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Short communication

Inflammasome genetics contributes to the development and control of active pulmonary tuberculosis

D. Souza de Lima [a,b,*], M.M. Ogusku [c], A. Sadahiro [b], A. Pontillo [a]

[a] Laboratório de Imunogenética, Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil
[b] Laboratório Imunologia Molecular, Departamento de Parasitologia, Universidade Federal do Amazonas, Manaus, AM, Brazil
[c] Laboratório de Micobacteriologia, Institut oNacional de Pesquisas da Amazônia, Manaus, AM, Brazil

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ABSTRACT

Tuberculosis (TB) continues to be a major public health problem. An estimated one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb) but remains asymptomatic (latent TB) and only 5% to 10% of these latent individuals will develop active pulmonary TB. Factors affecting the balance between latent and active TB are mostly unknown, even if host genome has been shown to contribute to the outcome of Mtb response. Acute inflammation and Th1 response are important in the early clearance of the bacteria as it was emphasized by the association between immune genes (i.e.: *HLA*, *IFNG*, *TNF*, *NRPAM1*, *IL10*) variants and the development of active pulmonary TB.

Recently, the role of the inflammasome in experimental TB has been demonstrated, however, to our knowledge, no data still exist about the contribution of inflammasome genetics to Mtb susceptibility and/or to the development of active TB.

For this reason, selected polymorphisms in inflammasome genes were analysed in a case/control cohort of individuals with active pulmonary TB from an endemic area of Brazil Amazon.

Our data evidence the novel association between polymorphisms in NLRP3-inflammasome encoding genes and active pulmonary TB, and replicated the association between *P2X7* and TB observed in other populations.

These results emphasize the role of NLRP3-inflammasome also in human TB, and contribute to our knowledge about pathways involved in the development of active TB, even if deeper investigation are needed to fully elucidate the role of the complex in Mtb infection.

1. Introduction

Tuberculosis (TB), one of the most important infectious diseases worldwide, continues to be a major public health problem, with about 9.6 million new cases of active TB with million deaths/year (WHO, 2015). TB is caused by lung macrophages infection with *Mycobacterium tuberculosis* and it is predominantly a pulmonary disease (O'Garra et al., 2013). Of note, an estimated one-third of the world's population is infected with Mtb but remains asymptomatic (latent TB) and only 5% to 10% of these latent individuals will develop active pulmonary TB in their lifetimes (Bellamy, 1998).

Acute inflammation (i.e.: production of IL-1β and TNF) and Th1 response (i.e.: production of IFN-γ) are important events in the early clearance of the bacteria (Verrall et al., 2014) and in the containment of the infection eventually contributing to the development of latent TB (O'Garra et al., 2013).

Factors affecting the balance between latent and active TB are mostly unknown, even if host genome has been shown to contribute to the outcome of immune response against Mtb and polymorphisms in immune genes such as *HLA*, *IFNG*, *TNF*, *NRPAM1* and *IL10* (Dubaniewicz et al., 2000; Etokebe et al., 2006; Lü et al., 2014; Sahiratmadja et al., 2007; Vejbaesya et al., 2007; Zhang et al., 2011) have been associated with the development of active TB.

In the last years, inflammasomes gain attention in the context of TB and in Mtb-macrophages interplay. Inflammasomes are cytoplasmic complexes that mediate innate immune response against pathogen- or danger-derived signals (PAMPs and DAMPs, respectively) leading to the caspase-1-mediated processing and production of pro-inflammatory cytokines IL-1β and IL-18. Several intracellular receptors, such as NLRP1, NLRP3, NAIP/NLRC4, AIM2, are able to activate an inflammasome, however, the NLRP3-inflammasome is the best characterized. PAMPs and DAMPs can activate NLRP3-inflammasome by interfering with cell homeostasis through several mechanisms, such as ATP-mediated K+ influx (mediated by the channel P2X7)

* Corresponding author at: Laboratory of Immunogenetics, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes 1730, Cidade Universitária, São Paulo, SP, Brazil.
E-mail address: dhemersonsouzalima@gmail.com (D. Souza de Lima).

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(Peng et al., 2015; Qu et al., 2007) and lysosomal damage and cathepsin-B release (Hornung et al., 2008). NLRP3 activation is strictly regulated by post-transcriptional mechanisms (i.e.: miR-223) (Yang et al., 2015) as well as by endogenous proteins, such as CARD8 (Ito et al., 2014).

The contribution of inflammasomes activation in infection diseases (i.e.: *Leishmania*, HIV-1, HSV) is well known (Hernandez et al., 2014; Johnson et al., 2013; Lima-Junior et al., 2013), and it has been reported also for TB. Mtb seems to be able to activate NLRP3- and AIM2-inflammasomes in murine peritoneal macrophages as well as in bone marrow-derived macrophages leading to IL-1β production (Dorhoi et al., 2012; Saiga et al., 2012). On the other hand, mice lacking the ATP-dependent K+ channel P2X7 (P2X7 KO) resulted highly susceptible to Mtb (Santos et al., 2013), emphasizing the involvement of the NLRP3-inflammasome in Mtb-host interaction.

Human association studies revealed that loss-of-function single nucleotide polymorphisms (SNPs) in *P2X7* are associated with susceptibility to active pulmonary TB (Shemon et al., 2006), suggesting that a less ATP-activated NLRP3-inflammasome contribute to a worse clearance of bacteria also in humans.

Moreover, individuals carrying gain-of-function SNPs in *NLRP3* (i.e.: rs35829419/Q705K) in combination with the non-sense variant rs2043211 (C10X) in NLRP3 inhibitory protein CARD8 presented a more activated NLRP3-inflammasome and a consequent increased processing/production of IL-1β and/or IL-18, and appeared to better control Mtb growth compared to non-carrying ones (Eklund et al., 2014).

All this considered and convinced that host genetic background affects the balance between latent and active presentation of TB, we hypothesised that variants in inflammasome genes could contribute to the individual response to Mtb and, at least in part, could determine the outcome of infection and the development of active TB.

With the aim to evaluate inflammasome genetics contribution to the development of active pulmonary TB, selected SNPs in inflammasome genes (*NLRP1*, *NLRP3*, *AIM2*, *CARD8*, *IL1B*, *IL18*) and in inflammasome-related components (*IL1R1*, *CTSB*, *P2X7*) and were analysed in a case/control cohort of individuals with active pulmonary TB from an endemic area of Brazil Amazon.

2. Material and methods

2.1. Case/control cohort

288 unrelated (not Amazon indigenous) individuals with active pulmonary TB (male/female: 193/95; 38.2 ± 13.2 years) were recruited from the Reference Center for Sanitary Pneumology "Policlínica Cardoso Fontes," (Manaus, AM, Brazil). The diagnoses of active pulmonary TB were carried out by sputum smear microscopy (Salem et al., 1990) and/or culture method (Salem et al., 2007), together with clinical evaluation and X-ray examination.

288 unrelated individuals (male/female: 155/133; 35.8 ± 12.0 years) that were in direct contact with TB patients, but without signs and symptoms of active pulmonary TB and negative to sputum smear were included in the study as controls. PPD test showed that controls are an admixture of PPD− and PPD+ subjects. Individuals with other diseases than TB (autoimmune diseases, cancer, diabetes, HIV-1) were excluded from the study.

All participants provided written informed consent as approved by the Human Research Ethics Committee of the Federal University of Amazonas (reference number: 0017.0.115.000-08).

2.2. SNPs selection

14 SNPs in 9 inflammasome genes (*NLRP1*, *NLRP3*, *AIM2*, *CARD8*, *IL1B*, *IL18*, *IL1R1*, *P2X7*, *CTSB*) were selected according to previously reported association studies (Eklund et al., 2014; Pontillo et al., 2012; Verma et al., 2012) and public databases (HapMap, SNPbrowser).

2.3. Genomic DNA isolation and genotyping

Genomic DNA was extracted from 4 mL of peripheral blood leucocytes using the rapid technique based on tetramethylammonium bromide salts (DTAB/CTAB) precipitation (Gustincich et al., 1991). SNPs genotyping was performed using commercially available TaqMan assays (Applied Biosystems/AB) using StepOne Real-Time platform (AB). Allelic discrimination was performed using the StepOne software (AB).

2.4. Association analysis

R software version 3.2.2 (www.r-project.org) was used to perform genotypes association and inheritance modelling (package "SNP assoc" version 1.9-2). Data were adjusted for sex and age. Haploview software (Barrett, 2009) was used to investigate the linkage disequilibrium (LD) pattern and for deriving the haplotypes. A formal Bonferroni correction for the number of SNPs analysed would require a significant threshold of $p = 0.004$ ($p0/n$, $p0 = 0.05$, $n = 14$ SNPs).

3. Results and discussion

SNPs frequencies were found to be in Hardy–Weinberg equilibrium in both cases and controls (Supplementary Table 1). Brazilian Amazon SNPs frequencies appeared to be similar ($p > 0.05$) to those present in public databases for Caucasian populations (Supplementary File 1).

All but 5 SNPs resulted similarly distributed in patients and controls (Table 1).

rs10754558 in *NLRP3*, rs8898 in *CTSB*, rs2043211 in *CARD8*, rs5744256 in *IL18* and rs2230911 in *P2X7* were differently distributed between patients and controls ($p < 0.05$), however only *NLRP3* rs10754558 and *P2X7* rs2230911 continued significantly associated with active pulmonary TB after Bonferroni correction ($p < 0.004$) (Table 1).

NLRP3 rs10754558 G/G genotype was significantly less frequent in patients (active pulmonary TB) than in controls (exposed individuals) (0.11 versus 0.04; $p_{adj} = 0.004$), suggesting a protective role of G allele against the development of active pulmonary TB ($OR_{adj} = 0.38$, according to a recessive model of inheritance) (Table 1). This 3′UTR polymorphism increases the stability of *NLRP3* mRNA and individuals carrying this variant present a higher availability of NLRP3 and a consequently increased processing rate of IL-1β and IL-18 (Hitomi et al., 2009). Moreover, rs10754558 affects the binding site of the micro-RNA (miR)-223, which is known to down-regulate NLRP3 expression and to consequently inhibit inflammasome activation (Yang et al., 2015). Therefore, individuals with rs10754558 are less susceptible to this regulating mechanism. Based on these functional data, we hypothesised that an increased NLRP3 availability is important for controlling Mtb infection and beneficial against the development of active pulmonary TB.

Of note, a similar association result has been observed for other types of intracellular chronic infection such as HIV-1 (Pontillo et al., 2012) and HTLV-1 (Kamada et al., 2014) reinforcing the idea that NLRP3-inflammasome represents an important tool in innate immune response against intracellular pathogens.

The missense variation rs35829419 (Q705K), responsible for increasing IL-1β and IL-18 secretion (Verma et al., 2012), was not statistically associated with the development of active TB (Table 1), maybe due to the low frequency in the general population (2–4%), especially in American individuals (Genomes Project et al., 2012). However, the frequency of minor A allele is higher in controls than in individuals with active pulmonary TB (0.04 versus 0.02) (Table 1), partially confirming that augmented NLRP3-inflammasome activation is protective against the development of active pulmonary TB.