CCL20 is overexpressed in *Mycobacterium tuberculosis* infected monocytes and inhibits the production of reactive oxygen species (ROS)

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Summary

CCL20 is a chemokine that attracts immature dendritic cells. We show that monocytes, cells characteristic of the innate immune response, infected with *Mycobacterium tuberculosis* express the CCL20 gene at much higher level than the same cells infected with non-tuberculous mycobacteria. INFγ, a fundamental cytokine in the immune response to tuberculosis, strongly inhibits both the transcription and the translation of CCL20. We have also confirmed that dendritic cells are a suitable host for mycobacteria proliferation, although CCL20 does not seem to influence their intracellular multiplication rate. The chemokine, however, downregulates the characteristic production of reactive oxygen species (ROS) induced by *M. tuberculosis* in monocytes, which may affect the activity of the cells. Apoptosis mediated by the mycobacteria, possibly ROS-dependent, was also inhibited by CCL20.

Introduction

Tuberculosis remains as a leading infectious disease and a major cause of death worldwide [1]. The main route of entry of *Mycobacterium tuberculosis* is via the respiratory track, until it reaches the pulmonary alveoli. Initially the innate immunity may abort the infection, through the activities of alveolar macrophages, and other cells which are recruited like neutrophils and NK cells. When the innate immunity fails the bacteria multiply intracellularly and the adaptive immunity determines the formation of the tuberculous granuloma. The arrival of macrophages and lymphocytes controls the bacterial proliferation, although some bacilli will survive in a latent form. Early in the primary infection of a "naïve" host bacteria are transported to regional lymph nodes, causing an intense reaction. The granulomatous reaction and necrosis in the lymph nodes are known as the Ranke complex, characteristic of tuberculosis in childhood [2]. The main candidates to carry the pathogen to the lymph nodes are macrophages and dendritic cells. Dendritic cells are specialized for the presentation of antigen to T cells and have been observed in the tuberculous granuloma, apparently migrated from the peripheral blood [3]. Once infected, they may be attracted by chemokines to the lymph nodes and become a reservoir for mycobacteria [4]. Chemokines are small chemotactic cytokines produced by many cellular types, including *M. tuberculosis* infected macrophages which express members of the chemokine ligand family like CCL2, CCL3, CCL4 and CCL5 [5].

Förstch *et al.* found that dendritic cells are appropriate hosts for *M. tuberculosis* in which bacilli multiply [6]. At the beginning of the infection bacteria reach the pulmonary alveoli where dendritic cells may find and phagocyte them. The epithelial transmigration of dendritic cells into the alveoli seems to be determined by CCR6, as suggested by experiments made in $CCR6^{-/-}$ mice [7]. The only known ligand for this

receptor is CCL20, which is expressed constitutively in many cell types and plays an important role in the immunity of mucosal-associated tissues, including lung and gastric mucosa [8]. When dendritic cells respond to a pathogen they mature and the expression of CCR6 is down-regulated. Instead, mature dendritic cells express CCR7, and its ligands, CCL19 and CCL21 promote their migration to the lymph nodes [9]. A recent report has shown that CCL20 is expressed during human tuberculosis, and macrophages from patients activated with an antigen from *M. tuberculosis* produce a higher amount of the chemokine than macrophages from healthy volunteers [10]. Furthermore, several studies have shown that other bacteria regulate the transcription of the *CCL20* gene [11-13].

Besides their chemotactic role, chemokines exhibit several other functions including leukocyte degranulation, NK cells proliferation, dendritic cell maturation, B and T cell development, angiogenesis or tumor growth [9;14]. Pervushina *et al.* have recently reported that the chemokine PF4 (CXCL4) induces the generation of reactive oxygen species (ROS) metabolites in monocytes [15]. Although ROS production is not associated to killing of *M. tuberculosis* [16], it may have a large influence in the macrophage signaling [17] and function. Thus, it has been known for a long time that ROS induces apoptosis [18], and a relevant example is the programmed cell death mediated by the strong generation of ROS in neutrophils infected with *M. tuberculosis* [19]. Apoptosis has been regarded as a host mechanism of defence because H37Rv, a virulent strain of *M. tuberculosis*, induces less apoptosis in macrophages than H37Ra, which is avirulent. It has also been argued that apoptotic infected cells slow the dissemination of the bacteria and that these cells are phagocyted by fresh macrophages which may be active against the bacteria [20].

In the present study we show that *M. tuberculosis* increases dramatically the expression of *CCL20* in human monocytes, even at a higher degree than other non-tuberculous mycobacteria. We have confirmed that the bacteria survive in dendritic cells, although CCL20 does not seem to promote an antimycobacterial activity. We have found, however, that CCL20 inhibits the generation of ROS, which may affect the activity of infected macrophages. Additionally, CCL20 inhibits the apoptosis mediated by *M. tuberculosis*, possibly as a consequence of the downregulation of ROS production.

Materials and methods

Bacterial strains

Mycobacterium tuberculosis HL186T, *M. kansasii* HL228K and *M. avium* HL70A were isolated at the Hospital de León (Microbiology Service) and kindly provided by Julio Blanco and Manuela Caño. They were grown on 7H11 agar supplemented with 0.2% glycerol and 10% Middlebrook enrichment OADC (Becton Dickinson Microbiology Systems, San Agustín de Guadalix, Madrid, Spain). *Legionella pneumophila* Philadelphia, ATCC 13151, generously provided by Carmen Pelaz, was grown on buffered charcoal yeast extract (BCYE) agar plates. Bacteria from fresh culture in agar plates were suspended in the serum free medium Macrophage-SFM (GIBCO, Invitrogen, Prat de Llobregat, Barcelona, Spain). To obtain isolated mycobacteria, they were sonicated using an S-450 digital ultrasonic cell disruptor (Branson Ultrasonics, Danbury, CT, USA). Pulses of 10 s were applied with a microtip at an amplitude of 10% (2 W), and sonicated bacteria were centrifuged at 100 × g for 1 min at room temperature. After recovering the supernatants, sonications were repeated as many times as necessary to obtain individualized bacteria, usually three or four rounds. At the end most bacteria were alive and very few groups remained, with ≤ 5 bacteria per group, as

determined by the LIVE/DEAD Baclight bacterial kit (Molecular Probes, Invitrogen, Prat de Llobregat, Barcelona, Spain). This treatment was not necessary for *Legionella*. After addition of glycerol to 20%, single use aliquots were frozen at – 80ºC.

Monocytes, human monocyte-derived macrophages and dendritic cells

Peripheral blood was obtained from healthy volunteers following informed consent and approval of the protocol by the Hospital of León Clinical Research Ethics Board, and each experiment was performed with cells from a different volunteer. Peripheral blood mononuclear cells were isolated by Ficoll-Paque Plus density gradient sedimentation (GE Healthcare, Life Sciences, Uppsala, Sweden), and $CD14^+$ cells (monocytes) were purified by magnetic cell separation (StemCell Technologies, Grenoble, France). We ascertained the purity of cells by flow cytometry with appropriate labelled antibodies (Becton Dickinson) and > 94 % of cells in the monocyte preparation were $CD14^+$. For studies of ROS formation excluded $CD14^-$ cells were used. The number of cells was calculated by counting in a Neubauer chamber and were cultivated, within 4 hours from blood collection, in the serum free medium Macrophage-SFM.. For antimicrobial activity assays monocytes were differentiated to macrophages for five days. Dendritic cells were also obtained by incubation of monocytes for five days in the presence of 20 ng/ml IL-4 ($\geq 5 \times 10^6$ units/mg), 10 ng/ml GM-CSF ($\geq 1 \times 10^7$ units/mg) and 5 ng/ml TGFβ1 ($\geq 2 \times 10^7$ units/mg). All cytokines were from Peprotech (London, UK). We checked the differentiation process by CD209 (DC-SIGN) expression. Cells changed from $CD14^+$ $CD209^-$ to > 98% $CD14^-$ $CD209^+$ (labelled antibodies from Becton Dickinson). All cells were incubated at 37ºC in 95% air/5% CO_2.

Cellular infection

For antimicrobial activity determination, macrophages and dendritic cells were differentiated as indicated above for five days in 96-well plates, always in a total volume of 100 μl. 10^5 cells were infected with 10^3 bacteria (Multiplicity of Infection, MOI = 0.01) in Macrophage-SFM. For dendritic cells, at the moment of infection fresh IL-4, GM-CSF and TGFβ1 were added. When indicated, cells were incubated in either 20 ng/ml of CCL20 or 100 ng/ml of IFN-γ (> 2×10^7 units/mg, Peprotech). Cells were lysed after four days by sonication with a microtip, at an amplitude of 10% (2 W) for 3 s, to release bacteria. At this setting, ultrasounds were able to lyse cells without affecting the bacterial viability. Decimal dilutions of the sonicates were inoculated and incubated at 37ºC in either BCYE agar plates (*Legionella*) for four days or 7H9 broth supplemented with 0.2% glycerol and 10% Middlebrook enrichment ADC (mycobacteria) for ten or less days. CFU were determined for mycobacteria under an inverted microscope at × 100 magnification [21].

For total RNA purification, and supernatant recovery for chemotaxis experiments and CCL20 quantification, immediately after purification, 5×10^5 monocytes were infected with 5×10^5 bacteria (MOI = 1) for 18 hours in a volume of 1,100 μl (24-well plates). When indicated, 100 ng/ml of IFN-γ were added.

Quantitative polymerase chain reaction (qPCR)

Total RNA from infected cells was prepared using the Ultraclean Tissue RNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, US), and reverse transcribed into cDNA by qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD; US). Real-time PCR was performed on a Bio-Rad iCycler system (El Prat de Llobregat, Barcelona, Spain) using SYBR-Green (Molecular Probes, Invitrogen, Prat de Llobregat, Barcelona, Spain). The threshold cycle (Ct) values for each of the target genes were normalized to the Ct of the reference gene *EF-1α* (elongation factor 1α). The efficiency (E) of the

PCR reaction for each gene was calculated using the slope of the standard curve obtained from the Ct of 1/8 dilutions of each amplicon ($E = 10^{-1/slope}$). Gene expression in infected cells (test samples) relative to non-infected cells (control sample), was calculated as the following ratio [22]: $[(E_{ref})^{Ct\ test}/(E_{target})^{Ct\ test}]/ [(E_{ref})^{Ct\ control}/(E_{target})^{Ct\ control}]$. Although data did not follow a normal distribution, a log transformation allowed statistical parametric testing. The primers used for *EF-1α* and *CCL20* were as follows: *EF-1α* forward 5'-TGTTCCTGTTGGCCGAGTG-3'; reverse 5'-ATTGAAGCCCACATTGTCCC-3'; *CCL20* forward 5'-GGCTGCTTTGATGTCAGTGC-3'; reverse 5'-GATGTCACAGCCTTCATTGGC-3'.

CCL20 and CCL2 quantification

To remove bacteria from supernatants, samples were centrifuged for 3 min at 8000 × g at room temperature in ultrafree-MC filter units (Millipore Iberica, Madrid, Spain) of 0.45 μm and frozen at –80ºC. CCL20 was quantified by the human CCL20/MIP-3α DuoSet ELISA development system (R&D systems, Minneapolis, MN, USA) and CCL2 by the BD OptEIA human MCP-1 ELISA set (Becton Dickinson).

Chemotaxis

5×10^4 monocyte-derived dendritic cells, differentiated as indicated above, were suspended in Macrophage-SFM and placed in BD Falcon Cell Culture Inserts (pore size 8.0 μm) in a volume of 200 μl (24-well plates, Becton Dickinson). The lower wells were supplied with 750 μl of either supernatants from infected cells or 10 ng/ml of CCL20. As controls we included supernatants of non-infected cells or medium without CCL20, respectively. When indicated, 6 μg of anti-CCL20 neutralizing antibody or 2 μg of anti-CCL2 neutralizing antibody (both rabbit polyclonal, Peprotech) were added. As a mock control 6 μg of a purified rabbit polyclonal anti-GST antibody (obtained at

our laboratory) were used. Plates were incubated for 16 hours at 37ºC. Migrated cells to the lower wells were counted under an inverted microscope. For statistical analysis, the migration index was calculated by dividing the number of migrated dendritic cells upon treatment by the number of migrated cells in controls.

Analysis of reactive oxygen species (ROS) formation

Intracellular production of ROS was measured as luminol-enhanced chemiluminiscence. Either 10^5 or 5×10^5 bacteria (MOI = 1 or 5, respectively), luminol 500 μM (Sigma-Aldrich Spain, Tres Cantos, Madrid, Spain) and Hanks balanced salt solution were added to 10^5 monocytes in a total volume of 100 μl, and incubated for 30 min at 37ºC. For some treatments 20 ng/ml CCL20, 150 nM phorbol-12-myristate-13-acetate (PMA, Calbiochem, Merck Spain, Madrid, Spain), 3 mg/ml Zymosan (Sigma-Aldrich Spain) or 10^5 $CD14^-$ mononuclear cells were added. Emitted light was measured at 21ºC at 30 min in a TopCount-NXT microplate scintillation and luminescence counter (Packard, Perkin Elmer España, Tres Cantos, Madrid, Spain) and ROS formation was expressed as counted photons per second (cps).

Apoptosis quantification

Apoptosis was detected with the fluorescein FragEL DNA fragmentation kit (Calbiochem), which is based on the fluorescent labeling of DNA ends cleaved during apoptosis. 2×10^5 cells were seeded on sterile 11 mm diameter cover glasses, infected with 10^6 bacteria, and incubated in 24 well-plates. When indicated 20 ng/ml CCL20, 5 μM diphenyleneiodonium chloride (DPI, Sigma Aldrich Spain), 6 μg anti-CCL20 antibody or 6 μg anti-GST mock antibody were added. The infection was stopped at 20 h by removal of the culture medium and fixing in 4% formaldehyde/phosphate buffered saline for 15 min. Preparations were conserved in 80% ethanol at 4ºC. Next day cells

were labelled according to manufacturer's instructions, and the total number of cells was determined by DNA fluorescent staining with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI). The proportion of apoptotic cells from DAPI stained cells was determined by fluorescent microscopy, and expressed as percentage.

Statistical Analysis

CFU and relative gene expression data followed a normal distribution when log transformed. Normally distributed data was analyzed by paired Student *t*-test or ANOVA. Pairwise comparisons for ANOVA were performed by HSD Tukey´s tests or Dunnett's T3 tests when variances were not homogeneous. Data non-normally distributed were analyzed by Kruskal Wallis test and the corresponding pairwise comparisons by Dunn's test. In all cases, a p value < 0.05 was considered significant. Analysis was performed with SPSS v. 14.0 (SPSS Ibérica, Madrid, Spain) and G-Stat v. 2.0 (Dunn's test, GlaxoSmithKline, Brentford, Middlesex, UK).

Results

CCL20 gene is differentially expressed in monocytes infected with tuberculous and non-tuberculous mycobacteria

In the initial steps of an infection the first response corresponds to the innate immunity, represented at the cellular level by monocytes/macrophages, neutrophils and NK cells. The main *M. tuberculosis* cellular targets are macrophages, which upon infection produce many cytokines that influence the immune response. CCL19, CCL20 and CCL21 may be very important at the beginning of the development of the disease. We analyzed them in monocytes infected with MOI = 1 and found no expression of *CCL21*, and no expression differences between resting monocytes and infected monocytes for *CCL19* (data not shown). On the contrary, the small basal level of expression of *CCL20*

in resting monocytes increased dramatically when they were infected with *M. tuberculosis*, ranging the increase from 4 to 39 fold (Fig. 1A). To investigate whether the observed increase was specific of *M. tuberculosis* we analyzed *CCL20* expression in monocytes infected with other non-tuberculous mycobacteria, namely *M. kansasii* and *M. avium* and observed that in both cases this gene was expressed at a much lower level. Although gene expression was higher in *M. kansasii* than in *M. avium* infected monocytes, no statistically significant differences were found between any of them and non-infected macrophages (Fig. 1A).

The importance of IFNγ in the immunity to tuberculosis is widely acknowledged [23]. We used this cytokine to activate monocytes in our preliminary experiments and soon observed that it inhibited the constitutive expression of *CCL20*. In fact, this effect was complete for one of the samples in which the gene expression was totally abolished in non-infected macrophages (data not shown). In other samples inhibition of *CCL20* expression in the presence of IFNγ also occurred, although it did not reach the low level of expression in non-infected cells (Fig. 1A).

Another cell that may contribute to the innate response in tuberculosis is the NK. Esin *et al.* have recently shown that NK cells ($CD56^+CD3^-$) bind directly to mycobacteria [24]. Indeed, we observed that *M. tuberculosis* also induced the activation of *CCL20* transcription in $CD56^+$ cells (data not shown).

CCL20 is produced by *M. tuberculosis* infected monocytes

Although the expression of *CCL20* is constitutive in monocytes, we were not able to detect the protein in many of the culture supernatants. CCL20 was, however, present in all supernatants of *M. tuberculosis* infected monocytes and in three and two out of five supernatants of *M. kansasii* and *M. avium* infected monocytes, respectively. In the presence of IFNγ the median of CCL20 production by infected monocytes was smaller.

Significant differences in the amount of CCL20 were only observed between cells infected with *M. tuberculosis* and both non-infected cells and infected cells in the presence of IFNγ (Fig. 1B). These results closely mirrored the observed *CCL20* expression: higher gene expression corresponded to higher protein production.

CCL20 influences the migration of immature dendritic cells

CCL20 represents a link between the innate and the adaptive immunity, because it attracts immature dendritic cells, which presents antigens to lymphocytes. Supernatants of infected monocytes were used to test the chemotactic activity of secreted CCL20 in monocyte-derived dendritic cells. We did not find significant differences in the migration index of dendritic cells exposed to supernatants of monocytes infected with either *M. tuberculosis* or *M. kansasii* as compared with non-infected cells. Nevertheless, the index value for both mycobacteria was higher than for non-infected cells, following the pattern of CCL20 production. Anti-CCL20 antibodies seemed to block the activity present in supernatants from *M. tuberculosis* infected monocytes, which suggests that in cell supernatants CCL20 is the most abundant chemokine which is active for dendritic cells. This observation provides an appropriate control of the bioactivity of the CCL20 protein detected by ELISA. As an additional control we have used a mock antibody (a non-specific polyclonal rabbit antibody), and observed that it did not inhibit the cell migration. Perhaps the best studied CC-chemokine in tuberculosis is CCL2, also known as MCP-1 [25], which also attracts immature dendritic cells [26]. We verified by ELISA the presence of CCL2 in supernatants of *M. tuberculosis* infected monocytes (366·9 SD 274·6 pg/ml, n = 3), but blocking of the chemokine with a neutralizing antibody did not produced any change in the chemotactic index (Fig. 2A). This data supports the evidence of the predominant role of CCL20 in the chemotaxis of dendritic cells in our model. A surprising result was the induction of a similar migration index by

supernatants from *M. kansasii* and *M. tuberculosis* infected monocytes, despite the strong differences in the production of CCL20 depending on the infecting bacteria. Because results did not reach statistical significance this observation may not correspond to a true biological effect. But it is also possible that *M. kansasii* induces the expression of an unknown chemokine responsible for this migration index.
When we added 10 ng/ml of purified CCL20 to the same dendritic cells, and blocked it with anti-CCL20 antibodies, we did obtain migration indexes significantly different (Fig. 2B). We therefore think that the reason that explains the small migration index observed when using supernatants is the low amount of CCL20 present (<100 pg/ml), enough to appreciate differences, although not statistically significant.

CCL20 does not augment antimicrobial activity of either macrophages or dendritic cells

Chemokines are known to have biological activities other than chemotaxis. We asked whether the increase of CCL20 production was a defence mechanism of infected cells that might promote increased antimycobacterial activity. Alveolar macrophages are critical for the innate immune response, and their production of CCL20 may be important for the recruitment of immature dendritic cells, which would be increasingly exposed to the chemokine. To test whether CCL20 have any influence in the intracellular multiplication of mycobacteria, macrophages and dendritic cells were obtained in vitro from monocytes and their killing activities in the presence of 20 ng/ml of CCL20 were measured. To mimic as closely as possible the natural course of infection, a very low MOI (0,01) was used. The macrophage antimicrobial activity was assessed with *L. pneumophila* infections which allowed us to confirm that IFNγ activated macrophages were able to restrict bacterial growth. We already knew that the CCL20 used was biologically active because it promoted migration of dendritic cells

(Fig. 2B). All three *M. tuberculosis*, *M. kansasii* and *M. avium* multiplied intracellularly in both macrophages and dendritic cells, reaching statistical differences for growth of *M. tuberculosis* in both macrophages and dendritic cells, and for non-tuberculous mycobacteria in dendritic cells. Addition of CCL20 did not decrease, however, the intracellular multiplication rate of the bacteria. On the contrary, CCL20 seemed to slightly favour the proliferation of bacteria, although differences were only marginal and statistically not significant when compared with cells infected in the absence of the chemokine (Table 1). Therefore, CCL20 does not seem to play any role in the antimycobacterial activity of human macrophages and dendritic cells.

CCL20 inhibits the *M. tuberculosis* mediated generation of ROS

The generation of ROS is among the cellular functions that may be influenced by chemokines like PF4, which has been found to promote ROS production in monocytes. We tested whether the well characterized induction of ROS production by *M. tuberculosis* could be increased in the presence of CCL20. Surprisingly, we observed that the effect of CCL20 was in fact inhibitory (Table 2). At MOI = 1 there was an increase in the production of ROS above basal levels. Although CCL20 inhibited the total amount of ROS generated, differences did not reach statistical significance. Only when MOI = 5, with a higher production of ROS, the inhibition of CCL20 was more noticeable and statistically significant. This and much larger MOI will occur in vivo when the innate response has failed, and the bacterial load increases progressively. At this point we hypothesized that other strong inducers of ROS would suffer similar levels of inhibitions and tested the phorbol ester PMA and the particulate stimulus zymosan. Indeed, the amount of detected ROS was much larger, but the level of inhibition by CCL20 was relatively smaller as compared with inhibition in infected monocytes and statistically not significant. These results suggest that the level of inhibition of CCL20

was dependent on the inducer, and that *M. tuberculosis* mediated production was particularly affected. We remained concerned, however, about the purity of the monocyte preparation, because we have indicated that monocytes were < 100% of the total number of cells. Most of the contaminating cells in the purification of monocytes are lymphocytes, which are known to express CCR6, the receptor of CCL20. The addition of 10^5 $CD14^-$ mononuclear cells, however, did not change markedly the amount of both ROS production and inhibition. We conclude that the inhibition of ROS formation was not mediated by $CD14^-$ cells. A stimulus comparable to *M. tuberculosis* is *L. pneumophila*. The level of ROS production was lower and we observed again an inhibition, although it was not statistically significant.

CCL20 inhibits *M. tuberculosis* mediated apoptosis

Although highly produced in monocytes infected with *M. tuberculosis*, ROS does not promote antimycobacterial activity. Nevertheless, it may have a large influence in other biological mechanisms like apoptosis, which has been described to be mediated by ROS in some circumstances. We decided to test the development of apoptosis mediated by *M. tuberculosis* under the influence of CCL20. We first confirmed that *M. tuberculosis* induces statistically significant levels of apoptosis in monocytes and, as expected, the addition of CCL20 inhibited apoptosis to levels close to the observed for non-infected cells. The inhibition induced by CCL20 was blocked by a neutralizing antibody but not by a non-specific mock antibody. Furthermore, the NADPH oxidase inhibitor DPI also decreased the number of apoptotic cells infected with *M. tuberculosis* (Fig. 3). These results suggest that also in monocytes apoptosis is dependent on ROS production, and inhibited by CCL20.

Discussion

Approximately 10% of people who become infected with *M. tuberculosis* will develop the disease. The rest are able to control tuberculosis through an efficient adaptive immunity [27] which, in the first stages, needs the presentation of bacterial antigens by either macrophages or dendritic cells to lymphocytes. There are evidences in the mouse model that infected dendritic cells, rather than macrophages, disseminate to draining lymph nodes [28]. Immature dendritic cells transform into mature cells after phagocytosis of bacteria. They begin to express CCR7, the specific receptor of CCL19 and CCL21 [9]. Infected cells do not seem to present antigen in the lungs but in the lymph nodes where they migrate through a CCL19/CCL21 dependent mechanism [29]. We have not detected the expression of CCL21, and very low levels of CCL19 which, in any case, was not differentially expressed when monocytes were infected with mycobacteria (data not shown). Nevertheless, immature non-infected dendritic cells do not express CCR7. Instead, they express CCR6, the receptor of CCL20 [8], the chemokine responsible for transversing the alveolar epithelium [7]. An important source of CCL20 monocytes and Lee *et al.* have showed that an antigen of *M. tuberculosis* activates them to produce the chemokine [10]. They did not test, however, live bacilli. We show that *M. tuberculosis* also induces a marked increase in *CCL20* expression. This induction had been already observed for *M. avium* [30], but we have found that both *M. avium* and *M. kansasii*, another non-tuberculous mycobacteria, induce a much lower expression of the gene. Although CCL20 is constitutively expressed in many cell types, *M. tuberculosis* seems to favour a strong increment of its transcription levels. Furthermore, we have found evidence that the attraction of immature dendritic cells by supernatants of *M. tuberculosis* infected cells was fully abolished by anti-CCL20, suggesting that it is the main chemokine produced for this purpose.

We do not know whether CCL20 represents a successful mechanism in the immune response to tuberculosis, although it participates in the first steps of an adaptive response [8], which is thought to be responsible for the appropriate control of the disease in most of the infected people [27]. When tuberculosis may not be controlled, the ability of *M. tuberculosis* to multiply in dendritic cells attracted by CCL20 may contribute to the survival and dissemination of the bacilli. It has been shown in the murine model that exposure to *M. tuberculosis* determines the accumulation and infection of dendritic cells in the lungs [28]. Nevertheless, there is some controversy regarding the ability of mycobacteria to multiply inside these cells. Förtsch *et al.* [6] and Buettner *et al.* [31] have reported the intracellular proliferation of *M. tuberculosis*. In contrast, these findings were not corroborated by Tailleux *et al*. who concluded that intracellular survival of *M. tuberculosis* was reduced in these cells. They argued that the discrepancy with Förtsch *et al*. might be the consequence of removing the dendritic differentiating agents (IL-4 and GM-CSF) at the moment of infection, which induced a reversion of cells to a macrophage-like phenotype [32]. We have kept, however, the cytokines during the infections, with no adverse effect in the intracellular multiplication of the microorganism. Moreover, we have also determined that non-tuberculous mycobacteria also proliferate, confirming the results obtained for *M. avium* by Mohagheghpour *et al.* [33]. Consequently, the infection of dendritic cells may be advantageous for *M. tuberculosis* in some circumstances.

It is very interesting, although difficult to interpret, the inhibition of *CCL20* expression by IFNγ. Lee *et al*. found that IFNγ increased the production of CCL20 in monocytes activated with the 30-kDa antigen of *M. tuberculosis* [10]. Our results with live bacilli differ, because we observed inhibition of its expression. In fibroblasts, a different cellular model, the production of CCL20 induced by IL-1β is also inhibited by IFNγ

[34]. It is possible that although the production of CCL20 may be important in the onset of the infection, it plays a less significant role afterwards and is downregulated by IFNγ, a critical cytokine in the response to tuberculosis [23].

We have also found that CCL20 affects other biological activities of infected cells, including the inhibition of ROS production. ROS may modulate macrophages by affecting many signalling molecules as has been shown for transcription factors, protein tyrosine kinases, MAP kinases or small GTPases [35]. In fact, experiments with murine macrophages deficient in phagocyte oxidase revealed that the NADPH oxidase activity has a major influence in the transcriptome resulting from *M. tuberculosis* infection [17]. Although the participation of ROS in mycobacteria killing is uncertain, CCL20 inhibition of ROS generation may be very important for other monocyte/macrophage activities. The inhibitory effect of CCL20 is noteworthy considering that other chemokines, including PF-4 (CXCL4), MCP-1 (CCL2), MIP-1α (CCL3) and RANTES (CCL5), enhance ROS production in monocytes [15;36].

Possibly as a consequence of the inhibition of ROS production, CCL20 also inhibits apoptosis in infected monocytes. Although infected murine macrophages have been described to undergo apoptosis through a ROS-independent mechanism [37], we present evidence that this may not be the case in human monocytes, because we show that the NADPH oxidase inhibitor DPI decreases the number of apoptotic cells. A similar situation has been described for *M. tuberculosis* infected neutrophils [19]. Apoptosis has been considered a mechanism of host defence because it denies the bacilli a favourable environment, and because infected apoptotic cells in contact with fresh macrophages reduce the viability of mycobacteria. In this context the inhibition of the apoptotic activity by CCL20 may be viewed as detrimental to the host, because it allows the prolonged survival of the bacillus in its target cell, and the evasion from the

phagocytosis by fresh activated macrophages. In contrast, at high MOI cell death help *M. tuberculosis* exit the macrophage to infect new cells [20]. Therefore, although the inhibition of the apoptotic activity by CCL20 may favour the pathogen, it is difficult to state that this phenomenon is only advantageous to the bacteria.

Other groups have reported that CCL20 is produced in patients that do not control the disease. Lee *et al*. have detected the presence of CCL20 in bronchoalveolar lavage of tuberculosis patients. Moreover human monocyte-derived macrophages from tuberculosis patients produce more CCL20 when activated with the 30-kDa antigen of *M. tuberculosis* than cells from healthy controls [10], which might reflect the expression of a chemokine from patients in which the immune response has failed. This idea is in line with the results obtained by Thuong *et al.*, who analyzed the gene expression profile of monocyte-derived macrophages stimulated with whole cell lysates of *M. tuberculosis* H37Rv. They compared the profiles of macrophages obtained from three groups: volunteers heavily exposed to *M. tuberculosis* with no active disease, patients with pulmonary tuberculosis and patients with meningitis tuberculosis, and found that, among other genes, *CCL20* was more expressed in the stimulated macrophages from patients with any of the two forms of the disease as compared with persons with no active tuberculosis [38]. The correlation between CCL20 production and severity of the disease may not have a cause-effect relationship, but it should not be ruled out that in a chronic disease like tuberculosis CCL20 may have some negative influence.

Although CCL20 may be essential in the development of the adaptive immunity to tuberculosis, which is successful in most cases, there is a possibility that *M. tuberculosis* may benefit from some of the chemokine biological activities. The understanding of the fine regulation of CCL20 production in tuberculosis patients may help to underscore new pathogenic mechanisms of the bacilli.

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Disclosure

All authors declare no conflicts of interest.

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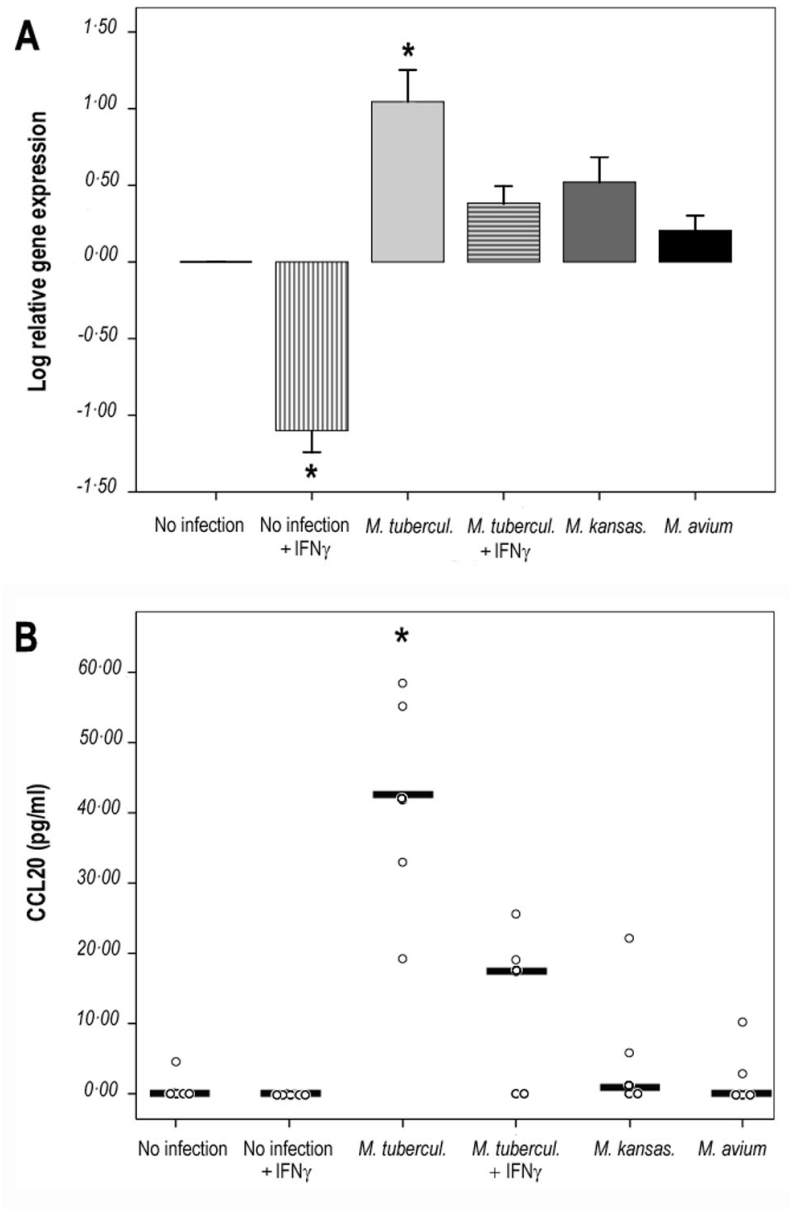

Fig. 1. Measurement of CCL20 expression. (A) Relative gene expression measured by qPCR. Data represent the mean + S.D. of the relative gene expression log in the infected groups in comparison with the control groups (non-infected cells) in monocytes (n = 4). Relative gene expression level in the control groups is always 1·0, its log 0·0, and has no S.D. HSD Tukey's test was performed for pairwise comparisons. (B). Protein expression measured by ELISA. Bars represent the median. Dunn's test was performed for pairwise comparisons. Comparisons vs. "No infection" group with *P < 0·05 is considered significant.

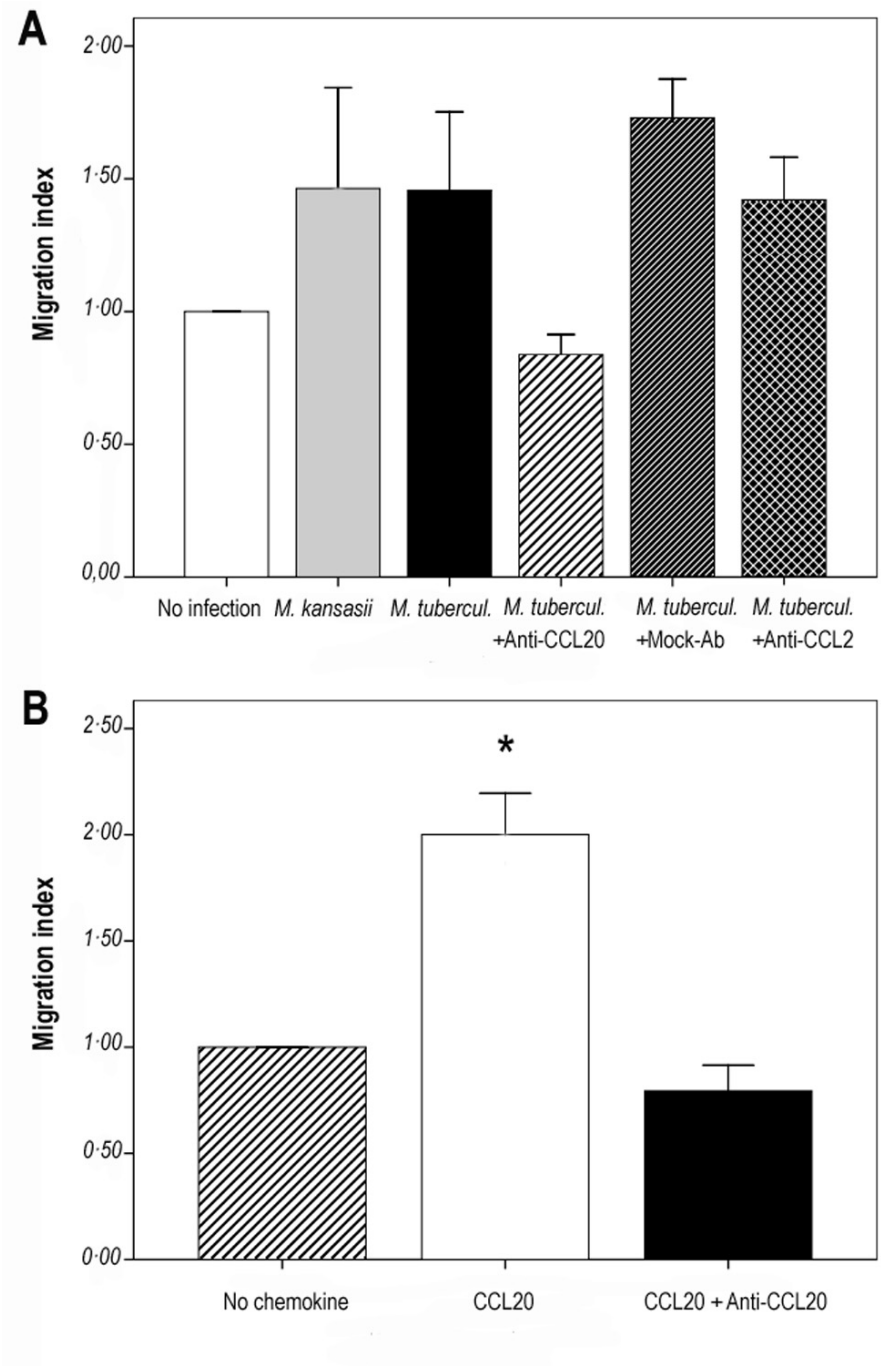

Fig. 2. Chemotactic response of dendritic cells. Data represent the migration index mean + S.D. from supernatants of infected cells (A, n = 3) or from purified CCL20 (B, n = 4). Controls are the supernatant from non-infected cells (A) and medium without chemokine (B), respectively. Migration index in the control group is always 1·00 and has no S.D. Dunnett's T3 test for pairwise comparison in (B) vs. control group and vs. "CCL20 + anti-CCL20" group with $*P < 0{\cdot}05$ is considered significant.

Table 1. Intracellular survival of mycobacteria in macrophages and dendritic cells activated with CCL20

	Inoculated bacteria	Macrophages		Dendritic cells	
		No treatment	Cytokine[a]	No treatment	Cytokine[a]
M. tuberculosis	2·91 ± 0·06	3·52 ± 0·06[b]	3·60 ± 0·12[b]	3·79 ± 0·25[b]	3·83 ± 0·31[b]
M. kansasii	2·78 ± 0·06	3·30 ± 0·25	3·34 ± 0·31	3·37 ± 0·24[b]	3·40 ± 0·24[b]
M. avium	3·08 ± 0·06	3·74 ± 0·28	3·76 ± 0·31[b]	3·83 ± 0·43[b]	3·93 ± 0·51[b]
L. pneumophila	2·75 ± 0·11	6·59 ± 0·32[c]	5·46 ± 0·49[c]		

Data are the average of the number log of CFU ± S.D. from five independent experiments. All ANOVA tests were significant ($P < 0{\cdot}05$). The antimicrobial activity of macrophages was controlled by infection with *L. pneumophila*.

[a] Mycobacteria infected cells were treated with CCL20 and *L. pneumophila* infected cells with IFNγ.

[b] Pairwise comparisons were significant only vs. "inoculated bacteria" ($P < 0{\cdot}05$).

[c] All pairwise comparisons were significant ($P < 0{\cdot}05$).

Table 2. Influence of CCL20 in *M. tuberculosis* mediated ROS production

	No chemokine		CCL20		p
Non-infected monocytes	4 888 ± 4 537				
Non-infected monocytes + PMA	342 154 ± 231 468		329 078 ± 224 854		0·311
Non-infected monocytes + Zymosan	424 823 ± 275 434		403 337 ± 246 498		0·446
M. tuberculosis (MOI 1)	12 965 ± 5 391		11 667 ± 5 161		0·102
M. tuberculosis (MOI 5)	28 360 ± 11 698		17 423 ± 10 492*		0·017
M. tuberculosis (MOI 5) + $CD14^-$ cells	21 759 ± 8 005		16 155 ± 6 458*		0·038
L. pneumophila (MOI 5)	5 771 ± 6 069		4 077 ± 3 669		0.350

Data are the average of cps ± S.D. from four independent experiments.
* Paired Student *t*-test was significant ($P < 0·05$).

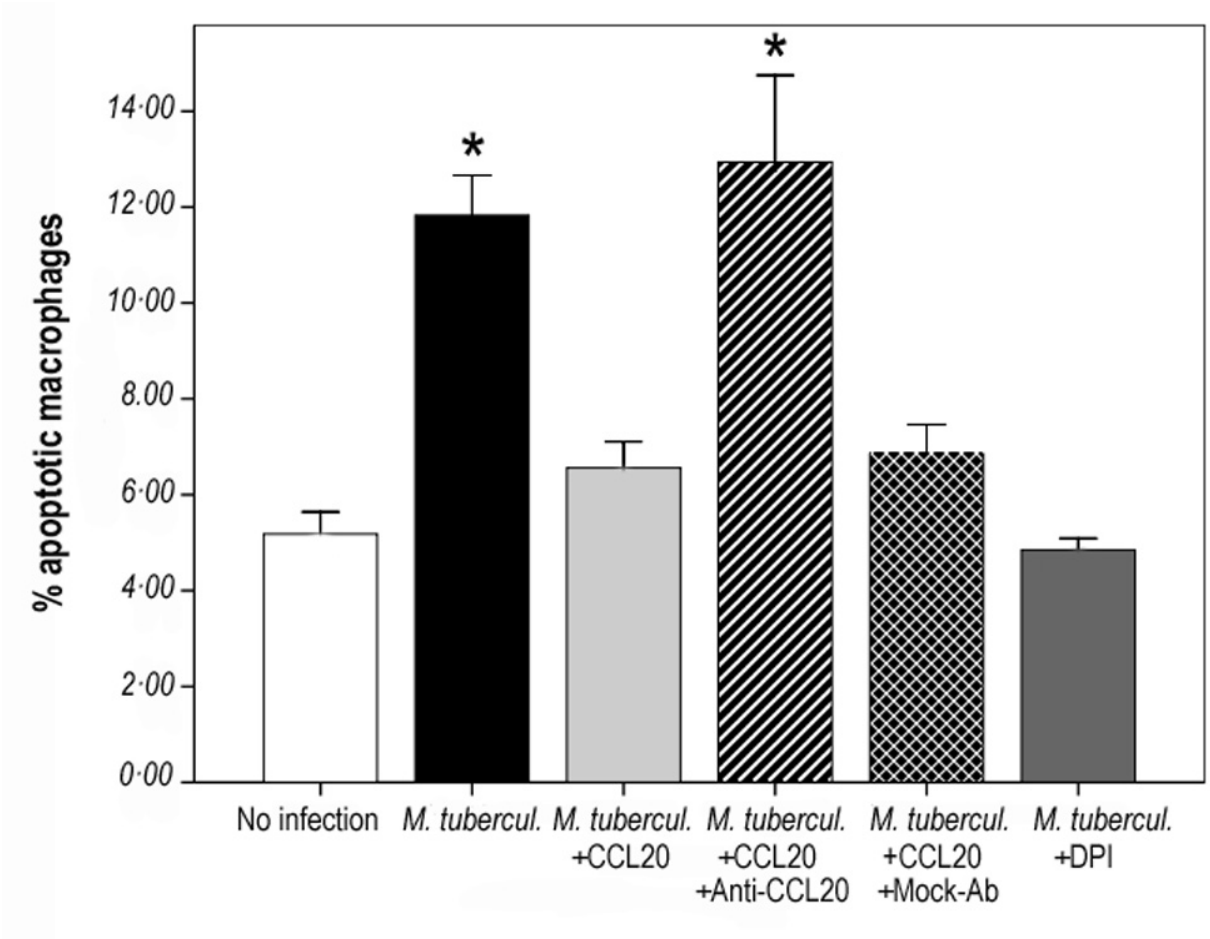

Fig. 3. Influence of CCL20 in the apoptosis mediated by *M. tuberculosis* in infected monocytes. Monocytes were infected with a MOI of 5, and a negative control (no infection) was included. Data are the proportions (expressed as percentage) of fluorescein labelled cells (apoptotic) with respect to DAPI labelled cells (total) and represent the mean + S.D. of five independent experiments. HSD Tukey's test was used for comparisons between the negative control and the other groups. *$p < 0{\cdot}05$ is considered significant.