Clinical Immunology 175 (2017) 56–68

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Pristane induces autophagy in macrophages, promoting a STAT1-IRF1-TLR3 pathway and arthritis

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ARTICLE INFO

Article history:
Received 3 August 2016
Received in revised form 22 November 2016
accepted with revision 29 November 2016
Available online 7 December 2016

Keywords:
Autophagy
TLR3
Macrophages
Experimental arthritis
Gene expression

ABSTRACT

Autophagy is involved in both innate and adaptive immune regulation. We propose that autophagy regulates activation of TLR3 in macrophages and is thereby essential for development of pristane-induced arthritis. We found that pristane treatment induced autophagy in macrophages *in vitro* and *in vivo*, in spleen cells from pristane injected rats. The induced autophagy was associated with STAT1 phosphorylation and expression of IRF1 and TLR3. Blocking the pristane activated autophagy by Wortmannin and Bafilomycin A1 or by RNAi of Becn1 led to a downregulation of the associated STAT1-IRF1-TLR3 pathway. Most importantly, the development of arthritis was alleviated by suppressing either autophagy or TLR3. We conclude that pristane enhanced autophagy, leading to a STAT1-IRF1 controlled upregulation of TLR3 expression in macrophages, is a pathogenic mechanism in the development of arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune inflammatory disease. Both adaptive and innate autoimmune processes are considered to be involved into the disease pathogenesis [1–3]. The innate immune response could further enhance the adaptive immunity, including the polarization of naive T cells and induction of autoreactive B cells [4,5]. One important receptor family of the innate immune system, Toll-like receptors (TLRs) have been confirmed to play an essential role in RA and experimental arthritis [6]. In our previous studies in pristane-induced arthritis (PIA) in rats, which is a MHC-II restricted, T cell dependent, model and fulfills the clinical criteria of RA, we have found that pristane could activate macrophages *in vitro* and spleen cells *in vivo* through regulating TLR3 expression, which further contributes to arthritis development [7]. TLR3 but not other TLRs could be uniquely induced by pristane in rat macrophages, however the mechanism how this innate immune reaction is triggered remains unclear.

Autophagy, a fundamental and evolutionary conserved phenomenon in eukaryotes, contributes to the elimination of pathogens, degrades cytoplasmic components, and maintains cellular homeostasis. Its physiological functions involve a wide variety of aspects such as the maintenance of amino acid pool, selective degradation, development and cell death, tumor suppression and anti-aging [8–11]. Of particular interest is that autophagy was recently proposed to regulate both innate and adaptive immunity [12]. Autophagy plays an important role not only in the defense against infections but also in the development of chronic inflammatory and autoimmune disease [13,14].

As a typical autoimmune disease, RA is likely to be closely associated with autophagy [14]. However, there is limited knowledge on the role of autophagy in RA, and most are based on experiments with fibroblast-like synoviocytes (FLS). It has been reported that autophagy in FLS contributes to the resistance to ER stress induced cell death [15,16], and with decreased apoptosis in RA synovium [17]. Recently, a dual role of autophagy in stress-induced cell death in RA FLS has been suggested, in that autophagy promotes cell death under ER stress but protects against apoptosis induced by proteasome inhibition [18]. Besides, in

Abbreviations: BMM, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; FLS, fibroblast-like synoviocyte; PIA, pristane-induced arthritis; RA, rheumatoid arthritis; RT-qPCR, realtime quantitative PCR; TLRs, toll-like receptor.

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http://dx.doi.org/10.1016/j.clim.2016.11.017
1521-6616/

osteoclasts derived from RA patients autophagy is activated in a TNF-α dependent manner and could regulate osteoclast differentiation and bone resorption [19]. In T cells derived from RA affected individuals, autophagy is impaired due to phosphofructokinase deficiency [20]. In antigen-presenting cells, such as dendritic cells and macrophages, autophagy has been found to participate in presentation of citrullinated peptides to CD4 T cells [21], whereas protein citrullination in RA synovial fluid cells is not induced by autophagy [22]. Obviously, the above studies indicate that autophagy might play a critical role in RA, and autophagy has been implicated in different aspects of the pathogenesis of RA. However, so far conclusive evidence of a regulatory role of autophagy on immune regulation of arthritis has been lacking.

In the present study, we induced PIA in DA rats to investigate role of autophagy, and focused on the autophagy induction and its immune regulatory role in macrophages. We found that autophagy level could be induced both in pristane stimulated macrophages and in the spleen of PIA rats, accompanied by upregulation of TLR3 expression. Blocking autophagy in cells *in vitro* reduced pristane-enhanced TLR3 expression and blocking either autophagy or TLR3 *in vivo* could alleviate arthritis.

2. Material and methods

2.1. Rats

DA rats were bred in a specific pathogen-free animal house of Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center. All animal experiments were approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University (No.2012-094 and 2013-013).

2.2. Induction of arthritis

To induce PIA, DA rats at age of 8–12 weeks were given a single intradermal injection of 300 μl of pristane (Acros Organics, Morris Plains, NJ, USA) at the base of tail as described previously [23]. Six age- and sex-matched rats per group were used. Rats were sacrificed at 0, 1, 6 and 12 days after pristane injection respectively, and spleens were collected and used for electron microscope observation and expression detection of autophagy related genes and proteins.

2.3. Stimulation of macrophages with pristane

Rat macrophage cell line NR8383 was cultured in F-12K medium (Sigma-Aldrich) with 15% FBS (Hyclone). Emulsion of pristane was made by repeated aspiration with complete medium respectively, in a 1:1 ratio, and used in the theoretical concentrations indicated. Half-million cells per well were seeded in 6-well plate overnight, and medium was replaced by fresh-made oil emulsion. Then cells were collected at indicated time points for assays.

Bone marrow cell were isolated from DA rats and seeded at the density of 2×10^6/ml in L929-conditioned medium to differentiate into bone marrow-derived macrophages (BMM) using Cold Spring Harbor Protocols [24]. After 7 days, BMM were stimulated by 1 mM pristane for 6 or 24 h, and protein was isolated for gene expression detection.

2.4. mRNA expression analysis

Total RNA from spleen tissues of rats and cells was isolated by using TRIzol® Reagent (Invitrogen), and cDNA was synthesized by First Strand cDNA Synthesis Kit (Fermentas). Realtime quantitative PCR (RT-qPCR) was performed by iQ5 (BIO-RAD) with FastStart Universal SYBR Green Master (Roche) for mRNA quantitation. The relative gene expression normalized by β-actin was calculated with $2^{-\Delta\Delta CT}$ method. The information of primers, products and annealing temperatures is depicted in Table 1.

2.5. Western blotting

Total protein lysates from spleen tissues and cells were extracted by using the RIPA solution (Beyotime, China) with a cocktail of protease and phosphatase inhibitors (Roche). The final protein concentration of each sample was determined by a BCA kit (Thermo Scientific).

The supernatants (20 μg total protein) from protein lysates were subjected to SDS-PAGE gel according to standard procedures in Bio-Rad system. The primary antibody including rabbit anti-TLR3 antibody (5 μg/ml, BIOSS, China), rabbit anti-LC3 antibody (1:1000, CST, #4108), rabbit anti-Becn1 antibody (1:2000, CST, #3495), rabbit anti-p-STAT1 antibody (1:1000, CST, #7649), rabbit anti-STAT1 antibody (1:1000, CST, #9172), rabbit anti-IRF1 antibody (1:1000, CST, #8478) and anti-β-actin (1:1000, CST, #4970) overnight. The signal was further detected by using the secondary antibody of goat anti-rabbit IgG conjugated with HRP (0.4 μg/ml, Abcam). Signal intensity was determined by Supersignal® West Pico Kit (Thermo Scientific). Data are expressed by showing one representative image whereas all performed experiments are included in accompanying graphs in which the results have been normalized with the values of the control group that are set to 1.

2.6. Autophagy assay

The analysis of autophagy was done according to the guidelines [25]. First, spleen tissues and NR8383 cells stimulated with pristane was fixed with ice-cold 2% glutaraldehyde in 0.1 M phosphate buffered saline, postfixed with 1% osmium tetroxide, dehydrated with graded ethanol concentrations, and finally embedded in 1:1 propylene oxide/

Table 1
Information of primers for RT-qPCR.

Gene name	NCBI Accession No.	Sequence(5′-3′)		Size (bp)	Annealing temperature (°C)
LC3b	NM_022867	forward	CTGCGGGTTGAGGAGACA	321	60
		reverse	AGGAGGAAGAAGGCTTGGTT		
Becn1	NM_053739	forward	CAGTGGCGGCTCCTATTC	320	60
		reverse	CTACGGCAGGGCTCTTTG		
TLR3	NM_198791	forward	GATTGGCAAGTTATTCGTC	205	54
		reverse	GCGGAGGCTGTTGTAGG		
TNF-α	NM_012675	forward	TCAGCCTCTTCTCATTCCTGC	203	60
		reverse	TTGGTGGTTTGCTACGACGTG		
IL-6	NM_012589	forward	AAGAAAGACAAAGCCAGAGTC	263	60
		reverse	CACAAACTGATATGCTTAGGC		
IL-1β	NM_031512	forward	CTGTGACTCGTGGGATGATGAC	322	54
		reverse	CTTCTTCTTTGGGTATTGTTTGG		
IFN-β	NM_019127	forward	CTTGGGTGACATCCACGACTAC	92	54
		reverse	GGCATAGCTGTTGTACTTCTTGTCTT		
β-actin	NM_031144	forward	GAGGGAAATCGTGCGTGAC	157	60
		reverse	GCATCGGAACCGCTCATT		