Vaccine 29 (2011) 6446–6450

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

Patterns of in vitro cell-death, metaloproteinase-9 and pro-inflammatory cytokines in human monocytes induced by the BCG vaccine, Moreau strain

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ARTICLE INFO

Article history:
Received 24 March 2011
Received in revised form 17 May 2011
Accepted 24 June 2011
Available online 13 July 2011

Keywords:
BCG vaccine
Tuberculosis, Monocyte
Apoptosis, Cytokine

ABSTRACT

Mononuclear cells have been implicated in the primary inflammatory response against mycobacteria. Yet, little is known about the interaction of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) with human monocytes. Here, we investigated the potential of BCG Moreau strain to induce in vitro specific cell-death utilizing a flow cytometry approach that revealed an increase in apoptosis events in BCG-stimulated monocytes from healthy adults. We also detected a concomitant release of interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), but not metalloproteinase (MMP)-9. In addition, annexin V-propidium iodide double staining demonstrated an enhancement of monocytes necrosis, but not apoptosis, following BCG Moreau strain stimulation of umbilical vein cells from naïve, neonate. This pattern was paralleled by different pro-inflammatory cytokine levels, as well as MMP-9 induction when compared to the adults. Our findings support the hypothesis that BCG induces distinct cell-death patterns during the maturation of the immune system and that this pattern might set the stage for a subsequent antimycobacterial immune response that might have profound effects during vaccination.

1. Introduction

In the early 90s, the World Health Organization selected tuberculosis (TB) as a public health priority because it is the second leading cause of death worldwide among infectious diseases. TB is mostly concentrated in the developing world, with roughly 80% of all TB cases occurring in the 22 highest-burden countries, including Brazil. Although the worldwide TB incidence has decreased at a rate of less than 1% per year in many settings over the past decade, case numbers and overall burden continue to rise in a number of countries, as a result of the rapid growth of the world population [1]. This is directly associated with poor treatment outcomes resulting in multidrug-resistance strains [2]. Despite the immunological parameters associated with pathogenesis of the disease being extensively studied, we still do not fully understand the signaling mechanisms, transcriptional responses, sub-cellular processes, and cell–cell interactions that follow *Mycobacterium tuberculosis* infection, particularly in the monocyte lineage. The currently vaccine in use is *M. bovis* bacillus Calmette-Guerin (BCG) which results in a strong cellular immune response against *M. tuberculosis*, although protection is highly variable [3]. Thus, BCG vaccine, despite being cheap and protective against severe forms of TB, it is not effective against pulmonary TB in hyper-endemic countries [4]. The BCG strain currently used in Brazil is Moreau (RDJ), but very little is known about its long-established protective properties. In vivo, the BCG Moreau strain induces a good DTH skin test response and rarely causes local or systemic adverse reactions. There is a lack of in vitro studies to understand the basis of the protection induced by this stain. As the TB epidemic continues, more attention has been paid for direct applicability and improvement of existing strategies of vaccination and management.

Based on the limited data available and because macrophage/monocyte lineage in the lungs represent the first line of defense to be recruited into the developing granuloma against pathogens entering by the airways, the aim of this study focused on understanding the pathways related to in vitro cell-death pattern associated with the immune response to the BCG Moreau strain in human monocytes. Previous studies have shown that host cell apoptosis is an important defense mechanism against mycobacteria [5,Reviewed by 6]. Soluble factors released during BCG and monocyte interaction were also compared, since TNF-α has been shown to induce metalloproteinase (MMP)-9 expression, which, in turn, degrades extracellular matrix in the inflammatory responses [7]. A better understanding of the changes induced by BCG infection could help to identify the processes resulting in protection, thus opening up prospects for future vaccine improvement. Furthermore, this work should result in better overall understanding of the pathogenesis of tuberculosis.

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0264-410X
doi:10.1016/j.vaccine.2011.06.093

2. Patients and methods

2.1. Study participants

Two groups of donors that may represent a distinct cellular immune response resulting from a previous exposure to mycobacterial antigens were enrolled from different settings of Rio de Janeiro: Healthy donor adults (HD; $n = 18$) vaccinated with BCG during childhood (BCG vaccination in Brazil is mandatory after birth) from the blood bank of Clementino Fraga Filho Federal University Hospital (anonymous donation policy, but included individuals age ≥18-years old), and newborn umbilical veins (UV; $n = 8$) of naïve individuals (3 boys) who have never been exposed to mycobacteria obtained by ex utero umbilical cord blood puncture of non-smoker, disease free mothers (all cesarean section at full terms: 37–42 weeks) from the Gaffree Guinle State University Hospital. The ex utero umbilical cord blood collection procedures were as follows: post baby delivery, the placenta and cord were placed into a sterile basin, 30 mL of blood was regularly taken from the umbilical cord, immediately transferred to heparinized tubes and maintained at room temperature before processing. Exclusion criteria for those individuals utilized HIV-seronegative status, a negative history of malignant, degenerative, or transmitted diseases, diabetes mellitus, and use of corticosteroids or other immunosuppressive agents at the time of the study. In addition, the UV group also excluded fetal distress, mothers with a history of TB and any other maternal infection. This study was approved by the respective Institutional Review Boards of both sites. All study participants provided written informed consent forms.

2.2. Mononuclear cells purification and culture

Both Peripheral and Cord Blood Mononuclear Cells (MC) were separated (>92% purity) within 24 h of obtaining the blood specimens from all study participants using a Ficoll density gradient. The collected cells were first washed 3-fold with endotoxin-free phosphate buffered saline (PBS 50 mM, pH 7.2), then suspended in DMEM medium (Sigma Immunochemicals, MD, USA) supplemented with 20% autologous serum. Cell cultures (1×10^6) were kept at 37 °C in a humidified 5% CO_2 atmosphere in individual 12 mm × 75 mm sterile polystyrene tubes (Falcon, Corning Inc., NY, USA). Previous experiments with these tubes showed a better viability of cells when compared to conventional culture plates (data not shown). Cells were used for subsequent cell death analysis, and the supernatants were stored at −70 °C.

2.3. In vitro MC infection with BCG Moreau strain

The BCG Moreau (RDJ) strain used through was a gift of the Ataulpho de Paiva Foundation (Rio de Janeiro, Brazil). Individual batches of sealed, single dose glass vials containing lyophilized BCG (approximately 1×10^7 viable bacilli) were maintained at 2–8 °C. The same batch was used for each infection. Upon receipt, ampoules were suspended in water (provided separately by the manufacturer) shortly before the infection of cells. The effectiveness of BCG Moreau infection was previously determined using a titration curve in order to establish the multiplicity of infection (MOI) ratio that would be used through the entire study, and accordingly the MOI of 2:1 (bacilli:mononuclear cell ratio) was chosen. The viability of the bacilli was promptly assessed by immunofluorescence kits (LIVE/DEAD® BacLight, Invitrogen Co., USA).

2.4. Cell death assay by fluorescence activating cell sorting (FACS)

MC from each donor were left in culture for 24 and 48 h. Tubes assigned as negative controls remained uninfected for the same period. Positive control cells were subjected to heating just before staining in order to force cell necrosis. After incubation, cells were labeled with TACS kits as specified by the manufacturer (TACS, R&D, USA) and immediately analyzed by flow cytometry (FACScalibur, BD, USA).

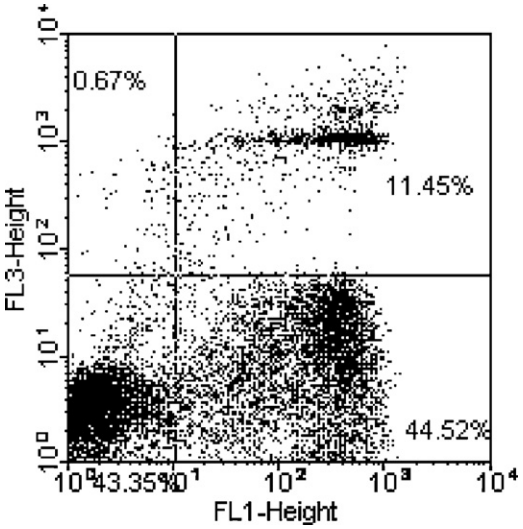

Fig. 1. Flow cytometry double staining demonstrates typical profile from one representative experiment. Healthy donor's peripheral blood MC stimulated with BCG Moreau strain for 24 h was used to set the gates for viable (lower left), apoptotic (lower right), and necrotic cells (upper right). Negligible dyes incorporation was seen in the viable, live cells. As denoted in the dot plot, the percentages of cells are indicated in each quadrant. The monocyte population was previously gated on the basis of their light scattering properties. FL-1 and FL-3 represent annexin-V-FITC and propidium iodide stainings, respectively.

2.5. Detection of metaloproteinases (MMP) using zymography and ELISA

The MMP activity in cell culture supernatants was analyzed using substrate gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) zymography. After titration and linearization at a maximum of 15 μg of total protein, the samples loaded in each slot were resolved in 10% polyacrylamide gels containing 1% of gelatin per mL at 100 V for about 3 h. The gels were then incubated for 1 h on a rotating platform in TBS (10 mM Tris–HCl, 0.15 M NaCl, pH 7.6) containing 2.5% Triton X-100. Gels were washed three times in TBS and then incubated for 24 h at 37 °C in TBS containing 5 mM CaCl2, 1% Triton X-100, and 0.02% NaN3. Coomassie blue staining revealed the presence of gelatinolytic activity as clear bands against the blue background. Cell-free supernatants were also assayed for total (active and pro) MMP-9 quantification by an enzyme-linked immunosorbent assay (ELISA) human commercial kit as specified by the manufacturer (R&D Systems, USA).

2.6. Cytokine detection

Cell-free supernatants were thawed out and subsequently assayed for determination of the concentration of human TNF-α and IL-1β by ELISA commercial kits as specified by the manufacturer (R&D Systems, USA).

2.7. Statistical evaluation

Data were analyzed by GraphPad Instat software, using the student t test to compare both groups of individuals. MMP-9 production was represented as the mean ± standard error of mean

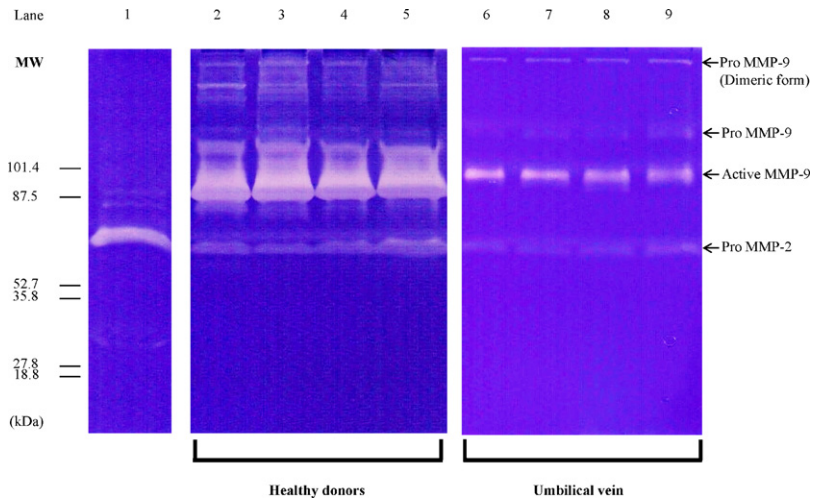

Fig. 2. In-gel gelatin zymography was performed on samples from healthy donor and umbilical vein groups in baseline (lanes 2, 4, 6 and 8) and 24-h of infection with BCG, Moreau strain (lanes 3, 5, 7 and 9). A recombinant human MMP-9 was loaded (lane 1) to represent the active enzyme. Bands shown in the gel (representative of 2 patients in each group) correspond to the characteristic 72-kDa MMP-2 and 92-kDa MMP-9. MW = molecular weight.

(SEM). The p value was scored and considered significant when ≤ 0.05.

3. Results

3.1. Cell-death ratio

We have enrolled two groups of donors for this particular study: A group of healthy donor adults (HD), and another group of naïve individuals using umbilical vein (UV) cells promptly collected after birth. Cells were infected with BCG Moreau for 24 and 48 h (after reconstitution, yielding an average of 87% of live bacilli), or were resting (baseline) uninfected cells with no stimuli. After lymphocyte population exclusion based on light scattering properties, cell-death events were analyzed using annexin-V and propidium iodide, which detect apoptosis (single positive) and necrosis (double positive; Fig. 1). Table 1 summarizes those findings (some individuals were excluded). After BCG Moreau infection at both time-points, we observed a significant increase in apoptotic events only in the HD group ($p \leq 0.001$). On the other hand, UV cells showed a significant increase of necrotic events at 24 h of infection, when compared to negative control ($p \leq 0.006$). As expected, the positive control cells (heating samples was used to artificially induce necrosis) showed increased necrotic events in both groups, and similar differences were found when the 2 distinct cell-death patterns were compared (Table 1).

3.2. Detection of metaloproteinases (MMP) using zymography and ELISA

Fig. 2 shows a representative gelatin zymography of the 2 cohorts studied. In the typical pattern, a middle, thick band contained active MMP-9 (92 kDa), and the weak, bottom band contained the pro-active MMP-2 (72 kDa). We did not observe the MMP-2 fully-active bands. The HD group did not show any significant change during the course of BCG infection (24 h), when compared the baseline cells. A similar pattern was seen in the UV group, although with a much lower intensity and there was no change in the MMP-2 and MMP-9 bands when compared to baseline cells (Fig. 2).

In addition, we evaluated the in vitro total MMP-9 levels in the 2 groups using ELISA. After BCG infection, there was no difference in induced levels of MMP-9 in either cohort. In the UV group, BCG-induced MMP-9 levels remained undetectable (0.6 ± 0.1 and 0.5 ± 0.2 μg/mL, for 24 and 48 h, respectively) which is similar to baseline levels (0.6 ± 0.2 μg/mL). However, the HD group did show much higher productions when compared to the UV group ($p \leq 0.002$), regardless of the stimuli, i.e.: BCG infection (13.0 ± 2.6, 12.8 ± 1.0 and 9.9 ± 1.3 μg/mL, for baseline, 24 and 48 h, respectively). This data mirrored the zymographic analysis results.

3.3. Cytokine detection

It was shown earlier that pro-inflammatory cytokines, such as TNF-α and IL-1β, play a key role during in vitro apoptosis induction [5,8], particularly when monocytes are infected with mycobacteria [9]. We therefore assayed the supernates from groups undergoing enhanced apoptosis for those 2 cytokines (some individuals were excluded), and a proportional increase of TNF-α levels was evident only for the HD group (Fig. 3a; $p < 0.004$). However, this finding did not mirror that of the UV group since the rates of TNF-α remained undetectable even in the presence of BCG infection at both time-points. Also, there was a statistically significant difference at 24 h of infection when HD and UV groups were compared ($p = 0.03$). The pro-inflammatory cytokine IL-1β, for which cell-death induction is also one of its main functions [8], was also assayed. There was a marked increase in IL-1β levels that were directly proportional to the time of BCG infection in the HD group (Fig. 3b; $p \leq 0.02$). This pattern was also a trend in the UV group, but opposite to TNF-α, although it did not attain a statistically significant difference when compared to the baseline condition. Also, no discrepancy was found when evaluating the IL-1β levels between the 2 cohorts in this last, resting condition ($p = 0.85$).

4. Discussion

It has been previously shown that mycobacteria are able to induce macrophage apoptosis, and the inhibition of this critical mechanism might be considered an evasive strategy of the pathogen [Reviewed by 6]. Evasion of apoptosis by *M. tuberculosis* can be achieved in human macrophages by enhanced release of sTNFR2 [6], Mcl-1 [10], bcl-2 and Rb [11], and lower productions of prostaglandin E_2 [12], bad and bax, and caspases-1, -3 and -10 [11]. On the other hand, necrosis can be looked at as a good strategy induced by pathogenic mycobacteria to skew the protective host immune response. Since 2005, a novel form of

Table 1
Average (mean ± SEM) of specific cell-death (%) in monocytes of healthy donor (HD, $n = 16$) and umbilical vein (UV, $n = 5$) groups.

	Apoptosis		Necrosis	
	HD	UV	HD	UV
Negative control	7.9 ± 1.3	9.7 ± 3.1	7.0 ± 1.3	6.7 ± 2.1
BCG 24 h	32.7 ± 6.4[a,b]	19.6 ± 4.9	9.1 ± 1.7	28.0 ± 5.4[a]
BCG 48 h	29.5 ± 4.9[a,b]	nd	11.0 ± 1.6	nd
Positive control[c]	21.3 ± 5.6	9.5 ± 2.3	58.5 ± 6.8[a,b]	73.8 ± 10.9[a,b]

[a] $p \leq 0.006$, when compared to negative control.
[b] $p \leq 0.002$, when compared to distinct cell-death pattern.
[c] Heating samples was used to artificially induce necrosis.

proinflammatory programmed cell death, or pyroptosis, has been identified to be uniquely dependent on caspase-1, which is not involved in apoptosis, and prototypically induced by infection with flagellin-expressing bacteria, such as *Salmonella* and *Shigella* species [13]. To date, pyroptosis seems to play a significant role in specific biological systems. It has been previously shown that this mechanism releases bacteria from macrophages and exposes the bacteria to uptake and killing by reactive oxygen species in neutrophils [14]. Similarly, activation of caspase-1 cleared intracellular *Legionella pneumophila* and *Burkholderia thailandensis* in vivo by IL-1β-independent mechanisms, an efficient bactericidal mechanism by the innate immune system [14]. In this study, we did not check whether pyroptotic cell death takes place in our system; however, based on the latest notion highlighted by those authors, the increased IL-1β levels found in the cultures could not support this possibility. With this in mind, and regarding *M. bovis* BCG as a low virulent organism, we can hypothesize high in vitro apoptosis levels when healthy donor monocytes are infected in a short-time manner. Actually, this is true only in previously exposed, adult individuals in which a BCG vaccination scar was present along with a history of living in a setting of environmental mycobacteria, such as Brazil. We were not, however, able to reproduce those findings in monocytes from naïve individuals; rather, necrosis was quite evident, particularly at 24 h of infection. The reasons behind this are speculative; perhaps this is due to a higher amount of circulating immature immune cells or to a lack of exposure to mycobacterial antigens. In fact, because of decreased production of Th1-cell-associated cytokines, it is thought that the neonatal innate immune system is generally impaired or depressed. The bias against Th1-cell-polarizing cytokines leaves the newborn susceptible to microbial infection and contributes to impairment of the neonatal immune responses to most vaccines, thereby frustrating efforts to protect this vulnerable population [15]. The ability of pro-inflammatory cytokines to induce spontaneous abortion is likely to be an important reason for the strong bias of the maternal and fetal immune systems of many mammalian species towards Th2-cell-polarizing cytokines [Reviewed by 16]. After birth, there is an age-dependent maturation of the immune response. Thus, the higher necrosis levels in these subjects might reflect still very immature monocytes in which BCG could behave as a moderate virulence organism. In fact, in immune compromised individuals, such as those co-infected with HIV, BCG is considered a life-threaten organism due to impairment of the immune response [17].

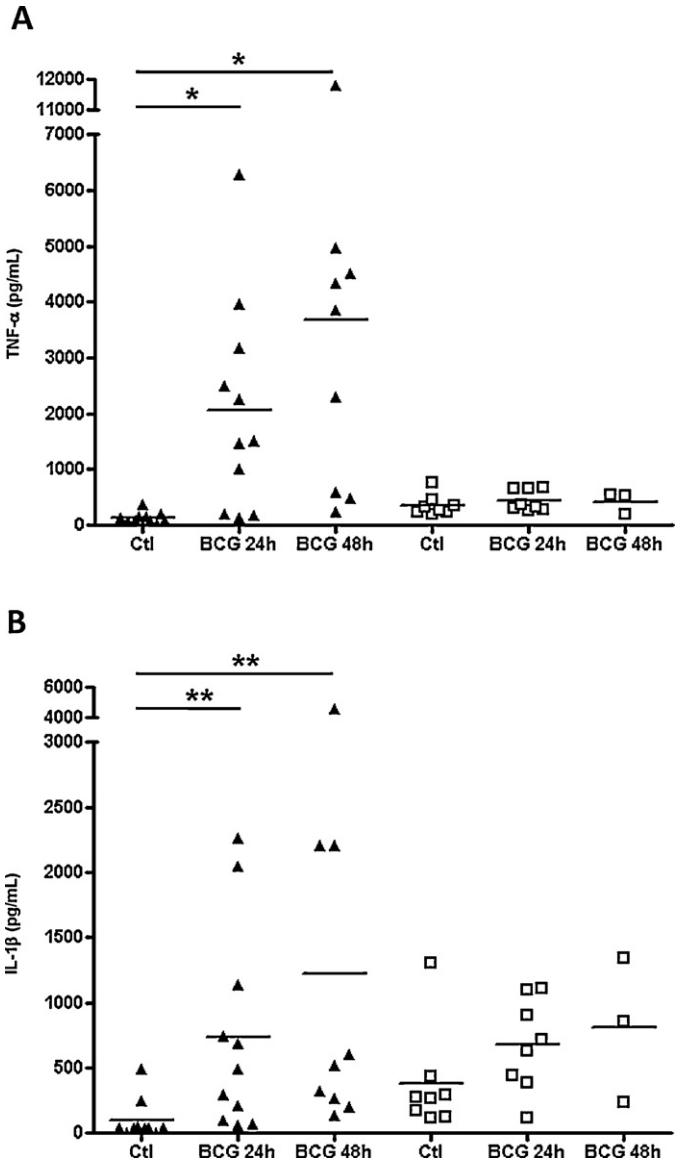

Fig. 3. (a) TNF-α and (b) IL-1β levels (pg/mL) in healthy donor (▲) and umbilical vein (□) groups representing different times of in vitro BCG Moreau strain infection in human monocytes. Horizontal bars represent mean cytokine values in each condition. $^{*}p < 0.004$, $^{**}p \leq 0.02$ vs. Ctl.

In an attempt to better explore the apoptosis and necrosis findings, we also measured levels of pro-inflammatory cytokines, the key components during cell-death induction. TNF-α is a pleiotropic cytokine during Th1 immune responses and it is also closely connected to mechanism of cell death, given this cytokine is intrinsic ability to activate caspases and thus induce apoptosis [Reviewed by 18]. This topic was considered in a previous study, where *M. avium*-induced macrophage apoptosis was dependent on the function of TNF-α because it was inhibited by the presence of anti-TNF-α antibodies [5]. In fact, true TNF-α bioactivity was actually reduced in supernatants from *M. tuberculosis*-infected cell cultures due to neutralization when soluble TNFR2, but not TNFR1, was released during macrophage infection [Reviewed by 6]. Accordingly, we observed a significant and progressive increase in the levels of TNF-α and IL-1β during in vitro BCG infection of monocytes from

HD individuals that was consistent with the increased rate of apoptosis in this group. This phenomenon was also supported by the fact that the apoptosis levels were not dominant in the immature, naïve group. There, TNF-α level is unchanged, while IL-1β tends to increase over the time during BCG infection. It is noteworthy that at 24 h of infection, both groups produced comparable IL-1β levels (HD = 757.7 ± 258 pg/mL vs. UV = 676.8 ± 124 pg/mL; p = n.s.).

There are both quantitative and qualitative differences between monocytes from newborns and adults. Qualitative differences are evident in utero, as human fetal circulating monocytes reveal reduced levels of MHC class II molecules. Also, the addition of endotoxin to whole cord blood from human newborns results in diminished production of TNF when compared with adult peripheral blood [19]. Indeed, newborn-derived monocytes cultured in whole blood or purified and cultured in autologous, newborn blood plasma show a 1-3-log impairment in TNF production in response to agonists of toll-like receptors [Reviewed by 16]. Thus, it has been confirmed that cells from umbilical cord produce fewer cytokines, such TNF-α, when compared to adult cells [19]. Another pattern was found when studying MMP-9 levels induced by BCG-infected monocytes. MMP-9 is a metalloproteinase with pro-inflammatory properties and some specific functions, such as a reducing response to IL-2, generating similar fragments of angiostatin, having a high affinity for collagen, and stimulating secretion of cytokines, among them TNF-α and IL-1β [20]. Strikingly, virtually no production was found only in the naïve group, but again BCG was not able to distinguish resting, baseline levels found in the HD group. This observation can also be explained by circulating immature cells of the naïve group, as opposed to the already sensitized adults, to promptly produce MMP-9. As expected, this pattern was in agreement with the in-gel gelatin data, although those techniques are not related, and thus, the results are not directly compared because they have distinct sensitivities. On the other hand, Quiding-Jarbrink and colleagues in 2001 [20] showed increasing rates of MMP-9, but this may be related to different MOI ratio between theirs and the present study (10:1 vs. 2:1 respectively).

In summary, one could conclude that the necrosis pattern found in monocytes from naïve group correlates well with IL-1β levels, but not with TNF-α and MMP-9, induced when those cells are BCG infected. Additional studies are warranted to rule out other mechanisms, such as pyroptosis. These findings support the hypothesis that BCG Moreau strain induces distinct cell-death patterns involving maturation of the immune system and that this pattern might set the stage for a subsequent antimycobacterial immune response, which may have profound effects during vaccination.

Acknowledgments

The authors are grateful to Dr. Stuart Krassner (UCI, Irvine, USA) for text editing. We also thank Paulo Redner and Ariane L. de Oliveira (Leprosy Laboratory, IOC/FIOCRUZ), and Luana T.A. Guerreiro and Prof. Dasio Marcondes (Gaffree Guinle State University Hospital) for their help during technical procedures. This work was partly supported by IOC/FIOCRUZ; CJAS is the recipient of a FAPERJ scholarship, DPHS is the recipient of PEC/FIOCRUZ scholarship, and PRZA is granted with a CNPq research fellowship (PQ-2).

This work was presented at the 2010 Keystone Vaccine Symposium, Oct 27–Nov 01, 2010, Seattle, USA. Abstract # 109.

Conflict of interest statement: None declared.

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