Involvement of ABC-transporters and acyltransferase 1 in intracellular cholesterol-mediated autophagy in bovine alveolar macrophages in response to the Bacillus Calmette-Guerin(BCG) infection

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Abstract

Background: Mycobacterium tuberculosis (M. tuberculosis, Mtb) is the pathogen causing human Tuberculosis (TB). Mycobacterium bovis (M. bovis) infection is the cause of bovine TB, which occasionally infects other species of mammals including humans and wildlife, a main reason of the difficulty to eradicate TB around the world. Recent studies in immunometabolism demonstrated that host cholesterol levels have implications in the establishment of Mtb infection, which lead to the development of host-directed therapy (HDT) strategies for treatment of TB. Methods: In the present report, the involvements of ATP-binding cassette transporters (ABC-transporters)- and cholesterol acyltransferase1 (ACAT1)-mediated intracellular cholesterol in autophagy of bovine macrophages induced by Bacillus Calmette-Guerin (BCG) were interrogated by enzymatic and biochemistry assays i n vitro, including quantitative RT-PCR and immunoblotting. Results: The results showed that a down-regulated expression of the ABC-transporters and ACAT1 in primary bovine alveolar macrophages (AMs) and murine RAW264.7 cells in response to the BCG infection. The inhibited expression of ABC-transporters and ACAT1 was associated with the reduction of intracellular free cholesterol, which in turn induced autophagy in macrophages upon to the Mycobacterial infection. These results strongly suggest an involvement of ABC-transporters and ACAT1 in intracellular cholesterol-mediated autophagy in macrophages in response to Mycobacterial infections. Conclusion: This study thus provides an insight into mechanism of immunometabolism of macrophages in response to the Mtb infection, and informative data for development of HDT in TB treatments.

Background

The tuberculosis (TB) remains the top global public health burden of infectious disease [1], which is caused by the infection of *Mycobacterium tuberculosis* (*Mtb*), an intracellular pathogen able to survive and grow within host cells, predominantly alveolar macrophages (AMs) [2]. Despite the employment of various anti-*Mtb* agents and *Bacillus Calmette-Guerin* (BCG) vaccine in the treatment and control of TB, the TB-control programs have been failed to reduce public health burden in most developing countries [3], in part owing to the difficulty to eradicate *bovine TB,* a zoonotic infectious disease in cattle caused by *Mycobacterium bovis* (*M. bovis*) infection that occasionally infects other species of mammals including humans and wildlife. The bovine TB currently remains a great public health concern in the world, particularly in developing contraries. Epidemiologically, bovine TB is able to transmit within and between, livestock, wildlife and human. The interspecies transmission of infection increases the incidence of human TB in affected population such as dairy farmers, as well as causes significant economic losses in diary and meat industry [4]. Therefore there is a need to investigate mechanisms of TB in bovines.

Upon the infection of *Mycobacterium tuberculosis*, the host innate immune is normally initiated through an bacterial phagocytic mechanism of residential macrophages, which further recruited immune cells such as neutrophils, dendritic cells and macrophages in the lung [5]. In this context, the Mycobacterium enters host macrophages and targets cellular organelles for their own benefit. In this regard, the alveolar macrophages (AMs) are the main target cells and immune cells of *Mtb*, which play critical roles in anti-

Mtb infection by eliminating pathogens through mechanisms including phagocytosis such as the autophagy, initiation of protective acquired immune responses *via* antigen presentation to T cells. The fate of Mtb in AMs is a key to determine the final outcome of *Mycobacterium tuberculosis* infection [6].

Indeed, the *Mtb*-induced programmed cell death and autophagy is fundamental cellular processes intimately involved in the interaction between the pathogen and immune phagocytes, including the macrophages, and other immune cells such as dendritic cells (DC) and neutrophils [7]. With this respect, autophagy plays key roles in immune defenses, in which it acts as an important immune barrier to participate in cellular immune responses to protect host cells from invaded pathogens, through mechanisms including inflammatory regulation, antigen presentation, microorganism capture and degradation [8]. In the case of invaded intracellular pathogen *Mtb*, the host cells such as macrophages can encapsulate the bacteria through a mechanism of autophagy and present the pathogen to lysosomes for degradation, ultimately eliminate the intracellular bacteria and maintain the cellular homeostasis of host cells [9].

Recent studies in immunometabolism demonstrate the intimate link between the metabolic states of immune cells in *Mtb* infections [10], in which the host lipid metabolism is associated with the *Mtb*-induced cell autophagy [11]. Several lines of evidence have suggested that host lipids have significant impacts on *Mtb* survivals in cells [12]. In this regard, the metabolic imbalance caused by an infection of *Mtb* leads the formation of lipid droplets in macrophages, and the accumulation of lipids forms foam cells, in order to provide a sufficient energy source for the Mycobacteria survival in cells [13]. Among these lipids, the cholesterol, a multifunctional lipid plays important metabolic and structural roles in the eukaryotic cells [14]. A compelling body of evidence has shown that the systemic cholesterol level is associated with the host immunity. Indeed, in addition to atherosclerosis and Alzheimer's disease, an abnormal cholesterol metabolism has been implicated in several lung diseases, including the development of TB [15].

It has been well documented that ATP-binding cassette transporters (ABC-transporters) [16], and cholesterol acyltransferase [17] play crucial roles in cellular cholesterol balance of immune cells such as macrophages and monocytes [18]. In this regard, ABC-transporters are a family of proteins that utilize the ATP-hydrolyzed energy to pump substrates across lipid bilayers [19]. In monocytes/macrophages, ABC-transporters have been demonstrated to involve in reverse cholesterol transport (RCT) and forestall atherosclerotic lesion progression [20]. In this context, the PPARγ-LXRα-ABCA1/ABCG1 signaling is involved in regulating cholesterol efflux in macrophages, where an activation of liver X receptor alpha (LXRα) directly induced the expression of the membrane ATP-binding cassette transporters, such as ABCA1 and ABCG1, which in turn pump cholesterol out of cells [21]. Similarly, the cholesterol acyltransferases (ACATs) are exclusively intracellular enzymes that produce cholesteryl ester utilizing free cholesterols as the substrates, which is a key procession in maintaining cellular cholesterol homeostasis [22]. To date, two mammal ACATs, the ACAT1 and ACAT2 have been identified [23]. The ACAT1 is an enzyme that resides in the endoplasmic reticulum (ER) membrane of cells. It is also a major isoenzyme in macrophages [24]. An increased number of evidences showed that the ACAT1 was involved in the

pathogenesis of many human diseases such as atherosclerosis, Alzheimer's disease (AD) and cancers, and it has been investigated as a potential target for treatment of these diseases [25].

With regard to bovine alveolar macrophages (AMs), our previous RNA-Seq analysis revealed an alteration of ABC-transporters and ACAT1 in BCG-infected primary bovine AMs as compared to the naïve AMs. Together with aforementioned findings, we therefore hypothesize that the BCG-altered ABC-transporters and ACAT1 may have an important implication in the regulation of intracellular cholesterol level and autophagy in macrophages in response to Mycobacterial infections.

Methods

Primary bovine alveolar macrophages and RAW264.7 cells

This study was approved by the ethics committee for use and care of animals at Ningxia University (Yinchuan, China). Bovine alveolar macrophages (AMs) were obtained from lungs of one- to two-year-old Simendal cattles from a tuberculosis-free herd. The entire lung was sterilely removed post-mortem with a portion of trachea, and was intratracheally infused with 500 mL of D-Hank's solution (Biotopped, Beijing, China) containing 50μg/mL of gentamicin, 2.5μg/mL of amphotericin and 100μg/mL of penicillin and streptomycin (Hyclone, Logan, USA). The recovered bronchoalveolar lavage fluid (BALF) was filtered by passing a 70 μm-pore nylon cell strainer prior to being collected into sterile beakers and centrifuged at 1000 rpm for 10min. The cell pellet was then resuspended in 20 mL RPMI-1640 medium (HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA), 100 U/mL of penicillin and 100 U/mL of streptomycin. 3 mL of red blood cell (RBC) lysate solution was then added into the cell suspension and gently mixed well for 3min at room temperature (RT) before the cells were recollected by centrifugation at 1000 rpm for 10min. The cells were resuspended with RPMI-1640 medium with 10% FBS and counted. The resultant primary cells were seeded at a density of $5´10^7$ per tissue culture dish with diameter of 140 mm and cultured in RPMI-1640 medium with 10% FBS for 6 h to 8 h. The unattached cells were removed by rinsing the culture with pre-warmed PBS, and the attached monolayer of primary AMs were dissociated with Tryple^{TM} Express (Thermo Fisher Scientific, Shanghai, China) and harvested by centrifugation. The isolated cells were then re-plated in 6-well plates at a density of $5×10^6$/well in RPMI-1640 medium with 10% FBS and cultured for 16-18 hours for subsequent experiments. The murine macrophage cell line RAW264.7 was purchased from Shanghai Academy of Life Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM medium supplemented with 10% FBS and penicillin/streptomycin. All cells were cultured in humidified incubators with 5% CO_2 atmosphere at 37°C.

BCG culture and infection

The *M. bovis BCG* vaccine strain was purchased from Chengdu Institute of Biological products (Chengdu, China). BCG bacterial cells were grown in the Middlebrook 7H9 medium (BD Difco, San Jose, CA, USA) supplemented with 10% albumin-dextrose-catalase (ADC) enrichment medium (BD Difco, San Jose, CA,

USA) and 0.05% Tween 80 (Sigma, St. Louis, MO, USA) at 37°C with slow shaking for 2 weeks. The bacteria cells were harvested by centrifugation and re-suspended in the culture medium. The bacterial cell number was titrated by spectrophotometer at wavelength of 600 nm based on an OD600nm of 1.0 equivalent to 1×10^8 mycobacterial cells [26]. The bacteria stocks were aliquot and stored at −80 °C freezer for subsequent uses. For infection, the macrophage cells were cultured with 6-well plates for 6-8h before they were infected with BCG at a multiplicity of infection (MOI) of 10 bacteria and then incubated for additionally various times.

Quantitative RT-PCR

The total RNA of cells was isolated using Trizol reagent per manufacturer's instruction (Invitrogen, Grand Island, NY, USA). The reverse transcription of first-strand cDNA synthesis was generated using M-MLV reverse transcriptase (TaKaRa, Dalian, China). The quantitative reverse transcription PCR (qRT-PCR) was performed in the Roche Lightcycler 2.0 using TaKaRa SYBR Green I kit (Takara, Dalian, China). The primer sets used for RT-PCR were designed and synthesized in Shanghai Sangon Biotech Inc. (Shanghai, China) by bioinformatics tools using available mRNA sequences (Suppl. Table 1). The relative expression of genes of interest was calculated by accessing the efficiencies and the crossing point deviation of a given gene *vs* housekeeping β-actin gene. In each independent experiment, The relative changes of gene expression were represented by folds of change over its respective uninfected control cultures by a $2^{-\Delta\Delta CT}$ method.

Immunoblotting analysis

Whole cell extracts were prepared by lysing cell cultures in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) for 60 min on ice. The concentration of soluble protein was determined with Bio-Rad Protein Assay based on the method of Bradford (Bio-Rad Laboratories, Richmond, CA, USA). The clarified lysates (100 μg) were resolved in 8% or 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes for immunoblotting assay probed with antibodies to proteins of interest. The primary antibodies used in this study were listed in Suppl. Table 2. All these primary antibodies were applied in a dilution of 1:500-1000. Following extensively washing, protein of interest was detected or visualized with an appropriate HRP-labelled or fluorescence-labelled IRDye (Li-Cor Biosciences, Lincoln, NE, USA) secondary antibody. The blots were then developed using the enhanced Western Bright ECL reagent (Advansta, Menlo Park, CA, United States) or Li-Cor Odassay Scanner (Li-Cor Biosciences). The relative expression of protein was semi-quantified by optical densitometry using ImageJ Software version 1.46 (http://rsb.info.nih.gov/ij/). The densitometric arbitrary unit (A.U.) was used for determined the ratio between the net intensity by calculating values of each sample divided by the β-actin internal control.

Determination of cholesterol and cholesterol ester

The contents of intracellular cholesterol and cholesterol ester were determined by commercially available cholesterol quantitative assay kit according to manufacturer's instructions (Sigma, St. Louis, MO, USA).

The absorbance was then detected at 570nm. Finally, the contents of intracellular total cholesterol and free cholesterol were calculated comparing to its standard curve, and the amount of cholesterol ester was calculated by the substrate of the content of total cholesterol to the content of free cholesterol.

Generation of RAW264.7 cell lines overexpressing or silencing ACAT1

To generate a lentiviral vector overexpressing ACAT1, cDNA of murine *acat1* gene (NM_144784) was cloned downstream of the CMV promoter of GV492 lentiviral proviral backbone plasmid (Genechem Co., Ltd., Shanghai, China); to generate lentiviral vectors knocking down endogenous ACAT1 expression, shRNAs targeting sequences of 5'TCGGTCTGGCTAGTATTTG3', 5'CGTACCTAAGGTTCTTAAA3' and 5'TAACTGATGTCTACAATAA3' of murine *acat1* gene (NM_144784) were respectively cloned downstream of the U6 promoter of GV493 lentiviral proviral backbone plasmid (Genechem Co.,Ltd., Shanghai, China). The constructed proviral plasmids were used for generation of respective VSV-G pseudotyped lentiviral vectors Lenti-ACAT1 and Lenti-shRNA-ACAT1 as described elsewhere. To generate murine macrophage RAW264.7 cells overexpressing and silencing ACAT1, RAW264.7 cells were infected with Lenti-ACAT1 and Lenti-shRNA-ACAT1, respectively. The virally transduced cells were then cultured for 72 h following the infection before they were refreshed with a selective medium containing purinomycin for additional 4-5 days. The cell pools with were then used for further experiments after functional determination.

Suppression of of ACAT1 using inhibitor K604

RAW264.7 cells were seeded in a 6-well plate culture dish at a density of 2×106/well in DMEM-10% FBS culture medium containing 10 μM of ACAT1 protein inhibitor K604 (MedChemExpress, USA) and BCG at a multiplicity of infection of 10:1. The cells were and cultured for 12 h before they were used for analysis.

2.8 Statistical analysis.

All data collected in this study were from at least three independent biological repeated experiments, which were analysed using SPSS statistics 22.0 (SPSS Inc., Chicago, IL, USA). The data was presented as the mean ± SD. Statistical differences between groups were analysed by one-way analysis of variance (ANOVA), followed by post-hoc Tu-key's test. The data was presented as the mean ± SD. A P values < 0.05 were considered as statistically significant.

Results

Alterations of ABC-transporters and ACAT1 in BCG-infected macrophages.

A down-regulated ABC-transporters ABCA5, ABCA6, and ABCA10 was found in primary bovine alveolar macrophages (AMs) infected with BCG as determined by RNA-seq analysis (data not shown). Among them, the ABCA5 was reported correlated with cholesterol efflux in macrophage, while little is known about functions of ABCA6 and ABCA10 [27], suggesting the BCG-altered ABC transporters may have an important implication in the regulation of intracellular cholesterol in macrophages. In order to validate the RNA-Seq findings and explore the changes of other ABC transporters in macrophages, the abundance of

transcripts of *ABCG1, AB1, ABCA5* and *ABCA6*, as well as the *acyltransferase 1* (*ACAT1*), a gene related to intracellular cholesterol, in both primary bovine AMs and RAW264.7 cells with BCG for 12h was evaluated by a qRT-PCR assay (Suppl. Fig. S1). As expected, the expression of all above tested genes was strikingly down-regulated in macrophages in response to the BCG infection (Suppl. Fig. S1). In agreement with the finding of transcripts, the down-regulated ABC transporters and ACAT1 was further corroborated by an immunoblotting assay, i.e. less abundant ABCG1, ABCA1, ABCA5, ABCA6 and ACAT1 proteins were observed in both BCG-infected primary bovine AMs (Fig. 1A) and murine RAW264.7 cells (Fig. 1B), in comparison with the naïve macrophages. Of note, the least ABCA1 and ACAT1 proteins in BCG-infected bovine AMs were observed at post infection 6h, while the ABCG1 was at 12h, and the least ABCA5 and ABCA6 were at 24h post infection (Fig. 1A). Accordingly, the least ABCG1, ABCA1 and ACAT1 proteins in BCG-infected RAW264.7 cells were found at 12h post infection, while the least ABCA5 and ABCA6 were at 24h post infection (Fig. 1B).

The BCG Infection increases intracellular cholesterol in macrophages

The ABC-transporters ABCG1, ABCA1, ABCA5 participate in the reverse cholesterol transport from macrophages and ACAT1 converts free cholesterol to cholesteryl esters [28]. A down regulation of these proteins may imply an increase of intracellular cholesterol. Indeed, a dynamic change of intracellular cholesterol AND cholesterol ester with time was observed in both bovine AMs and macrophages in response to BCG infection (Fig. 2). In this context, intracellular levels of free cholesterol (Fig. 2A) and cholesterol ester (Fig. 2B) of primary bovine AMs were gradually increased and decreased, respectively. The intracellular free cholesterol (Fig. 2A) and cholesterol ester (Fig. 2B) reached their peaks at 6h post infection before they respectively decreased and increased from 12h afterward. Consistently, the dynamic changes of intracellular free cholesterol (Fig. 2C) and cholesterol ester (Fig. 2D) were also detected in BCG-infected RAW264.7 cells. The intracellular free cholesterol was increased and reached its peak at 12h but reduced at 24h post infection (Fig. 2C); while the intracellular cholesterol ester was significantly decreased and reached the lowest level at 12h but increased at 24h post infection (Fig. 2D). These results may indicate that the increased intracellular cholesterol is a consequence of BCG-inhibited expression of ABC-transporters and ACAT1.

Infection of BCG induces autophagy in macrophages.

Accumulating evidences have demonstrated that autophagy can be induced by cholesterol in Mycobacteria-infected macrophages [29]. In line with other studies, a significant increased expression of autophagy markers LC3II/I and Beclin1 was observed in primary bovine AMs (Fig. 3A) and RAW264.7 cells (Fig. 3B). Interestingly, the BCG-induced expression of autophagy markers showed a comparably dynamic trend with the change of intracellular cholesterol in both cell types (Fig. 2), indicating that the intracellular cholesterol may play a role in regulating autophagy in BCG-infected macrophages, which needs further investigation.

The involvement of ACAT1-mediated intracellular cholesterol in autophagy of macrophages in response to BCG Infection.

The ACAT1 gene has been demonstrated to correlate with intracellular cholesterol and autophagy [25]. In order to further validate the involvement of the altered ACAT1 in BCG-infected macrophages, RAW264.7 stable cell lines overexpressing and silencing ACAT1 were generated by lentiviral vector-mediated gene transduction (data not shown). As expected, the overexpression of ACAT1 significantly decreased the BCG-induced intracellular free cholesterol, while the silence of ACAT1 expression led an increased BCG-induced intracellular free cholesterol (Fig. 4A). In consistence, an overexpression of ACAT1 restored the BCG-inhibited intracellular cholesterol ester, which a silence of ACAT1 aggravated the suppression of BCG-reduced intracellular cholesterol ester (Fig. 4B). Of importance, the ACAT1-altered intracellular cholesterol and cholesterol ester were correlated with the expression of protein markers of autophagy in macrophages infected with BCG (Fig. 5). An overexpression ACAT1 reduced the BCG-induced expression of autophagy-related proteins ATG5, ATG7 LC3II/I and Beclin1 (Fig. 5A), while knocking-down of ACAT1 expression enhanced the BCG-induced autophagy proteins in RAW264.7 cells (Fig. 5B). This funding was further corroborated the experiment using an ACAT1 specific inhibitor K604 (Fig. 6). In the presence of K604, the BCG-induced autophagy-related proteins ATG5, ATG7, LC3II/I and Beclin1 were significantly increased in both primary bovine AMs (Fig. 6A) and murine RAW264.7 cells (Fig. 6B), as compared with their respective controls in the absence of K604. These results strongly suggest an involvement of ACAT1 in regulation of intracellular cholesterol and autophagy of macrophages in response to BCG Infection.

Discussion

Tuberculosis (TB),remains a major global public health burden in the World. *M. tuberculosis* (*Mtb*) is the pathogen of human TB, and *M. bovis* is highly pathogenic mycobacterium that may infect many animal species, including bovine and humans [30]. Alveolar macrophages activated by mycobacteria infection can effectively transfer the phagocytosed pathogens to the destructive microenvironment of lysosomes. In this context, autophagy, a lysosome-dependent degradation process plays a crucial antibacterial effect that prevents against *Mtb*, by promoting an innate immune response [31]. In the case of *Mtb* infection, the bacteria-induced autophagy can target *Mtb* and promote phagosomal maturation, thus inhibiting the replication of intracellular bacilli [32]. However, the bacilli have an ability to escape from lysosome and survive within their host macrophages by utilizing host resources as early niches for their replication and growth in cells [33]. In this regard, virulent *Mtb* are able to utilize a variety of strategies to survival in macrophages, by preventing the fusion of phagosomes with late endosomes or lysosomes, limiting phagosomal acidification, and targeting cellular organelles such as mitochondria, and interfering the balance of pro- and anti-apoptotic factors to escape the host immune response [34].

A growing number of findings highlight the crucial role of metabolic reprogramming in macrophage activation. It is increasingly evident that macrophages undergo extensive lipid remodelling upon an infection, during which macrophages exhibit an M1 pro-inflammatory polarization state or M2 anti-inflammatory polarization state. Interestingly, the intracellular cholesterol of macrophages was processed differently between the M1 and M2 polarization states [35]. Cholesterol, a major structural component of animal cell membranes, is thought to be involved in immune regulations [36] and the development of *Mtb* infection [29]. In this regard, *Mtb* is able to import and metabolize host cholesterol during infection, which

is critical for the maintenance of *Mtb* infection [29]. As an important component of mammalian cells with many basic cellular functions, the intracellular cholesterol level is regulated many factors such as Acyl-CoA:cholesterol acyltransferase 1 (ACAT1) and ABC transporters [36],. Functionally, the ACAT1 is the major isoenzyme in macrophages [24], which converts cholesterol to cholesterol esters and plays important roles in lipoprotein assembly, dietary cholesterol absorption, and intracellular cholesterol metabolism [37]. An inhibition of ACAT1 by small molecule inhibitor K604 retarded macrophage foam cell formation in cell culture, and diminishes the presence of lesion macrophages in atherosclerotic Apoe mice [38]. In accordance with these findings, an overexpression or silence of ACAT1 in murine RAW264.7 cells led a respectively decreased and increased intracellular ACAT1 intracellular free cholesterol, but an opposite effect in intracellular cholesterol ester in response to BCG infections. Interestingly, the ACAT1-mediated alterations of intracellular cholesterol were positively correlated with the BCG-induced autophagy in macrophages. Of note, the ACAT1 inhibitor K604 exhibited a similar effect to shRNA-mediated ACAT1 knockdown in RAW264.7 cells, suggesting an involvement of ACAT1-regulated intracellular cholesterol in BCG-induced autophagy. In addition to the ACAT1, ABC transporters, ABCA1 and ABCG1 showed an ability to pump cholesterol out of macrophages [21]. In this study, we also found that ABC-transporters were involved in the alteration of intracellular cholesterol in in both bovine alveolar macrophages and murine RAW264.7 macrophages infected with BCG, suggesting a regulatory role of efflux transporters of macrophages in maintaining intracellular cholesterol and cell autophagy following a *Mtb* infection [39].

Conclusions

In summary, in the present study, we explored the regulatory roles of ABC-transporters and ACAT1 in cholesterol metabolism of bovine macrophages in response to an infection of *Mycobacterium bovis* vaccine stain BCG. Our results demonstrated that the infection of BCG inhibited the expression of ABC-transporters and ACAT1 in primary bovine AMs and murine RAW264.7 cells, which in turn reduced the intracellular free cholesterol and increased cholesterol ester. Importantly, the BCG-inhibited expression of ABC-transporters and ACAT1, and its consequently reduced intracellular cholesterol was correlated with the BCG-induced autophagy in macrophages, clearly indicating an involvement of ABC-transporters and ACAT1 in intracellular cholesterol-mediated autophagy in macrophages in response to *Mycobacterium tuberculosis* infections. This study thus highlights an importance of intracellular cholesterol of immunometabolism in macrophages, aids our better understanding in the innate immune mechanisms during mycobacterial infection and eventually provides useful information for development of host-directed therapy (HDT) strategies in TB treatments.

Abbreviations

ABCA1: ATP binding cassette subfamily A member 1 ABCA5: ATP binding cassette subfamily A member 5 ABCA6: ATP binding cassette subfamily A member 6 ABCA10: ATP binding cassette subfamily A member 10 ABCG1: ATP-binding cassette subfamily G member 1 ABC-transporters: ATP-binding cassette

transporters ACAT : Acyl coenzyme A:cholesterol acyltransferase ACAT1: Acyl coenzymeA:cholesterol acyltransferase 1 ACAT2: Acyl coenzymeA:cholesterol acyltransferase 2 AD: Alzheimer's disease AM: alveolar macrophages ATG5: Autophagy-Related Protein 5 ATG7: Autophagy-Related Protein 7 BCG: Bacillus Calmette-Guerin DC: dendritic cells ER: endoplasmic reticulum HDT: host-directed therapy LC3: Microtubule associated protein light chain 3 LXRα: liver X receptor alpha M. bovis: Mycobacterium bovis MOI: Multiplicity of infection Mtb: Mycobacterium tuberculosis qRT-PCR: The quantitative reverse transcription PCR RCT: reverse cholesterol transport TB: Tuberculosis

Declarations

Ethics approval

The experiments involving bovie were performed according to protocols approved by the Institutional Animal Care and Use Committee of Ningxia University (NXU-2017-069). The need for consent is deemed unnecessary according to national regulations, but an informed verbal consent was obtained from the farmers. The ethics committee for the use of animals of Ningxia University approved this study.

Consent for publication

Not applicable

Availability of data and materials

All the data supporting our findings are contained within the article.

Competing interests

The authors declare that they have no conflicts of interest.

Authors' contributions

YW and XL conceived and designed the experiments; JX, YY, YZ, and CL performed the experiments and acquired data, ZYB and YY analyzed the data; LCP and XJR drafted the manuscript; YW and XL interpreted data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Figures

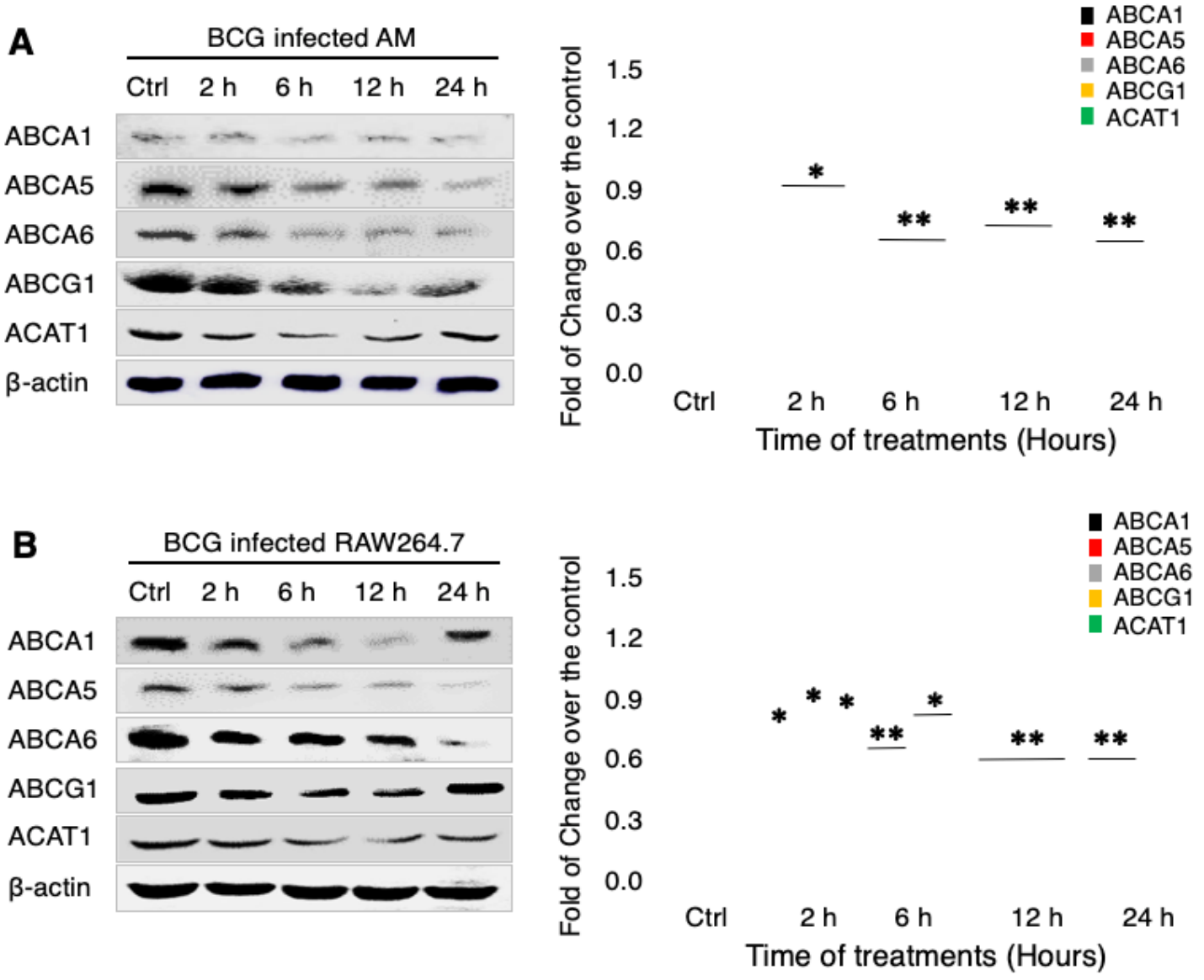

Figure 1

The dynamic changes of the expression of ABC-transporters and ACAT1 in macrophages n response to a BCG infection. Primary bovine alveolar macrophages (AMs) and murine macrophage RAW264.7 cells were infection with BCG at a dose of 10 for indicated time periods, abundances of ABC-transporters and ACAT1 were determined by an immunoblotting (IB) assay. (A) Representative images of blots for indicated proteins of interest of BCG-infected primary bovine AMs probed with corresponding antibodies

(Left panel), and the relative expression of proteins semi-quantified by an optical densitometry analysis (Right panel) demonstrated a dynamic change of protein expression (B) Representative images of blots for indicated proteins of interest of BCG-infected murine macrophage RAW264.7 cells probed with corresponding antibodies (Left panel), and the relative expression of proteins semi-quantified by an optical densitometry analysis (Right panel) demonstrated a dynamic change of protein expression. Both the BCG-infected bovine AMs and RAW264.7 cells exhibited the lowest expression of ABCA1, ACAT1 ABCG1, ABCA5 and ABCA6 at 6, 6, 12, 24 and 24h post the BCG infection, respectively. Data were expressed as mean ± SEM from three independent experiments. Compared to non-infection controls, *: $p<0.05$; **: $p<0.01$.

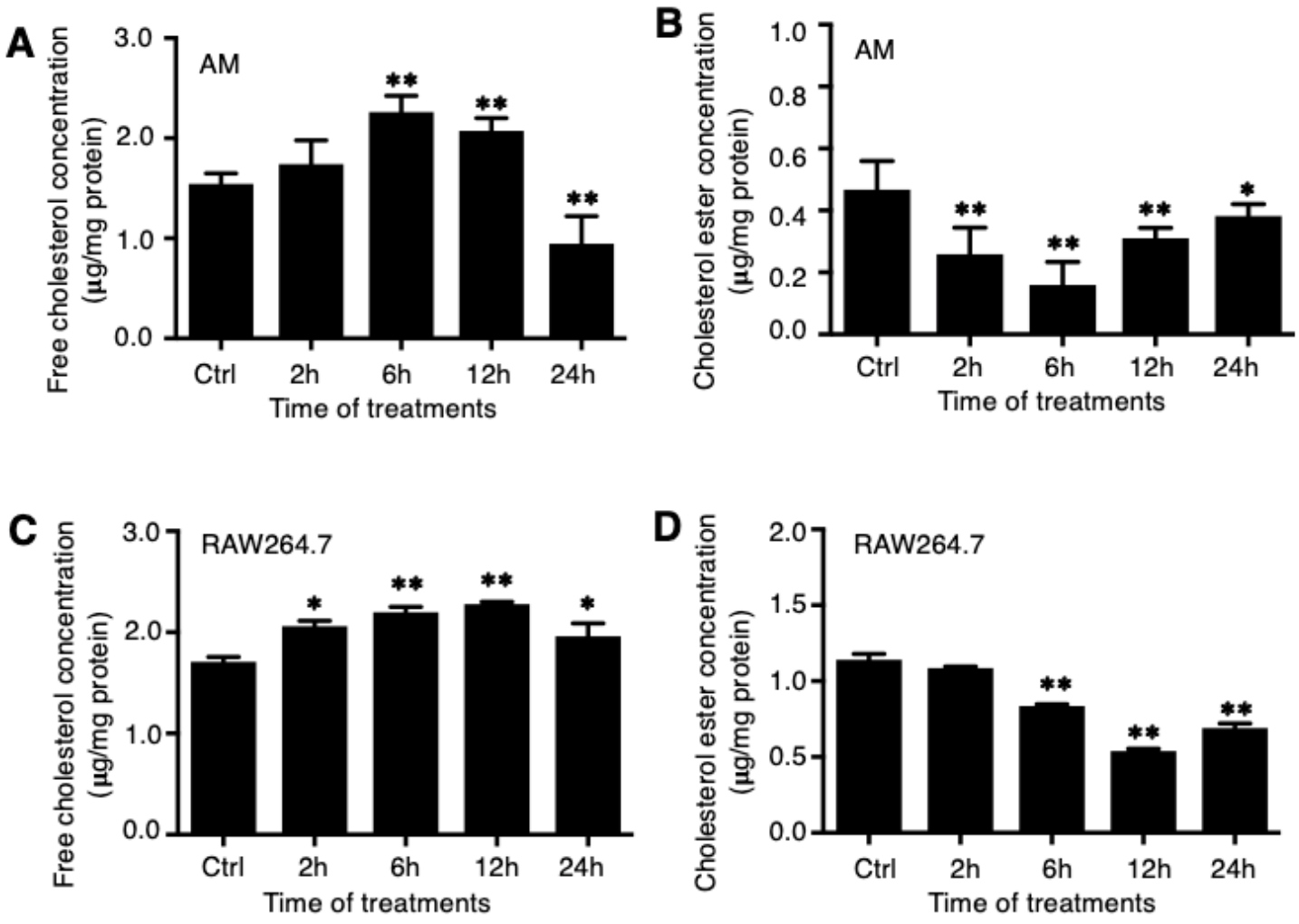

Figure 2

The BCG infection altered intracellular levels of cholesterol and cholesterol ester in macrophages. Bovine AMs and murine macrophage RAW264.7 cells were infected with BCG at a dose of 10 for indicated times before their intracellular levels of cholesterol and cholesterol ester were ascertained. (A-B) Intracellular levels of cholesterol (A) and cholesterol ester (B) of primary bovine AMs altered by the infection of BCG. (A) The content of intracellular free cholesterol was increased gradually and reached its peak at 6h post infection before it significantly decreased from 12h afterward; (B) while the intracellular cholesterol ester

content in AMs was significantly decreased and reached the lowest level at 6h and then dramatically increased at 12 and afterward. (C-D) Intracellular levels of cholesterol (C) and cholesterol ester (D) of murine RAW264.7 cells altered by BCG. (C) The intracellular free cholesterol was increased and reached its peak at 12h but reduced at 24h post infection. A significant change of intracellular cholesterol was observed between 2h, 6h and 12h after infection as compared with the control group; in contrast, (D) the intracellular cholesterol ester was significantly decreased and reached the lowest level at 12h but increased at 24h post infection. A significant change of intracellular cholesterol ester was determined between cells at 6h, 12h and 24h following the infection, as compared with the control group. Data were expressed as mean ± SEM from three independent experiments. Compared to non-infection control, *: $p<0.05$, **: $p<0.01$.

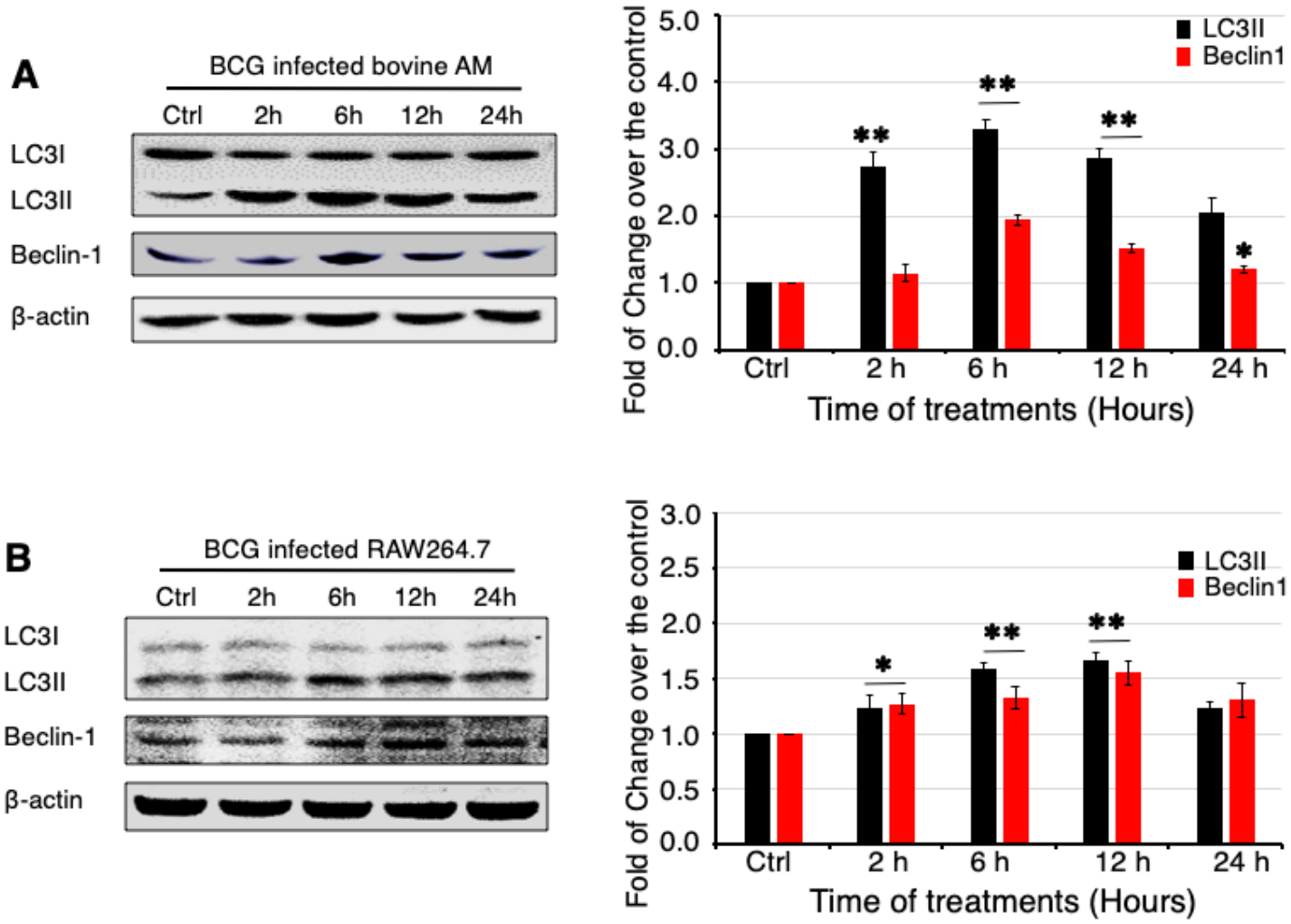

Figure 3

BCG infection induced the expression of autophagy-related proteins in macrophages. Bovine AMs and murine macrophage RAW264.7 cells were infected with BCG at a dose of 10 for indicated times, and the expression of autophagy-related proteins LC3I/II and Beclin1 was determined by an immunoblotting (IB) assay. (A) Representative images of blots for LC3I/II and Beclin1 in BCG-infected primary bovine AMs (left panel), and the relative expression of proteins semi-quantified by a densitometry analysis (right

panel) demonstrated an increased expression of autophagy-related proteins. (B) Representative images of blots of LC3I/II and Beclin1 in BCG-infected murine macrophage RAW264.7 cells (left panel), and their relative expression semi-quantified by a densitometry analysis (right panel) indicated an induced autophagy by BCG infection. Data were expressed as mean ± SEM from three independent experiments. Compared to non-infection controls, *: p<0.05; **: p<0.01.

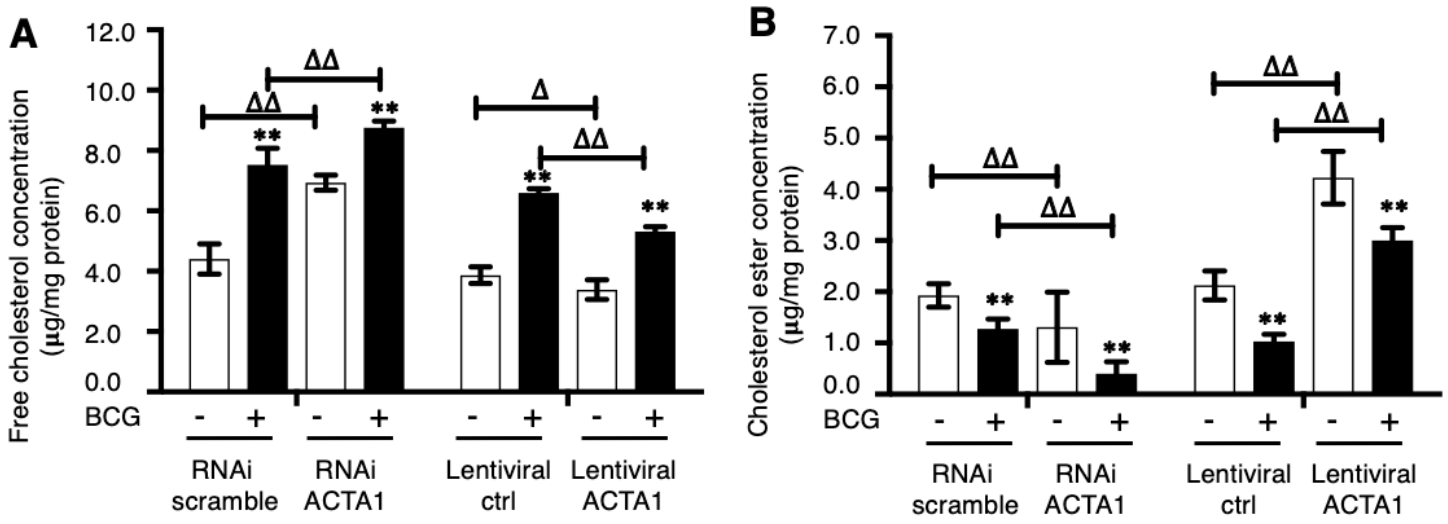

Figure 4

An ACAT1-mediated alteration of intracelluar cholesterol and cholesterol ester in macrophages in response to BCG infection. Murine RAW264.7 cell lines overexpressing and silencing ACAT1 were generated by lentiviral infections with vectors overexpressing ACAT1 or shRNA to ACAT1, respectively. Control RAW264.7 cell lines infected with appropriate control lentiviral vectors were also generated. The transgenic RAW264.7 cells were infected with BCG at a dose of 10 for 12 h prior to being harvested for assessment of intracellular cholesterol (A) and cholesterol ester (B). (A) An overexpression of ACAT1 exhibited an ability to significantly suppress BCG-induced intracellular cholesterol, while knockdown of ACAT1 showed an enhanced level of intracellular cholesterol in RAW264.7 cells. (B) An overexpression of ACAT1 significantly restored BCG-repressed intracellular cholesterol ester, while knockdown of ACAT1 further reduced the BCG-repressed cholesterol ester in RAW264.7 cells. Data were expressed as mean ± SEM from three independent experiments. Compared to non-BCG-infected cells, *: p<0.05; **: p<0.01; compared to non-control lentivirus-infected cells, Δ: p<0.05; ΔΔ: p<0.01.

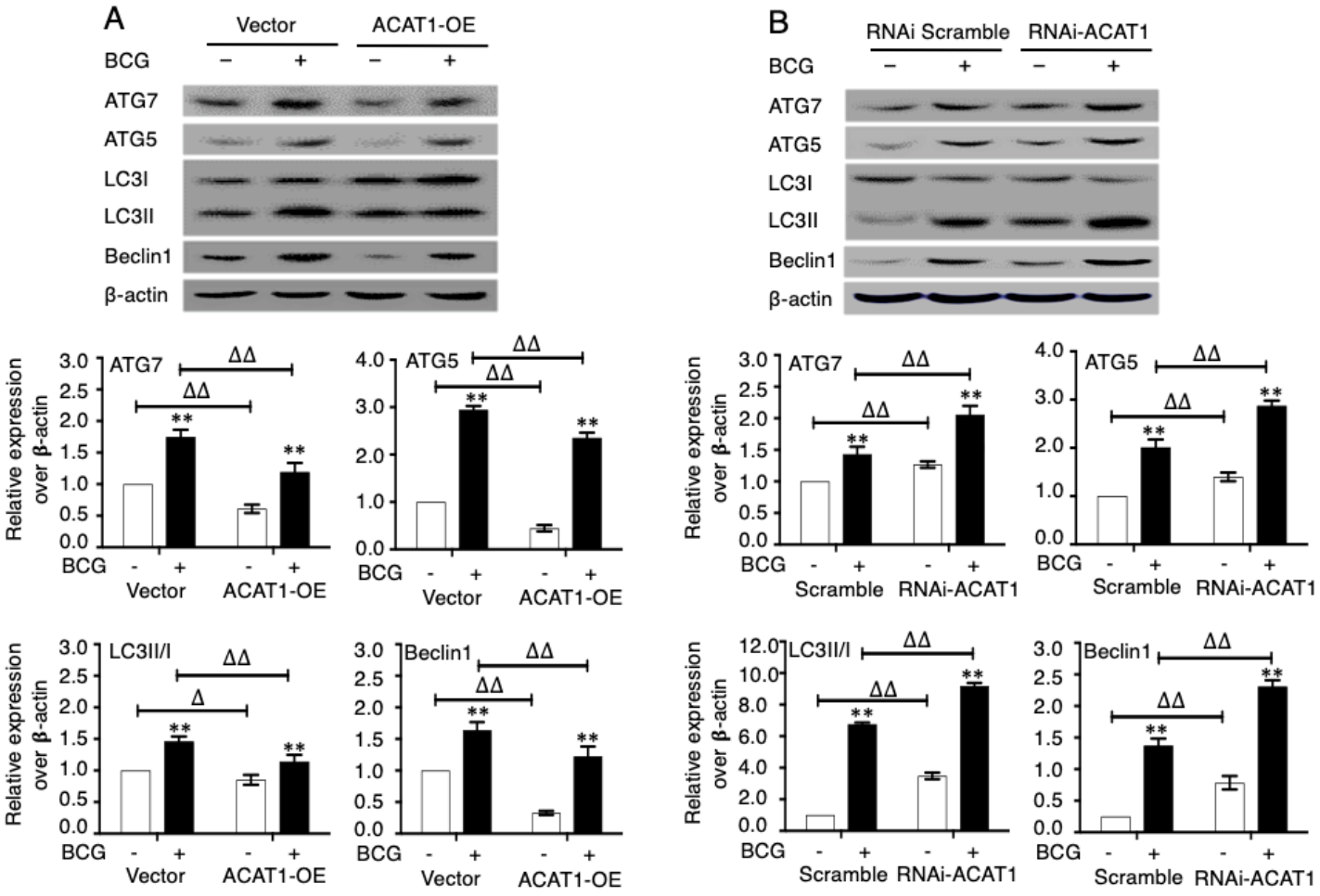

Figure 5

The impact of ACAT1 on the BCG-induced autophagy in RAW264.7 cells. The ACAT1-overexpressed and silenced RAW264.7 cells were infected with BCG at a dose of 10 for 12h before the pathogen-induced autophagy was determined by accessing autophagy-related proteins. (A) Representative blots showed an inhibition of BCG-induced autophagy-related proteins ATG7, ATG5, LC3II/I and Beclin1 in RAW264.7 cells overexpressing ACAT1 (top panel), and the relative expression semi-quantified by a densitometry analysis (bottom panel) demonstrated an significant reduction of autophagy-related proteins in comparison with control cells. (B) Representative blots displayed an increase of BCG-induced autophagy-related proteins ATG7, ATG5, LC3II/I and Beclin1 in RAW264.7 cells with a silence of ACAT1 (top panel), and the relative expression semi-quantified by a densitometry analysis (bottom panel) demonstrated an significant increase of autophagy-related proteins in comparison with uninfected control cells. Data were expressed as mean ± SEM from three independent experiments. Compared to non-BCG-infected cells, *: $p<0.05$; **: $p<0.01$; compared to non-control lentivirus-infected cells, Δ: $p<0.05$; ΔΔ: $p<0.01$.

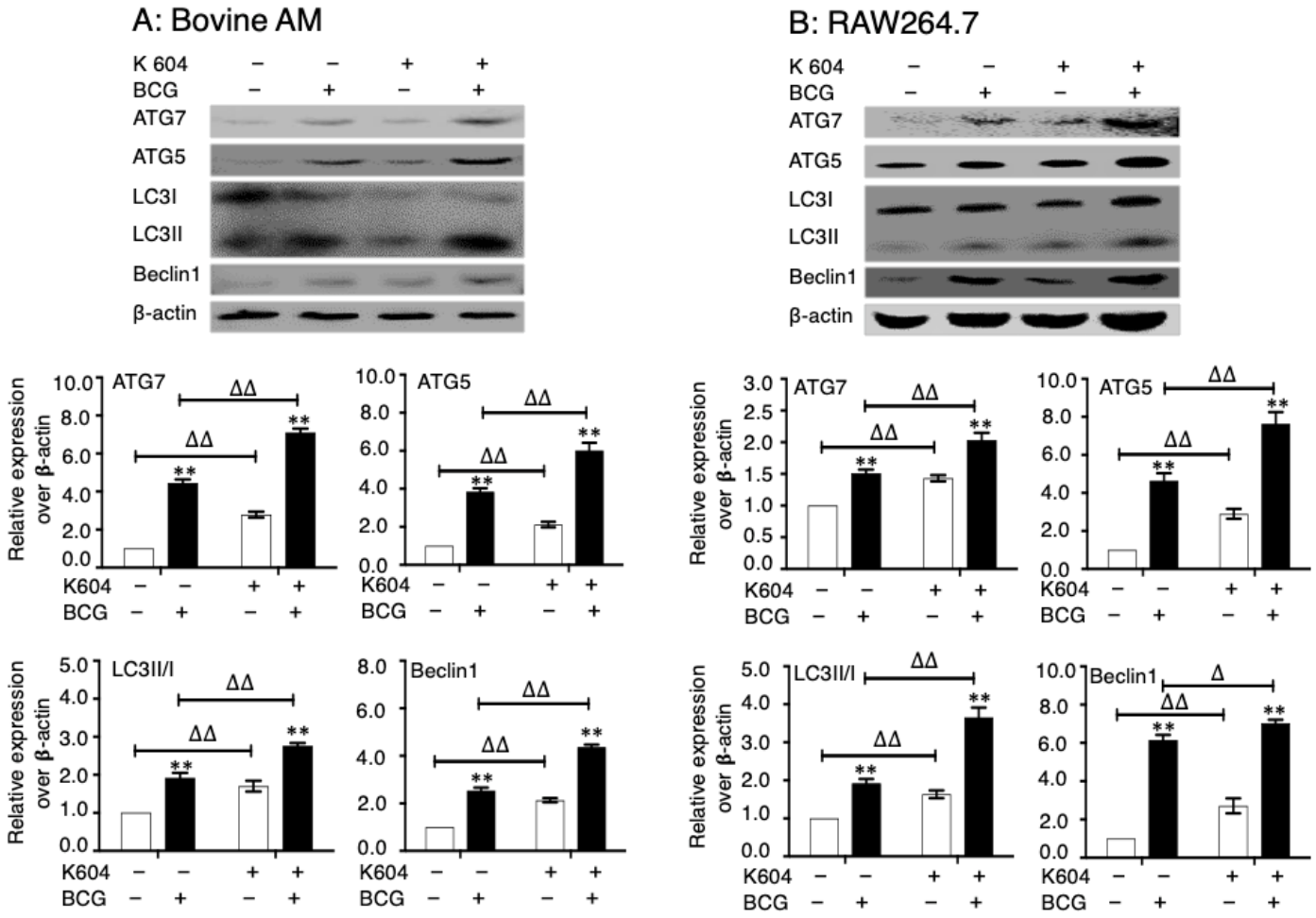

Figure 6

The impact of ACAT1 signaling on the BCG-induced autophagy in RAW264.7 cells. The primary bovine AMs and RAW264.7 cells were infected with BCG at a dose of 10 in the presence of ACAT1 signaling inhibitor K604 for 12h before the autophagy was determined by accessing autophagy-related proteins. (A) Representative blots demonstrated an increased expression of BCG-induced autophagy-related proteins ATG7, ATG5, LC3 and Beclin1 in primary bovine AMs in the presence of K604 (top panel), and the relative expression semi-quantified by a densitometry analysis (bottom panel) demonstrated an significant increase of autophagy-related proteins in comparison with cells without K604. (B) Representative blots demonstrated an increased expression of BCG-induced autophagy-related proteins ATG7, ATG5 LC3II/I and Beclin1 in RAW264.7 cells in the presence of K604 (top panel), and the relative expression semi-quantified by a densitometry analysis (bottom panel) demonstrated an significant increase of autophagy-related proteins in comparison with cells without K604. Data were expressed as mean ± SEM from three independent experiments. Compared to non-BCG-infected cells, *: $p<0.05$; **: $p<0.01$; compared to cells in the absence of K604, Δ: $p<0.05$; ΔΔ: $p<0.01$.

Supplementary Files

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- supplement1.pdf