Non-chemotactic influence of CXCL7 on human phagocytes. Modulation of antimicrobial activity against *L. pneumophila*.

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Abbreviations: β-TG, β-thromboglobulin; CFU, colony forming units; Ct, threshold cycle; CTAP-III, connective tissue-activating peptide-III; CXCL7, CXC chemochine ligand 7; F, forward; FBS, fetal bovine serum; IR, interquartile range; MDM, monocyte derived macrophages; MOI, multiplicity of infection; NAP-1 and NAP-2, neutrophil activating protein-1 and -2; PBP, platelet basic protein; PPBP, pro-PBP; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophils; qPCR, quantitative polymerase chain reaction; R, reverse.

A B S T R A C T

We have investigated the role of CXCL7 in the immune response of human phagocytes against the intracellular bacteria *Mycobacterium tuberculosis* and *Legionella pneumophila*. We have observed that polymorphonuclear neutrophil (PMN) chemotaxis induced by the supernatants of infected monocyte derived macrophages (MDM) may be attributed to CXCL8 rather than CXCL7, although both chemokines are present in large quantities. We have also found that CXCL7 is present not only in the supernatants of MDM, but also in the supernatants of PMN of some, but not all, individuals. Western blot analysis revealed that, in both MDM and PMN supernatants appeared two bands with molecular weights consistent with the platelet basic protein (PBP) and the neutrophil activating protein-2 (NAP-2) sizes. Regarding the influence on infected cells, recombinant NAP-2 enhanced the antimicrobial activity of IFNγ activated MDM against *L. pneumophila*, but not against *M. tuberculosis*. In addition, U937 cells transfected with a NAP-2 construct inhibited the intracellular multiplication of *L. pneumophila,* supporting its role in the modulation of the antimicrobial activity. Finally, U937 cells transfected with the NAP-2 construct showed an adherence that was dramatically enhanced when the substrate was fibronectin. We conclude that human phagocytes produce CXCL7 variants that may have a significant influence on the immune response against bacterial pathogens.

Introduction

Mycobacterium tuberculosis and *Legionella pneumophila* are both bacteria implicated in pulmonary diseases. An average of 356 cases of legionellosis per year reported in the United States is only a fraction of the 8,000 to 18,000 cases which are estimated to occur annually. The epidemiology of Legionnaires' disease is usually related to community outbreaks, frequently linked to cooling waters and whirlpool spas (Fields et al., 2002). As regards tuberculosis, it remains a first order health problem. The global burden of disease in 2009 was 14 million prevalent cases and 1.68 million deaths (World Health Organization, 2010). Although both bacteria share the attributes of being intracellular pathogens that infect alveolar macrophages and are responsible for important respiratory infections, their pathophysiologies are quite different (McChlery et al., 2009). For this reason, comparison of the biological effect induced by either microorganism on the host cells may help exam the mechanisms involved in the success or failure of the immune response against their infection

Cytokines are critical modulators of the immune response. Among them, the chemokines constitute a group of small chemotactic cytokines divided into four families (CXC, CC, C and CX3C) on the basis of the pattern of cysteine residues in the ligands (Zlotnik and Yoshie, 2000). The role of chemokines in both tuberculosis and legionellosis is poorly characterized. *L. pneumophila* induces the production of IL-8 (CXCL8) in human lung epithelial cells (Teruya et al., 2007), but we are not aware of any studies on chemokine induction by this bacterium in human macrophages. On the other hand, *M. tuberculosis* induces CXCL chemokines in human macrophages, including CXCL2, CXCL5 and CXCL8 (Volpe et al., 2006). Furthermore, virulent strains induce CCL2, CCL3, CCL4 and CCL5 expression, a response that is attenuated in avirulent strains (Saukkonen et al., 2002).

CXCL7 is the most abundant chemokine in platelets, where it is stored in their α-granule compartment, and may occur within a range from 1.6 to 4.8 μM in normal serum. There are several variants proteolytically derived from the precursor molecule pro- platelet basic protein (PPBP, 128 aa). Amino-terminal truncations include platelet basic protein (PBP, 94 aa), connective tissue-activating peptide-III (CTAP-III, 85 aa), β-thromboglobulin (β-TG, 81 aa) and neutrophil activating protein 2 (NAP-2, 70 aa). In addition, the CTAP-III and the NAP-2 variants may also have carboxi-terminal truncations of either 4 or 7 aa. CXCL7 has a tripeptide motif (ELR) which is required for binding to and cell activation through the CXCR-1 and CXCR-2 receptors (Brandt et al., 2000). Besides platelets, CXCL7 is also known to be expressed in T cells (Skerka et al., 1993) and monocytes (El-Gedaily et al., 2004). With the exception of PF-4 (CXCL4) (Brandt et al., 2000), all ELR+ CXC chemokines attract neutrophils, but in the case of CXCL7 only the NAP-2 variant exhibits this activity and although NAP-1 (CXCL8) shows only one chemotactic peak at nM concentrations, NAP-2 has two peaks at 4nM and 3 μM, respectively (Ludwig et al., 1997). Both NAP-2 and NAP-1 bind the same receptors and attract the same cells, but it seems that NAP-2, rather than NAP-1, is the chemokine that chemoattracts neutrophils about monocytes (Malawista et al., 2002). Several other biological activities have been studied in CXCL7 variants, including enzyme release in human neutrophils (Brandt et al., 1991). NAP-2 does not promote a respiratory burst in monocytes, and only a small activity is observed in neutrophils, ten times lower than that of NAP-1 (Walz et al., 1991). It is particularly interesting the microbicidal activity that chemokines like the CXCL7 variants PBP and CTAP-III exert, with mechanisms of action similar to defensins (Tang et al., 2002). In a recent report Khajoee *et al.* describe the differential gene expression of *CXCL7* in GM-macrophages (granulocyte-macrophage colony-stimulating factor-induced human

monocyte derived macrophages) and M-macrophages (macrophage colony-stimulating factor-induced human monocyte derived macrophages) infected with *M. bovis* bacillus Calmette-Guérin (BCG). Furthermore, they found that CXCL7 might have a role in the resistance against mycobacteria and in the production of reactive oxygen intermediates (Khajoee et al., 2006).

The immunologic response of monocytes to *M. tuberculosis* and *L. pneumophila* is very different when they are activated with IFNγ. While *L. pneumophila* is readily inhibited (Bhardwaj et al., 1986), *M. tuberculosis* survives the antimicrobial activity of the phagocyte (Douvas et al., 1985). In order to investigate the differences in the gene expression of infected monocyte derived macrophages (MDM), we have constructed a new subtraction library of cDNA from human IFNγ activated MDM infected with either *M. tuberculosis* or *L. pneumophila*. This library has been partially characterized, and a clone representing *CXCL7* was scored as positive for differential expression in these macrophages. Subsequently, we performed a series of experiments to analyze the role of CXCL7 in infection. We describe for the first time that polymorphonulear neutrophils (PMN) produce CXCL7, and that this protein enhances the antimicrobial activity induced by IFNγ in MDM against *L. pneumophila*. Although it had already been reported that NAP-2 induces cellular adhesion through CD11b/CD18, we characterize fibronectin as a new adhesion substrate specifically recognized after NAP-2 induction.

Materials and methods

Bacterial strains

M. tuberculosis HL186T was isolated at the Hospital de León (Microbiology Service) and kindly provided by Julio Blanco and Manuela Caño. It was grown on 7H11 agar supplemented with 0.2% glycerol and 10% Middlebrook enrichment OADC (Oleic acid, albumin, dextrose, catalase; Becton Dickinson Microbiology Systems, San Agustín de Guadalix, Madrid, Spain). *L. pneumophila* Philadelphia, ATCC 13151, generously provided by Carmen Pelaz, was grown on BCYE (buffered charcoal yeast extract) agar plates. Bacteria from fresh culture in agar plates were suspended in RPMI medium without serum. 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 until individualized bacteria were obtained, 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 *L. pneumophila*. After addition of glycerol to 20%, single use aliquots were frozen at – 80ºC.

MDM, PMN and cell lines

Peripheral blood was obtained from healthy volunteers following informed consent and approval of the protocol by the Hospital of León Clinical Research Ethics Board. 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). Neutrophils were isolated by dextran and Ficoll-Paque Plus sedimentation (GE Healthcare). > 94 % of cells in the monocyte preparation were $CD14^+$ and > 99 % of cells in the neutrophil preparation were $CD66b^+$ (labelled antibodies from Becton Dickinson). The number of cells was calculated by counting in a Neubauer chamber and cells were cultivated within 3 h from blood collection. The human histiocytic lymphoma cell line U937 was kindly provided by Ignacio Rodríguez, and the human chronic myelogenous leukemia cell line K562, by Carmen Marín. All primary cells and cell lines were grown in RPMI/10% FBS with 100 U/ml penicillin and 250 μg/ml fungizone, at 37ºC and 5% CO_2.

Plasmids and transfection

For K562 transient transfection, *CXCL7* was cloned by PCR using the following primers: (F) ATCAAAGCTTATGAGCCTCAGACTTG and (R) ATCACTCGAGTTAATCAGCAGAT, which include the Hind III and Xho I restriction sites, respectively. This PCR product was subcloned in pcDNA3 (Invitrogen) and is translated into a 128 aa product that includes the leader sequence (aa 1 to 34). 2×10^6 K562 cells in RPMI without serum were transiently electroporated with 10 μg of pcDNA3-*CXCL7* at 260 v, 1 mF, using a Gene-Pulser II (Bio-Rad). Cells were grown in RPMI/10% FBS (3 ml) for two days in 6 wells plates.

For U937 cells stable transfection we constructed a *NAP-2* form obtained by deleting the sequence that codify aa 35 to 59 by a two-step PCR that keeps the leader sequence. Using as template the complete *CXCL7* sequence, the deletion was obtained with the following primers: (F) GCTCTGGCTTCTGAACTCCG and (R) CGGAGTTCAGAAGCCAGAGC. The recombinant plasmid was tested by sequencing.

U937 cells were transfected as described above, and cells were selected with 200 μg/ml G418 (Gibco, Invitrogen) for two months.

Quantitative polymerase chain reaction (qPCR)

5×10^5 monocytes were infected with 5×10^5 bacteria (multiplicity of infection, MOI = 1) for 18 hours in a volume of 400 μl (48-well plates). 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). Threshold cycle (Ct) values for *CXCL7* were normalized to the Ct of the reference gene *EF-1α* (elongation factor 1α), using the following ratio: $(E_{EF1\alpha})^{Ct\ EF1\alpha}/(E_{CXCL7})^{Ct\ CXCL7}$, where E is the efficiency of the reaction (1.93 for *EF1α* and 2.01 for *CXCL7*). The primers used for *EF-1α* and *CXCL7* were: *EF-1α* (Forward, F) TGTTCCTGTTGGCCGAGTG; (Reverse, R) ATTGAAGCCCACATTGTCCC; *CXCL7* (F) GCGAAAGGCAAAGAGGAAAGT; (R) TCTGGGAGCATCTGGGTCC.

CXCL7 and CXCL8 quantification

Supernatants were obtained from cells cultured as indicated for qPCR. To remove bacteria, samples were centrifuged 3 min at 8000 × g at room temperature in ultrafree-MC filter units of 0.45 μm (Millipore Ibérica, Madrid, Spain) and frozen at –80ºC. Chemokines were quantified by the human CXCL7/NAP-2 and human CXCL8/IL-8 DuoSet ELISA development system (R&D Systems, Minneapolis, MN, USA).

Western blot analysis

CXCL7 was immunoprecipitated with GammaBind G Sepharose (GE Healthcare), with 2 μg of a monoclonal anti-human NAP-2 antibody (R&D Systems). Samples were used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 16% Tris/Tricine mini-gels under denaturing conditions (6 M urea) (Schägger, 2006). Before electrophoresis, samples were diluted in sample buffer and incubated for 10 min at 95ºC. To keep the proteins in a reduced form, 4% (final concentration) iodoacetamide (Sigma-Aldrich, Tres Cantos, Madrid, Spain) was added, and incubated for 30 min at room temperature. For molecular weight standards, the Peptide Marker Kit was used (GE Healthcare). After electrophoresis and blotting onto PVDF (Millipore), membranes were incubated with 1 μg/ml of polyclonal anti-human NAP-2 antibody (Peprotech, Rocky Hill, NJ, USA), that also recognizes the PPBP, PBP and β-TG variants of CXCL7. Blots were developed with the ECL Advance Western Blotting Detection Kit (GE Healthcare) using the Chemidoc-XRS image analyzer (Bio-Rad).

Chemotaxis

10^5 PMN in a volume of 100 μl were placed in BD Falcon Cell Culture Inserts (pore size 8.0 μm) (24-well plates, Becton Dickinson). The lower chamber was supplied with supernatants from cells (250 μl) cultured as indicated for qPCR, that were diluted in RPMI/10% FBS (500 μl). As the reference control we included RPMI/10% FBS. When indicated, 1 μg of anti-human NAP-2 neutralizing antibody or 1 μg of anti-human IL-8 neutralizing antibody (both monoclonal, R&D Systems) were added. Plates were incubated for 30 min at 37ºC. Migrated cells were counted in a Neubauer chamber under an inverted microscope. For statistical analysis, the migration index was calculated by dividing the number of migrated PMN upon treatment by the number of migrated PMN in the RPMI/10% FBS control.

Antimicrobial activity assays

Experiments were made in 96-well plates, always in a total volume of 100 µl. 10^5 cells were infected with 10^3 bacteria (MOI = 0.01) in RPMI/10% FBS. When indicated, 25 ng/ml was added of either interferon gamma (IFN-γ, > 2×10^7 units/mg, Peprotech) or neutrophil activating protein-2 (NAP-2, Peprotech). MDM were lysed after 96 h and PMN after 24 h by sonication at an amplitude of 10% (2 Watts) for 3 s, to release bacteria. Decimal dilutions were incubated at 37ºC in either BCYE agar plates (*L. pneumophila*) for four days or 7H9 broth supplemented with 0.2% glycerol and 10% Middlebrook enrichment ADC (albumin dextrose catalase, *M. tuberculosis*) for seven days. Colony forming units (CFU) were determined for mycobacteria under an inverted microscope at × 100 magnification (Fazal et al., 1992).

pcDNA3-*NAP-2* stable transfected U937 cells were cultured in G418 free medium for four days and differentiated overnight with 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). Infection, cell lysis and colony counting were performed as described.

Cell adhesion assay

50 µl of human fibronectin, mouse collagen, type IV or mouse laminin (100 µg/ml, Becton Dickinson) were added to corresponding wells well (96 well plates) and incubated overnight. After three washes with PBS, 2×10^4 U937 cells were inoculated to either coated or uncoated wells, and incubated for 90 min at 37ºC. In inhibition experiments 1 mg/ml of the peptide with the sequence GRGDSP (Calbiochem, Merck Chemicals, Nottingham, UK) was added to the cells 30 min before inoculating the plates. Non-adherent cells were removed by three washes with PBS. Remaining cells were counted by cell lysis and naphtol blue black staining of nuclei (Nakagawara and

Nathan, 1983). Briefly, 50 µl of lysis buffer (1% Triton X-100, 0.05% naphtol blue black, 0.1 M citric acid, pH 2.2, all reagents from Sigma-Aldrich) were added for 5 min at room temperature. Stained nuclei were counted in a Neubauer chamber under an inverted microscope.

Statistical analysis

Data followed a normal distribution (Shapiro-Wilk test), with homogeneous variance (Levene test) and were expressed as means with standard deviation (SD). Comparisons of two groups were performed by Student's *t*-test, and more than two groups by one-way ANOVA. Pairwise comparisons were performed by the HSD Tukey's procedure. When variance was not homogeneous, comparisons were performed by the Dunnett's T3 procedure. A p value <0.05 was considered significant. Data was analysed with PASW Statistics 18 (SPSS Ibérica, Madrid, Spain).

Results

CXCL7 expression in infected human MDM

To characterize differences in MDM gene expression we constructed a cDNA subtracted library from IFNγ activated MDM infected with either *M. tuberculosis* or *L. pneumophila*. One of the positive clones of this partially characterized library corresponded to *CXCL7*, which was isolated from the *M. tuberculosis* subtracted pool. To confirm this finding we analyzed cDNA from an independent sample of MDM from healthy volunteers activated with IFNγ and infected with either bacterium. Indeed, we appreciated that *CXCL7* was more expressed in MDM infected with *M. tuberculosis* as compared with *L. pneumophila* (Fig. 1) and this observation explained why this clone was scored as positive from the subtracted library, although the difference did not reach significant levels ($p = 0.13$). The protein expression detected by ELISA in the cellular supernatants was high with very small variations between *M. tuberculosis* (median = 12.7 ng/ml, Interquartile Range (IR) = 52.4 ng/ml) and *L. pneumophila* infected MDM (median = 11.3 ng/ml, IR = 53.9 ng/ml, n = 5). Regarding the production of CXCL7 by non-infected cells, given for comparison purposes, values were very similar with a median = 12.6 ng/ml, IR = 53.9 ng/ml. At this point, even though our results did not seem so encouraging in terms of differences, we speculated that such an elevated protein production, at ng/ml concentration levels, should have a biological significance. Since very little information was available about the role of CXCL7 in infection, we decided to analyze its participation in the response of MDM and PMN to infection by intracellular bacteria.

PMN produce CXCL7

The production of CXCL7 by monocytes has been known for some time (El-Gedaily et al., 2004), but we have not found in the literature any reliable description of CXCL7 production in PMN, despite their use in the studies of CXCL7 post-translational modifications. For this reason, it was unexpected to find that PMN from two out of five volunteers expressed the *CXCL7* gene. We also studied the protein production, and confirmed that the supernatants from the two PMN that had gene expression and had been infected with *M. tuberculosis* had a median of 7.8 and 30.9 ng/ml, and infected with *L. pneumophila*, 10.3 and 32.1 ng/ml, respectively, similar to the amounts in MDM. Supernatants from the other three volunteers did not have CXCL7, indicating that PMN are not constitutively expressing this gene and hence may not be helpful in chemotaxis.

To explore the possibility that polymorphisms in the proximal region of the *CXCL7* promoter explained these differences of expression among individuals, we amplified the gene promoter by PCR (Transcriptional Regulatory Element Database, Accession number 32462), using the following primers: TACACTCGAGGGTACTCTTAGGTGGTAG (F) and TACAAAGCTTGCAGATAAGTGGCTTCTC (R), which include a Xho I and a Hind III restriction sites, respectively. The sequence between these primers ranges from -14 to -670 from the transcription start nucleotide and in all five cases the nucleotide sequence of the promoter was identical to that of the database. We therefore concluded that mechanisms other than the transcriptional regulation in the proximal region of the promoter accounted for the observed differences.

Given the number of CXCL7 variants that has been reported, we studied which form of the protein was present in both MDM and PMN supernatants. To help in the identification of variants we constructed a plasmid expressing PPBP and transfected it

into the myeloid leukemia K562 cells. We chose this cell line because they exhibit megakaryocyte markers when differentiated with PMA (Alitalo, 1990) and platelets, which store large amounts of NAP-2 (Brandt et al., 2000), come from megakaryocytes. Supernatants of the transfected cells were immunoprecipitated and analyzed by Western blot (Fig. 2, lane 3). The PPBP construct generated three protein products with molecular weights consistent with those of the proteins PPBP (14.0 kD), PBP (10.3 kD) and NAP-2 (7.6 kD). This result demonstrates the capacity of K562 cells to proteolyze higher molecular weight variants, although these cells may not be differentiated to macrophages (Koeffler et al., 1981). An additional higher band could be detected, but its molecular weight does not correspond to any of the CXCL7 variants. We ignore the nature of this band, although we suspect it could be an oxidized form of PPBP which have resisted the strong reducing conditions of the electrophoresis. Although this pattern is suggestive, we may not assign a definitive identity to each of the bands unless they are sequenced, because post-translational modifications, even involving a few amino acids, may not be ruled out.

When we analyzed both PMN and MDM supernatants (lanes 1 and 2, respectively), we observe a protein which migrates like the protein of the same molecular weight of PBP recognized in the K562 supernatant. MDM has traces of the higher molecular weight band and, as it would be expected, both MDM and PMN produce a smaller molecular weight variant, most likely NAP-2, but in very small amounts. The same pattern was observed in the supernatant of phagocytes infected with either *M. tuberculosis* or *L. pneumophila* (data not shown).

CXCL7 induces limited chemotaxis in PMN

The best characterized biological activity of several of the CXCL proteins is the induction of chemotaxis in PMN. We consequently analyzed the level of chemotaxis

induced by the CXCL7 protein present in the supernatant of MDM infected *M. tuberculosis*. It was known that *M. tuberculosis* induces the production of CXCL8 (Zhang et al., 1995) a chemokine that binds to CXCR1 and CXCR2, the same CXCL7 receptors (Moser et al., 1991). Therefore, a comparison between both chemokines was warranted and we confirmed by ELISA their presence in the supernatants of infected cells, with a median of 8.3 ng/ml for CXCL7 and 102 ng/ml for CXCL8. CXCL8 was also present in the supernatant of non-infected cells (median = 66.9 ng/ml), less than the observed amount in the supernatant of infected cells. While only a small level of chemotaxis was observed in the supernatant of non-infected cells, the supernatant of MDM infected with *M. tuberculosis* could attract efficiently PMN, showing statistically significant differences in comparison with the negative control (medium without cells). To investigate the molecules involved in this activity we used an anti-human NAP-2 neutralizing antibody that recognizes all variants of CXCL7 and an anti-human IL-8 neutralizing antibody. Some inhibition of chemotaxis was detected in the presence of the anti-NAP-2 antibody, but this activity was completely abolished in the presence of the anti-IL-8 antibody, with a calculated migration index even below the one observed for the supernatant of non-infected cells (Fig. 3). These results suggest that CXCL8 is the main chemokine that attracts PMN in this setting. Whatever the actual identity of the CXCL7 variants presents in the supernatant, their induced chemotaxis was small. From this observation it is difficult to conclude that the main biological function of CXCL7 in these cellular models is chemotaxis for PMN, because CXCL8 seems to play this role predominantly. This deduction brought about the question about additional biological roles for CXCL7.

NAP-2 enhances the antimicrobial activity against L. pneumophila induced by IFNγ in MDM

With the aim of investigating possible additional biological roles for CXCL7, we tested whether NAP-2 was able to affect microbial killing, one of the more important activities in phagocytes. *M. tuberculosis* multiplies in both MDM (Crowle and May, 1981) and PMN (Jones et al., 1990). Indeed, we observed a significant growth of the bacteria in macrophages, and a slight increase in their number in PMN, although not statistically significant. Nevertheless, we did not detect any influence of either IFNγ or NAP-2 on the growth rate of the bacilli (Fig. 4). A different situation was, however, observed for *L. pneumophila*. As expected (Horwitz and Silverstein, 1981), PMN kill a small proportion of the bacteria after one day of infection, because there is a significant decrease in the number of bacteria recovered as compared with the number in the inoculum. In contrast, no differences were detected when cells were treated with either IFNγ or NAP-2. Regarding MDM, after four days of infection, *L. pneumophila* multiplied 3 orders of magnitude. NAP-2 itself did not alter this level of multiplication but it seems to cooperate with IFNγ to kill them. When only IFNγ is added, a decrease of viable bacteria is observed, as compared with the inoculum, but it does not reach statistical significance. This result implies a bacteriostatic effect of this cytokine, because it inhibits the intracellular multiplication of *L. pneumophila*. The antimicrobial activity reaches statistical significance in the presence of both IFNγ and NAP-2, with a further 0.5 log decrease in the number of recovered bacteria (Fig. 4). This result suggests that although IFNγ is the critical cytokine that controls *L. pneumophila* infections, other factors, like NAP-2, may synergize with it.

To gain further evidence of the influence of NAP-2 in the antimicrobial activity of macrophages, we stably transfected the monocytic cell line U937 with the NAP-2 construct. Cells were differentiated to macrophages with PMA and infected with *L. pneumophila* in the presence of IFNγ (Table 1). Cells transfected with the pcDNA3

vector (negative control) allowed the multiplication of the bacteria nearly 3 orders of magnitude. IFNγ inhibited this level of multiplication, but differences were not statistically significant. In cells transfected with the NAP-2 construct *L. pneumophila* growth was, however, significantly inhibited, although IFNγ did not show much activity in these cells. We consequently conclude that NAP-2 also enhances antimicrobial activity in transfected U937 cells. This result does not perfectly mirror the data obtained with primary cells, but we need to consider that the transfected cells constitutively express NAP-2 (not PBP), and that they do not behave exactly like MDM, because in these cells, IFNγ only marginally inhibits the multiplication of *L. pneumophila*.

U937 cells transfected with a NAP-2 construct have increased adherence to fibronectin

While culturing U937 cells, we noticed that cells transfected with the NAP-2 construct, but not with the empty vector pcDNA3, adhered to the culture vessel surface. This is an additional biological activity that may confer CXCL7 an important role in infection, and had been previously described (Detmers et al., 1991). We found that U937 cells are an excellent model for the study of this phenomenon. We analyzed the adhesion properties of transfected cells and observed a dramatic enhancement when the substrate was fibronectin, as compared with plastic (culture vessel surface), laminin or collagen type IV. To further characterize this activity we used a fibronectin binding inhibitor, a peptide with the sequence GRGDSP, which includes the fibronectin RGD motif recognized by some integrins (Ruoslahti and Pierschbacher, 1987). This peptide successfully competed with fibronectin for cell binding (Fig. 5).

Discussion

From a subtraction library we have cloned *CXCL7* as a candidate gene that may influence the antimicrobial activity of human phagocytes against *M. tuberculosis* or *L. pneumophila*. It was surprising to find out that CXCL7 was constitutively produced in MDM and that the amount of protein in supernatants did not vary after infection regardless of the bacterial species. There is not always a perfect correspondence between gene expression and the amount of the extracellular protein detected. For example, although *Lactobacillus casei* induces the CXCL10 (IP-10) gene expression, it also inhibits the extracellular secretion of the protein (Hoermannsperger et al., 2009). Conversely, *M. tuberculosis* increases IP-10 protein production despite inhibition of *IP-10* transcription (Bai et al., 2010). From these data we infer that post-transcriptional regulation seems to play an important role in the production of these proteins.

The first interesting finding that we report is that the PMN of some, but not all, individuals produce CXCL7, which may explain negative results in other studies in which only an isolated individual was examined. To our knowledge, our results constitute the first report in which the nature of CXCL7 variants is characterized in phagocyte supernatants. Although El-Gedaily *et al.* had detected PBP only in monocyte lysates (El-Gedaily et al., 2004), we demonstrate the presence of CXCL7 derivatives in the supernatants of both PMN and MDM. The ability of both PMN and monocytes to proteolyze PPBP derivatives to NAP-2 had already been described (El-Gedaily et al., 2004; Walz and Baggiolini, 1990) and therefore, the presence of this band in supernatants was expected. Iida *et al.* identified a protein that crossreacted with a platelet derived growth factor (PDGF) antibody, and that they termed leukocyte derived growth factor (LDGF). Using this antibody they cloned the corresponding gene and found that it represented what is now regarded as *CXCL7*. This antibody was able to

recognize a protein with a molecular weight close to 16 kD in PMN supernatants. Instead, in the supernatants of monocytes and T lymphocytes a smaller protein (~ 14 kD) was identified (Iida et al., 1996). They did not detect any other smaller protein, like NAP-2. It is very difficult to ascertain the nature of the 16 kD protein in PMN supernatants, because the antibody is not specific for CXCL7, and no other means were used to characterize this band. We have not observed a protein of such a size produced by PMN. Iida *et al.* report provides the only reference to the presence of CXCL7 in PMN supernatants. Other authors have analyzed mRNA from PMN and found no *CXCL7* expression (El-Gedaily et al., 2004). Furthermore, granulocytic differentiation of the promyelocytic HL60 cell line with dimethyl sulfoxide did not induce its expression (Skerka et al., 1993).

We have showed that infection with either *M. tuberculosis* or *L. pneumophila* does not increase the production of CXCL7. In contrast, *M. tuberculosis* has been described to increase the *CXCL8* expression and the CXCL8 production (Zhang et al., 1995), a result that we have confirmed. When analyzing the chemotactic properties of supernatants from non-infected and *M. tuberculosis* infected cells, the different migration indexes observed were consistent with the amounts of CXCL8, but not of CXCL7. Namely, the chemotaxis induced by supernatants of non-infected cells is lower than the induced by the supernatant of *M. tuberculosis* infected cells.CXCL7 exhibits two different chemotactic peaks (Ludwig et al., 1997), so it is possible that the amount of CXCL7 present in the supernatants is not optimal. On the other hand, it is also possible that the particular variants of CXCL7 in the supernatants do not promote chemotaxis. All these reasons may explain that only the neutralization of CXCL8 completely abolishes the chemotaxis of PMN, and that CXCL7 variants may have other little investigated biological roles.

We have found that NAP-2 influences the microbicidal activity of human MDM. In a recent report, Khajoee *et al.* show that in granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced MDM, CXCL7 inhibits the growth of *M. tuberculosis* (Khajoee et al., 2006). We have not observed such effect, but there are several differences in our experimental conditions. First, we use IFNγ activated MDM, and not GM-CSF differentiated MDM. Second, their bacterial model is *M. tuberculosis* H37Ra, an attenuated strain, whereas we have chosen a virulent clinical isolate. Third, they lyse the infected cells after 6 days, while we do it at 4 days. In their hands, osteopontin, a protein with chemokine properties, also inhibits *M. tuberculosis* growth. In contrast, the effect in the antimicrobial activity that we detect is against *L. pneumophila*. Although it does not promote any change in their multiplication in MDM, it enhances the bactericidal activity of IFNγ, a cytokine already reported to induce macrophages to inhibit the growth of this bacteria (Bhardwaj et al., 1986). Tang *et al.* have shown that the CXCL7 variants PBP and CTAP-III are also antimicrobial peptides (Tang et al., 2002). Furthermore, Schaffner *et al.* have also identified PBP and CTAP-III as monocyte products with antimicrobial activity (Schaffner et al., 2004), which may explain the killing of *L. pneumophila* that we have observed. Nevertheless, NAP-2 has not been tested in either study. In contrast, Krijgsveld *et al.* have not detected antimicrobial activity for NAP-2 (Krijgsveld et al., 2000). We do not believe that the effect that we observe against *L. pneumophila* depends on the antimicrobial activity of NAP-2 because in the absence of IFNγ it does not exert any apparent activity in either MDM or PMN. To analyze in different conditions whether NAP-2 may modulate the growth inhibition of the bacteria we used a classical model in *L. pneumophila* infection, the monocytic cell line U937 (Pearlman et al., 1988) and we have observed that in cells

transfected with the NAP-2 construct 3.5-fold less bacteria were recovered as compared with cells transfected with the empty vector, with log CFU from 5.77 to 5.23.

The influence of CXCL chemokines in cellular adhesion has already been reported. Detmers *et al.* found that the increase of adherence induced was dependent on the integrin CD11b/CD18 (αMβ2) (Detmers et al., 1991). We have expanded our knowledge about the influence of NAP-2 in the induction of cell adhesion. We found that the adherence of transfected cells was specific, as they adhered to fibronectin, but not to laminin or collagen type IV. Integrins that mediate the binding to fibronectin are different from CD11b/CD18, because they do not use the β2 subunit (CD18). Fibronectin is present in plasma, basal lamina or cell surfaces and fibronectin peptides inhibit leukocyte integrin binding and block recruitment to limit inflammation (Wahl et al., 1996), which supports that it is involved in the immune response. Moreover, binding of monocytes to fibronectin enhances IFNγ-induced signalling events (McCarthy et al., 1997). We find intriguing the possibility that there may be a relationship between the adherence promoted by NAP-2 and the observed enhancement of the antimicrobial activity induced by IFNγ.

In conclusion, our data indicates that CXCL7 play important roles additional to chemotaxis that may help phagocytes fighting intracellular bacteria. These roles include the enhancement of IFNγ induction of antimicrobial activity and cellular adhesion. Susceptibility to tuberculosis and legionellosis has a very complex nature and we need to increase our knowledge of the different factors that influence the final outcome of these diseases.

Acknowledgments

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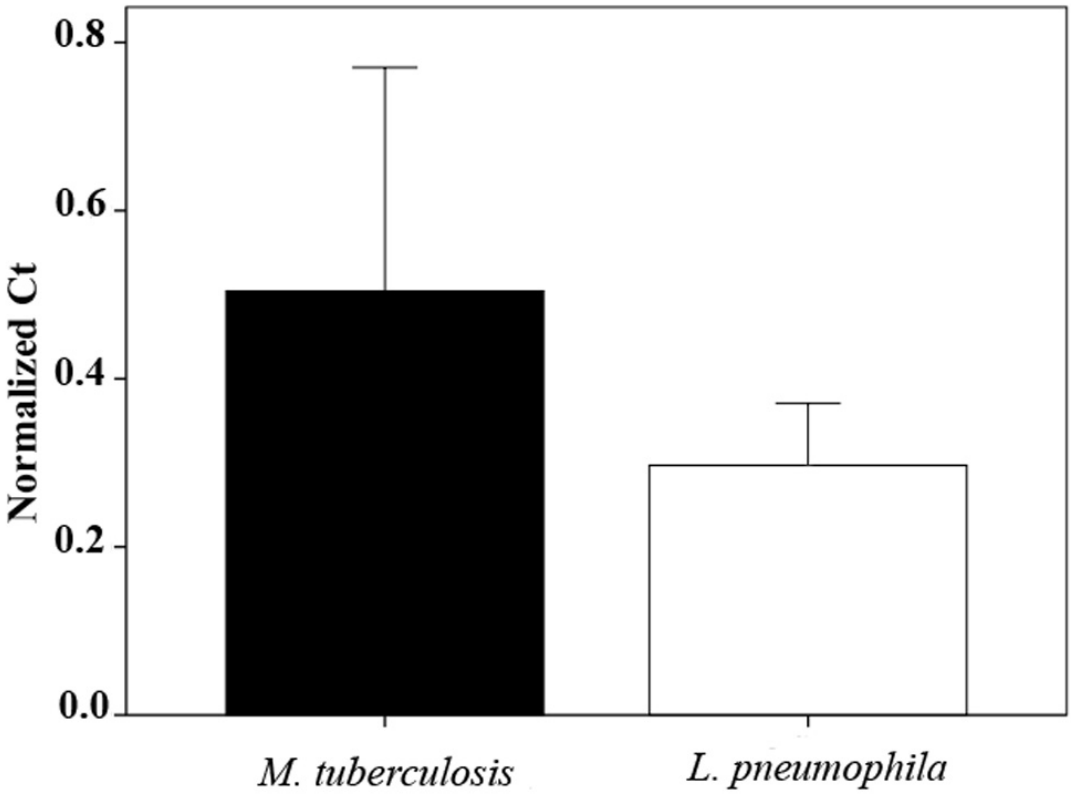

Fig. 1. *CXCL7* expression measured by qPCR. Data represent the mean ± SD of the normalized C_t in IFNγ activated MDM infected with *M. tuberculosis* or *L. pneumophila* (n = 5). Student's *t*-test comparison was not statistically significant ($P > 0.05$).

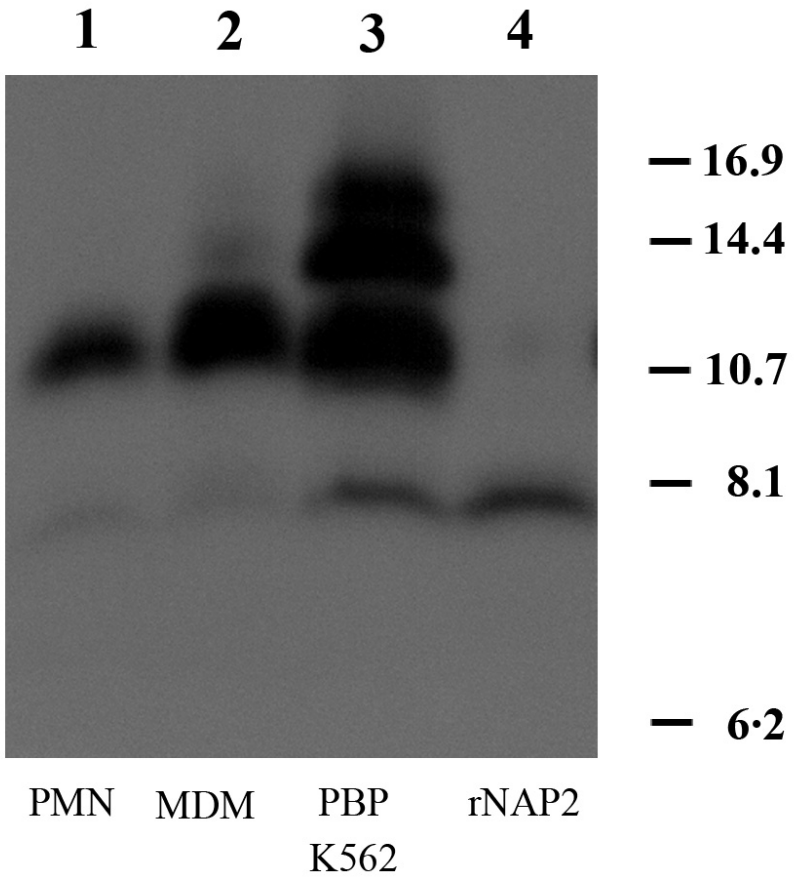

Fig. 2. Western blot analysis of supernatants from phagocytes. PMN and MDM, immunoprecipitations of 600 µl of *M. tuberculosis* infected PMN or MDM supernatants, respectively; PPBP K562, immunoprecipitation of 75 µl of transfected K562 supernatant; rNAP-2, 2 ng of recombinant NAP-2.

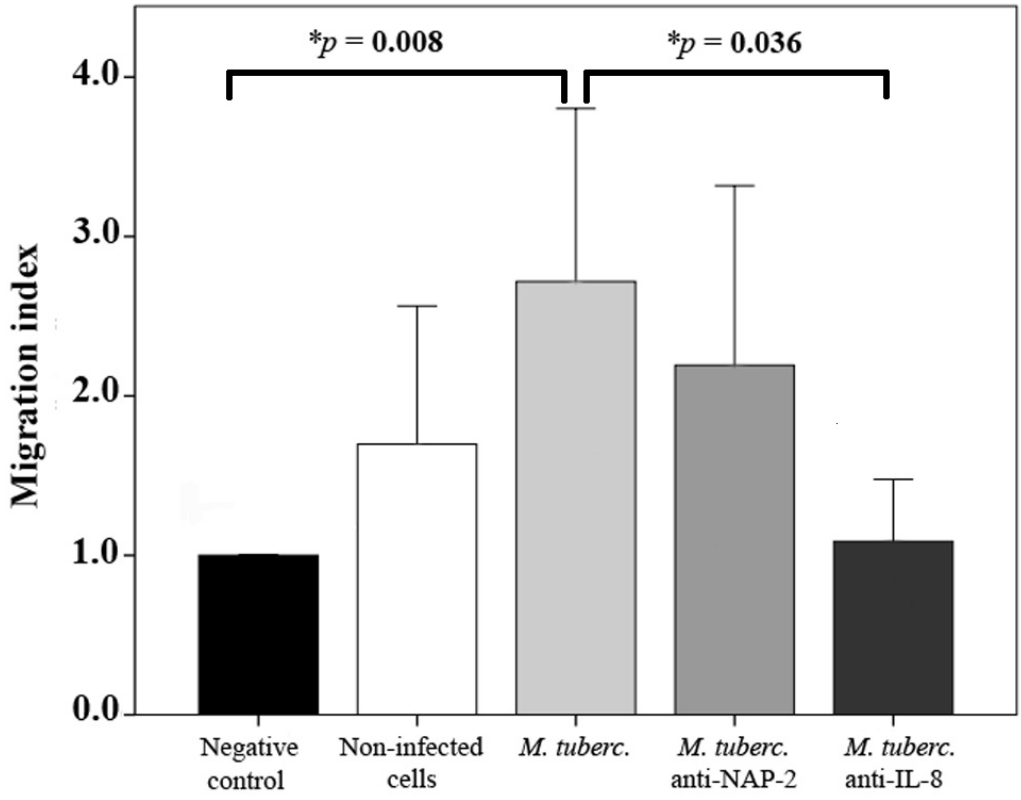

Fig. 3. PMN chemotactic response. Data represent the migration index mean ± SD of PMN to supernatans of *M. tuberculosis* infected MDM. The reference control, medium without cells, is always considered to have an index = 1 (SD = 0). Pairwise comparisons with the supernatant from *M. tuberculosis* infected cells group with $*P < 0.05$ are considered significant.

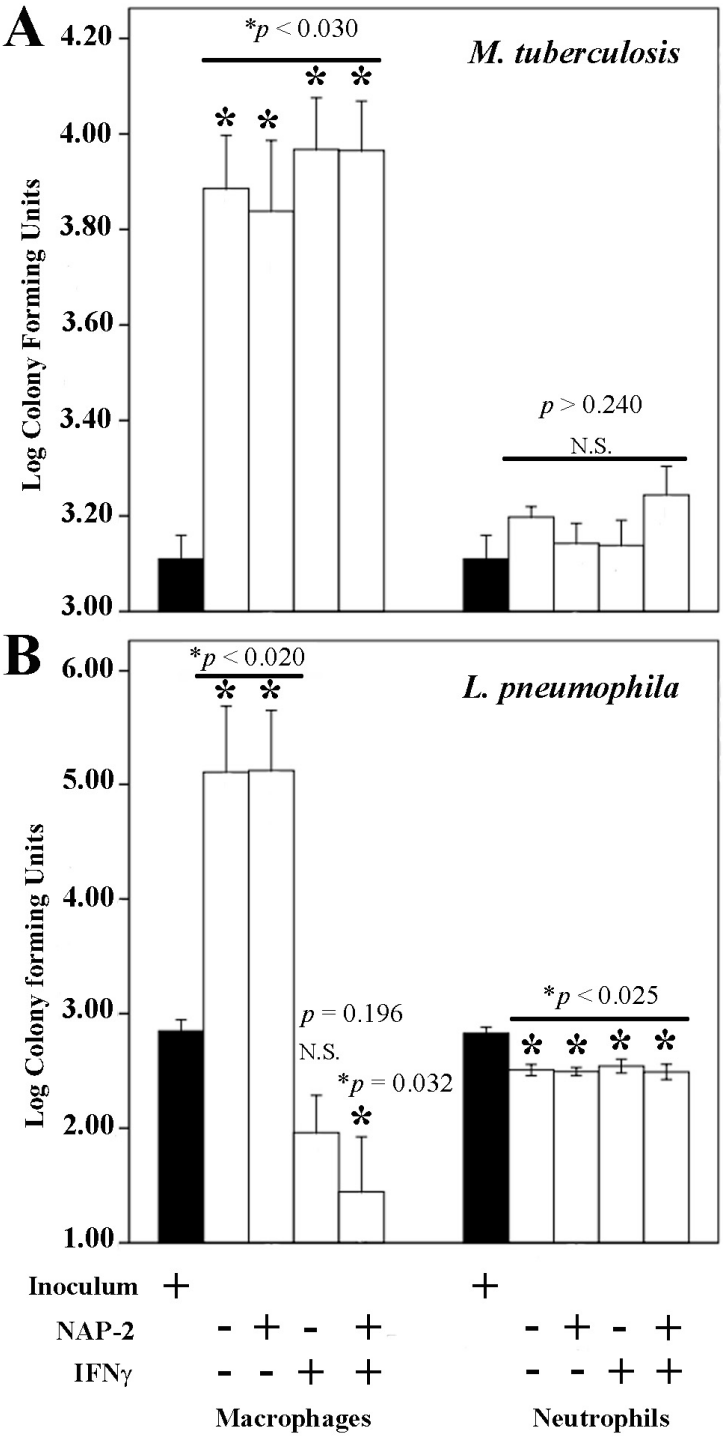

Fig 4. Influence of NAP-2 on the antimicrobial activity of phagocytes. Data represent the average of the log number of colonies with standard deviation from four independent experiments. A) *M. tuberculosis* infection. B) *L. pneumophila* infection. Pairwise comparisons versus "Inoculum" with $*P < 0.05$ were considered significant. N.S. stands for Non-Significant.

Table 2. Antimicrobial activity of NAP-2 transfected U937 cells against *L. pneumophila*

	Log CFU	P
Inoculated bacteria	2.88 SD 0.23 (2.51-3.26)*	< 0.001
pcDNA3	5.77 SD 0.26 (5.35-6.19)	
pcDNA3 + IFNγ	5.60 SD 0.13 (5.40-5.80)	0.798
pcDNA3-NAP-2	5.23 SD 0.24 (4.86-5.61)*	0.023
pcDNA3-NAP-2 + IFNγ	5.22 SD 0.19 (4.91-5.52)*	0.018

Data are the average of the log number of colonies with SD (95% confidence interval) from four independent experiments. Pairwise comparisons versus the pcDNA3 group with *$P < 0.05$ were considered significant.

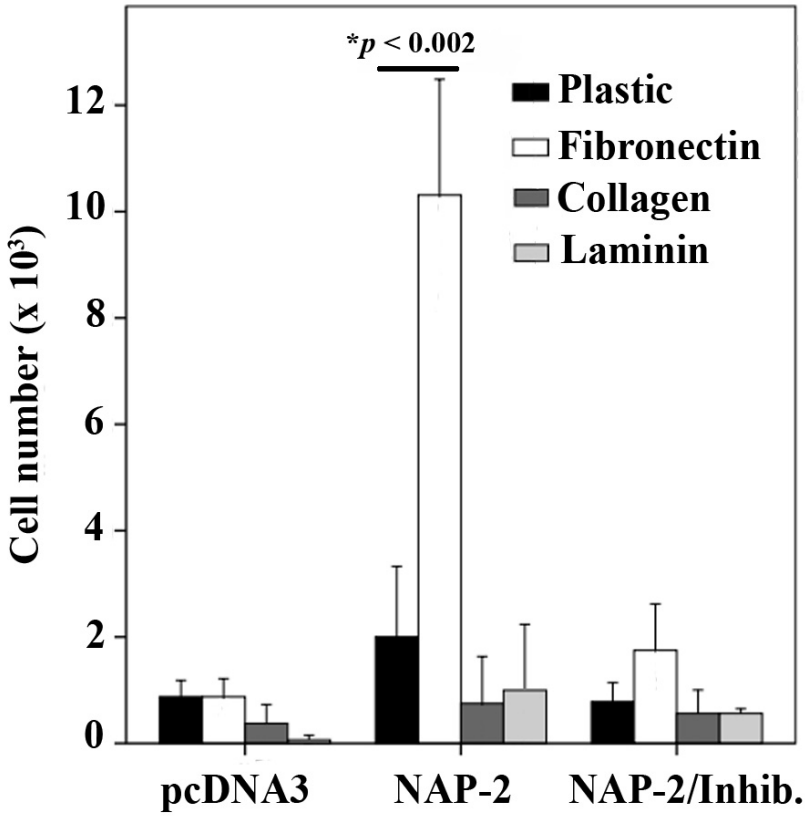

Fig. 5. Adhesion of NAP-2 transfected cells. Wells were either uncoated (plastic), or coated with fibronectin, collagen type IV or laminin. Data represent the mean of the number of cells ± SD remaining in the wells after washing non-adherent cells. Transfected cells with the pcDNA3 vector represented the negative control. Inhibition of fibronectin binding was accomplished with the incubation of cells in the presence of a GRGDSP peptide. Pairwise comparisons versus "Plastic" with $*P < 0.05$ are considered significant.