

## CONCISE COMMUNICATION

# Analysis of inflammasomes and antiviral sensing components reveals decreased expression of NLRX1 in HIV-positive patients assuming efficient antiretroviral therapy

Milena Nasi<sup>a</sup>, Sara De Biasi<sup>a</sup>, Elena Bianchini<sup>b</sup>, Margherita Digaetano<sup>c</sup>,  
Marcello Pinti<sup>b</sup>, Lara Gibellini<sup>a</sup>, Simone Pecorini<sup>a</sup>,  
Gianluca Carnevale<sup>a,e</sup>, Giovanni Guaraldi<sup>c,d</sup>, Vanni Borghi<sup>d</sup>,  
Cristina Mussini<sup>a,d</sup> and Andrea Cossarizza<sup>a,e</sup>

**Objective:** Few studies have investigated the importance of different components of the inflammasome system and of innate mitochondrial sensing (IMS) pathways in HIV infection and its treatment. We analysed the expression of several components of the inflammasome and of the IMS in HIV-positive patients taking successful combination antiretroviral therapy (cART).

**Methods:** We enrolled 20 HIV-positive patients under cART, who achieved viral suppression since at least 10 months and 20 age and sex-matched healthy donors. By RT-PCR, using peripheral blood mononuclear cells (PBMCs), we quantified the mRNA expression of 16 genes involved in inflammasome activation and regulation (*AIM2, NAIP, PYCARD, CASP1, CASP5, NLRP6, NLRP1, NLRP3, TXNIP, BCL2, NLRCA, PANX1, P2RX7, IL-18, IL-1 $\beta$ , SUGT1*) and eight genes involved in IMS (*MFN2, MFN1, cGAS, RIG-I, MAVS, NLRX1, RAB32, STING*).

**Results:** Compared with controls, HIV-positive patients showed significantly lower mRNA levels of the mitochondrial protein NLRX1, which plays a key role in regulating apoptotic cell death; main PBMC subpopulations behave in a similar manner. No differences were observed in the expression of inflammasome components, which however showed complex correlations.

**Conclusion:** The decreased level of NLRX1 in HIV infection could suggest that the virus is able to downregulate mechanisms linked to triggering of cell death in several immune cell types. The fact that HIV-positive patients did not show altered expression of inflammasome components, nor of most genes involved in IMS, suggests that the infection and/or the chronic immune activation does not influence the transcriptional machinery of innate mechanisms able to trigger inflammation at different levels.

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<sup>a</sup>Department of Surgery, Medicine, Dentistry and Morphological Sciences, <sup>b</sup>Department of Life Sciences, <sup>c</sup>Department of Medical and Surgical Sciences of Mother, Child and Adult, University of Modena and Reggio Emilia, <sup>d</sup>Infectious Diseases Clinics, Azienda Ospedaliero-Universitaria Policlinico di Modena, and <sup>e</sup>Dipartimento Sperimentale Interaziendale, Campus San Lazzaro, University of Modena and Reggio Emilia, Modena, Italy.

Correspondence to Andrea Cossarizza, MD, PhD, Department of Surgery, Medicine, Dentistry and Morphological Sciences via Campi, 287 - 41125 Modena, Italy.

Tel: +39 059 205 5415; fax: +39 059 205 5426; e-mail: andrea.cossarizza@unimore.it

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## Introduction

Pattern recognition receptors (PRRs) are proteins that respond to pathogen-associated molecular patterns, and endogenous stress signals termed danger-associated molecular patterns. PRRs comprise toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), absent in melanoma (AIM)-2-like receptors (ALRs) and C-type lectin receptors (CLRs). These molecules are central to the rapid innate immune response and are crucial for starting adaptive immunity responses [1]. Most intracellular NLRs [NOD, LRR and pyrin domain-containing 1 (NLRP1), NLRP3, NLRP6, or NOD, LRR and CARD-containing 4 (NLRC4)] can assemble the inflammasome, which is a cytosolic, multi-molecular platform that activates proinflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 via caspase-1 (CASP1). Other proteins are able to assemble an inflammasome, such as AIM2 or RIG-I (also known as DDX58), that sense double-stranded DNA and RNA, respectively [2]. RIG-I is also able to induce type I interferon (IFN) responses via mitochondrial antiviral signalling protein (MAVS) [3].

HIV infection is characterized by a persistent inflammatory status, and indeed a low-grade, chronic level of inflammation can be observed even in patients successfully treated with combination antiretroviral therapy (cART) [4]. The molecular mechanisms of this phenomenon, and those that trigger or maintain inflammation are however not clear. Until now, only few studies have investigated the role of different components of the inflammasome system, or the importance of innate mitochondrial (mt) sensing pathways in HIV infection and its treatment. The aim of the study was to analyse the expression of several, main components of the inflammasomes and of the antiviral mt sensing in HIV-positive patients taking successful cART.

## Materials and methods

### Patients

The study was approved by the Institutional Review Board of the Department of Surgery, Medicine, Dentistry and Morphological Sciences of the University of Modena and Reggio Emilia. We enrolled 20 HIV-positive patients (18 men and two women;  $47.5 \pm 11.9$  years old; mean  $\pm$  SD) who took cART for  $4.3 \pm 1.6$  years, had a CD4<sup>+</sup> T cell count of  $638.9 \pm 310.4$  cells/ $\mu$ l and have

achieved viral suppression (i.e. undetectable plasma viral load, below 50 copies/ml) for at least 10 months. All but one patients were taking at least one nucleoside reverse transcriptase inhibitors (NRTIs): eight abacavir/lamivudine, two only lamivudine, 10 tenofovir/emtricitabine and one zidovudine. As controls (CTR), we studied 20 age and sex-matched healthy donors (12 men and eight women;  $42.8 \pm 7.7$  years old). All patients and controls gave written informed consent for the studies described here, according to the Italian laws.

Twenty millilitre of blood was collected from each individual. Peripheral blood mononuclear cells (PBMCs) were isolated, pelleted and stored at  $-80^{\circ}\text{C}$ , as described [5]. For the analysis of gene expression in different cell subsets, CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B cells, CD4<sup>+</sup> T and CD8<sup>+</sup> T cells were isolated from PBMCs of three healthy donors and three HIV-positive patients by using magnetic sorting (MACS, by Miltenyi, Bergisch Gladbach, Germany), pelleted and stored at  $-80^{\circ}\text{C}$ . Purity was always more than 95%. Total RNA was extracted from thawed cells using RNeasy Mini Kit (Qiagen Inc., Valencia, California, USA). Cell viability, assessed with Live-dead AQUA (Thermo Fisher, Waltham, Massachusetts, USA), on Attune NxT Acoustic Focusing Cytometer (Thermo Fisher) was more than 95% in all samples.

### Gene expression analyses

We simultaneously quantified the mRNA expression of 16 genes involved in inflammasome activation (*AIM2*, *NAIP*, *PYCARD*, *CASP1*, *CASP5*, *NLRP6*, *NLRP1*, *NLRP3*, *IL-18*, *IL-1 $\beta$* ) or regulation (*TXNIP*, *BCL2*, *NLRC4*, *PANX1*, *P2RX7*, *SUGT1*), and eight genes involved in antiviral mitochondrial sensing (*MFN2*, *MFN1*, *cGAS*, *RIG-I*, *MAVS*, *NLRX1*, *RAB32*, *STING*) by using a prevalidated set of real-time PCR assays (PrimePCR assays Bio-Rad Laboratories, Hercules, California, USA), following provided instructions. We also measured three housekeeping genes (HKG), that is *ACTB*, *GADPH* and *RLP13*, and have chosen *ACTB* as the most stable gene, required to calculate the relative mRNA gene expression.

### Statistical analyses

Quantitative variables were compared with nonparametric Mann-Whitney or Kruskal-Wallis tests. The correlations between clinical parameters and molecular data were performed by Spearman correlation test and linear regression analysis. *P* values less than 0.05 were considered statistically significant. Statistical analyses were

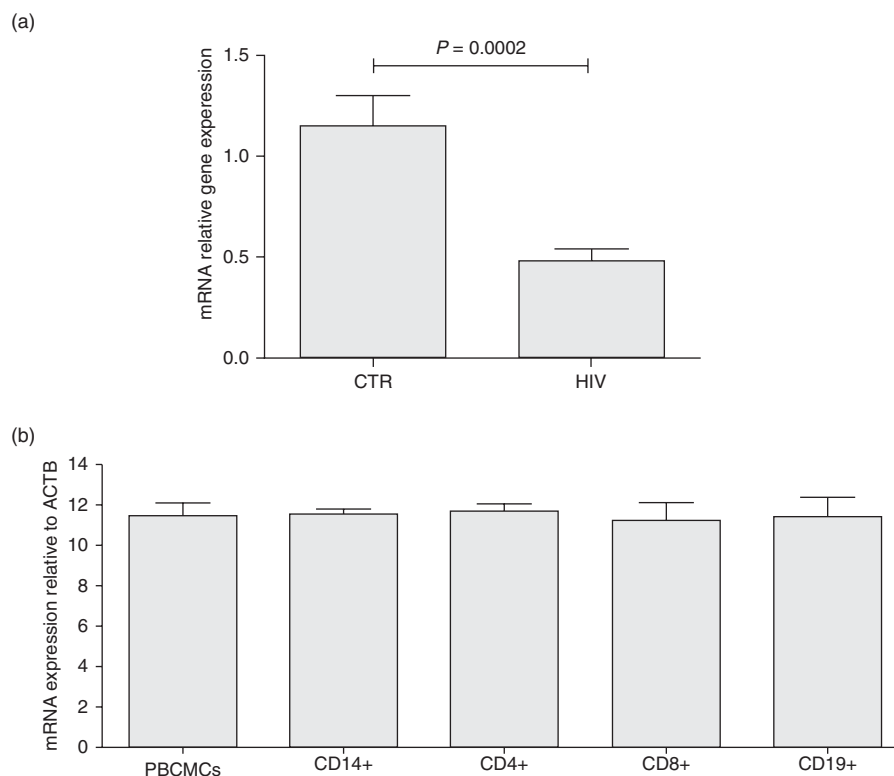
performed using Prism 6.0 (Graphpad Software Inc., La Jolla, USA).

## Results and discussion

Inflammasomes are macromolecular platforms able to control maturation and secretion of the proinflammatory interleukins IL-1 $\beta$  and IL-18, and play a crucial role in innate immune defenses. Inflammasomes exert several other actions, such as the activation of inducible nitric oxide synthase, regulation of autophagy or pyroptosis, help in production of antibodies and activation of T cells. However, only few data exist on the importance of inflammasomes in HIV infection [6,7]. Thus, we investigated mRNA expression of main components of inflammasomes (sensors, adaptors, regulators and downstream signalling) and of the mitochondrial antiviral signalling in PBMC from HIV-positive patients with undetectable viremia.

Among genes involved in antiviral signalling and regulation, we found that NLRX1 was differentially expressed: HIV-positive patients showed significantly lower levels of NLRX1 than healthy donors (Fig. 1a). We then investigated different PBMC subpopulations and found that NLRX1 expression was similar among

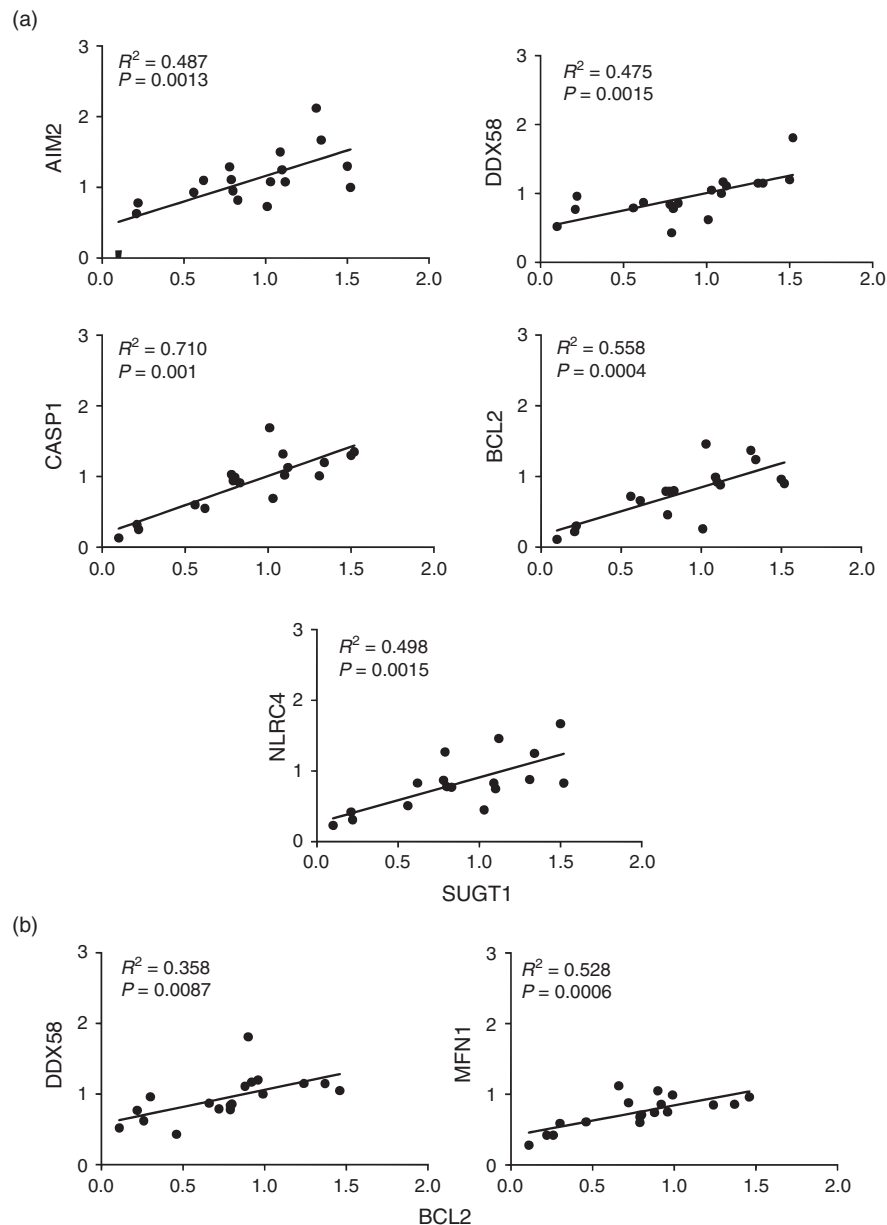
CD14<sup>+</sup> monocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells, either from HIV-positive patients (Fig. 1b), or from healthy donors (not shown). NLRX1 is a still poorly characterized protein expressed in mitochondria, previously identified as a negative regulator of mitochondrial antiviral signalling proteins through a direct interaction on the cytosolic side of the mitochondrial outer membrane [8]. Recently, different roles and functions of NLRX1 have been identified, such as overexpression of NLRX1 causes the generation of reactive oxygen species [9]; NLRX1 controls apoptotic cell death in transformed cells [10]; NLRX1 can bind a small death-inducing protein from influenza A virus and can protect immune cells against virus-driven apoptosis [11]; NLRX1 dually regulates IFN-I and autophagy through the engagement of the mitochondrial protein named 'Tu translation elongation factor' (TUFM). Reduction of both NLRX1 and TUFM resulted in enhanced IFN-I activation, demonstrating a partnership between NLRX1 and TUFM to control host antiviral responses [12]. Thus, during HIV infection, the decreased expression of NLRX1 could represent a yet unknown mechanisms of viral escape from host restriction factors. It is noteworthy that the decreased expression was present at the same level in the main lymphocyte populations (i.e. CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as in CD19<sup>+</sup> B lymphocytes) and in CD14<sup>+</sup> monocytes.



**Fig. 1. Relative mRNA expression of the genes of the inflammasomes and mitochondrial viral sensing systems.** Relative mRNA expression of NLRX1 in healthy donors (CTR) and patients with HIV infection (HIV) taking effective cART. *P* value by Mann–Whitney test. mRNA expression of NLRX1 relative to ACTB in PBMCs, CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B cells and CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocytes in patients with HIV infection. Bars indicate mean values with S.E.M.; *P* = NS, as revealed by Kruskal–Wallis test.

The expression of genes involved in inflammasome pathways, including NLRP3 (the scaffold component of the best characterized inflammasome complex), was not significantly different between HIV-positive patients and controls (not shown). This could indicate that the infection and/or the consequent chronic immune activation do not influence the transcriptional machinery of innate mechanisms able to trigger inflammation at different levels. In other words, patients taking efficient cART display the same level of basal inflammasome activation of healthy individuals. In mice, nucleosidic NRTIs block inflammation triggered by NLRP3 inflammasome by inhibiting the activity of P2RX7 [13].

Blocking inflammation implies that the production of pro-inflammatory cytokines is reduced. Accordingly, it has been found that treating HIV-positive patients with cART can reduce plasma levels of IL-18 [14]. Further data, also performed by using functional assays, are needed to better correlate the importance of inflammasome activation and the production of proinflammatory cytokines during HIV infection. We did not find any correlation between the expression of the genes we have analysed and the viro-immunological parameters that were collected during the routine clinical practice (CD4<sup>+</sup> T cell count and percentage, CD8<sup>+</sup> T cell count and percentage, CD4<sup>+</sup> T cell nadir and CD4<sup>+</sup>/CD8 ratio).



**Fig. 2. Correlations between the relative mRNA levels in peripheral blood mononuclear cell from HIV-positive patients.** Correlations between the relative mRNA levels of SUGT1 (a) and BCL2 (b) versus the relative mRNA levels of the indicated transcripts.  $P$  values have been obtained by linear regression analysis.

We have also found that mRNA levels of some genes, whose expression is not linked to IFN activity, showed significant correlations (Fig. 2a and b, referred to as the negative regulators SUGT1 and BCL2, respectively). SUGT1 forms a chaperone complex with HSP90, which maintains inactive, misfolded or used NLR-type sensors. Similarly, the antiapoptotic BCL2 regulates the NLRP1 inflammasome [15]. Further studies are needed to understand the reasons of such correlations that could include the possible use of the same transcription factors, or the presence of mutual interaction(s) between these genes. Finally, we found that in HIV-positive patients, other genes, belonging to the family of IFN-stimulated genes (i.e. *AIM2*, *CASP1*, *IL18*, *PANX1*, *MAVS*, *MFN1*, *DDX58*, *CASP5*, *NAIP*, *PYCARD* and the small GTP-binding protein of the RAB family, RAB32), were positively correlated among them (not shown).

We are well aware that this study presents some limitations, first of all the relatively small sample size in which however a broad panel of genes have been analysed. In any case, we find that PMBC from HIV-positive patients taking successful treatment display a markedly different expression of the gene for the mitochondrial protein NLRX1 and did not show a differentially expression of components of the inflammasome pathway, as well as of genes involved in the antiviral mitochondrial sensing. Mitochondria are emerging as a central platform for the assembly of signalling complexes regulating inflammatory pathways. Changes in the expression of the NLRX1, which is able to regulate the cellular sensitivity to apoptotic signals, suggest a critical role for this protein in several physiological processes and pathological conditions, including HIV infection.

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A.C., M.N., C.M. and G.G. contributed to the study's concept. V.B. and M.D. performed the selection and enrolment of patients and controls. M.N., S.D.B., E.B., G.C. and S.P. performed the blood sample processing and the gene expression analysis. M.N., M.P. and L.G. contributed to the analysis and interpretation of the data. M.N., A.C. and M.P. contributed to writing the manuscript. All authors contributed to the critical review of the manuscript.

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## Conflicts of interest

The authors do not have any conflicts of interest.

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