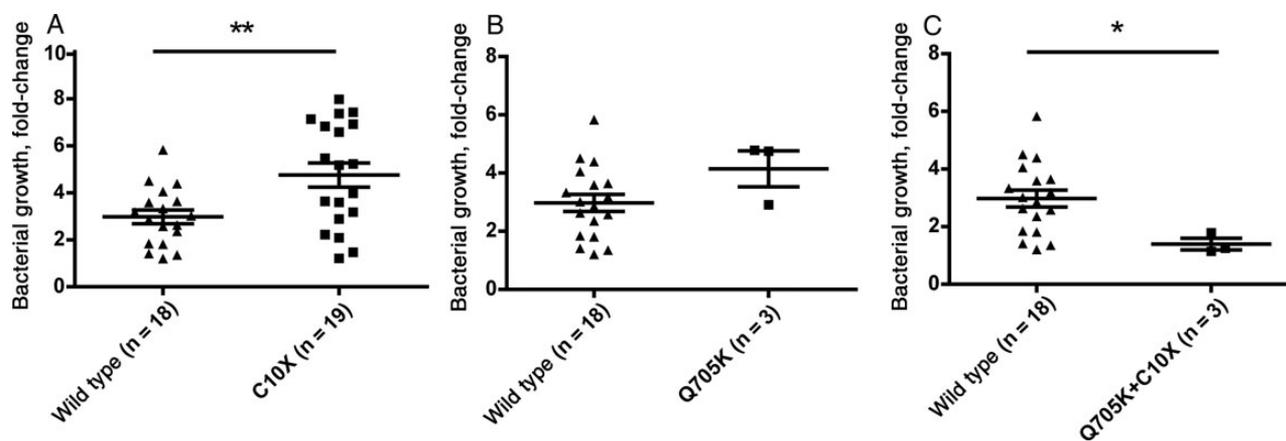


Figure 2. Macrophages harboring both *CARD8* C10X and *NLRP3* Q705K polymorphisms display increased growth control of *M. tuberculosis*. Human monocyte-derived macrophages from healthy blood donors ($n = 43$) were infected with H37Rv-lux at multiplicity of infection 10 for 1 hour before luminescence was measured (ie, on day 0). Forty-eight hours after infection (ie, on day 2), luminescence was measured again. Bacterial growth was calculated as the day 2 value divided by the day 0 value. The same donors were genotyped for 2 polymorphisms (Q705K and C10X), and the mean bacterial growth was compared between different groups. The figure shows the comparison between both heterozygous and homozygous individuals for *CARD8* C10X ($n = 19$) and individuals with none of the studied polymorphisms (wild type; $n = 18$; *A*), individuals heterozygous for *NLRP3* Q705K ($n = 3$) and wild-type individuals (*B*), and individuals heterozygous for *CARD8* C10X plus *NLRP3* Q705K ($n = 3$) and the wild-type population (*C*). The Student's *t* test was used for comparison in all panels. * $P < .05$, ** $P < .01$.

which were infected with H37Rv-lux. The blood sample from each donor was subjected to gene analysis, and the allele frequencies are shown in [Supplementary Table 1](#). Because the data presented in [Figure 1](#) suggest that *CARD8* C10X might be the sole determinant and that polymorphisms in *NLRP3* are redundant for increased *M. tuberculosis* control, we first compared the *M. tuberculosis*-restricting ability of hMDMs from subjects who carried *CARD8* C10X (i.e., C10X/Q705K individuals were excluded) to that in hMDMs from wild-type individuals. hMDMs from 19 donors harboring *CARD8* C10X were less able to control growth ($P = .005$; [Figure 2A](#)). Further comparison of the 3 individuals carrying *NLRP3* Q705K but lacking the *CARD8* C10X polymorphism against the wild-type population did not reveal any significant differences ([Figure 2B](#)). However, comparison of bacterial growth between the wild-type population and the 3 individuals heterozygous for C10X plus Q705K ([Figure 2C](#)) revealed significantly reduced bacterial growth ($P = .04$).

DISCUSSION

Carriage of a combination of *NLRP3* Q705K and *CARD8* C10X has been linked to increased risk or severity of several inflammatory diseases [12] and to spontaneous production of IL-1 β in plasma in patients and asymptomatic carriers [7]. Asymptomatic individuals carrying only one of the polymorphisms display the same low basal level of IL-1 β as healthy wild-type individuals, whereas asymptomatic double carriers have slightly higher plasma levels of IL-1 β than healthy wild-type individuals [13], which further underlines the synergistic relationship between these polymorphisms. Although the current study includes a limited number of individuals and caution should be taken when drawing conclusions, the data suggest that this synergy extends not only to increasing the risk of inflammatory diseases, but also confers protection against intracellular infections. Increased generation of IL-1 β constitutes an attractive explanation of the decreased bacterial growth, but adding exogenous IL-1 β did not affect bacterial growth. We addressed this by inhibiting the generation of IL-1 β by YVAD. However, the lack of effect could be explained by incomplete inhibition of caspase-1 by YVAD [9]. In contrast, blocking of the IL-1 receptor by using anakinra increased bacterial growth in macrophages infected at multiplicity of infection of 1. Blocking of the IL-1 receptor would not only serve to decrease the direct effects of IL-1 β , it would also inhibit the IL-1 receptor-driven transcription of proIL-1 β . However, the IL-1 receptor also binds IL-1 α , which can be released upon inflammasome activation, and a role for IL-1 α during infection with *M. tuberculosis* cannot be ruled out. These data collectively show that although IL-1 receptor signaling is a prerequisite for the control of bacterial growth, the lack of correlation between the absolute levels of IL-1 β and increased growth control in macrophages suggests that inflammasome-dependent mechanisms other than IL-1 β production are involved in the

beneficial effects on bacterial growth in macrophages of individuals carrying the studied genes. Whether IL-1 β plays a role in the beneficial effects remains to be elucidated, but irrespective of the mediator, we show that combined gene variants in *NLRP3* and *CARD8* result in increased phagolysosomal fusion in hMDMs, giving an explanation for the differences in bacterial growth.

The *CARD8* C10X polymorphism leads to decreased expression of functional *CARD8* [8] and thereby to the loss of *CARD8*-mediated inhibition of caspase-1 and NF κ B, leading to increased levels of IL-1 β [3], as well as to caspase-1-dependent cell death, termed pyroptosis, in *Salmonella*-infected cells [8]. Pyroptosis is accompanied by increased IL-1 β levels and might be beneficial to the host during *Salmonella* infection, but the necrotic nature of pyroptosis favors bacterial spreading to neighboring cells and is detrimental for the *M. tuberculosis*-infected host [14]. A recent study showed that autoproteolyzed caspase-1 generated by an ASC-containing inflammasome (ie, *NLRP3* inflammasome) is responsible for the generation of IL-1 β , whereas alternatively activated caspase-1 interacts with other inflammasomes to cause pyroptosis [15]. This might explain why *CARD8* C10X alone contributes to increased growth of *M. tuberculosis* by increasing the levels of alternatively activated caspase-1 and pyroptosis, whereas in conjunction with gain-of-function variants in *NLRP3*, caspase-1 is autoproteolyzed and redirected from causing pyroptosis to other effects, including generation of IL-1 β .

In summary, although the role of *NLRP3* and inflammasome activation during in vivo infection has been questioned, our data suggest that enhanced inflammasome activation (eg, by activating polymorphisms/mutations) leads to increased anti-*M. tuberculosis* activity in macrophages from both patients with inflammatory disorders and healthy individuals. Activation of *NLRP3* by a small peptide, acALY18, has been suggested as a broad-spectrum treatment against other intracellular pathogens, strengthening the idea of increased *NLRP3* activity as a protective player in infected macrophages [16]. Although we were able to identify only a small number of healthy double carriers of Q705K and C10X, it is tempting to speculate that these genetic variants predispose for increased inflammatory dysfunction but simultaneously lead to decreased capacity of intracellular bacteria such as *M. tuberculosis* to establish infection. The enormous selective pressure posed by *M. tuberculosis* through history might explain the high frequency of the studied polymorphisms and suggests a role for *NLRP3* inflammasome variants in protection against tuberculosis. Therefore, extending our studies to include patients with tuberculosis and their healthy household contacts in tuberculosis-endemic settings would be of great interest.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The

posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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