

The *Mycobacterium tuberculosis* glycoprotein Rv1016c protein inhibits dendritic cell maturation, and impairs Th1 /Th17 responses during mycobacteria infection

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ABSTRACT

The mycobacterial factors and the associated mechanism by which *Mycobacterium tuberculosis* (Mtb) evades the host immune surveillance system remain widely unexplored. Here, we found that overexpressing Rv1016c, a mannosylated protein of *M. tuberculosis* in BCG (rBCG-Rv1016c) led to increased virulence of the recombinant BCG in the severe-combined immunodeficient (SCID) mice model and to a loss of protective efficacy in a zebrafish-*M. marinum* model, compared to wild type BCG. Further investigations on the effects of rBCG-Rv1016c on the host innate immunity revealed that rBCG-Rv1016c decreased the production of cytokines IL-2, IL-12p70, TGF- β , IL-6 as well as of the co-stimulatory molecules CD80, CD86, MHC-I and MHC-II by the infected DCs. These effects were mimicked by rBCG-Rv1016cHis, which carried an extra 6-His tag at the C-terminus of Rv1016c. Relatively to BCG infected DCs, the rBCG-Rv1016c-infected DCs failed to polarize naïve T cells to Th1- and Th17-type cells to secrete IFN- γ and IL-17. Additionally, T lymphocytes from BCG- infected mice showed significantly less proliferation and production of IFN- γ and IL-17. Similarly, rBCG-Rv1016c mice released a higher level of IL-10 in response to rBCG-Rv1016c stimulation than wild type BCG infected mice. Furthermore, DCs from TLR-2 knockout mice showed no reduction in IL-6, IL-12 p70 and TGF- β secretion in response to rBCG-Rv1016c infection, compared to DCs infected with BCG. We propose that Rv1016c interferes in differentiation of the DCs by targeting suppressor of cytokine signaling (SOCS) 1 and SOCS3 expression, which subsequently leads to the reduction in STAT-1 and STAT-6 phosphorylation. These findings open new perspectives regarding the immunosuppressive strategies adopted by *Mtb* to survive in the host.

1. Introduction

Mycobacterium tuberculosis (Mtb) is considered a main cause of mortality worldwide, particularly due to the emergence of multiple-drug resistant strains and to the HIV- Mtb co-infection (Lawn and Zumla, 2011). Under natural conditions or following BCG vaccination, the host protection against Mtb infection depends on both the innate and the adaptive immune response, which trigger a robust Th1- or Th17- response towards the secretion of IFN- γ and IL-17 through antigen-specific CD4⁺ /CD8⁺ T cells (Kaufmann et al., 2014; Lewinsohn et al., 2017). *Mycobacterium tuberculosis* is capable of surviving for prolonged periods in the host by evading both the innate and the adaptive immunity systems. The mechanisms exploited by Mtb to trick the host immune response are: the inhibition of phagolysosome fusion (Mariotti et al., 2002), function impairment of antigen presenting cells by the obstruction of the expression of co-stimulatory molecules and

secretion of cytokines (Hickman et al., 2002), the hampering of antigen production and presentation to T cells (Noss et al., 2000), as well as the induction of T-cells exhaustion or apoptosis (Khan et al., 2016). However, only a few of Mtb proteins capable of subverting host immunity have been characterised. One of those proteins, the Acr1(Rv2031c) factor, a latency-associated protein, impairs the dendritic cells (DCs) functionality and the differentiation of naïve CD4⁺ T cells toward Th1- and Th17- type cells (Amir et al., 2017a). Thus, understanding the immunological mechanisms modulating DC activation and Th1/Th17 responses during Mtb infection (Khader et al., 2007) became an important research goal.

The early interactions of mycobacteria and mycobacteria-derived molecules with the host cells are thought to greatly affect the subsequent adaptive immune responses. DCs, being the most potent antigen presenting cells, play a central role in triggering the adaptive immune responses against Mtb by polarizing naïve T cells to effector/

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memory T cells (Cooper, 2009). Therefore, the cross-talk between Mtb and the DCs is considered to have profound consequences for the development of the immune response. Several Mtb proteins, e.g. ESAT-6 (Wang et al., 2012) and RpfE (Choi et al., 2015), have been found to induce DC maturation and subsequently promote the differentiation of naïve T cells, mostly in a TLR2 or TLR4-dependent manner. Additionally, PstS1, a 38 kDa-lipoprotein of *Mtb*, interacts with DCs and induces the Th1/Th17-associated secretion of IFN- γ and IL-22 as well as the IL-17 production by CD4⁺ T cell (Palma et al., 2013). Conversely, a small group of Mtb proteins attenuated DCs function by inducing anti-inflammatory cytokines or inhibiting the presentation of Mtb antigens to CD4⁺ T cell. The DC inhibition effect of this small group of Mtb proteins provides a mechanism for increased mycobacterial survival (Ishikawa et al., 2017) in the host. For example, MTSa, a 10 kDa secretory antigen, impaired DCs differentiation providing a niche for the intracellular survival of Mtb (Sinha et al., 2006). Therefore, identification of Mtb antigens that target DC cells would enlarge the current understanding of the mechanism through which mycobacteria evade the immune surveillance during infection.

Complex lipids, carbohydrate moieties and glycosylated proteins located on the cell surface or membrane of pathogenic microbes (e.g. *Pseudomonas aeruginosa*, *Streptococcus parasanguis*, *Treponema pallidum*) play a crucial role in virulence, antigenic variation or immune regulation (Szymanski and Wren, 2005). Mannosyl-capped LAM (ManLAM), and its related cell wall-associated types of glycolipids/lipoglycans, namely phosphatidylinositol mannosides (PIMs) and lipomannan (LM), exhibit important and distinct immunomodulatory properties due to their structure, internal heterogeneity and abundance (Ishikawa et al., 2017). Recently, emerging interest has arisen regarding the glycoproteins capable of interfering with the host immunity. *Mycobacterium tuberculosis* genome encodes for at least 40 putative glycoproteins as revealed by mass spectrometric identification (Gonzalez-Zamorano et al., 2009). However, only a few glycoproteins have been well identified including Rv1860 (Dobos et al., 1996) and MPB83 (Michell et al., 2003), all of which containing 1–3 mannose residues linked to threonine residues. The characterization of mycobacterial glycosylated factors and the underlying molecular mechanisms exploited by Mtb to affect the innate and adaptive immune responses is poorly understood.

The Rv1016c protein from *Mycobacterium tuberculosis* is a component of the mycobacterial cell wall and is required for the prolonged survival of mycobacteria within the macrophage (Xiong et al., 2005; Gonzalez-Zamorano et al., 2009). The disruption of genes encoding Rv1016c reduced the *in vivo* growth of Mtb in C57BL/6 mouse (Sassetti and Rubin, 2003). Previously, we identified Rv1016c as a virulence factor which enhanced the survival of bacilli (Su et al., 2016). These findings indicated that Rv1016c may be a negative immunomodulatory factor and prompted us to further investigate the function of Rv1016c as a regulator of the innate immune cell response and its role in the differentiation of naïve T cells. Here, we found that the Rv1016c protein inhibited dendritic cell functionality through its interaction with the TLR2/STAT/SOC3 pathway, which subsequently impaired the differentiation of naïve T lymphocytes to Th1 and Th17 cells.

2. Materials and methods

2.1. Animals and ethics statement

C57BL/6, BALB/c and SCID mice (female, 6–8 weeks) were purchased from the Laboratory Animal Science of Guangdong (Guangdong, China). TLR2^{-/-} mice (female, 6–8 weeks old) were purchased from Sebia Biotechnology Co. Ltd. (Guangzhou, China). All the mice were kept under specific pathogen-free conditions in the Center of laboratory animal science of Guangdong (Guangzhou, China). All animal experiments and procedures on mice were performed in accordance with the Animal Ethics Committee-approved guidelines of Guangzhou Medical

University.

2.2. Antibody and reagent

A T lymphocytes cell enrichment kit was purchased from Miltenyi Biotec (Auburn, CA, USA). The recombinant proteins GM-CSF and IL-4 as well as the ELISA kits for TNF- α , IFN- γ , IL-6, IL-12p70, IL-17 were purchased from either BD Biosciences (San Diego, CA, USA) or from R&D Systems. The Rv1016c and Rv2145c antiserum were obtained from C57BL/6 mice or rabbits immunized with each respective protein. Antibodies for His, STAT-1, pSTAT-1, STAT-3, pSTAT-3, STAT-6, pSTAT-6 were obtained from Santa Cruz (CA, USA) while for those for SOS1, SOS2, SOS6 and HRP-conjugated secondary Abs were obtained from Sigma (St. Louis, MO, USA). All these antibodies were used to detect protein signaling during Rv1016c-modulated DC function. An endotoxin removal kit (Thermo Scientific, Rockford, IL, USA) was used to eliminate endotoxin contamination.

2.3. Flow cytometry analysis

To determine the expression of surface makers on BMDCs, the following antibodies were used: FITC anti-CD11c, PE anti-CD8 and PE anti-CD4, biotin CD80, CD86, MHC class-I, MHC class-II (BD Pharmingen or eBiosciences). Allophycocyanin-conjugated anti-CD3 and allophycocyanin-eFluor780 anti-CD8 were purchased from eBioscience. Biotinylated antibody (Ab) was analyzed with PerCP streptavidin (BD Pharmingen). Data were acquired and analyzed on a FACS system (BD Biosciences).

2.4. Dendritic cell culture

Dendritic cells were collected and cultured as previously described (Palma et al., 2013). Briefly, bone marrow cells were obtained from femurs and tibiae of 8–10 week old mice. BMCs were then cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM β -mercapto-ethanol, penicillin (100 U/ml) and streptomycin (100 mg/ml) and maintained at 37 °C in a humidified incubator (5% CO₂). GM-CSF (4 ng/ml) and IL-4 (6 ng/ml) were also added to the culture to induce immature DCs. After 7 days, the non-adherent cells were collected and examined by flow cytometry with cell surface markers (CD80, CD 86, MHCI, and MHCII). We found that over 90% of those cells were classical immature DCs. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA)

2.5. Cytokines analysis by ELISA

The culture supernatants were collected and the cytokine production was assessed. The levels of TNF- α , IL-4, IL-2, IL-12 p70, IL-10 using ELISA kits obtained from eBioscience and the level of IFN- γ was assessed using an Elisa Kit obtained from R&D Systems, according to manufacturer's instructions.

2.6. Antiserum generation and construction of recombinant mycobacterial strains

To obtain antiserum specific to Rv1016c, the expression of the recombinant Mtb Rv1016c protein was performed as previously described (Su et al., 2016). A total of 20 μ g of Rv1016c protein suspended in PBS combined with incomplete Freund's adjuvant was intraperitoneally injected into mice three times at 3-weeks intervals. Similarly, a total of 300 μ g Rv1016c suspended in PBS combined with incomplete Freund's adjuvant was injected into rabbits three times at 1-week intervals.

Wildtype BCG was grown in Middlebrook 7H9 liquid medium (Difco, Detroit, USA) supplemented with 10% albumin/dextrose/catalase (OADC) (Difco), 0.2% glycerol, and 0.05% Tween 80. Aliquots of log phase cultures were frozen at -80 °C and bacteria were enumerated

by plating serial dilutions on 7H10 agar plates supplemented with 10% OADC and 0.2% glycerol (1 A₆₀₀ nm = 10 (Khader et al., 2007) colony forming units [cfu]/ml). The recombinant BCG strains expressing *Mtb* Rv1016c, BCG-Rv1016c and BCG-Rv1016c His, were transformed with the plasmids pMV261-Rv1016c and pMV261-Rv1016c-6His, respectively as previously described (Su et al., 2016). After electroporation of wildtype BCG with the recombinant plasmids, using a BIORAD Gene electroporator (2.5 kV, 200 μF and 1000 Ohms), the recombinant BCG strains were selected with 100 μg/ml hygromycin on 7H10 agar plates. Western blotting was used to confirm the expression of Rv1016c or Rv1016c-6xHis with anti-Rv1016c or anti-His, respectively. Wildtype BCG was also transformed with an empty plasmid pMV261 (BCG-pMV261) used here as a control.

2.7. Immunoprecipitation and Western blotting

Bacterial lysates, dissolved in RIPA buffer containing a protease and phosphatase inhibitor cocktail, were used to perform Western blotting analysis. The total cell lysate and cell wall-associated fractions were incubated with protein A-Sepharose beads coated with rabbit anti-Rv1016c (25 μl) or anti-6xHis (5 μl) at 4 °C for 16 h. After three washes, beads were eluted with Laemmli buffer. The binding of the total cell-lysate or cell-wall fractions to Con-A beads was promoted in a buffer containing 20 mM Tris–HCl pH 7.4, 0.5 M NaCl, 1 mM MnCl₂, and 1 mM CaCl₂. After washed, samples were analyzed by SDS-10% PAGE and then transferred to a PVDF membrane. Anti-Rv1860, anti-6xHis followed by Con-A-HRP was used to detect the target protein binding, respectively. The Pierce ECL Western Blotting substrate was used to visualize target bands (Thermo Fisher Scientific, Waltham, MA).

2.8. Recombinant BCG infection experiments in BMDCs

Before BMDCs inoculation, BCG strains were sonicated to disperse clumps in an ultrasonic water bath (Grant) for 10 min (10 (Wang et al., 2012) cfu/ml, 40 s pulse). BMDCs were infected with recombinant BCG bacilli at a MOI of 10. Infection was confirmed by cell lysis in TritonX-100/PBS buffer and lysate transfer to 7H10 agar medium plates.

2.9. Recombinant BCG infection experiments in mice

To assess virulence of the rBCG strains in SCID mice, 6 weeks female SCID mice were intravenously infected via the tail vein with recombinant BCG strains (1.3–1.5 × 10 (Khan et al., 2016) CFU in PBS). At 24 h post infection, 3 mice were sacrificed to determine the optimum infection BCG dosage. Briefly, the lungs and spleens were collected, homogenized in PBS, and then plated on 7H10 agar medium. After 30 days under cell culture, the number of rBCG bacilli was counted. During the infection, the mice death rate was monitored, and Log-rank analysis was used to compare survival between experimental groups.

To examine the effect of recombinant BCG expressing Rv1016c on DC, 10 (Khan et al., 2016) CFU of rBCG strains in PBS were used to infect mice and the cytokine production and cell surface molecules were measured 24 h later by flow cytometry.

2.10. Protective efficacy of recombinant BCG in zebrafish- *M. marinum* infection model

A zebrafish *-M. marinum* infection model was established as previously described (Oksanen et al., 2013). Briefly, adult zebrafish were intraperitoneally immunized with 2.3 × 10 (Mariotti et al., 2002) CFU of recombinant BCG strains (PBS as a control). At 4 weeks after immunization, five zebrafish from each group were killed to determine the actual Colony-Forming Units (CFU) on Middlebrook 7H10 medium. The remaining zebrafish were intraperitoneally infected with 10–25 CFU of *M. marinum* bacilli for survival analysis.

2.11. T lymphocytes polarization analysis

In mixed lymphocytes reaction assays, naïve T cells from female C57/BL6 mice were purified by MACS, as per manufacturer's instructions (BD Biosciences, San Diego, CA, USA). BMDCs from BALB/c mice were infected with rBCG strains at an MOI of 10 for 24 h. and subsequently, gamma irradiated (15 Gy). BMDCs were then co-cultured with naïve T cells at the ratios of 1:10 and 1:50 in a 24 well plate. In syngeneic assays, C57/BL6 mice were infected with 10⁶ CFU of BCG-pMV261 or BCG-Rv1016c. At 3 weeks post-infection, BMDCs from uninfected C57/BL6 mice were co-cultured with naïve T cells from infected C57/BL6 mice at the ratios of 1:10 and 1:50 in a 24 well plate for 3 days. Supernatants were used for cytokine analysis. After pulsed with (Lewinsohn et al., 2017) H-thymidine (0.5 μCi/well) for 16 h, cells were counted in a liquid scintillation counter (Model 1209, LKB-Wallac, Turku, Finland). 10 μg/ml of Con-A was used as a control.

2.12. Statistical analysis

Results are presented as the means ± standard deviations (SD) of the results obtained from two or three replicate experiments. Results were analyzed by Student's test or by one Way analysis of variance (ANOVA) test with Tukey's correction for multiple comparisons using Origin 8.0 software (Origin Lab, Northampton, MA, USA). For all tests, P ≤ 0.05 was considered statistically significant.

3. Results

3.1. Construction of recombinant BCG strains and characterization of the mannosylated Rv1016c

Previously, the Rv1016c protein was identified by mass spectrometry as a putative glycoprotein probably mannosylated (Gonzalez-Zamorano et al., 2009). To examine whether the recombinant Rv1016c protein expressed by BCG-Rv1016c was mannosylated, plasmids pMV-Rv1016c, pMV-Rv1016c-6His, and pMV261 were initially electroporated into BCG to obtain recombinant rBCG-Rv1016c, rBCG-Rv1016cHis and BCG-pMV261 strains, respectively. Western blotting was used to detect the expression of Rv1016c anti-Rv1016c mouse serum. As shown in Fig. 1A and B, the expression of Rv1016c was up-regulated 3.14 fold and 3.57 fold in the rBCG-Rv1016c and rBCG-Rv1016c-6His strains, respectively compared to the BCG strain. Immunoblotting also detected the 6His-tagged protein band in the culture filtrates from rBCG-Rv1016c-6His with a rabbit anti-6His antibody, but not in the lysates from BCG-pMV261 (Fig. 1A). Next, immunoprecipitation was performed with the total lysates and culture filtrates from the BCG-pMV261 and rBCG-Rv1016c-6His strains with a mouse serum anti-Rv1016c or with Con-A sepharose beads. Subsequently, Western blotting was used to examine the target protein bands. As shown in Fig. 1C and D, the Rv1016c protein was only found in rBCG-Rv1016c-6His strain detected by an anti-6His antibody. Additionally, both the anti-Rv1016c immunoprecipitates, obtained with Con-A-HRP, and the Rv1016c precipitation from the total cell lysate and culture filtrate fractions with Con-A beads were analyzed by immunoblotting, both confirmed the paucity of mannosylation on Rv1016c (Fig. 1C and D). Furthermore, immunoprecipitation with mannosylated form of Rv1016c protein from the total lysates and culture filtrates fraction of rBCG-Rv1016c-6His by anti-6His mouse serum revealed that its glycosylation was based on Con-A-HRP reactivity (Fig. 1E). Together, these results strongly suggested that the recombinant Rv1016c-6His expressed on wild type BCG was mannosylated.

3.2. Rv1016c attenuated the protective efficiency

As previously reported (Su et al., 2016), we also observed that

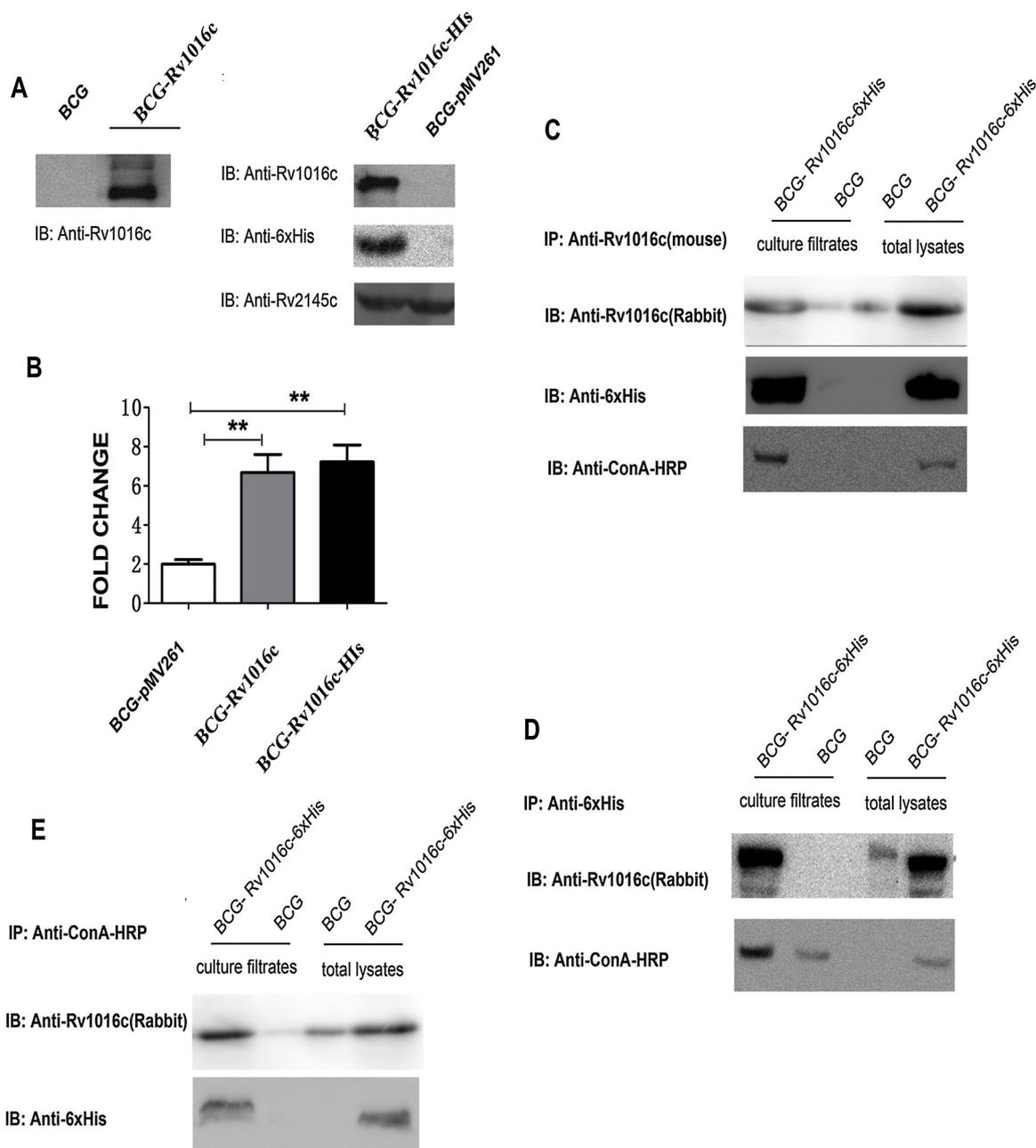


Fig. 1. Characterization of recombinant BCG strains expressing MTB Rv1016c. (A) Quantification of Rv1016c expression. 10 µg protein from each of the strains BCG, BCG-pMV261, BCG-Rv1016c and BCG-Rv1016c-6xHis was electrophoresed in a SDS-10% PAGE, transferred to PVDF membrane and probed using a mouse anti-serum specific to Rv1016c (top panels), or rabbit antiserum specific to MTB Rv2145c (bottom panels); 80 µg lysates were probed with mouse anti-6 × Histidine antibody (middle panel on the right). Bands were detected by chemiluminescence. (B) Quantification of expression of Rv1016c in the 3 strains shown in (A). Mean of two or three replicate experiments was used for quantitation of pixels obtained from chemiluminescence detection analysis. (C, D and E) Immunoprecipitation of 20 ml culture filtrates and 500 µg lysates of BCG-Rv1016c-6xHis and BCG using rabbit anti-Rv1016c serum (C), and mouse anti-6 × Histidine (D), Con-A-sepharose beads (E). Samples electrophoresed on a SDS-10% PAGE were transferred to PVDF membrane and western blotted with mouse anti-Rv1016c (top panels, 10% in C and D and 50% in E), mouse anti-6 × His antibody (45%, middle panel in C and lower panel in D) or Con-A-HRP (lower panel, 45% in C and 50% in E). The lysate samples in E required much longer exposure time than the CF and were hence developed separately. **, p < 0.01; ***, p < 0.0001.

Rv1016c promoted the growth of the recombinant BCG strains in 7H9 broth and its survival in the BMDCs from C57BL mice (Fig. 2A and B), indicating that Rv1016c may be a virulence factor, consistent with previously published data on the growth of *Mycobacterium smegmatis* strain overexpressing Rv1016c. As mentioned above, the Rv1016c protein in mycobacterial was probably mannosylated. Therefore, to further determine the virulence of the glycosylated Rv1016c, PBS, BCG or rBCG-Rv1016c were intravenously injected into SCID mice lacking T- and B- lymphocytes, and their survivals were assessed. As shown in Fig. 2C, the survival time of rBCG-Rv1016c was significantly shorter

than that of the parental BCG (p < 0.01). These results suggest that the overexpression of Rv1016c in rBCG enhanced the virulence and promoted its growth.

We further determined whether the Rv1016c knock-in strains of BCG reduced the protective efficacy against mycobacterial challenge using a zebrafish-*M. marinum* infection model. Adult zebrafish (30/group) were intraperitoneally vaccinated with 3.0 × 10⁴ CFU of the parental BCG, rBCG-Rv1016c or PBS (as a negative control). Four weeks after vaccination, these fish were infected with 3.0 × 10⁴ CFU of *M. marinum* and all groups were then followed for 4 weeks for survival

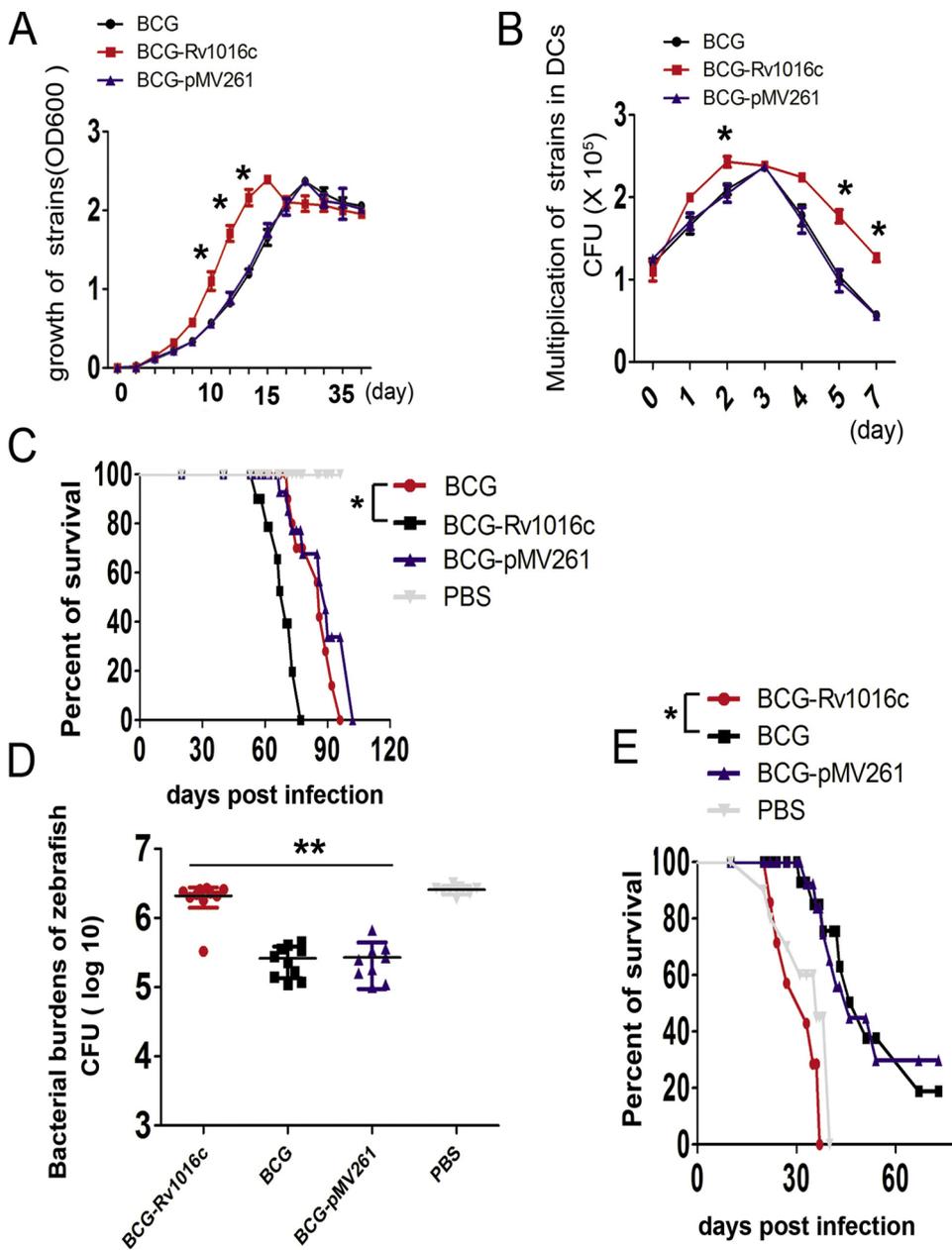


Fig. 2. Virulence of recombinant BCG expressing Rv1016c (A) In vitro growth of Rv1016c knock-in strains of *M. bovis* BCG. (B) Multiplication of RD4 knock-in strains of *M. bovis* BCG in DCs. DCs were infected with recombinant strains of BCG (MOI = 10). At various time points, the intracellular bacterial number was determined and plotted. Data are from triplicate samples of each strain (mean \pm S.D.). (C) Virulence of BCG-Rv1016c. Survival curves of SCID mice infected with recombinant BCG. Groups of SCID mice (n = 20) were intravenously infected with 10 (Khan et al., 2016) CFU of BCG strains and their survivals were monitored. (D) Survival curves of zebrafish vaccinated with recombinant BCG or PBS and challenged with *M. marinum*. Groups of adult zebrafish (n = 30) were vaccinated with 10 (Mariotti et al., 2002) CFU of BCG-Rv1016c, BCG, BCG-pMV261 or PBS. 4 weeks after vaccination, zebrafish were infected with 10 (Mariotti et al., 2002) CFU of *M. marinum* and were monitored for survival. Log-rank analysis was performed for statistical significance. (E) Bacterial burdens of zebrafish vaccinated with recombinant BCG-Rv1016c with *M. marinum*. Groups of zebrafish (n = 15) were vaccinated with 10 (Mariotti et al., 2002) CFU of BCG-Rv1016c, BCG, BCG-pMV261 or PBS. 4 weeks after BCG vaccination, zebrafish were infected with 10 (Mariotti et al., 2002) CFU of *M. marinum*. After 4 weeks of *M. marinum* infection, 10 live fish from each group were sacrificed and the number of *M. marinum* in each fish was determined. Kruskal-Wallis test followed by Dunn's Multiple Comparison test was performed. *, p < 0.05; **, p < 0.01.

analysis. The mortalities of the rBCG-Rv1016c vaccination group was 73.2%, whereas, in the BCG vaccination and in the PBS group mortalities were 25.7% and 91.4%, respectively (Fig. 2D). This result suggests that zebrafish vaccinated with rBCG-Rv1016c survived significantly less than those vaccinated with the parental BCG. Moreover, the bacterial infection caused-burdens were significantly higher in rBCG-Rv1016c-vaccinated group than in the wild type BCG-vaccinated group at 4 weeks after infection of the zebrafish (Fig. 2E). These results indicate that expression of *Mtb* Rv1016c in BCG diminishes the protective efficacy of the host immunity and promotes its virulence.

3.3. Rv1016c protein impedes cytokine secretion in BCG-infected dendritic cells

To further determine the molecular basis by which Rv1016c enhanced the virulence and reduced the protective immune response induced by BCG, we hypothesized that the glycosylated Rv1016c interfered with the host innate immunity during mycobacteria infection. BMDCs from C57BL mice were infected with BCG-pMV261, rBCG-

Rv1016c, or rBCG-Rv1016c-6His at MIO of 10 for 4 h, respectively. A group of BMDCs were kept in PBS, uninfected, as a control group. The culture supernatants were harvested 24 h later and used for cytokine detection using an ELISA assay. As shown in Fig. 3A, compared to BCG-pMV261 infected BMDC, the production of cytokines including IL-2, IL-6, IL-12 p70, TNF- α and TGF- β from BMDC infected with rBCG-Rv1016c was reduced by up to 70%. Similarly, rBCG-Rv1016c-6His infection led to the reduction of IL-2, IL-6, IL-12 p70, TNF- α and TGF- β secretion. In contrast, the level of IL-10 secreted by rBCG-Rv1016c infected BMDCs was up-regulated, compared to that secreted by BCG-pMV261-infected cells (Fig. 3A). After added to BMDCs, sonicated rBCG-Rv1016c preparations also reduced the levels of pro-inflammatory and regulatory cytokines compared to the sonicated BCG-pMV261 (Fig. 3B). Moreover, adding purified Rv1016c-6His to BMDC treated by BCG-pMV261 or to the sonicated BCG-pMV261 preparations also resulted in the decrease of cytokine production (Fig. 3A and B).

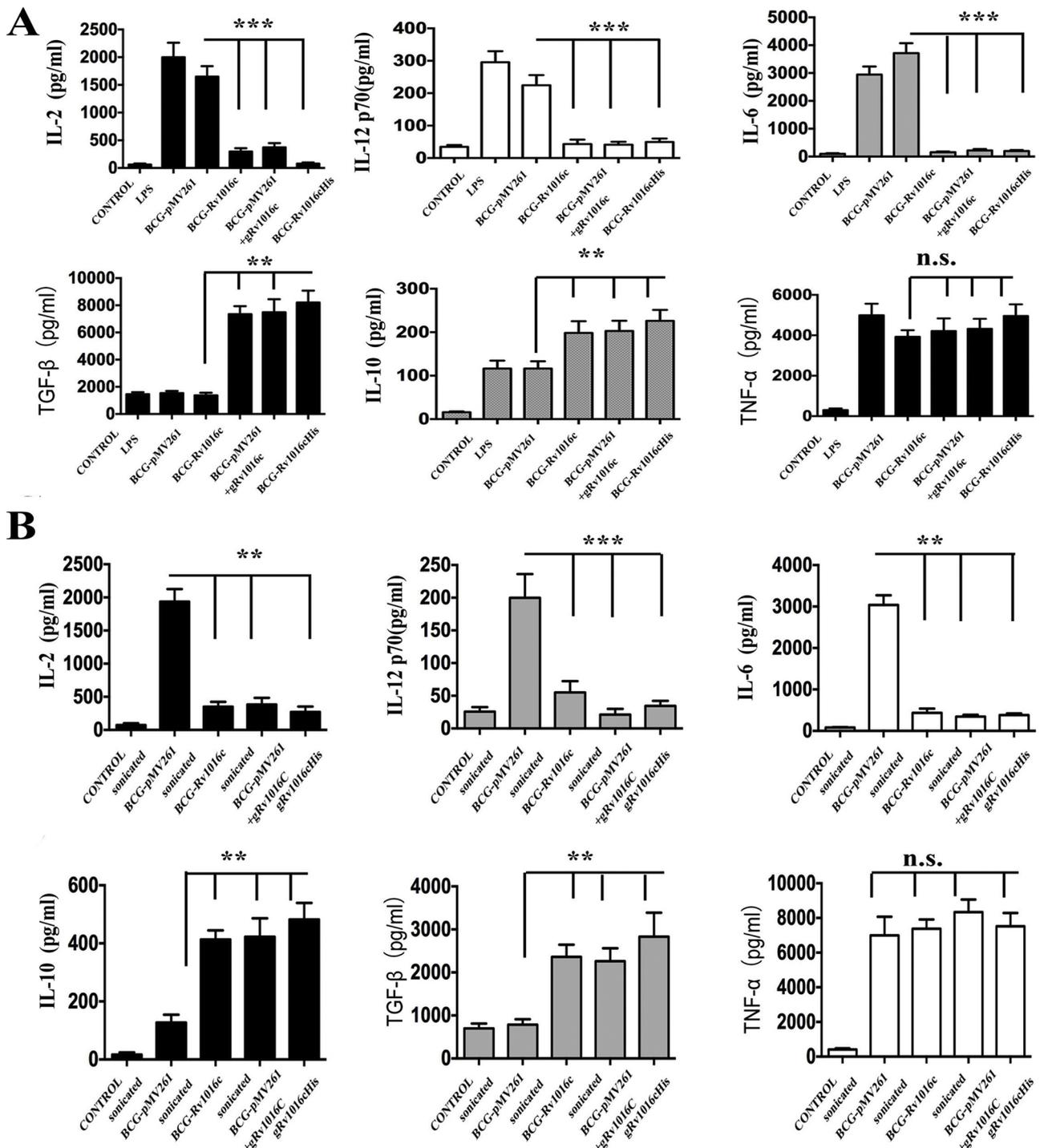


Fig. 3. Expression of MTB Rv1016c in BCG suppressed cytokine secretion from mouse BMDC. (A) C57BL/6 bone marrow differentiated for 7 days with GM-CSF into dendritic cells were infected of the indicated BCG strains at 10 MOI. Culture supernatants were harvested 8 h later and used for cytokine measurements. Values are mean \pm SD obtained from five mice. (B) C57BL/6 BMDCs were treated with sonicated preparations of the BCG strains indicated, comparable to infection at 10 MOI. Glycosylated native Rv1016c (g Rv1016c) was added at 10 ng/well, estimated to be consistent with to that coming from 10 MOI infection as described in Methods. Cytokines were measured after 8 h of incubation. Values are mean \pm S.D. from minimum of 3 mice. ** $p < 0.01$; *** $p < 0.001$.

3.4. Rv1016c reduces the up-regulation of co-stimulatory molecules by BCG-infected DCs

Co-stimulatory molecules on the surface of DCs play a crucial role in triggering an adaptive T cell immunity (Sia et al., 2015). We further examined whether Rv1016c adversely affected the up-regulated expression of co-stimulatory molecules on DC induced by BCG. LPS was used as a control. As expected, BCG-pMV261 infection dramatically

increased the expression of CD 80, CD86, MHC I and MHC II (Fig. 4A and B), whereas the rBCG-Rv1016c infection resulted in the significant reduction of those co-stimulatory molecules on DCs, down to levels comparable to those found on uninfected DCs (Fig. 4A and B). The reduction was significant for all the markers analyzed. Together, these findings suggest that Mtb Rv1016c protein interferes with DC maturation normally induced by BCG infection.

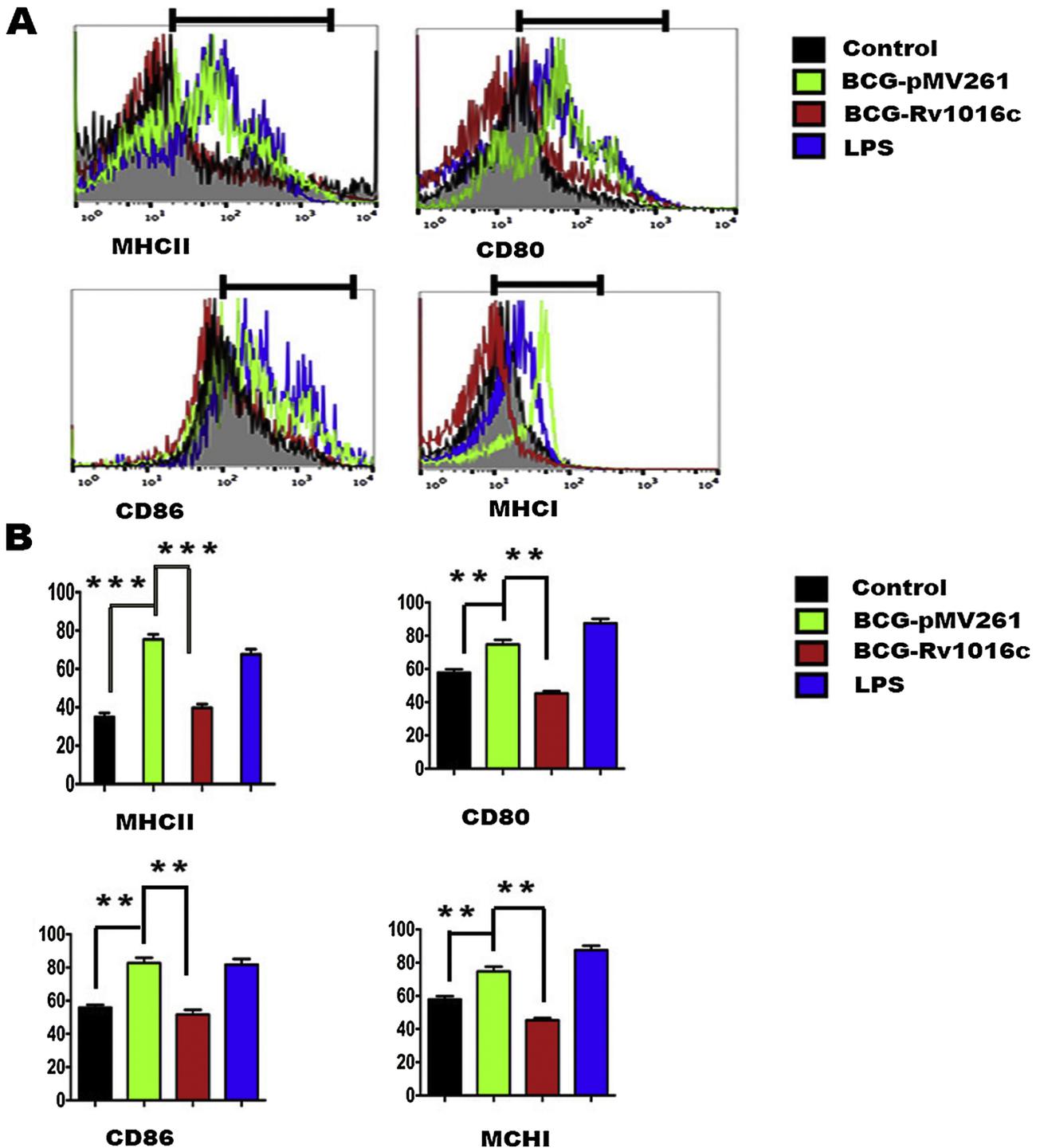


Fig. 4. Overexpressing MTB Rv1016c in BCG suppressed the expression of surface co-stimulatory molecules on infected BMDC. (A) C57BL/6 bone marrow differentiated for 7 days with GM-CSF into dendritic cells were infected at of the indicated BCG strains at 10 MOI. FITC and PE- conjugated antibodies specific to the indicated surface co-stimulatory molecules monitored by flow cytometry. Plots show the histogram for each marker on BMDC. Key describes the identity of each line. Values are mean \pm S.D. from 3 mice. (B) Data obtained from (A) for percentage of cells positive for each co-stimulatory marker mentioned below were analyzed using Graph Pad Prism software. P values for significant differences between the two strains of bacteria are indicated: **, $p < 0.01$; ***, $p < 0.001$.

3.5. Rv1016c inhibited DCs functionality by targeting TLR2/STAT/SOC3 pathway

We previously found that Rv1016c direct binds to TLR2 (Su et al., 2016). Consequently, we asked if the function of TLR2 involved the rBCG-Rv1016c- mediated inhibition of the DCs function. BMDCs from TLR2^{-/-} or wild type mice were infected with BCG-pMV261 or rBCG-Rv1016c and cytokine production was measured using ELISA. We found

that TLR2 deficiency stopped the reduction in the secretion of IL-6, IL-12 p70 as well as TGF- β in rBCG-Rv1016c-infected DCs, whereas BCG-pMV261 infection and LPS treatment promoted these cytokines production (Fig. 5A).

JNK/ STAT signaling regulate cytokines production and DC maturation (Amir et al., 2017b). We then examined the phosphorylation status of STAT1, STAT3 and STAT6 after rBCG-Rv1016c stimulation. As shown in Fig. 5B, the phosphorylation of STAT1 and STAT6

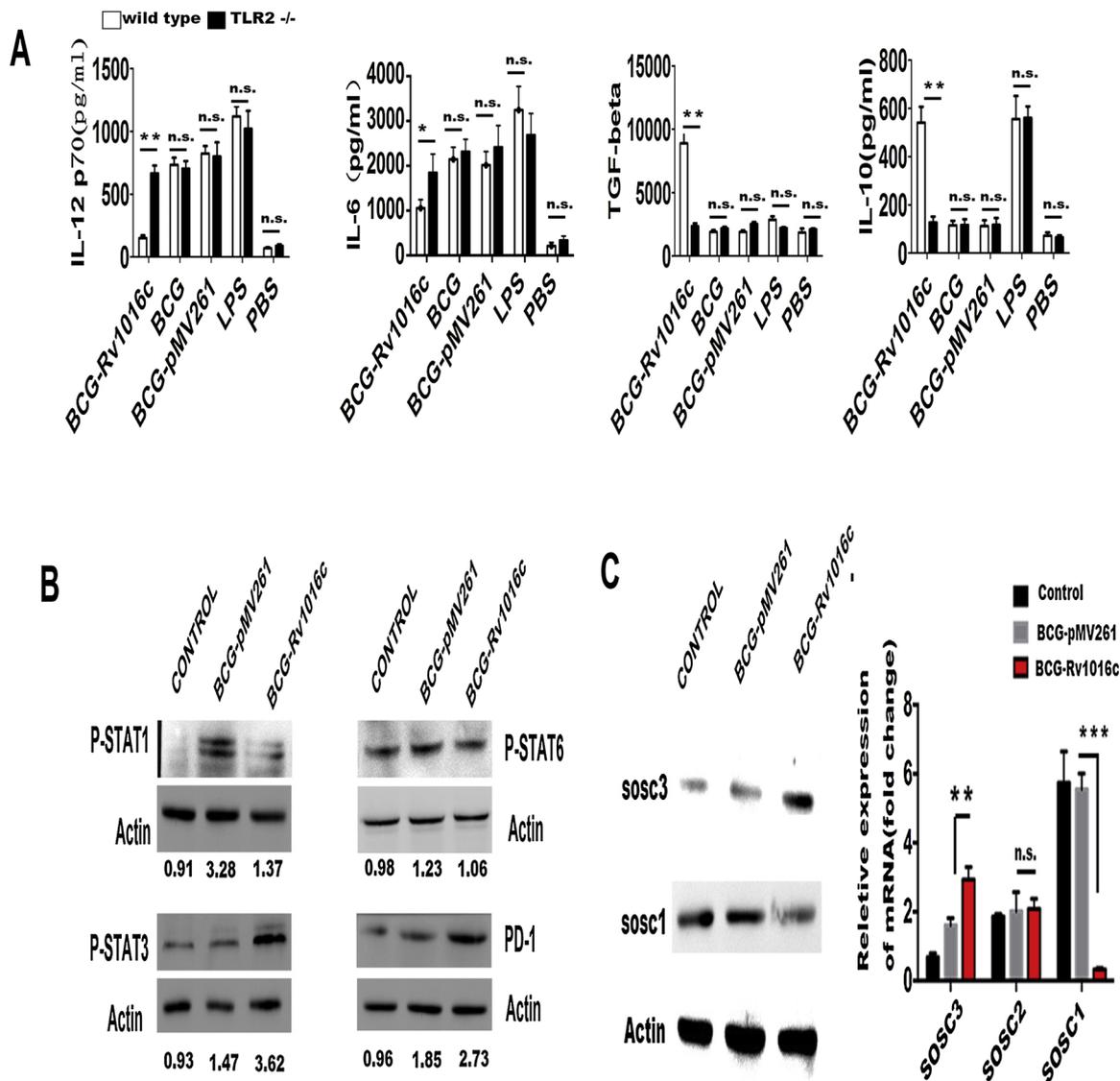


Fig. 5. Rv1016c modulates DCs functionality by targeting TLR2/STAT/SOC3 pathway.

(A) BMDCs from TLR2^{-/-} or wildtype mice were infected with BCG-pMV261 or rBCG-Rv1016c and cytokine production was measured using ELISA. (B) The phosphorylation status of STAT1, STAT3 and STAT6 after rBCG-Rv1016c stimulation was detected by Western blotting. The comparison was done against control DCs. (C) SOCS level as measured by Western blotting and densitometric analysis, expressed (mean \pm SD) after normalizing with loading control actin. The results shown are representative of two to three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s., no significance.

significantly decreased in rBCG-Rv1016c-infected DCs, compared to BMDCs infected with BCG-pMV261. Intriguingly, the expression and phosphorylation of STAT3 did not change significantly.

Suppressors of cytokine signaling (SOCS) were previously reported to regulate the JNK/ STAT pathway involved in DCs activation and T cell proliferation (Amir et al., 2017b). We observed that the expression of SOCS1 was decreased and expression of SOCS3 increased, whereas no change occurred in SOCS2 protein expression (Fig. 5C), compared to DC infected with BCG-pMV261. Thus, the results suggest that TLR2-STAT1/6-SOS3 signaling may be affected by the deleterious effects of Rv1016c on DCs maturation.

3.6. Rv1016c inhibits the Th1 and Th17 differentiation normally polarized by BCG-infected DCs

DCs play a crucial role in priming and polarizing naïve T cells toward effector/memory T cells (Sia et al., 2015). As observed above, Rv1016c protein adversely affected the DCs maturation normally induced by wildtype BCG. We further analysed the influence of the

Rv1016c protein on the ability of BCG-infected DCs to polarize naïve T cells to Th1 and Th17, and to change the cytokine profile, using an allogeneic mixed-lymphocytes reaction (MLR) assay. We found that C57BL/6 BMDC infected with BCG-pMV261 significantly promoted CD4⁺/CD8⁺ T splenocytes proliferation (Fig. 6A) and enhanced IFN- γ secretion (Fig. 6B) from BALB/c, whereas rBCG-Rv1016c-infected BMDC failed to promote the proliferation (Fig. 6A) or IFN- γ production (Fig. 6B) from BALB/c splenocytes. Additionally, the levels of IL-4 and IL-10 were significantly increased in BALB/c splenocytes co-cultured with rBCG-Rv1016c-infected DCs (Fig. 6C). Thus, these results indicated that Rv1016c inhibited the Th1 polarization and caused the Th2 response.

In the syngeneic polarization assays using lymphocytes from rBCG-Rv1016c- or BCG-pMV261-vaccinated C57BL/6 mice, BMDC infected with rBCG-Rv1016c significantly diminished CD4⁺ and CD8⁺ T splenocytes proliferation (Fig. 7A), the secretion of IFN- γ and IL-17 (Fig. 7B and C) compared to BCG-pMV261-infected BMDCs. In addition, splenic lymphocytes from BCG-pMV261-infected mice elicited a robust immune response, compared to rBCG-Rv1016c-immunized mice, as

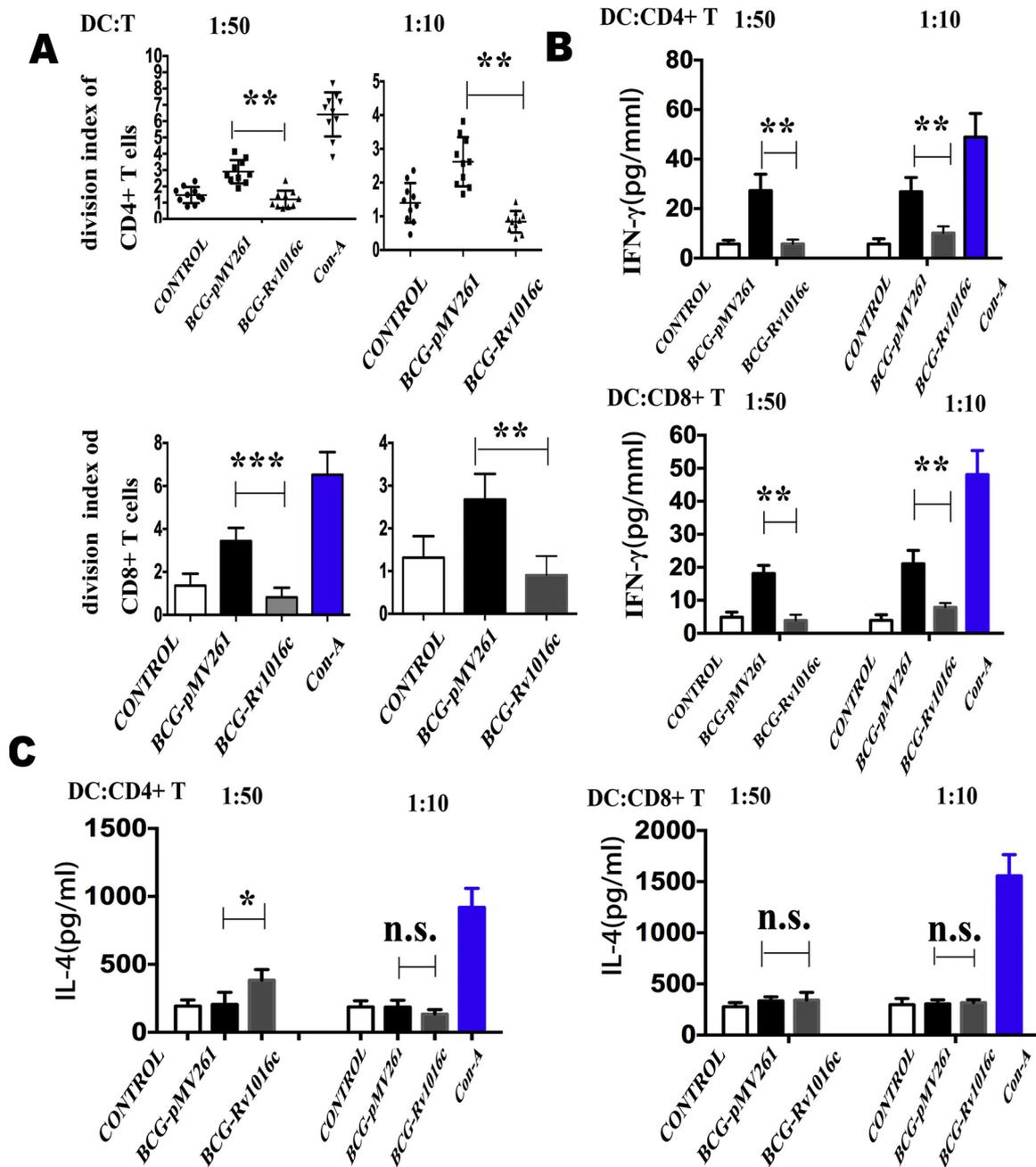


Fig. 6. Polarization of allogeneic splenocytes by BCG-infected BMDC. (A) 7 day differentiated C57BL/6 BMDC were either untreated (NT) or infected with the indicated BCG strains at 10 MOI for 24 h, gamma irradiated as described in methods section and co-cultured with BALB/c splenocytes at T cell/DC ratios of 10 and 50 for 72 h. Supernatants were then collected for measurement of IFN- γ (B) and IL-4 (C). Cells were pulsed with 0.5 μ Ci tritiated thymidine for 16 h, harvested and counted. Stimulation indices (A) were obtained by dividing cpm obtained for each stimulation by the sum of cpm obtained for corresponding DC plus T cells. Values are mean \pm S.D. from at least four mice. *, $p < 0.05$; **, $p < 0.01$; n.s., no significance.

referred to antigen processing and presentation (Fig. 7A–C).

Additionally, compared to BCG-pMV261, rBCG-Rv1016c inhibited *in vitro* TH1 and TH17 recall responses of splenocytes from BCG-immunized mice. Secretion of IFN- γ , IL-2 and IL-17 by splenocytes from mice immunized with wildtype BCG or infected *in vitro* with BCG-Rv1016c were significantly reduced comparatively to those infected with BCG-pMV261 while their levels of IL-10 were significantly increased (Fig. 7D). These findings demonstrated that rBCG-Rv1016c inhibited *in vitro* Th1 and Th17 recall responses of splenocytes from BCG-immunized mice.

4. Discussion

Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis (TB), has co-evolved with the human host for millennia. Hence, it is not surprising that mycobacteria have developed intricate mechanisms to interfere with the induction and course of the host immune response to the infection (Jozefowski et al., 2008). Mucosal T-helper cells producing IFN- γ (Th1) and IL-17 (Th17) are important protective factors against tuberculosis (TB), but the mechanisms by which *M. tuberculosis* impairs the function of antigen presenting cells (APCs) and induces suboptimal antigen-specific CD4 T cell immune responses are not well defined (Sutherland et al., 2009; Gallegos et al.,

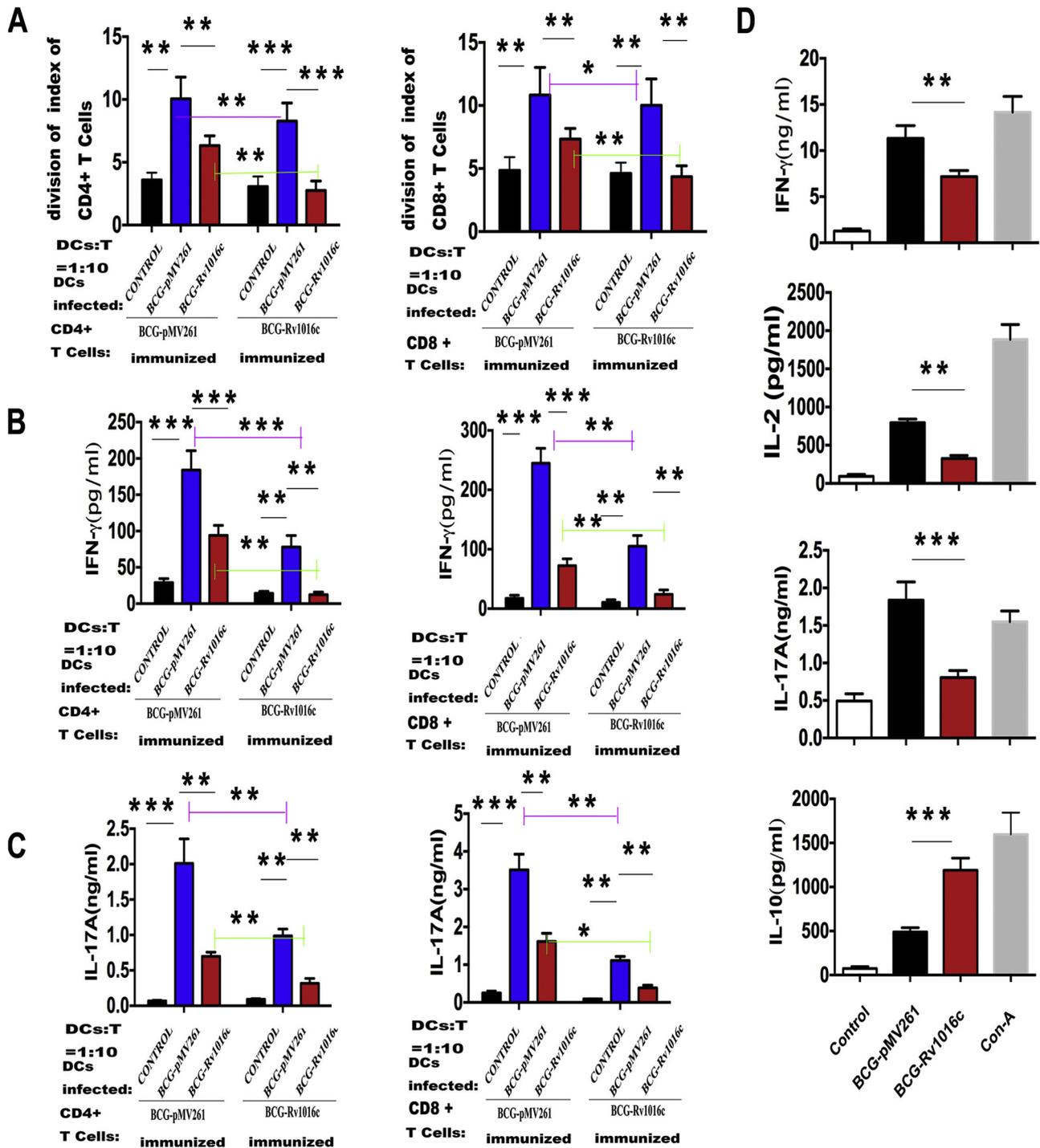


Fig. 7. Polarization of syngeneic splenocytes by infected BMDC. 7 day differentiated C57BL/6 BMDC were either untreated (control) or infected with the indicated BCG strains at 10 MOI for 24 h, gamma irradiated as described in the methods section and co-cultured with splenocytes from C57BL/6 mice infected with 10 (Noss et al., 2000) viable bacilli of BCG-pMV261 or rBCG-Rv1016c 3 weeks previously, at T cell/DC ratios of 10 and 50 for 72 h. Supernatants were then collected for measurement of IFN- γ (B) and IL-17 (C). Cells were pulsed with 0.5 μ Ci tritiated thymidine for 16 h, harvested and counted. Stimulation indices (A) were obtained by dividing cpm obtained for each stimulation by sum of cpm obtained for corresponding DC plus T cells. (D) In vitro recall responses of splenocytes from BCG-immunized mice. 6 week old C57BL/6 mice were immunized with 10 (Khan et al., 2016) viable BCG-SSI. Splenocytes recovered 3 weeks later, were stimulated in vitro at an MOI of 10 with BCG-pMV261 or BCG-Rv1016c for 72 h. Con-A at 10 μ g/ml was included as positive control. Supernatants were collected for the measurement of IFN- γ , IL-2, IL-17 and IL-10. Values are mean \pm S.D. from four mice. **, $p < 0.01$; ***, $p < 0.001$.

2011). We previously reported the immunosuppressive characteristics of Rv1016c in THP-1-differentiated macrophages or PBMCs derived from mice during the interplay between the host innate immune response and the bacterial (Su et al., 2016). In order to gain an understanding of this interplay it is of importance to analyze how *M. tuberculosis* subverts the bactericidal effects of APCs, and dampens

processes required for protective immunity, including cytokine and chemokine induction. Here, we deepened the understanding of the immunosuppressive properties of Rv1016c on DCs and observed that recombinant BCG expressing glycosylated Rv1016c is capable of: (1) enhancing the virulence of BCG in SCID mice and attenuating the protective immune response in Zebrafish-*M. marinum* infection model;

(2) functionally inhibiting DC maturation by the reduction of inflammatory cytokines production (IL-2, IL-12p70, TNF- α , IL-6, TGF- β), and co-stimulatory molecules (CD80, CD86, MHC-I and MHC-II) induced by BCG; (3) hampering the ability of DCs to polarize allogeneic / syngeneic T lymphocytes to produce IFN- γ and IL-17. We additionally propose that the mechanism underlying this immunostimulatory phenomenon is dependent on the TLR2/ STAT/ SOCS-3 pathways. Together, our results suggest that the glycosylated Rv1016c lipoprotein of MTB was able to functionally inhibit DC maturation by activating the antigen-specific CD4⁺ / CD8⁺ T cells to reduce IFN- γ and IL-17 production.

Dendritic cells are antigen presenting cells able to initiate innate and adaptive immune responses against invading pathogens (Lozza et al., 2014). Different properties such as an efficient Ag processing machinery, the high levels of expression of co-stimulatory molecules, and the production of cytokines contribute in making DCs potent stimulators of naive T cell responses (Lozza et al., 2014; Jonuleit et al., 2001). The numerous exquisite strategies that Mtb exploits to evade or circumvent the host innate immunity include the impairment of the dendritic cells function by the obstruction of cytokine secretion (Liu et al., 2017). Interleukin (IL)-2 is known to be crucial for T cell activation for DCs stimulation, and an IL-2 deficiency attenuates ability of DCs to induce CD4⁺ and CD8⁺ T cells differentiation (Granucci et al., 2001). In our study, the secretion of IL-2 was almost abolished in rBCG-Rv1016c-infected BMDCs. Among mycobacterial immunomodulatory compounds, glycolipids of the bacterial cell wall, particularly lipooligosaccharides (LAMs) play a crucial role in the virulence of a mycobacterial species and the outcome of the infection by the inhibition of IL-2 cytokine secretion of DCs (Kallénius et al., 2016). In addition, IL-6 is critical for the proliferation and differentiation of Th1 and Th17 effector cells (Pyle et al., 2017). Our results also suggest that rBCG-Rv1016c induced lower levels of IL-6 in BMDCs. Moreover, IL-12 increases the resistance of mice to *Mycobacterium tuberculosis* infection due to its key role in the DC migration and the initiation of Th1 cells response (Shafiani et al., 2013). The early migration of DCs to the draining lymph nodes and the subsequent priming of T cells determine the level of consequences of an Mtb infection. Thus, the attenuated secretion of IL-12p70 in rBCG-Rv1016c-infected BMDC would threaten the early activation of Th1 cell immunity. Previously, the phosphatidylinositol mannosides (PIMs) purified from the Mtb strains H37Rv were capable of reducing the production of cytokines IL-6 and IL-12 in DC, and acted as powerful virulence factors contributing to the pathogenesis of tuberculosis (Briken et al., 2004). Additionally, BMDCs infected with rBCG-Rv1016c produced high level of IL-10, compared to those infected by the BCG-pMV261 strain. IL-10, a Th2 type cytokine, has been known to promote the development of T regulatory cells differentiation (Shi et al., 2016). Furthermore, CD4⁺ T and CD8⁺ T cells from rBCG-Rv1016c-infected mice reduced the Th1 and Th17 cytokine production to level detected in BCG-pMV261-infected mice, which suggests that Rv1016c has a suppressive effect on the ability of dendritic cells to polarize naive T cells toward Th1 cells and Th17 cells, as observed by the deficiency of Th1 and Th17 cell immunity during human MTB infection.

Co-stimulatory molecules in conjunction with the MHC-antigen complex directly affect the delivery of the second signal, which is crucial for the initiation and activation of T cells (Langenkamp et al., 2000; Su et al., 2015). Thus, both the expression of the co-stimulatory molecules and the correct encounter of the MHC-antigen shape the nature of host immunity and determine the naive T cell fate, either polarization to effector/memory cell or differentiation to anergy T cells (Langenkamp et al., 2000; Dhodapkar et al., 2001). Hence, modulation of these molecules by Mtb can help them evade the immune system and establish their existence in the host (Natarajan et al., 2011). Thus, we examined the impact of glycosylated Rv1016c on the expression of co-stimulatory molecules (CD40, CD80, CD86, MHC I and MHC II). The level of CD40 on DCs surface was closely associated with the expression of CD80 and

CD86, both acting as a stimulus for the pro-inflammatory cytokines secretion and the Th1 cells priming (Lazarevic et al., 2003). CD40 expression on DCs is required for the IL-17 release and the interaction between CD40 on DCs and CD40L on CD4⁺ T cells is critical for generating antigen-specific IL-17 responses to Mtb (Sia et al., 2017). Here, the glycosylated Rv1016c employed by BCG could suppress co-stimulatory molecule expression and impair the function of DCs, as revealed by the attenuated ability of DCs to trigger the Th1/ Th17 responses. Therefore, the inhibition of processing and presentation of antigens caused by glycosylated Rv1016c seems to help the pathogen to evade the immune surveillance.

In addition to LAMs, various other mycobacterial compounds have been shown to have potent modulatory effects *in vitro* on cells of the host immune system (Chatterjee, 1997). These compounds include secreted or structural proteins such as ESAT-6 (Wang et al., 2012), 19-kDa lipoprotein (Pennini et al., 2006), hsp70 (Bulut et al., 2005) and cell wall associated glycolipids such as phenolic glycolipids (Reed et al., 2004). Some of these molecules, such as the 19-kDa lipoprotein, PIMs or LM, have been reported to bind to and signal through Toll-like receptors 2 and/or 4 (TLR2, TLR4) on host cells. Others however, such as ManLAM, are thought to bind DC-specific C-type lectin DC-SIGN (Geijtenbeek et al., 2003) and mannose receptors and to deliver negative signals that interfere with TLR-mediated signaling (Nigou et al., 2001). Thus, we further investigated the mechanism by which glycosylated Rv1016c functionally suppresses DCs maturation. Here, we found that TLR2 deficiency in mice abolished the suppressive secretion of IL-2 and IL-12 p70 in rBCG-Rv1016c infected BMDCs. These findings suggest that TLR2-dependent signaling was affected by the DCs function impairment caused by the glycosylated Rv1016c. This result is consistent with other obtained for MTB antigens, namely Acr1 (Amir et al., 2017a), Rv0577 (Byun et al., 2012). These antigens modulated the DC maturation and subsequently led to the altered Th1 and Th17 responses in a TLR2 dependent manner. The expression of the SOCS protein was shown to be significantly up-regulated in DCs by *M. tuberculosis* infection (Imai et al., 2003; Bartz et al., 2006). It is well accepted that the TLRs stimulation activates dendritic cells to transition from immature to mature DCs. However, ligands for TLR-4, TLR-2, and TLR-9 (murine system) were also effective to impede differentiation of DCs (Palucka et al., 1999; Xie et al., 2003). Additionally, TLR stimulation during the early stage of differentiation of human monocytes to DCs resulted in an increase of SOCS proteins transcription (Palucka et al., 1999; Alexander and Hilton, 2004). It has been known that SOCS proteins diminish the JAK/STAT signaling and are able to inhibit a variety of different cytokines, resulting in the inhibition of DC activation (Dalpke et al., 2003; Bode et al., 1999). Here, we found that Rv1016c significantly up-regulated the expression of SOCS-3, which can be explained by reduction of IL-12 p70 but increase of IL-10 amount. Previously, dendritic cells overexpressing SOCS-3 exhibited a tolerogenic/DC2 phenotype that showed a low level of expression of IL-12 but a high level of IL-10 (Li et al., 2006). Moreover, in this study, rBCG-Rv1016c infection in DCs induced a remarkable decline of the level of STAT-1 phosphorylation. Conversely, a significant increase was detected in the level of STAT-3 phosphorylation, which is critical for the formation of the tolerogenic DCs subset. The perturbed signaling due to Rv1016c stimulation of DCs was also observed in APCs treated by Mtb or Mtb components (Abebe et al., 2011).

In summary, these results suggest both the suppressive effect of glycosylated Rv1016c on the DC maturation and the impairment of polarization of naive T cells to Th1/Th17 cells, providing a niche for Mtb to persistently survive in the host. Therefore, we propose that Mtb may utilize its components including Acr1, Rv1016c, PIMs to interfere with the early stage of differentiation of DCs precursors, which has been considered as an immune evasion strategy (Grace and Ernst, 2016). Additionally, the inactivation of the Rv1016c gene may lead to an attenuated strain of *M. tuberculosis* for the development of new vaccine candidates.

Statement of ethics

All the mice were kept under specific pathogen-free conditions in the Center of laboratory animal science of Guangdong (Guangzhou, China). All animal experiments and procedures on mice were performed in accordance with the Animal Ethics Committee-approved guidelines of Guangzhou Medical University. Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

Competing interests

The authors declare no competing financial interests.

Author contributions

Haibo Su and Zhi Zhang conceived and designed the experiments; Haibo Su, Zijian Liu, Baozhou Peng, and Zhen Zhang performed the experiments; Zijian Liu and Baozhou Peng analyzed the data; Haibo Su wrote the manuscript; Haibo Su and Zhi Zhang reviewed the manuscript and supervised the research. All authors read and approved the final manuscript.

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