Inflammation Research (2018) 67:255–264
https://doi.org/10.1007/s00011-017-1119-2

Inflammation Research

ORIGINAL RESEARCH PAPER

Polymorphisms and expression of inflammasome genes are associated with the development and severity of rheumatoid arthritis in Brazilian patients

Catarina Addobbati[1,2] · Heidi Lacerda Alves da Cruz[2] · José Eduardo Adelino[1,2] · Amanda Luíze Melo Tavares Ramos[2] · Thiago Sotero Fragoso[3] · Alexandre Domingues[4] · Ângela Luiza Branco Pinto Duarte[4] · Renê Donizeti Ribeiro Oliveira[5] · Paulo Louzada-Júnior[5] · Eduardo Antônio Donadi[5] · Alessandra Pontillo[6] · Jaqueline de Azevêdo Silva[1,2] · Sergio Crovella[1,2] · Paula Sandrin-Garcia[1,2]

Received: 1 July 2017 / Revised: 22 October 2017 / Accepted: 21 November 2017 / Published online: 11 December 2017

Abstract

Objective In the present study, we analyzed the possible association of inflammasome gene variants and expression to rheumatoid arthritis (RA)'s development and severity in the Brazilian population.

Materials and methods Thirteen single nucleotide polymorphisms within six inflammasome genes (*NLRP1, NLRP3, NLRC4, AIM2, CARD8, CASP1*) as well as *IL1B* and *IL18* genes in two different Brazilian populations (from Northeast and Southeast Brazil) were analyzed. We also evaluated inflammasome gene expression profile in resting and LPS + ATP-treated monocytes from RA patients and healthy individuals. For genetic association study, 218 patients and 307 healthy controls were genotyped. For gene expression study, inflammasome genes mRNA levels of 12 patients and ten healthy individuals were assessed by qPCR.

Results Our results showed that rs10754558 *NLRP3* and rs2043211 *CARD8* polymorphisms are associated with RA development (p value = 0.044, OR = 1.77, statistical power = 0.999) and severity measured by Health Assessment Questionnaire (HAQ) (p value = 0.03), respectively. Gene expression analyses showed that RA patients display activation of *CASP1, IL1B* and *IL1R* genes independently of LPS + ATP activation. In LPS + ATP-treated monocytes, *NLRP3* and *NLRC4* expressions were also significantly higher in patients compared with controls.

Conclusions The first reported results in Brazilian populations support the role of inflammasome in the development of RA.

Keywords SNPs · Autoimmunity · Prognostic and monocytes

Responsible Editor: Jason J. McDougall.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00011-017-1119-2) contains supplementary material, which is available to authorized users.

✉ Catarina Addobbati
catarinaaddobbati@hotmail.com

[1] Department of Genetics, Federal University of Pernambuco, Av. Moraes Rego, 1235, Recife, Pernambuco CEP 50760-901, Brazil

[2] Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Recife, Pernambuco, Brazil

[3] Rheumatology Service, Clinical Hospital, Federal University of Alagoas, Maceió, Alagoas, Brazil

[4] Rheumatology Division, Clinical Hospital, Federal University of Pernambuco, Recife, Pernambuco, Brazil

[5] Clinical Immunology Division, Department of Medicine, Medicine Faculty of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

[6] Laboratory of Immunognetics, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, São Paulo, Brazil

Introduction

Rheumatoid arthritis (RA) is a complex and chronic inflammatory disease associated with progressive joint destruction, disabling and systemic complications. It is characterized by proliferation of synovial tissue, autoantibody production and systemic alterations such as cardiovascular, pulmonary and dermatological [1, 2]. RA is a disorder with an important sex bias, substantially more affecting women from 30 to 50 years old [2, 3]. Even though RA etiology remains unclear, it depends upon the interaction between genetic and environmental factors. The genetic component has a pivotal role in RA etiology with several genes contributing in disease´s triggering [2, 4]. The majority of genetic variations associated with RA development are within immune response-related genes, with the human leukocyte antigen (HLA) variants being the most well-known genetic risk factors [4].

The pathogenesis of RA encompasses abnormal innate and adaptive immune responses, being tumor necrosis factor and interleukin (IL)-6 the main pro-inflammatory cytokines involved in RA pathogenesis [5, 6]. High levels of IL-1β, secreted by monocytes, macrophages and dendritic cells, have also been involved in RA, revealing an important role in bone resorption and cartilage destruction [7]. Indeed, the therapeutic inhibition of IL-1 reduces signs and symptoms of RA as well as radiological damage. Animal models of RA, such as collagen-induced arthritis and antigen-induced arthritis, also respond to IL-1 inhibition [8, 9], underlining the importance of this cytokine in disease's development.

IL-1β secretion is induced by the activation of inflammasomes, which are multiprotein complexes capable of promoting the processing and maturation of IL-1β. Assembly of inflammasomes depends upon the activation of intracellular receptors belonging to Nod-like receptors and PHIN receptors families, such as NLR family pyrin domain-containing 1 (NLRP1), NLR family pyrin domain-containing 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4) and absent in melanoma 2 (AIM2), the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protein caspase-1. When sensing pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP), the intracellular receptors recruit the adaptor protein ASC, which triggers the cleavage of pro-caspase-1 into caspase-1. Activated caspase-1 processes pro-IL-1β and pro-IL-18 cytokines into their mature and secreted form. Inflammasome activation is strictly regulated by transcriptional mechanisms (i.e., NF-kB-dependent transcription of *NLRP3, AIM2, IL1B*), post-transductional modifications (i.e., ubiquitination, nitrosylation), as well as by endogenous proteins, such as caspase recruitment domain-containing protein 8 (CARD8), which specifically inhibit NLRP3-inflammasome activation. Pro-IL-1β production is dependent on NF-kB role, being the cytokine secretion a two-stage process [10–12].

Mutations in inflammasome genes may cause rare autoinflammatory disorders characterized by constitutively elevated level of IL-1β contributing to the systemic inflammatory presentation [13]. Polymorphisms in inflammasome genes have been associated to more common chronic inflammatory disorders, such as systemic lupus erythematosus [14], psoriasis [15], Crohn´s disease [16, 17], celiac disease [18, 19], type 1 diabetes [18, 20] and vitiligo [21]. Previous studies reported the association between *NLRP3* and/or *CARD8* and RA in different populations [22–27], and despite their heterogeneity, the results pointed out a major role of NLRP3-inflammasome dysregulation in RA pathogenesis.

Herein, we first performed in Brazilian populations a genetic association study to assess inflammasome and also *IL1B* and *IL18* gene polymorphisms and the susceptibility to RA and its clinical manifestations. Moreover, we analyzed inflammasome components genes as well as *IL1B* and *IL1R* expression profile in peripheral blood-derived monocytes from RA subjects with and without inflammasome activation in vitro.

Methods

Genotyping study

Subjects

For this study, a total of 218 RA patients and 307 healthy individuals, all from Brazil, were enrolled. The Northeastern sample comprised 128 patients (mean age 51.3 ± 11.7 years; mean age at diagnosis 42.1 ± 11.7 years; 122 females and 6 males) and 149 healthy individuals (mean age 39.2 ± 14.2 years; 122 females and 27 males) from the state of Pernambuco, Northeastern Brazil. The Southeast sample comprised 90 patients (mean age 55.7 ± 10.8 years; mean age at diagnosis 44.8 ± 13.2 years; 83 females and 7 males) and 158 healthy controls (mean age 37.4 ± 11.3 years; 76 females and 82 males) from state of São Paulo, Southeast Brazil. Patients from Northeastern Brazil were under care of the Division of Rheumatology of Hospital das Clínicas from Federal University of Pernambuco, whereas patients from Southeast Brazil were recruited from Division of Clinical Immunology of University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo. All RA patients were diagnosed according to the American College of Rheumatology (ACR) criteria [28]; control individuals were healthy blood donors without previous family history

of autoimmune diseases, as reported in appropriate questionnaire. The patients were evaluated for the presence of rheumatoid factor and bone erosions. Disease activity score in 28 joints (DAS28) [29, 30] and Health Assessment Questionnaire (HAQ) [31] were applied to patients as a measurement of disease activity and functional disability, respectively.

Aiming to maintain a good statistical power, and also to report the results as from the Brazilian population as a whole, we joined the two groups into one, and then correct the analysis by geographical origin and demographic data. The demographic and clinical features from all assessed RA patients and controls are shown in Supplementary data 1.

All procedures involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the participants provided a written informed consent approved by the local Research Ethics Committee (Southeast: HCRP 2981/2009 and Northeast: CAAE 03065312.3.0000.5208).

DNA isolation

Genomic DNA was isolated from whole blood samples, using DNA Wizard Genomic DNA Purification Kit (Promega, USA) according manufacturer's protocol or using a salting out method [32].

SNPs selection and genotyping

Thirteen SNPs within *IL1B, IL18, NLRP1, NLRP3, NLRC4, AIM2, CARD8* and *CASP1* genes were selected according to previously reported association studies [18, 19] and/or their functional effect on protein activity [16] or mRNA stability [33]. More details about all assessed SNPs are reported in Supplementary data 2.

SNPs genotyping was performed using fluorogenic allele-specific probes (Taqman Probes, Applied Biosystems, USA) on an ABI7500 sequence detection system (Applied Biosystems, USA).

Statistical analysis

Genotype distribution was compared for Hardy–Weinberg (HW) equilibrium using genotype transposer [34]. At a first sight, considering RA patients and controls independently of their geographical origin, the allele and genotype frequencies of controls and RA patients were compared using Chi-square test. Binary logistic regression was used to confirm the association between the polymorphisms and RA, adjusting for origin of sample and gender. Bonferroni's correction for multiple comparisons was applied (pBonf = observed *p* value x N, N = number of studied polymorphisms within the same gene). The possible association of the assessed polymorphisms with DAS28 and HAQ in RA patients was analyzed using the likelihood ratio test. The significance level was set at $\alpha = 0.05$ (two tailed). All statistical analyses were performed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The eventual presence of linkage disequilibrium (LD) between polymorphisms within the same gene and the association of haplotypes with RA susceptibility were evaluated using the online software SNPStats [35]. The power was verified using G*Power software 3.1.9.2 [36].

Inflammasome genes expression profile in peripheral blood monocytes

Heparinized whole blood samples were obtained from twelve post-menopausal RA female patients (mean age 50.42 ± 13.91 years) and ten healthy post-menopausal female controls (HC) (mean age 57.5 ± 8.14 years) from metropolitan area of state of Pernambuco. None of the patients or controls had received systemic treatment recently, as corticosteroids drugs. To evaluate the correlation of inflammasome mRNA levels and RA activity, measured by DAS28, patients were stratified as high disease activity (DAS28 > 5.1), moderate disease activity (3.2 < DAS28 < 5.1), low disease activity (2.6 < DAS28 < 3.2) or disease in remission (DAS28 < 2.6) [29, 30].

Peripheral blood monocytes were isolated by adherence from PBMCs obtained by centrifugation over Ficoll–Paque (Sigma-Aldrich, USA) gradient. An amount of 5×10^6 PBMCs/well was cultured in RPMI 1640 containing 10% fetal bovine serum in 24-wells microplate (Invitrogen, Life Technology, USA). For this assay, we performed biological replicates. Monocytes were stimulated with 1 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 4 h and 1 mM adenosine triphosphate (ATP; Sigma-Aldrich) for 15′. After incubation supernatants were collected for cytokines measurement, cells were lysed for mRNA isolation and gene expression analysis. RNA isolation from monocytes was performed using the RNAqueous micro kit (Ambion, Life Technologies, USA), following the manufacturer's instructions. The RNA samples were stored at − 80 °C until used and RNA integrity analysis was performed by gel electrophoresis and quantification by Nanodrop 2000 (Thermo Scientific, USA). cDNA synthesis was performed with SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Life Technology, USA).

The major inflammasome genes, *NLRP1, NLRP3, NLRC4, AIM2, ASC/PYCARD* and *CASP1*, and *IL1B* and *IL1R* genes were amplified with specific TaqMan Gene Expression Assays using the ABI 7500 SDS platform (Applied Biosystems, USA). *GAPDH* was the reference gene used for normalization. Relative quantitative expression was calculated comparing RA and healthy individuals cells (fold change (FC) = RA $2^{-\Delta Cq}$/HC $2^{-\Delta Cq}$) following the

indications by Schmittgen and Livak, 2008 [37]. Student's *t* test was applied to compare different groups.

IL-1β measurement

The IL-1β secreted by monocytes was measured with ELISA kit (R&D systems, USA) following manufacturer's protocols and results were expressed in picograms per milliliter. One-way ANOVA test with Bonferroni post-test was applied to compare IL-1β secretion in RA and healthy controls cells as well as stimulated and resting cells.

Results

Genetic association study

Genotype distributions were in Hardy–Weinberg equilibrium for the assessed SNPs with exception of *NLRP3* rs35829419 and *IL1B* rs1143643 SNPs in the controls (p value = 0.0001 and 0.0419, respectively). No association was observed among polymorphisms within *NLRP1, NLRC4, AIM2, CASP1, IL1B* and *IL18* genes and RA in studied population. Genotype and allele frequencies are shown in Table 1.

Considering RA patients and controls independently of their geographical origin, it showed a significant association between the *NLRP3* rs10754558 SNP [C > G] and RA susceptibility with a statistical power = 0.999. The C allele and C/C genotype were significantly more frequent in patients than in controls (67 vs 56%, p value = 0.005, OR = 1.59, 95% CI = 1.23–2.05; and 47 vs 32%, p value = 0.0036, OR = 2.23, 95% CI = 1.32–3.75, respectively). After applying Bonferroni's correction, the association remained statistically significant (pBonf = 0.01 and 0.007, respectively).

When we performed a binary logistic regression adjusting for gender and geographical origin of sample, the association of C/C genotype for *NLRP3* rs10754558 SNP with susceptibility to RA remained statistically significant (p value = 0.044, OR = 1.77, 95% CI = 1.23–3.09) (Table 1), suggesting that the association is not affected by gender or origin.

The association of the studied SNPs with Rheumatoid Factor production and bone erosions development was evaluated and no significant association was observed. Also, the association between SNPs and disease activity, measured by DAS28, was not detected (data not shown).

When we assessed the influence of the studied polymorphisms on disease activity (DAS28) and patient's functional disability (HAQ), the association between *CARD8* rs2043211 SNP [A > T] and a higher mean for HAQ was observed. Patients homozygous for T allele presented a higher functional disability (HAQ mean = 2.13 ± 0.20) when compared to homozygous for A allele (HAQ mean = 1.32 ± 0.11) and heterozygous patients (HAQ mean = 1.41 ± 0.10). This association was observed in both codominant [p value = 0.03, *Akaike Information Criterion* (AIC) = 309.2] and recessive model (p value = 0.01, AIC = 307.5) (Table 2).

Linkage disequilibrium was found for polymorphisms rs12150220 [A > T] and rs2670660 [A > G] within *NLRP1* (D' = 87), rs455060 [A > G] and rs212713 [T > C] within *NLRC4* (D' = 0.94) and also for polymorphisms rs1143643 [C > T] and rs1143634 [G > A] in *IL1B* gene (D' = 0.82). However, the observed haplotypes were not differentially distributed between RA patients and healthy individuals (Data not shown).

Inflammasome expression analyses

We evaluated the expression of major inflammasome genes (*NLRP1, NLRP3, NLRC4, AIM2, ASC* and *CASP1*) and of IL-1β cytokine and its receptor genes *(IL1B* and *IL1R*) in non-stimulated/resting and LPS + ATP-stimulated monocytes from twelve RA patients and ten healthy controls. First, basal as well as LPS + ATP-stimulated expression was evaluated comparing RA patients with controls. We found a statistical significant increase in the basal expression of *CASP1* (+ 499.02-fold, p value = 0.01), *IL1B* (+ 2.976-fold, p value = 0.003) and *IL1R* (+ 216.6-fold, p value = 0.013) genes in untreated monocytes from RA patients when compared with untreated monocytes from healthy individuals (Fig. 1a). In LPS + ATP-treated monocytes, *NLRP3* (+ 14.5-fold, p value = 0.002), *NLRC4* (+ 53.88-fold, p value = 0.04), *CASP1* (+ 19.1-fold, p value = 0.004), *IL1B* (+ 19.7-fold, p value = 0.004) and *IL1R* (+ 14.1-fold, p value = 0.007) genes were statistical significantly upregulated in monocytes from RA patients compared to monocytes from control individuals (Fig. 1b).

LPS + ATP-induced IL-1β secretion in monocytes from RA (LPS + ATP versus ATP: p = 0.007; LPS + ATP versus resting: p = 0.006) as well as from healthy individuals (LPS + ATP versus ATP: p = 0.002; LPS + ATP versus resting, p = 0.006). The stimulation with LPS induced a small increase in IL-1β secretion in controls; however, a greater augmentation was observed in LPS + ATP cells. In RA cells, LPS as well LPS + ATP were able to induce a similar increment in IL-1β secretion.

IL-1β secretion was incremented in RA patients compared to controls in resting (mean = 6.12 ± 10.8 vs. 2.13 ± 2.98 pg/ml), +ATP (12.6 ± 19.1 vs. 2.65 ± 3.26 pg/ml), +LPS (124.2 ± 129.7 vs. 16.12 ± 8.8 pg/ml) and in LPS + ATP cells (287.75 ± 175.63 vs. 273.7 ± 174 pg/ml, respectively). However, no statistical significance was observed between RA and healthy individuals (Fig. 2).

When we evaluated the correlation of inflammasome components and *IL1B* and *IL18* mRNA levels with RA

Table 1 Allele and genotype frequencies from RA patients and controls

SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI	SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI
NLRP1 rs12150220						*CASP1* rs572687					
A	383 (66)	288 (67)		1		G	493 (82)	359 (82)		1	
T	201 (34)	144 (33)	0.77	0.95	0.73–1.24	A	109 (18)	77 (18)	0.92	0.97	0.7–1.34
AA	132 (45)	96 (44)		1		GG	200 (66)	148 (68)		1	
AT	119 (41)	96 (44)	0.66	1.11	0.75–1.64	GA	93 (31)	63 (29)	0.72	0.92	0.61–1.37
TT	41 (14)	24 (12)	0.54	0.8	0.46–1.42	AA	8 (3)	7 (3)	0.96	1.18	0.42–3.33
NLRP1 rs2670660						*IL1B* rs1143643					
A	346 (58)	231 (55)		1		C	390 (65)	307 (71)		1	
G	248 (42)	187 (45)	0.38	1.13	0.88–1.45	T	214 (35)	127 (29)	0.05	0.75	0.57–0.99
AA	97 (33)	62 (30)		1		CC	134 (44)	111 (51)			
AG	152 (51)	107 (51)	0.71	1.1	0.72–1.68	CT	122 (41)	85 (39)	0.39	0.84	0.57–1.24
GG	48 (16)	40 (19)	0.39	1.3	0.77–2.21	TT	46 (15)	21 (10)	0.05	0.55	0.29–1.01
NLRP3 rs35829419						*IL1B* rs1143634					
C	585 (96)	427 (98)		1		G	489 (81)	347 (80)		1	
A	23 (4)	9 (2)	0.16	0.54	0.25–1.17	A	115 (19)	85 (20)	0.86	1.04	0.76–1.42
CC	284 (93)	209 (96)		1		GG	198 (65)	144 (67)		1	
CA	17 (6)	9 (4)	0.56	0.72	0.28–1.75	GA	93 (31)	59 (27)	0.56	0.87	0.58–1.31
AA	3 (1)	0 (0)	0.37	ND	ND	AA	11 (4)	13 (6)	0.35	1.63	0.71–3.73
NLRP3 rs10754558						*IL18* rs1946519					
G	269 (44)	144 (33)		1		C	165 (57)	237 (54)		1	
C	339 (56)	288 (67)	0.005[a]	1.59	1.23–2.05	A	125 (43)	199 (46)	0.54	1.1	0.81–1.51
GG	62 (20)	29 (13)		1		CC	51 (35)	66 (30)		1	
GC	145 (48)	86 (40)	0.43	1.27	0.38–0.85	AC	63 (44)	105 (48)	0.33	1.29	0.77–2.14
CC	97 (32)	101 (47)	0.0036[b, c]	2.23	1.32–3.75	AA	31 (21)	47 (22)	0.66	1.17	0.63–2.19
AIM2 rs35130877						*NLRC4* rs212713					
T	614 (100)	436 (100)		1		T	324 (53)	234 (54)		1	
G	0 (0)	0 (0)	ND	ND	ND	C	290 (47)	200 (46)	0.75	0.95	0.74–1.23
TT	307 (100)	218 (100)		1		TT	84 (27)	63 (29)		1	
TG	0 (0)	0 (0)	ND	ND	ND	CT	156 (51)	108 (50)	0.75	0.92	0.60–1.42
GG	0 (0)	0 (0)	ND	ND	ND	CC	67 (22)	46 (21)	0.8	0.92	0.54–1.55
AIM2 rs2276405						*NLRC4* rs455060					
C	597 (98)	431 (99)		1		A	403 (66)	271 (62)		1	
T	11 (2)	5 (1)	0.55	0.63	0.22–1.83	G	211 (34)	163 (38)	0.32	1.15	0.89–1.48
CC	293 (96)	213 (98)		1		AA	138 (45)	87 (40)		1	
CT	11 (4)	5 (2)	0.54	0.63	0.17–1.99	AG	127 (41)	97 (45)	0.37	1.21	0.82–1.79
TT	0 (0)	0 (0)	ND	ND	ND	GG	42 (14)	33 (15)	0.49	1.25	0.73–2.12

Table 1 (continued)

SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI	SNPs	Controls, *n* (%)	RA, *n* (%)	*p*	OR	95% CI
CARD8 rs2043211											
A	434 (72)	300 (69)		1							
T	168 (28)	136 (31)	0.28	1.17	0.89–1.53						
AA	157 (52)	99 (45)		1							
AT	120 (40)	102 (47)	0.13	1.35	0.94–1.94						
TT	24 (8)	17 (8)	0.87	1.12	0.57–2.2						

RA rheumatoid arthritis, *p* Chi-square test *p* value, *OR* odds ratio, *CI* confidence interval, *ND* not determined

*Due to technical issues, only 145 healthy individuals were genotyped for SNP rs1946519

[a]*p* value after Bonferroni correction = 0.01

[b]*p* value after Bonferroni correction = 0.007

[c]Binary logistic regression, adjusted by sex and origin of sample: $p = 0.044$, OR = 1.77, 95% CI = 1.23–3.09

activity, measured by DAS28, no statistical significance was observed (Data not show).

Discussion

In the last years, substantial information has emerged connecting deregulated inflammasome signaling to inflammatory diseases. In this study, we evidenced for the first time the association between SNPs in inflammasome genes and RA development in the Brazilian population. In addition, we demonstrated a dysregulated expression of some inflammasome components as well as IL-1β cytokine and its receptor genes *(IL1B* and *IL1R*) in RA patients.

The *NLRP3* rs10754558 [C > G] C allele seems to confer an augmented risk for the development of RA when in homozygosis (OR = 1.77) with a power up to 99.9%; the moderate value of OR suggest that being RA a multifactorial trait *NLRP3* SNP gives a partial contribution to diseases susceptibility. This SNP is located within the 3′UTR of *NLRP3* gene and according to the PolymiRTS Database 2.0 (http://compbio.uthsc.edu/miRSNP/) its occurrence affects the binding of miRNAs. In the presence of G allele, there is a binding site for miR-3529-3p and miR-549a, while the C allele abrogates such site, but gives rise to a binding site for miR-146a-5p, miR-146b-5p, miR-589-5p and miR-7153-5p. If and how rs10754558 SNP interferes with the binding of these miRNAs *in vivo* remains to be further elucidated. According to Hitomi et al., 2009, functional analyses of *NLRP3* rs10754558 SNP showed that allele G influences higher *NLRP3* expression (1.4-fold) by altering mRNA stability (34). The eventual effect of this 1.4-fold increasing in *NLRP3* expression on the inflammasome activation has not been investigated. The C allele was also associated to type 1 diabetes mellitus susceptibility [18] and to systemic lupus erythematosus development (personal communication, manuscript submitted), suggesting a role of this polymorphism in autoimmunity development.

When considering inflammasome SNPs in RA clinical manifestations, we observed the association between *CARD8* rs2043211 SNP [A > T] (p.C10X) and disease's severity. Patients homozygous for T allele presented a higher functional disability measured by HAQ. CARD8 interacts physically with caspase-1 and negatively regulates caspase-1-dependent IL-1β expression and nuclear factor NFκB activation [38–41]. The rs2043211 polymorphism introduces a premature stop codon (Cys > Stop), which results in the expression of a severely truncated protein [42]. The exact role of CARD8 in inflammasome biology is still unclear. It has been proposed that CARD8 acts as a modulator of NLRP3 activation or it exerts an inflammasome independent role, as NF-kB inductor [43]. CARD8 C10X variation leads to an increased secretion of IL-1β, especially in combination

Table 2 Association between *CARD8* rs2043211 SNP and a higher functional disability

	n	HAQ mean ± SD	dif	95% CI	*p*	AIC
Codominant model						
A/A	61	1.32 ± 0.11	0			
A/T	58	1.41 ± 0.10	0.09	− 0.20 to 0.37	0.03	309.2
T/T	8	2.13 ± 0.20	0.80	0.21 to 1.39		
Recessive model						
A/A–A/T	119	1.36 ± 0.07	0			
T/T	8	2.13 ± 0.20	0.76	0.19 to 1.33	0.01	307.5

dif HAQ mean difference in relation to A/A genotype, *CI* confidence interval, *p p* value, *AIC* Akaike information criterion

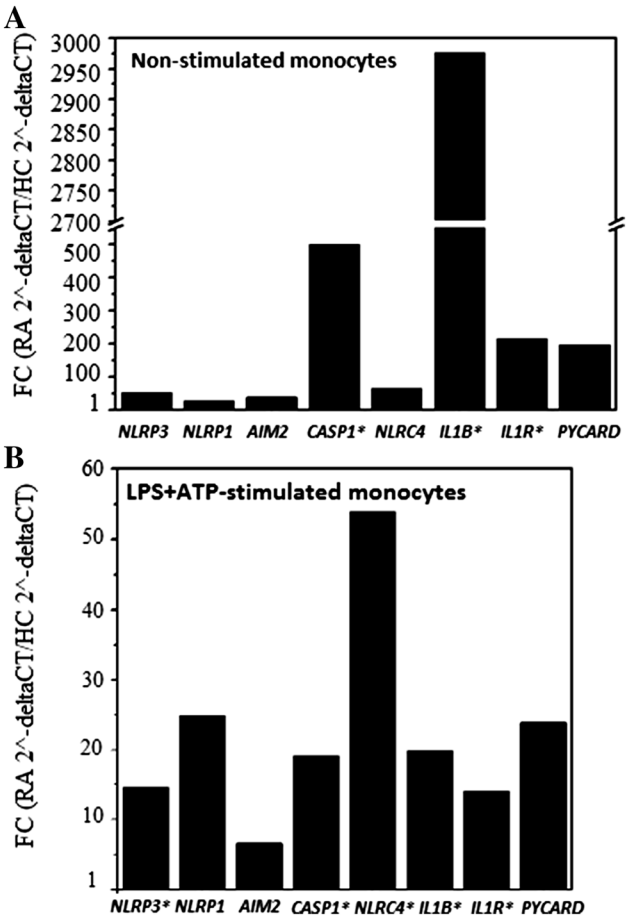

Fig. 1 *NLRP3, NLRP1, AIM2, CASP1, NLRC4, IL1B, IL1R* and *PYCARD* gene expressions in RA patients compared with healthy controls (HC). The results were normalized to *GAPDH* expression. Target gene expression in healthy controls was normalized to 1 (not reported in the graph) and fold change (FC) is reported as RA $2^{-\Delta CT}$/HC $2^{-\Delta CT}$. **a** Expression in non-stimulated monocytes. **b** Expression in LPS + ATP-stimulated monocytes. **p* value < 0.05

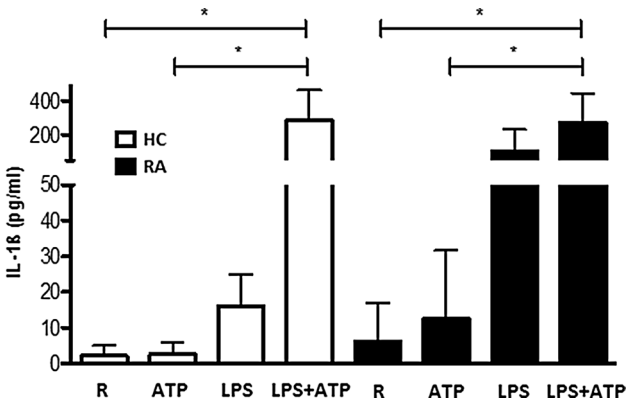

Fig. 2 IL-1β secretion in healthy controls (HC) and RA patients in non-stimulated monocytes (R); ATP-stimulated monocytes (ATP); LPS-stimulated monocytes (LPS); and LPS + ATP-stimulated monocytes (LPS + ATP). **p* < 0.05

with NLRP3 Q705K [16]. Moreover, this variation was associated to an increased induction of NFκB activity and its translocation to the nucleus [44], which leads to high constitutive levels of pro-IL-1β and tumor necrosis factor α, mediators of inflammation in RA [45]. Furthermore, NF-κB has been reported to contribute to the proliferation of synovial cells, and consequently, to bone and cartilage destruction [46, 47].

Kastbom et al., 2010 [27] and Fontalba et al., 2007 [44] also described an association between *CARD8* p.C10X polymorphism and RA severity in Swedish and Spanish patients, respectively. Different from our study, both studies assessed RA severity through cumulative amount DAS28 over 2 years, while we have chosen to measure the severity of the disease by the HAQ due to the fluctuation of the DAS28 during the course of the disease. However, our study corroborates the involvement of *CARD8* p.C10X polymorphism in RA severity and then its possible use as a genetic prognostic marker for RA.

The combination between the minor alleles for *NLRP3* rs35829419 (Q705K) and *CARD8* rs2043211 (p.C10X) polymorphisms was described as associated with delayed apoptosis of neutrophils [48] and RA susceptibility and severity [22]. However, it was not possible to confirm this association in our population, as the *NLRP3* A/A genotype was not found in our patients group. When we tested the

combination between the other genotypes for those polymorphisms, no significant association was observed.

Moreover, our results corroborate the findings from García-Bermúdez et al., 2013 [26], Hamad et al., 2011 [24] and Kastbom et al., 2010 [27], which demonstrated the lack of association of *CARD8* rs2043211 with RA susceptibility when individually analyzed in Spanish, French, Tunisian and Swedish populations. Despite the association between the rs2043211 SNP and RA severity, this SNP was not associated to bone erosions and rheumatoid factor in our population.

When we assessed the gene expression profile for RA patients, we found a statistical significant upregulation of *CASP1, IL1B* and *IL1R* genes in untreated monocytes from RA patients when compared with healthy individuals. This result suggests that RA patients are characterized by a chronic expression of *CASP1, IL1B* and *IL1R* genes. In LPS + ATP-treated monocytes from RA patients, the same genes were upregulated but accompanied by *NLRP3* and *NLRC4* genes. This increased expression of *CASP1, IL1B* and *IL1R* in stimulated monocytes from patients may be consequence of the previous upregulation in resting monocytes. The observed augmented expression of *NLRP3* and *NLRC4* genes in patients after stimulus prompt us to hypothesize that *NLRP3* and *NLRC4* transcriptions are dysregulated in RA patients, contributing to the establishment of the exacerbated inflammation observed in the disease. When we compared the gene expression between patients with disease in remission and patients with high disease activity, no statistical difference was observed, suggesting that the treatment of patients is efficient to control the disease symptoms but may be not able to control the deregulation of inflammasome.

Being aware that other mechanisms may influence upon IL-1β production overall, the observed differences in inflammasome components and IL-1β and its receptor genes expression between patients and healthy controls are accompanied by IL-1β secretion *ex vivo* monocytes. In all studied conditions, the RA monocytes secreted higher amounts of IL-1β. Basal secretion of IL-1β in RA monocytes was higher compared to healthy ones, indicating a constitutive secretion of pro-inflammatory cytokine.

The exact mechanisms responsible for the production and secretion of IL-1β remain unclear, but two signals are traditionally required. The first signal, in our case LPS, induces the transcription of pro-IL-1β and inflammasome subunits; the second signal promotes rapid activation of caspase-1 and then secretion of mature IL-1β [49, 50]. This second signal is provided by reduction of intracellular K + generated by ATP stimulus. Of note in both LPS-stimulated and ATP-stimulated RA monocyte, the signals individually are sufficient to induce an augment of IL-1β secretion, without the common first or second signal mediation (Fig. 2), suggesting an inflammasome complex more prone to activation in RA monocytes than in healthy ones.

Mathews et al., 2014 [23] also showed a higher *NLRP3* and *CASP1* expression in RA patients. However, in this study, the gene expression was characterized directly in peripheral blood mononuclear cells of patients. Thereby, the upregulation of these inflammasome components could be due to the abundant release of DAMPs upon tissue damage, typical in RA pathogenesis [51–53]. Differently, our study evaluated the inflammasome gene expression in RA and control individual's monocytes under the same conditions, proving indeed the dysregulated transcription of these inflammasome components.

The importance of *NLRP3* in RA pathogenesis is confirmed by the findings of increased *NLRP3* mRNA in the synovium of RA patients compared to individuals suffering from non-autoimmune osteoarthritis [25]. The NLRP3-inflammasome was described as an activator of both apoptotic and pyroptotic cell death [54]. Therefore, besides the excessive IL-1β secretion, the deregulated activation of this complex may exacerbate the cell death, contributing to the inflammatory process and its maintenance in RA disease.

Conclusion

In conclusion, herein, we provide enough data to infer that *CASP1, IL1B* and *IL1R* are activated in RA patients as well as *NLRP3* and *NLRC4* genes are dysregulated transcript upon stimulus. We also demonstrated that *NLRP3* and *CARD8* polymorphisms are associated to RA susceptibility and severity in our studied populations. These results are useful to help understanding the role of inflammasome complex in RA.

Acknowledgements This work was supported by the following Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPESP (Fundação Amparo à Pesquisa do Estado de São Paulo) and FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco).

Author contributions CAJC conducted experiments, wrote the manuscript and prepared the tables and figures; HLAC, JEA and ALMTR helped conducting the experiments and provided insightful suggestions to the manuscript; PSG, JAS, AP and SC assisted in the study design and coordination, and read, corrected and provided major suggestions to this manuscript; TSF, AD, LFRJ, ALBPD, RDRO, PLJ and EAD recruited patients and participated in data acquisition. All authors addressed important intellectual content and approved the final manuscript for publication.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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