Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 5 Issue 12 December 2022

Research Article

Autophagy Inducing Capacities of a Commercial Preparation Based on of *Bacillus thuringiensis* Cry1A-Cr2A Autophagy-Inducing Properties Toxins in Human Macrophages: Implication Against Mycobacterial Infections

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DOI: 10.31080/ASMI.2022.05.1178

Received: November 07, 2022

Published: November 18, 2022

Abstract

Autophagy is a highly conserved degradative and recycling program to maintain homeostasis. In particular, it plays an important role in the innate immune response against intracellular pathogens. Several studies have shown that BCG and/or *M. tuberculosis* block autophagosome formation, inhibiting thus, activation of the autophagy machinery, and survival of mycobacteria. Human macrophages preparation from Peripheral blood mononuclear cells (buffy coats of the healthy donor) (blood bank of INER. MX). Monocytes were further isolated by CD14 positive selection and differentiated into monocyte-derived macrophages. Infection of macrophages with *M. bovis* BCG vaccine (ATCC, Manassas, VA, US) at MOI 1:5. Stimulation with Cry1A-Cr2A (5 µg/ml Rapamycin (250 µg/ml) and/or Wortmannin (100 nM). Autophagy detection and fluorescence microscopy were made in the uninfected well and the infected-stimulated cells were stained with rabbit anti-LC3B coupled to Alexa Fluor 488. The percentage of cells with more than 5 LC3+ puncta (autophagosomes) was calculated as well as the percentage of bacteria co-localizing with LC3. The percentage of mycobacteria BCG co-localizing with light chain 3 (LC3-II) in human macrophages is greater in infected and stimulated human macrophages with the commercial preparation based on Cry1A-Cry2A Bt proteins versus rapamycin and/or Wortmannin. The commercial preparation of Cry1A-Cry2A in combination with the BCG vaccine represents a potential alternative to enhance the autophagy-mediated elimination of intracellular pathogens such as *M. tuberculosis*.

Keywords: *Bacillus thuringiensis*; Cry1A Toxins; Autophagy; Macrophages; Microtubule Light Chain 3 (LC3); Tuberculosis

Introduction

Tuberculosis is still a major health problem worldwide and specially in developed countries [1,2]. One serious problem that are facing public health systems is the increased appearance of multidrug resistance strains (MDR) and excessive use of medication against intracellular pathogens [1-3] like *Mycobacterium tuberculosis (Mtb*) the causal agent of Tuberculosis [1,2]. The actual BCG vaccine (*Mycobacterium bovis Bacillus Calmette Güerin)* (BCG) [3-8] is protective against some forms of Tuberculosis in children and variable immunoprotection or lack of immunological memory in young and adult people [9-13].

Autophagy is highly conserved process to maintain homeostasis at molecular and cellular level [14-17]. Any organelle damage, misfolded proteins or pathogens trigger and activate the machinery of autophagy [18-20]. In this sense it has been reported that autophagy can be used for therapeutic intervention against intracellular pathogens as *M. tuberculosis* [21-26]. Therefore, one promising alternative against Tuberculosis is autophagy inducers are a potential alternative to enhance BCG immunity. Indeed, a recent paper have shown that a recombinant BCG vaccine with a peptide, enhance BCG autophagy inducing properties. BCG vaccine

and *M. tuberculosis* interfere with phagosome maturation is through the secretion of sap M phosphatase, which dephosphorylates phosphatidyl-inositol-3-kinase (PI-3K), considered a key initial trigger for phagosome maturation [24,26,31,32]. BCG or *M. tuberculosis* block the fusion of the autophagosome and lysosome and therefore autophagy induction, hampering thus activation of the innate and cellular immune response [31-34]. How this can be overcome?. Autophagy, defined as the self control eating process (from the greek word "self-eating"), is a highly conserved intracytoplasmic process for degradation and recycling of components for cellular homeostasis [35-38]. It is characterized by the formation of double-membrane bound vesicles, "autophagosomes", able to engulf different types of cargo: protein aggregates or damaged, defective organelles (selective autophagy), or non-specific cellular material (bulk autophagy), transport it to the vacuole/lysosome for digestion and recycling for energy production [32,36,39]. Other types of autophagy is the Chaperone-mediate autophagy (CMA) for selective degradation of single proteins. Microautophagy or endosomal microautophagy (eMI), at basal or induced levels, is a direct engulfment of proteins into lysosomes. On referring specifically to Macroautophagy this mechanism during starvation or treatment with rapamycin or amino acid leads to the inhibition of mTOR (target of rapamycin) [33,40]. The target of rapamycin (mTOR), known as a conserved Ser/Th kinase that sense energy inputs and nutritional demands [33,40]. Furthermore, activation of mTOR stimulates anabolic processes and biomass production, while its inhibition enhances catabolic processes, including autophagy [33,40].

Autophagy, and autophagosome biogenesis is mediated by the action of the associated proteins, the ATG protein, in particular ATG9 and ATG16 (or the yeast orthologue), proteins that can be associated from different membrane sources [39,41-45]. Several studies form the literature suggest that autophagy represents an opportunity of host immune response to tackle and overcome resistant intracellular pathogens [25,46-48]. Indeed, autophagy inducing drugs has been recently described as one of the strategies that can circumvent *MTb* infection disease [49-52]. Pharmacological activation of autophagy is achieved by blocking the kinase activity of mammalian target of rapamycin (mTOR) enzymatic complex 1 (mTORC1) leading to remove the autophagy inhibition state of the autophagy under physiological conditions [39,40,53].

In a previous work i*n vivo* and *in vitro* assays either in the mouse model and/or in human cell lines (A549/THP-1 cell line) have shown that a commercial preparation of Cry1A-Cry2A Bt proteins enhanced BCG immunity. Thus, A549 or THP1 cells, infected with BCG vaccine and/or co-administered with Cry1A-Cry2A induced protective humoral (IgG antibodies) and cellular immune responses (IL-17 cytokine) even at low doses [54]. Interestingly at high concentrations of this commercial Cry1A preparation did not affect the A549 viability nor of THP-1 cells [54]. The mechanism of immunogenicity and adjuvanticity using protoxina CryAc has been proposed to be mediated by receptor like molecules [55,56]. In the present study, we are reporting that a commercial preparation of *Bacillus thuringiensis*, Cry1A-Cry2A combined with BCG vaccine enhanced autophagy induction, determined by surface co-localization of the microtububle associated light chain-3 (LC3-II) protein determined by contrast and immune fluorescence microscopy in human macrophages (derived from PBMCs of healthy donors).

Methods

Human macrophages preparation

Peripheral blood mononuclear cells were obtained from buffy coats of healthy donor to the blood bank of the Instituto Nacional de Enfermedades Respiratorias (INER, CDAD of MX) Ismael Cosío Villegas. Monocytes were further isolated by CD14 positive selection using magnetic beads from Miltenyi (Miltenyi Biotech, Auburn, CA, US). Cells' viability was 100% by tripan blue exclusion. Monocytes were seeded in 8-well chamber slides (3 x 10^5 cells/well) and were allowed to differentiate into monocyte derived macrophages by culturing in complete RPMI-1640 (Lonza, Walkersville, MD, US) supplemented with 200mM L-glutamine and 10% heat-inactivated human serum (Valley Biomedicals, Winchester, VA, US) for 7 days. Medium was refreshed at day 4.

Macrophage stimulation and infection

Cells were stimulated with 5 µg/ml of Cry for 24 h. For infection experiments macrophages were infected with *M. bovis* BCG (ATCC, Manassas, VA, US) at MOI 5 during 1 h in RPMI with 30% nonheat-inactivated human serum and without antibiotics. Cells were extensively washed to discard non phagocitozed bacteria and further stimulated with 5 µg/ml of Cry and cultivation for additional 24 h. Medium was used as negative control and 250 µg/ml of rapamycin (Sigma Aldrich, St. Louis, MO, US) were used as positive control. The autophagy inhibitor wortmannin (Fluka, Seelze, DE) was used at 100 nM 30 minutes prior stimulation when indicated. After the incubation period the medium was discarded, and cells were fixated with 4% parafolmadehyde and stored at 4ºC until fluorescence microscopy staining. In selected experiments, the supernatants were cryopreserved at -20ºC cells for further use.

Autophagy detection by fluorescence microscopy

The uninfected cells were stained with rabbit anti-LC3B coupled to Alexa Fluor 488 (Novus Biologicals, CA, US). Nuclei were stained with Hoechst (Enzo Life Sciences, NY, US) following the manufacturer's instructions. The slides were mounted with Prolong Gold antifade reagent (Molecular probes, Carlsbad, CA, US). The cells were visualized with a fluorescence Axio Scope A1 microscope (Carl Zeiss, Oberkochen, DE), and the images were

acquired and analyzed with ZEN Pro software (Carl Zeiss). At least 100 cells were counted for each condition. We considered 5 puncta as basal expression, and the percentage of cells with more than 5 LC3+ puncta (autophagosomes) was calculated.

When analyzing infected cells, and in-house developed antibody was used to stain the bacteria that was revealed using a secondary antibody coupled to Alexa Fluor 594. The percentage of cells with more than 5 LC3+ puncta (autophagosomes) was calculated as well as the percentage of bacteria colocalizing with LC3 determined by the merging of green and red fluorescence that produces yellow images.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 (CA, US) using a Friedman's non-parametric analysis of variance (ANOVA) followed by Dunn's multiple comparison post-test. A $p \leq 0.05$ was considered significant

Results

A commercial preparation of *Bacillus thuringiensis* Cry1A-2A proteins induce autophagy in human macrophages

Autophagy is induced as an immunological host response to intracellular pathogens as *Mycobacterium tuberculosis.* The commercial preparation based on Bt Cry1A-Cry2A proteins is able to enhance BCG immunity [54]. Thus, we tested whether in human macrophage stimulated with this commercial preparation, autophagy was induced. The effect was evaluated as described in material and methods. The percent of autophagic puncta induced in human macrophages stimulated by Cry1A-Cry2A, Rapamacyin and/or Wortmanin stimulation were calculated and plotted ($p < 0.05$). Macrophages activated with Cry1A-Cry2A show an increase in autophagic puncta labeled with microtubule light chain 3 LC versus medium, or Cry-Wortmanin. Rapamycin is the positive control of autophagy induction. Wortmanin is the autophagy inhibitor (Figure 1a) ($P < 0.05$).

Figure 1: Autophagy –inducing of a commercial preparation based on a commercial preparation of *Bacillus thuringiensis* Cry1A-Cry2A proteins in human macrophages.

Macrophages were stimulated with 5µg/ml of Cry in presence or absence of the autophagy inhibitor wortmannin (100nM) during 24 h. Medium and 250 µ g/ml of rapamycin were used as controls. Cell preparations were stained with anti-human LC3 antibody coupled to Alexa Fluor 488 and nuclei were stained with Hoescht. a) Cells were observed at 100X. Size bars indicate 10 µm. Depicted are representative images. b) Cells with LC3 puncta were counted and the percentage of cells with puncta were determined. Depicted are box plots with medians and quartiles, n = 8, *$p < 0.05$.

Commercial preparation of Cry1A-Cry2A of *Bacillus thuringiensis* combined with BCG vaccine enhance autophagy inducing in human macrophages

Next, since we observed a mild autophagy induction induced by the commercial preparation of Cry1A-Cry2A, we measured the possibility that the stimulation of BCG infected human macrophages showed an increase in the autophagy induction. From figure 2a-b), we found that the percentage of mycobacteria BCG co-localizing with light chain 3 (LC3-II) in human macrophages is greater in infected and stimulated human macrophages with the commercial preparation based on Cry1A-Cry2A BT proteins than with Rapamycin and/or Wortmanin. (Figure 2a-b) ($P < 0.05$).

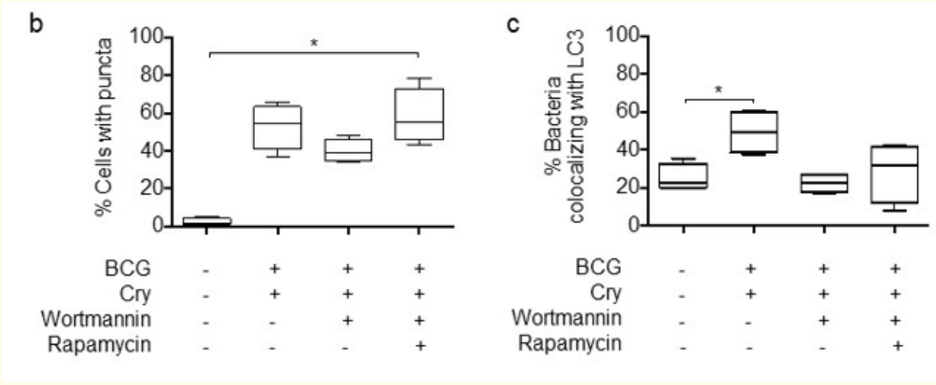

Figure 2: A commercial preparation based on *Bacillus thuringiensis* Cry1A-Cry2A proteins enhance BCG vaccine autophagy inducing properties in human macrophages.

Macrophages were infected with *M. bovis* BCG at MOI 5 during 2 1h after extensive washing of non phagocitozed bacteria. Further, the cells were stimulated with 5µg/ml of Cry in presence or absence of the autophagy inhibitor wortmannin (20 nM) and were incubated for additional 24 h. Medium and 250µg/ml of rapamycin were used as controls. Cell preparations were stained with anti-human LC3, BCG were stained with the proper antibodies and nuclei were stained with Hoescht. a) Cells were observed at 100X. Size bars indicate 10 µm. Depicted are

representative images. b) Cells with LC3 puncta were counted and the percentage of cells with puncta were determined. Depicted are box plots with medians and quartiles, n = 4, *p < 0.05. c) Bacteria were counted and the percentage of bacteria colocalizing with LC3 were determined. Depicted are box plots with medians and quartiles, n = 4, *p < 0.05.

Discussion and Conclusion

In the present work, we are reporting that a commercial preparation of *Bacillus thuringiensis* Cry1A-Cry2A co-administered with *Mycobacterium bovis Bacillus Calmette Guerin (BCG*) vaccine enhance surface co-localization of mycobacteria and the microtubule light chain (LC3II)) in human macrophages.

Autophagy is a highly conserved eukaryotic catabolic process that is fundamental to maintain homeostasis at cellular an physiological levels [14-17]. Autophagy activation leads to degradation and recycling of the excess of cytoplasmic cargo, damaged organelles, misfolded proteins, microbes [14-17,36]. The molecular machinery of the autophagy is under the control of the target of rapamycin kinase (mTOR) a conserved Serine/ Threonine kinase, the activated protein kinase (AMPK), and the set of proteins associated to autophagy, ATGs proteins [39,40,53]. In mammals around seven to eight proteins associated to autophagy has been described. Among the most common, are Atg8c (mAtgs, and the microtubule-associated light chain-3, -LC3A, LC3B, LC3B2 and LC3C [34,57-60]. LC3B usually is used as autophagosomal marker [20,21,27,28]. However, other members of this set of proteins can also be used as markers of membrane association or autophagosome formation and the lysosomes markers LAMP1 and CD68 [39,59-61].

How autophagy becomes pivotal for cellular homeostasis?, How is triggered?. It is known that autophagy is triggered in the presence of nutrients (i.e. aminoacids), starvation), and/or growth factors. In the absence of any of these components, mTOR1 is activated and autophagy is inhibited. MTOR and the transcription factor EB (TFEB) works together through collaboration of GABARAP members (mAtg8 family) and other LC3-positive membranes to maintain cellular function, lysosomal biogenesis, and the homeostatic mechanism to avoid any deleterious effects in the body tissues [26,40,62-64]. Furthermore, under starvation conditions, or during cellular stress as *MTb* infection, immunity related GTPase family M (IRGM) is activated [64,65]. IRGM is inhibitor of mTOR kinase activity, but it also promotes PPP3/calcineurin's protein phosphatase activity, which collective translates into effective TFEB activation. Thus, IRGM becomes the master regulation of the mTOR inhibition and autophagy induction [34,48,63,64]. Moreover, IRGM control phosphatase activity of PPP3CB, the translocation of TFEB to the nucleus, and therefore, have a role in the lysosomal biogenesis [40].

Of relevance is that the conection of immunity and autophagy has been suggested to occur through TLR signalization and engagement linking with the lysosomal pathways. Particulate antigens as for example bacteria can trigger on a human derived macrophages the autophagosome marker LC-3 to be rapidly recruited to the phagosome in a manner that depends on the autophagy pathway proteins ATG5 and ATG7 [14,26,37,61,65,66]. This process is preceded by recruitment of Beclin 1 and phosphoinositide-3-OH kinase activity, translocation of Beclin 1 and LC-3 to the phagosome, association with phagosome fusion with lysosome [14,26,37,65,66]. This will favor targeting the cargo or the intracellular pathogens to the proteasome lysosomal degradation, peptide presentation through MHC-II or MHC-Ito CD4+ or CD8+ T cellular activation [14,35,40,62].

Under the settings of BCG vaccination, autophagy is induced as a defense mechanism of the host to the infection. Briefly, BCG vaccine as antigen is phagocytose by the antigen presenting cells i.e. (macrophages), endocytosed, released by the ESX-1 secretion system to the cytoplasm. Mycobacterial DNA is recognized by the STING-dependent pathway, and the autophagic receptors p62 and NDSP52 [20,46,61,65,66]. Then, mycobacteria is ubiquitinated by the ubiquitin ligase Perkin, TRIM16, Galectin-3, ATG16L-1 and the Ubiquitin ligase Smurf1, activating autophagy, the lysosomal pathway for processing, and presented loaded on MHC-II to CD4+T cells or peptide-loaded on MHC-I loaded to CD8+ T cells [14,37,65,66]. However, the highly variability in the BCG vaccine immune protection can be due to defects in APCs presentation and T cell activation [7,29]. BCG vaccine sequestered in phagosomes inhibits digestion of immunodominant antigens (i.e Ag85B) leading to the impairment of antigen presentation, T cell activation, and the induction of long lasting immunological memory [14,26]. On the contrary BCG vaccine and/or *Mtb a*re capable to block or sequester autophagosome formation, maturation, and fusion with the lysosome pathways, leading to an incomplete or delay in the host immune response [14,26-28].

To take advantage of the autophagy machinery as an innate host response is that can be harnessing as a mechanism of delivering vaccines and/or cargo (microbes) that links lysosomal patways with antigen presentation and T cellular immune response [14,37,65,66]. At this point, is noteworthy recent on the effect of the combination of BCG vaccine and rapamycin. BCG-autophagy induction was enhanced gainst *M. tuberculosis* infection. Rapamycin enhanccd BCG vaccine delivery to lysosomes, and thereby increased BCG antigen presentation to CD4+ and/or CD8+ T cell activation [14,26,37,65,66]. Furthermore, a recombinant BCG vaccine that over express Ag85B and mixed with the C5 peptide derived from *MTb* CFP-10 protein elicited a strong Th1-type cellular response represented by the IL-12, IL1-β, and TNF-alpha, restoring antigen MHC-II presentation via TLR-2 and MyD88. phosphorylation of p38MAPK and ERK against *M. tuberculo*sis (Kahn., *et al.* 2019) [29]. In a previous work, we have shown that a commercial preparation of Cry1A-Cry2A Bt proteins, behaves as immunogen and adjuvant of BCG vaccine [54]. In the present study, human macrophages

derived from healthy patients PBMCs upon BCG and Cry1A-Cry2A, enhanced autophagy induction. Bt Cry proteins showed an increase in the colocalization of LC3 versus rapamycin and/or wortmannin (Figure 1A-B) In the combination of BCG vaccine, at low concentration it is increased the co-localization of LC3 in human derived PBMC macrophages (Figure 2A-B). From the data reported here, we think that this potential alternative can endowed to the actual BCG vaccine with autophagy inducing properties that can harnessed against either infectious diseases (i.e. mycobacteria of the complex of *M. tuberculosis*) or other chronic diseases (i.e. cancer).

Acknowledgments

In debt with the financial support of UAZ (Zacatecas, Zac. MX); INER (CDAD MX).

Conflict of Interest Statement

"I, as the corresponding author, declare, on behalf of all authors of the paper, that no financial conflict of interest exists in relation to the work described. "I, as the corresponding author, declare that the results/data/figures in this manuscript have not been published, nor are they under consideration for publication elsewhere".

Author Contribution Statement

G.G.G.M. conceptualization, data analysis, writing of the manuscript. A.D.R.H and E.J. Methodology, Data analysis, Research. Y.G. Research, Data analysis. JMFV. Research, "I certify that the above information is true and correct. All the authors contributed to the study and the manuscript.

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