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Calcimycin induced IL-12 production inhibits intracellular mycobacterial growth by enhancing autophagy

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ABSTRACT

Previously, we reported pivotal role of P2RX7 in augmenting autophagy in THP-1 cells upon Calcimycin treatment by modulating intracellular Calcium regulated ATP production but the role of immune modulators in Calcimycin induced autophagy is not known. In this study, we demonstrate that treatment with Calcimycin in PMA (Phorbol 12-myristate 13-acetate) differentiated THP-1 (dTHP-1) cells significantly induced interleukin (IL)-12 mRNA expression and its release. IL-12 receptor (IL-12Rβ1 and IL-12Rβ2) was also significantly expressed on the cell surface in dTHP-1 cells upon Calcimycin treatment. We report that small molecule or siRNA based P2RX7 inhibition abrogated IL-12 release upon Calcimycin treatment. P2RX7 inhibition also resulted in reduced Jun N-terminal kinase (JNK) activation, IκBα phosphorylation, p65 translocation and NF-κB expression. Further, inhibition of NF-κB activation or IL-12-IL-12R interaction led to down-regulation of the expression of autophagy related markers such as Beclin-1, autophagy-related gene (Atg) 3, Atg 7 and impairment of microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) to LC3-II conversion. Finally, blocking of autophagy led to significant growth of intracellular mycobacteria in Calcimycin treated macrophages. Overall, these results reveal that interaction of Calcimycin with P2RX7 modulates intracellular JNK-NF-κB signaling pathway. This modulation results in IL-12 release that restricts the mycobacterial growth in THP-1 macrophages.

1. Introduction

In 2016, Tuberculosis (TB) has afflicted almost 10.4 million people worldwide and close to 1.6 million people lost their lives that is caused by a deadly monster, *Mycobacterium tuberculosis* (*M. tb*) [1]. Due to this magnitude of destruction, TB has gained notorious distinction of being a leading cause of death than any other infectious agent [1]. Further, advent of drug resistant strains namely multidrug-resistant (MDR), extremely drug-resistant (XDR) and totally drug-resistant (TDR) along with ineffectiveness of *M. bovis* BCG in the adults has compounded the problem [2–4].

Upon entry of *M. tb* through respiratory tract into lung alveoli, alveolar macrophages phagocytose the bug and pose first line of defense by employing diverse host defense mechanisms [5,6]. These mechanisms include apoptosis induction, production of reactive nitrogen and oxygen intermediates (RNI and ROI), initiation of autophagy, phagolysosome fusion and secretion of proinflammatory cytokines [7–11]. Proinflammatory cytokines play a monumental role in determining the outcome of infection, initiation and sustenance of host anti-microbial responses [12]. Some of the prominent cytokines like tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-23, IL-27, IL-18, IL-1, IL-7, IL-15 and IL-12 have already been shown to be effective against *M. tb* in the published literature [13,14].

IL-12, a member of the IL-12 cytokine family belongs to IL-6 superfamily [15]. The members of this family are heterodimeric and show sundry and polyphonic functions [16,17]. IL-12 consists of IL-12p35 and IL-12p40, produced mainly by antigen presenting cells and bridges both innate and adaptive immunity [15,17–21]. IL-12 regulates processes such as T cell–dependent or independent macrophage activation, generation of cytotoxic T lymphocytes (CTLs) and Th1 cells, synthesis

*Abbreviations: M. bovis*BCG, *Mycobacterium bovis* BCG; *M. tb*, *Mycobacterium tuberculosis*; TB, Tuberculosis; P2RX7, Purinergic receptor P2X7; Atg, Autophagy-related gene; LC3, Microtubule-associated protein 1A/1B-light chain 3; 3-MA, 3-methyladenine; Baf-A1, Bafilomycin A1; KN-62, (1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine); BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra acetic acid tetra(acetoxy-methyl) ester; MDR, multidrug-resistant; XDR, extremely drug-resistant; TDR, totally drug-resistant; qRT-PCR, quantitative real-time PCR; JNK, Jun N-terminal kinase; TNF, tumor necrosis factor; CTL, cytotoxic T lymphocyte; Tyk, tyrosine kinase; hMDMs, human monocyte-derived macrophages

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of complement-fixing antigen-specific antibodies and hostility against intracellular infections [22,23]. The interplay of IL-12, IL-23 and IL-27 in generating effective cellular responses against TB is already highlighted [24]. Individuals with IL-12Rβ1, IL-12p40 and IFN-γR1 deficiencies are more prone to TB [15,25–31]. Mice studies have conclusively proven the role of IL-12 in reducing bacterial burden by modulating the immune responses [32–38]. In a separate study, IL-12 has been shown to mediate its antimycobacterial effect by enhancing phagosome-lysosome fusion through IFN-γ [39]. Autophagy, induced by vitamin D3, damage-associated molecular patterns (DAMP), reactive oxygen species (ROS), IFN-γ and ATP is known to be antimycobacterial by promoting phagosome-lysosome fusion [40–43] but the exact role of IL-12 in autophagy induction and its effect on mycobacteria is not yet clear.

Autophagy is a homeostatic process that maintains the intracellular milieu by maintaining the degradation of unwanted cellular components through formation of autophagosome [44,45]. This pathway accomplishes through different steps in a sequential way that involves initiation of autophagosome, its elongation and maturation leading to the degradation. Various proteins like autophagy-related genes (Atg) namely Beclin-1, Atg 5, Atg 3, Atg 12, Atg 7 etc. are known to coordinate autophagy [45,46]. Recently, we showed that calcium ionophore, Calcimycin, produced by *Streptomyces chartreusensis* restrict the intracellular mycobacterial growth by inducing autophagy through ATP-dependent P2RX7 pathway [47]. However, the interplay among immune regulators in Calcimycin mediated intracellular killing is not yet clear.

The present study was performed to understand the immune mechanism of Calcimycin mediated killing of mycobacteria. Here, we observed specific and significant increase in IL-12 transcript levels in Calcimycin treated macrophages. We show that P2RX7 mediated signaling results in IL-12 production through NF-κB signaling pathway. IL-12 regulates autophagy in Calcimycin treated macrophages through JAK2 dependent pathway. We also demonstrate abrogation in Calcimycin mediated killing by either inhibiting NF-κB/JAK2 signaling pathway or IL-12 interaction with its receptor. Hence, our findings support the role of innate immune response through IL-12 production in augmenting antimycobacterial response of Calcimycin treated macrophages by inducing autophagy.

2. Materials and methods

2.1. Culture of THP-1 cells and reagents

THP-1, human monocytic cell line was maintained and used for experiments as reported earlier [47]. Cell culture supplies were procured from Gibco Laboratories (Grand Island, NY, USA). ECL western blotting detection reagents and analysis system were obtained from GE Healthcare (Buckinghamshire, UK). Various antibodies like rabbit anti-Beclin-1, anti-Atg 7, anti-Atg 3, anti-β-actin, anti-LC3, anti-p65, anti-IκBα, anti-pIκBα, anti-Lamin B1, anti-JAK2 and anti-pJAK2 were acquired from Cell Signaling Technology (MA, USA). Human IL12p40/70 ELISA kit was bought from Diaclone (Besancon, France). pNF-κB-DD-ZsGreen1 reporter vector (Clontech; CA, USA) was a kind gift from Dr. C.V. Srikanth, Regional Centre for Biotechnology, Faridabad, Haryana, India. Phorbol 12-myristate 13-acetate (PMA), monodansylcadaverine (MDC, a fluorescent dye that stains autophagic vacoules), anti-rabbit HRP antibody, rabbit anti-p62 antibody, 1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxy-methyl) ester (BAPTA-AM, a cell permeable Ca^{2+} chelator), Bafilomycin A1 (Baf-A1, a vacuolar ATPase inhibitor), BCA kit, Calcimycin, KN-62 (a P2RX7 antagonist), 3-methyladenine (3-MA, a selective phosphatidylinositol 3-kinases inhibitor), NSC33994 (a potent and specific JAK2 inhibitor), 100 × protease inhibitor cocktail and Fluoromount aqueous mounting medium were procured from Sigma (St. Louis, MO, USA). Anti-IL-12 neutralization antibody was bought from R&D Systems (MN, USA). Rabbit anti-P2RX7 and FITC-anti-rabbit IgG antibodies were acquired from Origene (MD, USA). siRNA transfection kit, P2RX7 and control siRNA were purchased from Santa Cruz Biotechnology (CA, USA). All other reagents were obtained from HiMedia Laboratory (Mumbai, India). All experimental supplies were free from endotoxin.

2.2. Bacterial strain and infection

Maintenance and experimental use of *M. smegmatis* mc^2155 and *M. bovis* BCG Danish was followed as described previously [47]. Experiments involving mycobacterial infection to THP-1 cells were performed as reported earlier with difference that 0.5×10^6 THP-1 cells/well were plated in 12-well plates [47]. At the end of infection, cells were replenished with RPMI medium having different chemicals/agents according to the experiment.

2.3. MDC staining

THP-1 cells were differentiated overnight by PMA on glass cover slips at a density of 2.5 x 10^5 cells/well in 24-well plate. Next day, dTHP-1 (differentiated THP-1) cells were washed and given Calcimycin treatment for 12 h. In some of the experiments/combinations, either 3-MA was added 1 h before Calcimycin addition or Baf-A1 treatment was given 3 h before the conclusion of Calcimycin treatment. MDC (50 μM) was added to the wells 30 min. before the end of 12 h incubation and 4% p-formaldehyde was then used to fix the cells. Stained cells were mounted with Fluoromount aqueous mounting medium and visualized on confocal scanning laser microscope (CSLM) (Leica Microsystems, Wetzlar, Germany). Analysis of the acquired images was done with Adobe Photoshop (Adobe system).

2.4. mRNA expression analysis

dTHP-1 cells (3×10^6) were given Calcimycin treatment for different time points. Infection with *M. smegmatis/M. bovis* BCG or siRNA transfection was also done in these cells according to different experiments before treating with Calcimycin for 12 h. Total RNA was extracted as described previously [47]. The forward and reverse primer sets used were P2RX7: 5-TATGAGACGAACAAAGTCACTCG-3 and 5-GCAAAGCAAACGTAGGAAAAGAT-3 [47]; IL-12p40: 5-CCAAGAACTT GCAGCTGAAG-3 and 5-TGGGTCTATTCCGTTGTGTC-3; IL-12p35: 5-CCTCAGTTTGGCCAGAAACC-3 and 5-GGTCTTTCTGGAGGCCAGGC-3; TNF-α: 5-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3 and 5-GCAA TGATCCCAAAGTAGACCTGCCCAGACT-3; IL-12Rβ1: 5-TAGGGACCTG AGATGCTATCG-3 and 5-CCCGGAGCTAAGGCAACAC-3; IL-12Rβ2: 5-AAAATAGATGCGTGCAAGAGG-3 and 5-GGGGAAGACCTGTGACTT GAG-3; IL-10: 5-ATGATGGCTTATTACAGTGGCAA-3 and 5-GTCGGAG ATTCGTAGCTGGA-3; IL-1β: 5-GTGATGCCCCAAGCTGAGA-3 and 5-CACGGCCTTGCTCTTGTTTT-3; β-actin: 5-CATGTACGTTGCTATCCA GGC-3 and 5-CTCCTTAATGTCACGCACGAT-3, respectively. SYBR green (Thermo Fisher Scientific, IL, USA) methodology was used to perform quantitative PCR. $2^{-\Delta\Delta CT}$ method was employed to measure relative expression of different genes [48].

2.5. Quantitation of cytokine

Supernatants from different combinations were harvested at indicated time and stored at −70 °C until IL-12 amount was measured using human IL12p40/70 enzyme-linked immunosorbent assay (ELISA) kit as per supplied instruction manual (Diaclone; Besancon, France).

2.6. Flow cytometry

P2RX7 expression on the cell surface of Calcimycin treated dTHP-1 cells was evaluated by indirect surface staining using Rabbit anti-P2RX7 and FITC-anti-rabbit IgG antibodies. Briefly, Calcimycin treated