MYCOBACTERIUM AVIUM SPP. *PARATUBERCULOSIS* UP-REGULATES CD40 EXPRESSION AND DOWN-REGULATES IL-10 PRODUCTION BY HUMAN MACROPHAGES

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RESUMO

Mycobacterium avium spp *paratuberculosis* (MAP) tem vindo a ser sugerido como um agente etiológico da doença de Crohn. Este estudo teve por objectivo avaliar os efeitos imunomoduladores do MAP em macrófagos humanos. Os macrófagos foram obtidos a partir do sangue de controlos normais ou pacientes com doença de Crohn, e infectados *in vitro* com MAP ou *Mycobacterium avium* spp. *avium* (MA). Os efeitos observados na produção de IL-10 e expressão de CD40 sugerem que a infecção micobacteriana pode contribuir para a imunopatologia observada na doença de Crohn.

PALAVRAS-CHAVE

Doença de Crohn, Macrófagos, *Mycobacterium avium* spp *paratuberculosis*

ABSTRACT

Mycobacterium avium spp *paratuberculosis* (MAP) has been suggested as one etiologic agent of Crohn's disease. In this study, we aimed to evaluate immunomodulating effects of MAP on human macrophages. Macrophages were obtained from the blood of normal controls or Crohn's patients, and were infected *in vitro* with MAP or *Mycobacterium avium* spp. *avium* (MA). The effects observed on IL-10 production and CD40 expression, point to a role of mycobacteria in the pathology of Crohn's disease.

KEYWORDS

Crohn's disease, macrophages, *Mycobacterium avium* spp *paratuberculosis*

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1. INTRODUCTION

Crohn's disease is an inflammatory bowel disease that may affect any part of the digestive tract (from mouth to anus) although, in most cases, the ileum or the first part of the colon are affected (Chacon et al. 2004). Lesions extend through all intestinal linings, differing from ulcerative colitis, another form of inflammatory bowel disease. Since this pathology was first described, the similarity of Crohn's disease with intestinal tuberculosis and with Johne's disease (a pathology affecting ruminants caused by *Mycobacterium avium* spp *paratuberculosis* (MAP)), led to the hypothesis that this mycobacterium could be the etiologic agent of Crohn's disease. In fact, not only ruminants can be infected with MAP; other mammals, as deers, rabbits, sheep, and even primates (Glawischnig et al. 2006; Judge et al. 2005; McClure et al. 1987) are also affected. An increasing number of genetic loci associated with Crohn's disease, suggests that the genetic background of an individual is determinant in the development of the disease (Van Limbergen et al. 2009), although environmental conditions are also involved.

Through the years, it has been difficult to prove Crohn's disease etiology. Numerous reports have shown an association between MAP colonization and the disease (Abubakar et al. 2008; Feller et al. 2007). However, the difficulty in MAP detection and the results of clinical antimicrobial trials, have been unable to help clarify MAP's role in Crohn's disease.

It is known that pathogenic mycobacteria are able to survive for a long time inside macrophages, while an inflammatory response is mounted by the host in an attempt to control the infection. It is possible that the inflammatory response to MAP might be exacerbated in susceptible human hosts, leading to an inflammatory disease, as it is the case for Johne's disease (Chacon et al. 2004).

IL-10 is a key cytokine in the control of inflammatory responses by the host, as it down-regulates Th1 responses, and co-stimulatory molecules in macrophages. It is produced by monocytes and macrophages, Th2 and Treg lymphocytes, NKT cells and activated B lymphocytes.

It was reported that Crohn's disease patients (mainly subjects homozygous for 3020insC NOD2 mutation and subjects with severe types of Crohn›s disease) have an impaired ability to produce IL-10, and that this impairment may be important in the exacerbation of inflammation observed in these patients (Correa et al. 2009; Marrakchi et al. 2009; Noguchi et al. 2009). In bovine macrophages, MAP has been reported to increase IL-10 production locally, and to take advantage of the low inflammatory response in order to grow (Khalifeh and Stabel 2004). MAP up-regulated IL-10 production by human mononuclear cells isolated from controls and Crohn›s patients bearing wild type NOD2 (Ferwerda et al. 2007). However, this increase in IL-10 production was abrogated in patients homozygous for 3020insC NOD2 mutation. Induction of IL-10 by MAP during infection of human mature macrophages was still not reported.

CD40 is a surface receptor of monocytes, macrophages, B and T lymphocytes and dendritic cells. It binds CD154 (CD40L), a receptor for co-stimulatory signal for B cells, T cells and other cell types (van Kooten and Banchereau 2000). It is a key molecule in activation of immunity and inflammation, and it is known to be over-expressed in inflammatory bowel disease patients (Battaglia et al. 1999; Danese et al. 2006; Polese et al. 2002).

In this study we assayed MAP-induced IL-10 production and CD40 expression in human peripheral blood monocyte-derived macrophages obtained from normal controls or from

patients with Crohn's disease. We aimed to evaluate possible immunomodulating effects of MAP that could have a role in the pathogenesis of Crohn's disease.

2. MATERIALS AND METHODS

2.1. PATIENTS AND SAMPLES

In this study, 38 participants were included: 19 with Crohn's disease and 19 healthy individuals. Informed consent was obtained in accordance with institutional review board regulations at the Universidade Fernando Pessoa and Hospital de São João. The patients with Crohn's disease were recruited from the Gastroenterology Service in Hospital de São João. The diagnosis of Crohn's disease was established on standard clinical, endoscopic, histologic and radiographic criteria. Normal controls (healthy individuals) were recruited from the academic community at Faculdade de Ciências da Saúde, Universidade Fernando Pessoa. Characterization of patients and controls is included in table 1.

Table 1. Patients and controls used in this study.

Group and gender	Age (years)	Disease location	Therapeutics		
			5-ASA	Corticoids	Immunosupression
Crohn's disease			Y	Y	Y
M	28	ileum	Y	Y	Y
F	32	ileum	Y	Y	Y
M	45	ileum+colon	Y	Y	Y
F	31	colon	Y	Y	Y
M	27	ileum+colon	Y	Y	Y
M	24	ileum+colon	Y	N	N
M	24	ileum+colon	Y	N	N
F	28	ileum+colon	Y	N	Y
M	26	ileum+colon	Y	N	Y
M	43	ileum	Y	N	N
F	45	ileum	Y	N	N
F	32	ileum+colon	Y	N	Y
F	33	ileum+colon	Y	N	N
M	23	ileum	Y	N	N
M	31	ileum	Y	Y	Y
F	42	ileum	Y	Y	N
F	62	ileum+colon	Y	N	Y
M	73	ileum+colon	Y	N	Y
F	25	ileum+colon	Y	N	N
Controls					
F	31				
F	20				
M	27				
F	20				
F	20				
F	25				
M	43				
M	29				

F	20
M	30
F	49
F	64
M	38
M	48
M	70
M	53
M	49
F	44
F	36

Whole blood samples (13.5 ml) were obtained from every participant, and isolation of peripheral blood mononuclear cells was performed.

2.2. **CELL CULTURE**

The blood collected from each participant was diluted 1:2 in phosphate buffered saline (PBS) and placed on Histopaque 1077 (SIGMA) in 15 ml sterile tubes. Mononuclear cells were separated according to Histopaque manufacturer instructions. The mononuclear cells present in the monolayer were collected, washed 3x in Hank's Balanced Salt Solution (HBSS, SIGMA), and suspended in RPMI 1640 (SIGMA) supplemented with L-glutamine, antibiotic/antimycotic solution, and 10% heat inactivated fetal bovine serum (FBS). Cells were counted in a Neubauer chamber. One ml of the cell suspension ($3x10^6$ cells/ml) was seeded in each well of 24-well multidish culture plate. Cultures were incubated for 7 days at 37 ºC and 5% CO_2, with the medium changed at day 3 after culture establishment. At day 7, monocytes have differentiated into mature macrophages, and cultures were infected.

2.3. ***IN VITRO*** **INFECTION OF MONOCYTE-DERIVED MACROPHAGES**

Macrophage cultures were either left uninfected or were infected with MAP (ATCC 43015, an isolate from a Crohn's patient), or *Mycobacterium avium* spp *avium* (MA) (strain 101, an isolate from an AIDS patient) at infection ratio of 10 bacteria: 1 cell. Cultures were incubated for 3 hours to allow phagocytosis of the bacteria. After 3h (T0), supernatants were collected from some wells and used for determination of IL-10. Unphagocytosed bacteria were washed off, and cells present in the wells used to collect supernatants were detached by incubation with trypsin-EDTA (SIGMA), for assessment of CD40 expression. Cells in other wells were allowed to incubate for further 3 days (T3) or 7 days (T7). At this time-points, collection of supernatants for IL-10 determination and detachment of cells to assay CD40 expression were also performed.

2.4. **IL-10 DETECTION**

After collection, supernatants were centrifuged at 10 000 rpm for 5 minutes to sediment remaining bacteria. The pellet was rejected and supernatants were aliquoted and stored frozen at -70 ºC until assay performance. IL-10 concentration was measured with human IL-10 ELISA Ready-Set_Go (eBioscience), according to manufacturer instructions.

2.5. **CD40 EXPRESSION**

After detachment from the culture plates wells, macrophages from each well were centrifuged at 200g for 10 minutes and ressuspended in 100 ml PBS. A volume of 3 ml of Fc blocking antibody (IgG2a) (CD32, Santa Cruz Biotechnology) was added, and macrophages were incubated on ice for 10 minutes. A volume of 5 ml of FITC-conjugated human CD40 antibody was then added and cells were further incubated for 30 minutes on ice, in the dark. After this incubation period, cells were centrifuged at 200g for 10 minutes and ressuspended on 300ml PBS supplemented with 3% FCS and 0.1% sodium azide. Acquisition was performed immediately in an EPICS-XL cytometer (Beckman-Coulter).

2.6. **STATISTICAL ANALYSIS**

Differences observed between two different treatments were compared with unpaired T student test. A p value of less than 0.05 was judged to be significant.

3. **RESULTS**

3.1. **IL-10 EXPRESSION BY INFECTED MACROPHAGES**

Peripheral blood monocyte-derived macrophages (PBM) were cultured *in vitro* and infected with MAP or MA. At 3h (T0), day 3 (T3) or day 7 (T7) post-infection, supernatants were removed and IL-10 concentration was determined by ELISA (Fig 1 A and B).

Mycobacterial infection of macrophages obtained from normal controls caused a significant decreased in IL-10 production, at T0 (MAP) and T3 (MAP and MA), as compared to uninfected macrophages. At T7 differences observed were insignificant (Fig.1 A). Both MAP and MA infected macrophages show, however, an increase in IL-10 production at T3 and T7 as compared to T0.

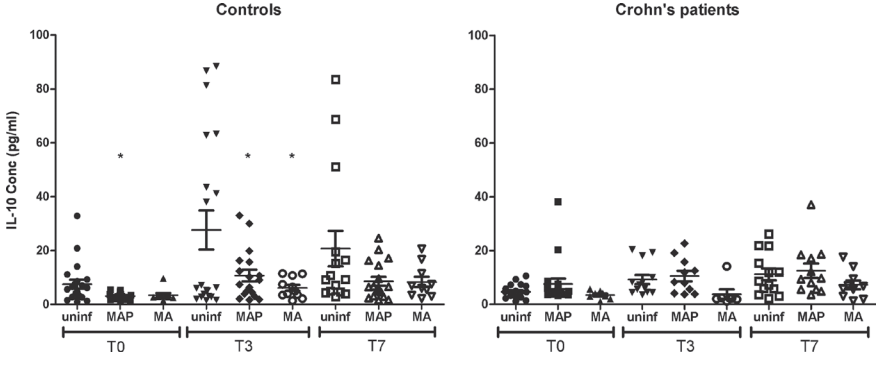

Figure 1. IL-10 production by PBM obtained from normal controls (A) or Crohn's patients (B). At different time-points after infection, supernatants were collected and and IL-10 determination was carried out by ELISA. Results obtained with infected macrophages were compared to those obtained with uninfected cells, at each time-point. *P<0.050.

Uninfected macrophages obtained from Crohn's patients showed decreased IL-10 production as compared to macrophages from normal controls. No differences were observed, in IL-10 production, between uninfected and infected macrophages from Crohn's patients (Fig. 1B).

3.2. **CD40 EXPRESSION BY INFECTED MACROPHAGES**

CD40 expression was measured in macrophages isolated from controls and Crohn's patients (Fig.2). The percentage of CD40 positive cells was above 95% for all situations tested. However, macrophages isolated from Crohn's patients and infected *in vitro* with MAP showed higher CD40 expression at T3 and T7, as measured by FL1 (FITC) median fluorescence intensity. MA infection also induced higher CD40 expression at T7 (Fig.2B). Macrophages isolated from normal controls and infected with MAP showed a significant increase in CD40 expression only at T3.

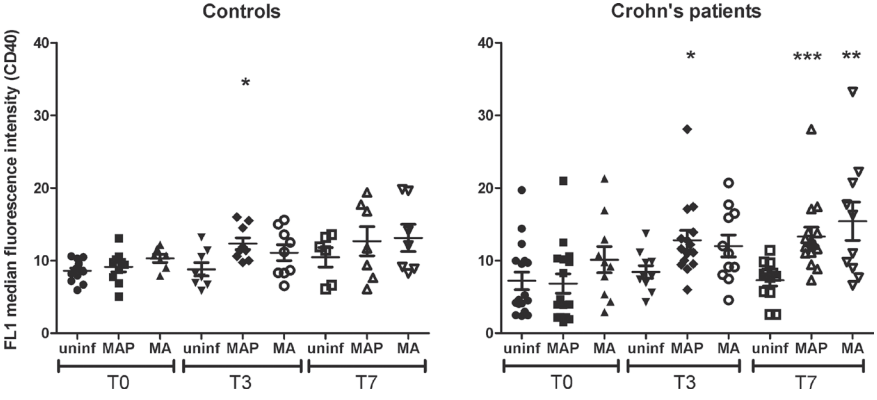

Figure 2. CD40 expression by PBM obtained from normal controls (A) or Crohn's patients (B). Macrophages were detached from multidish culture plates and were stained with FITC-conjugated human CD40 antibody. Results are expressed as FL1 medium fluorescence intensity (CD40 expression). Results obtained with infected macrophages were compared to those obtained with uninfected cells, at each time-point. *P<0.050; **P<0.010; ***P<0.001.

4. **DISCUSSION AND CONCLUSIONS**

The aim of this study was to evaluate imunomodulating effects of MAP infection on human macrophages isolated from normal controls and Crohn's disease patients, in an attempt to further clarify MAP involvement on the pathology of Crohn's disease.

Although Crohn's patients used in this study were under different therapeutic regimens, the results obtained did not differ substantially amongst the patients studied, and so they were grouped together. It is feasible to think that the long cell culture period (7 days of maturation and another 7 days of infection) was sufficient to abolish differences related to therapeutics.

A striking difference was observed in IL-10 production between PBM isolated from controls and from Crohn's patients. In fact, MAP and MA down-regulated IL-10 production by PBM from controls at T0 and T3 of infection, although no effect was observed on PBM isolated

from Crohn' disease patients. However, uninfected PBM from these patients already showed reduced IL-10 expression as compared to PBM from controls, which is in accordance with previous reports (Correa et al. 2009; Marrakchi et al. 2009). In bovine macrophages, MAP was found to stimulate IL-10 production, and it was suggested to be a virulence mechanism, that allowed bacterial proliferation under a down-regulated inflammatory state. This seems not to be the case for human macrophages. On the contrary, we cannot exclude whether MAP can be responsible for the diminished IL-10 production in Crohn's patients, as previous MAP detection studies were not undertaken in those patients.

MAP and MA infection caused a significant increase in CD40 expression by PBM isolated from Crohn's patients. Only a transient increase at T3 was observed in MAP-infected PBM from controls. Uninfected macrophages from both controls and Crohn's patients exhibited comparable CD40 expression. CD40 expression is important to the establishment of humoral and cell-mediated immunity, as well as inflammation, and it is known to be elevated in inflammatory bowel disease patients (both Crohn's disease and ulcerative colitis). Although we did not observe differences on CD40 expression between uninfected PBM from controls and Crohn's patients, the up-regulation observed in infected PBM from Crohn's patients points to a role of mycobacteria in the exacerbation of inflammatory reaction in Crohn's disease.

Finally, MA is a known human pathogen, infecting immunocompromised hosts, as AIDS patients. Inhibition of IL-10 production and up-regulation of CD40 expression occurred in MAP-infected, as well as in MA-infected macrophages. In the experiments here reported, using PBM, we did not observe a differential macrophage response to MAP infection as compared to MA infection. However, as the tropism of MAP organisms to intestinal lamina propria is known (MA intestinal colonization occurs only in disseminated infection of AIDS patients), one cannot exclude the possibility that MAP, but not MA, may be able to proliferate locally in Crohn's patients, and induce a differential inflammatory response. Further studies concerning production of other cytokines and surface markers are needed, using both PBM and intestinal lamina propria macrophages, in order to better evaluate the local inflammatory response to mycobacteria by normal controls and Crohn's patients.

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