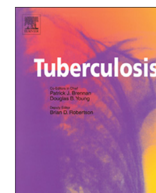
Tuberculosis 94 (2014) 207–218

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



IMMUNOLOGICAL ASPECTS

Differentiation of human mononuclear phagocytes increases their innate response to *Mycobacterium tuberculosis* infection

Diana Castaño [a,c], Luis F. García [a,c], Mauricio Rojas [a,b,c,*]

[a] *Grupo de Inmunología Celular e Inmunogenética, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia*
[b] *Unidad de Citometría de Flujo, Sede de Investigación Universitaria, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia*
[c] *Centro Colombiano de Investigación en Tuberculosis, Medellín, Colombia*

ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form
20 December 2013
Accepted 8 January 2014

Keywords:
Mycobacterium tuberculosis
Monocyte
Macrophage
Mononuclear phagocyte
Differentiation
Phagocyte heterogeneity

SUMMARY

The heterogeneity of mononuclear phagocytes, partially explained by cell differentiation, influences the activation of innate responses. It has been reported that *Mycobacterium tuberculosis* inhibits monocyte differentiation into either dendritic cells or macrophages. To evaluate whether the activation of effector mechanisms against *M. tuberculosis* differ between less and more differentiated mononuclear phagocytes, we compared monocytes differentiated *in vitro* for 24 h (MON24) and 120 h (MDM120) infected with *M. tuberculosis* H37Rv, H37Ra and the clinical isolate UT127 at different multiplicity of infection. MDM120 phagocytosed more *M. tuberculosis*, inhibited mycobacterial growth and did not die in response to the infection, compared with MON24. In contrast, MON24 become Annexin V and Propidium iodide positive after 36 h of *M. tuberculosis* infection. Although, there were striking differences between MON24 and MDM120, there were also some differences in the response to the mycobacterial strains used. Finally, in MDM120 infected with *M. tuberculosis* H37Rv, a lower percentage of mycobacterial phagosomes accumulated transferrin and a higher percentage co-localized with cathelicidin than in MON24. These results demonstrate that innate responses induced by *M. tuberculosis* depends upon the stage of differentiation of mononuclear phagocytes and support that terminally differentiated cells are more efficient anti-mycobacterial effectors than the less differentiated ones.

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1. Introduction

Tuberculosis (TB) is still one of the main causes of morbidity and mortality worldwide, due to a single infectious agent [1,2]. *Mycobacterium tuberculosis* infects and survives inside macrophages altering different cellular processes; such as inhibiting phagosome–lysosome fusion [3,4], allowing bacilli to remain in compartments with characteristics of early and recycling endosomes, avoiding the activation of bactericidal mechanisms [5,6] and favoring the access to the nutrients and oligoelements needed for its metabolism [7,8]. *M. tuberculosis* is also capable to eventually induces the death of its host cell [9].

Besides being the principal reservoirs of *M. tuberculosis*, macrophages are also considered the first line of defense against this pathogen, as they are the cells responsible for containing mycobacterial replication [10,11]. The ability of mononuclear phagocytes (monocytes and macrophages) to activate innate mechanisms against *M. tuberculosis* depends on the interaction of several factors, such as the genetic background of the host, bacterial virulence and inoculum size [11–13]. Also, there is evidence that the stage of differentiation of the mononuclear phagocytes may affect the activation of an efficient innate response [14–16].

The differentiation of monocytes into macrophages is characterized by changes in the activation of transcription factors [17] and gene expression [18], that results in changes in the expression of adhesion molecules [19], membrane receptors [20], cytokine production and morphology [21,22]. These phenotypic changes occurring during differentiation should be reflected in the effector responses of the mononuclear phagocytes against pathogens. Our group has previously reported that monocytes differentiated in the presence of *M. tuberculosis* have alterations in their differentiation process [23]. However, it still remains unclear how the stage of

* Corresponding author. Sede de Investigación Universitaria, Universidad de Antioquia, Carrera 53 No. 61-30, Laboratorio 420, Medellín, Colombia. Tel./fax: +57 4 219 6463.
E-mail addresses: dmcastano@gmail.com (D. Castaño), lfgarcia@udea.edu.co (L. F. García), mrojaslop@hotmail.com (M. Rojas).

1472-9792/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tube.2014.01.001

differentiation of mononuclear phagocytes influences their effector capacities against *M. tuberculosis* and how it may affect the course of infection.

At the early stages of *M. tuberculosis* infection, resident alveolar macrophages are considered the main effector cells against mycobacteria. In the murine model it has been demonstrated that, besides macrophages, there is a reservoir of immature monocytes in the lung parenchyma [26]; these cells, like the circulating monocytes [24], can be recruited to the site of infection and become infected with *M. tuberculosis* before differentiate into macrophages. It has been reported that in murine pulmonary TB, monocytes ($CD11b^{+/mid}$/$CD11c^{-}$) and undifferentiated macrophages (with low levels of F4/80, CD86, MHC class II and MAC-3 expression) are present in the lungs until 21 days post-infection [24,25]. Thus, it is possible that some monocytes enter into the granulomatous lesions at later time points of the infection, and that some of them may have not completed their differentiation. In the zebra fish model, the circulating monocytes that enter to the site of infection rapidly become part of the granuloma [26,27]. In humans, macrophages obtained from bronchoalveolar lavages of TB patients exhibit characteristics of immature cells [28]. In addition, TB patients have increased numbers of circulating $CD14^{+}CD16^{++}$ monocytes [29] with characteristics of cells at a lower stage of differentiation.

Several studies have contradictory results regarding the capacity of human mononuclear phagocytes to contain mycobacterial replication. Whereas some authors reported that less differentiated mononuclear phagocytes (2 h of culture *in vitro*) are more permissive for *M. tuberculosis* growth than those with 3 or 7 days of *in vitro* differentiation [30]; others have found comparable mycobacterial growth in phagocytes of 2 and 7 days of differentiation [31]. Our group reported a higher replication of *M. tuberculosis* in the U937 promonocytes than in the macrophages derived from this cell line [15], suggesting that the stage of differentiation of the mononuclear phagocytes plays a critical role in the control of the infection by *M. tuberculosis*. Vogt and Nathan have reported differences in the antimycobacterial activity of monocyte-derived macrophages (MDM) with a variety of approaches to the *in vitro* differentiation, such as the source or serum, percentage of oxygen and presence of cytokines and colony stimulating factors (GM-CSF, TNF-α, IFN-γ). Actually it was observed a higher mycobacterial replication in undifferentiated (0 and 3 days) than differentiated cells (7, 14, 21 and 28 days) [32].

The present study aimed to determine whether phagocyte responses against *M. tuberculosis* vary with the host cells stage of differentiation. For this purpose, we compared *in vitro* the activation of innate mechanisms and the anti-*M. tuberculosis* effector capabilities of human monocytes differentiated for 24 h (MON24) and 120 h (MDM120). The results show that MDM120 have a higher expression of phagocytic receptors CD11b, CD11c, CD16, CD18, CD64, CD44 and CD206; MDM120 bind and internalized more latex beads and *M. tuberculosis* H37Rv, H37Ra and the clinical isolate UT127, and more effectively control replication of *M. tuberculosis* than MON24. Control of *M. tuberculosis* replication by MDM120 was neither associated with the level of cytokines (IL-12p70, TNF-α, IL-1β, IL-10 and IL-8), H_2O_2 production, nor with the percentage of acid phagosomes containing *M. tuberculosis*. However, it was associated with a lower percentage of mycobacterial phagosomes that accumulated transferrin and a higher percentage that co-localized with cathelicidin, as well as a less proportion of cell death. These results demonstrate that differentiated human mononuclear phagocytes have more efficient anti-mycobacterial machinery than the less differentiated cells.

2. Materials and methods

2.1. Culture of mononuclear phagocytes and differentiation

Peripheral blood mononuclear cells (PBMC) were isolated from defibrinated blood from healthy individuals by centrifugation on Histopaque-1077 (Sigma Aldrich, St. Louis, MO). PBMCs containing 2.5×10^5 $CD14^+$ cells/ml in RPMI-1640 (Gibco-BRL, Gran Island, NY) supplemented with 0.5% heat inactivated pooled human serum (PHS), were enriched by adherence to plastic plates (Corning Incorporated Life Science, Lowell, MA). Wells were extensively washed to remove non-adherent cells. Adherent cells were cultured in RPMI-1640 supplemented with 10% heat inactivated PHS for 24 h (MON24) and 120 h to allow differentiation into monocyte-derived macrophages (MDM120). At baseline differentiation, more than 90% of the cells were CD14+ (clone RMO52, Immunotech, Beckman Coulter, Miami, FL). Adherent cells numbers were determined after scraping or lysing to count cells and nuclei, respectively, as described [33]. There were not significant changes in the number of adherent cells during 120 h of culture.

2.2. Expression of phagocytic receptors

MON24 and MDM120 monolayers were washed with PBS plus 1% BSA (bovine serum albumin, Sigma–Aldrich) and 0.1% NaN_3 (Sigma–Aldrich), and blocked with PBS plus 2% PHS. Cells were independently stained with anti-CD11b (-PE, clone VIM12, Invitrogen), anti-CD11c (-PE, clone B-ly6, BD-Pharmingen), anti-CD16 (-PE, clone 3G8, BD-Pharmingen), anti-CD18 (-FITC, clone L130, BD-Pharmingen), anti-CD44 (-FITC, clone L178, BD-Pharmingen), anti-CD64 (-PE, clone 10.1, BD-Pharmingen), and anti-CD206 (-RPE, clone 19.2, BD-Pharmingen) or their respective isotype controls for 30 min. Cells were then washed, fixed with 2% PFA for 20 min and scraped with a rubber policeman. Ten thousand cells were acquired in a BD FACS Canto II (Becton Dickinson Biosciences. San Diego, CA). The percentage of stained cells and the mean fluorescence intensity (MFI) were estimated using FlowJo 7.6.1 software (Tree Star, Inc. Ashland, OR).

2.3. Culture of M. tuberculosis and labeling with FDA and CFSE

M. tuberculosis H37Rv and H37Ra, obtained from the Instituto Nacional de Salud (Bogotá, Colombia), and UT127 (LAM family), a drug-sensitive clinical isolate from a Colombian HIV negative TB patient (characterized and followed in a 2–3 years cohort study) [34], were grown in Middlebrook 7H9 liquid media (Becton Dickinson, Cockeysville, MD) supplemented with OADC (Oleic Acid Albumin Dextrose Catalase complex, Becton Dickinson). Mycobacteria were harvested after 3 weeks, extensibility washed with PBS (phosphate-buffered saline, Gibco-BRL) and labeled or not with 250 ng/ml FDA (fluorescein diacetate, Invitrogen, Eugene, OR) or 200 μM CFSE (5-(and-6)-carboxyfluoresceinsuccinimidyl ester, Invitrogen) for 60 min at 37 °C. The CFSE-labeled mycobacteria were killed with 1.4% PFA (paraformaldehyde, Fisher Scientific, Pittsburgh, PA) for 18 h at room temperature. Labeled and non-labeled mycobacterias were slightly sonicated to disrupt clumps at 204,8 Watts (Sonics Vibra Cell, model CV33. Newtown, CT). Mycobacterial suspension was centrifuged for 5 min at $200 \times g$ and aliquots from the supernatants were frozen at −70 °C in a solution containing 20% glycerol. The mycobacterial concentration was calculated by spectrophotometry at 600 nm and verified by counting the number of colony-forming units (CFUs) [11]. The percentage and intensity of FDA and CFSE staining were measured by flow cytometry (FACSort flow cytometer, Becton Dickinson). For

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