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IMMUNOLOGICAL ASPECTS

PD-L2 induction on dendritic cells exposed to *Mycobacterium avium* downregulates BCG-specific T cell response

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SUMMARY

The exposure to certain species of Nontuberculous Mycobacteria (NTM) can modulate the immune response induced by *Mycobacterium bovis* BCG. *Mycobacterium avium* has been postulated as a weak inducer of dendritic cell (DC) maturation. However, how the DC exposure to *M. avium* could contribute to the modulation of a BCG-specific CD4+ T cell response and the molecules involved remain unknown. Here, we exposed bone marrow–derived DCs (BMDCs) to *M. avium* either prior to exposure to BCG or as a unique stimulus. We found that *M. avium* induces high expression of PD-L2 (B7-DC) in BMDCs. This was dependent on IL-10 production through the TLR2-p38 MAPK signaling pathway. Exposure to *M. avium* prior to BCG results in BMDCs that do not express co-stimulatory molecules and pro-inflammatory cytokines, while the expression of PD-L2 and IL-10 was maintained. BMDCs exposed to *M. avium* impaired the activation of BCG-specific T cells through the PD-1: PD-L interaction. This suggests that a *M. avium*-induced phenotype in DCs might be implicated in the induction of mechanisms of tolerance that could impact the T cell response induced by BCG vaccination.

1. Introduction

The only available vaccine to prevent tuberculosis (TB) in humans that is routinely used is the attenuated *Mycobacterium bovis* strain Bacillus Calmette-Guérin (BCG). However, according to a meta-analysis of trials involving BCG, the efficacy of this vaccine in conferring protection against pulmonary TB is approximately 50%.[1] A remarkable aspect of this vaccine is its variable efficacy, as observed in some tropical regions.[2–4] While the cause of this variability in populations is not well understood, it may be related to prior exposure to Nontuberculous Mycobacteria (NTM). Populations in which vaccination with BCG induced modest or no protection may have had contact with NTM, as evidenced by skin responsiveness to tuberculin.[5] As a result of this study, it has been suggested that prior exposure to NTM may reduce the protective efficacy of the BCG vaccine. Furthermore, several studies in animal models have provided additional evidence that prior contact with NTM may interfere with the effective generation of adaptive immunity to BCG[6,7] through the down-regulation of the interferon-gamma (IFN-γ) responses induced by vaccination.[8–11] Nevertheless, the interaction of NTM with innate immune cells, particularly dendritic cells (DCs), and the contribution of these cells to the regulation of the immune response to BCG have not been extensively studied.

DCs are antigen-presenting cells (APCs) that integrate a variety of incoming signals and orchestrate the immune response. The central role of DCs in the induction of immunity and in the silencing of T cell immune responses has been well documented.[12,13] DC activation is crucial for the effective stimulation of antigen-specific effector T cell responses.[14] This activation is characterized by the upregulation of MHC class II (MHC II) and co-stimulatory molecules (e.g., CD86, CD80) as well as CD40 and cytokine secretion (e.g., IL-12 and TNF).[12] However, hypo-responsive or unresponsive effector T cells are observed following stimulation with partially matured DCs.[15–17] Partially matured DCs express low to intermediate levels of co-stimulatory molecules and pro-inflammatory cytokines, but high levels of the anti-inflammatory cytokine IL-10.[16] In addition to co-stimulatory molecules, activated DCs can also express PD-L1 (B7-H1) and PD-L2 (B7-DC), which are both considered co-inhibitory molecules due to their capacity to bind Programmed Death 1 (PD-1), which is expressed on activated T cells. PD-L2 has been shown to have potent co-inhibitory properties for T cell responses.[18] DCs with partially mature phenotype can been

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generated *in vitro* by treatment with IL-10, transforming growth factor-β (TGF-β), vitamin D_3 and glucocorticoids. A tumoral microenvironment can also generate this partially mature DC phenotype.[15,17,19–21] In the case of mycobacteria, previous studies have demonstrated that the interaction of human DCs with *Mycobacterium tuberculosis* or BCG results in cell maturation and activation.[22,23] Moreover, DCs infected with BCG are potent inducers of a cellular immune response against *M. tuberculosis in vivo*.[24] In contrast, infection with the *Mycobacterium avium* subspecies *paratuberculosis* induces DC maturation, but the resultant phenotype is similar to partially mature DCs.[25] An additional report indicated that *M. avium* (WAg206) is unable to induce significant upregulation of activation markers on BMDCs or the release of pro-inflammatory cytokines in murine DC-macrophage co-cultures compared with lipopolysaccharide (LPS). This report also showed that after pre-immunization with *M. avium*, mice do not to respond efficiently to BCG.[11] These findings suggest that exposure to NTM could modulate the activation of DCs and, subsequently, the adaptive immune response. However, the relationship between the DC phenotype and the modulation of the T cell response as well as the possible mechanism has not been further evaluated.

On the other hand, it has been documented that the maturation and function of DCs may be suppressed by continual exposure to antigen, in particular bacterial LPS, and this exposure may induce a hypo-responsive state in DCs and modify their capacity to direct Th1 responses.[26] The lack of the DC response to subsequent stimuli has also been observed after exposure to tumor cells.[27] Therefore, it is possible that the prior exposure of DCs to NTM could induce a hypo-responsive state in the DCs, thereby affecting their capacity to induce an appropriate T cell response.

In this study, we characterized the interaction between an NTM *M. avium* and BMDCs and found that *M. avium* induces high levels of PD-L2, which was dependent on IL-10 production through the TLR2-p38 MAPK signaling pathway. This was not observed after BCG stimulation; however, the phenotype induced by *M. avium* is maintained even in the presence of BCG. We show that PD-L2 expression in BMDCs exposed to *M. avium* and their interaction with PD-1 of T cells is crucial in the down-modulation of the BCG-specific CD4+ T cell response.

2. Materials and methods

2.1. Mycobacteria

Previous reported that *M. bovis* BCG Phipps substrain led to the grastest and most persistent reduction of area of pneumonia and a significant drop in CFU at 2 and 4 months in Balb/c model of progressive pulmonary tuberculosis and it was recently analyzed by proteomic analysis.[28,29] According to the refined genealogy of the BCG vaccines based on regions of differences markers, *M. bovis* BCG Phipps has been positionated in DU2 Group IV (late strain).[30] *M. bovis* BCG Phipps was grown in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase and 0.05% Tween 80 (Sigma, St. Louis, MO) until the mid-log phase, when the cells were harvested and frozen in 1 mL aliquots.

M. avium subspecies *hominisuis* (*M. avium*) was isolated from water from the Xochimilco channel (Mexico City, México). We performed standard decontamination of samples using 1% sodium hydroxide and cultured at 37 °C on Löwenstein–Jensen medium to isolate the organisms. The identity of the bacteria was confirmed using standard biochemical identification tests for mycobacteria and PCR-RFLP analysis of the *hsp65*, *recA*, *ropB*, and *16S rRNA* genes (Castillo-Rodal, submitted manuscript). *M. avium* was grown at 37 °C in Middlebrook 7H10 broth (Difco, Detroit, MI) and supplemented with oleic acid-albumin-dextrose-catalase (BBL, Cockeysville, MD) in order to obtain pure colonies. The pure cultures were subsequently cultured in Sauton broth until the mid-log phase of bacterial growth, and then they were harvested and frozen in 1 mL aliquots. The CFU present in each aliquot were determined by thawing the sample and plating dilutions onto Middlebrook 7H10 agar plates. The colonies were counted after 21 days.

2.2. Mice

Groups of five male wild type BALB/c mice (6–8 weeks-old) were immunized subcutaneously (s.c.) at the base of the tail with 1×10^5 CFU of live *M. bovis* BCG Phipps or with *M. avium* diluted in 100 μL of phosphate-buffered saline (PBS). Mice were sacrificed 4 weeks after immunization.

2.3. Generation of bone marrow-derived DCs (BMDCs)

Bone marrow was obtained from the femurs and tibias of 6–8-week-old male Balb/c mice. Bone marrow cells were harvested as previously described[31] and washed with PBS. The cells were resuspended in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FCS (GIBCO BRL), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO BRL), and 10% supernatant from a granulocyte-macrophage colony-stimulating factor (GM-CSF)–producing cell line[32] and cultured in 100 mm diameter plates. On day 3, the cells were fed with fresh RPMI supplemented with 10% FCS containing GM-CSF. On day 5, the bone-marrow cells were examined for the expression of CD11c and CD11b. For this, the cells were stained with an APC-conjugated anti-mouse CD11c monoclonal antibody and a FITC-conjugated anti-CD11b monoclonal antibody (Pharmingen-BD Biosciences, Mountain View, CA, USA). The final purity of the DC cultures was 60% CD11c positive, as determined by flow cytometry.

2.4. Culture of BMDCs with BCG or M. avium

On day 5 of culture, the bone-marrow cells in suspension were harvested and counted, and 5×10^6 cells were cultured in the absence of stimuli or with live *M. avium* or BCG Phipps at a 1:1 ratio (DCs : Mycobacteria), or with 0.1 μg/mL *Salmonella Typhimurium* LPS, kindly donated by Dr. Rodolfo Pastelín (Faculty of Chemistry, UNAM) for 24 h. The LPS was used as a positive control for activation. All cultures were performed in 100 mm diameter plates with 10 mL of fresh RPMI. After 24 h, DCs were harvested, and surface marker expression and intracellular cytokine production was assessed using flow cytometry.

For the re-stimulation assays, on day 5 of culture the BMDCs were harvested and counted, and 5×10^6 cells were cultured in the absence of stimuli or with live *M. avium* or BCG at a 10:1 ratio (DCs : Mycobacteria) for 3 h. After stimulation, the cells were harvested and positively selected using anti-mouse CD11c MACS MicroBeads (Miltenyi, Biotec, Auburn, CA, USA). The cells were then plated and re-stimulated with live BCG at a 1:1 ratio for 18 h. After this second incubation, the surface phenotype was examined using flow cytometry. Supernatants were also harvested for cytokine detection.

In some experiments, BMDCs were incubated with or without a blocking monoclonal antibody against TLR2 (10 μg/mL, T2.5, BioLegend), selective p38 MAPK inhibitor (SB203580, 2 μg/mL, InvivoGen) or isotype control for 1 h prior to exposition with *M. avium* or BCG. Additionally, BMDCs were incubated with or without a blocking monoclonal antibody against IL-10R (10 μg/mL, 1B1.3a, BioLegend) or an isotype control.

2.5. Flow cytometry

For surface molecule staining, BMDCs stimulated and re-stimulated with live BCG Phipps or *M. avium* were stained with APC-