Microarray analysis of *Mycobacterium tuberculosis*-infected monocytes reveals *IL-26* as a new candidate gene for tuberculosis susceptibility

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Summary

Differences in the activity of monocytes/macrophages, important target cells of *Mycobacterium tuberculosis*, might influence tuberculosis progression. With the purpose of identifying candidate genes for tuberculosis susceptibility we infected monocytes from both, healthy elderly individuals (a tuberculosis susceptibility group) and elderly tuberculosis patients with *M. tuberculosis*, and performed a microarray experiment. We detected 78 differentially expressed transcripts and confirmed these results by quantitative PCR of selected genes. We found that monocytes from tuberculosis patients showed similar expression patterns for these genes, regardless of whether they were obtained from younger or older patients. Only one of the detected genes corresponded to a cytokine: *IL-26*, a member of the IL-10 cytokine family which we found to be downregulated in infected monocytes from tuberculosis patients. Non-infected monocytes secreted IL-26 constitutively but they reacted strongly to *M. tuberculosis* infection by decreasing IL-26 production. Furthermore, IL-26 serum concentrations appeared to be lower in the tuberculosis patients. When whole blood was infected, IL-26 inhibited the observed the pathogen-killing capability. Although lymphocytes expressed *IL26R*, the receptor mRNA was not detected in either monocytes or neutrophils, suggesting that the inhibition of antimycobacterial activity may be mediated by lymphocytes. Additionally, IL-2 concentrations in infected blood were lower in the presence of IL-26. The negative influence of IL-26 on the antimycobacterial activity and its constitutive presence in both serum and monocyte supernatants prompt us to propose *IL-26* as a candidate gene for tuberculosis susceptibility.

Introduction

Although recent advances in diagnostics, drugs, and vaccines and the enhanced implementation of existing interventions have increased the prospects for improved clinical care and global tuberculosis control, the disease remains a major global health problem[1]. The appearance of extremely resistant strains has led to an additional concern due to the low success rate of treatment. Thus, global efforts to control resistant strains can no longer focus on high-risk patients and require the development of preventive and management strategies. Treatment regimens for these strains include the use of second line or new antibiotics, surgery and immunotherapy[2]. Adjunctive immunotherapy is based on the strengthening of the immune system to fight the infection, and promises to become an important complement to antibiotic treatments. The fact that nearly 50% of individuals exposed to *Mycobacterium tuberculosis* never become tuberculin skin test positive[3] may indicate that the bacterium is eliminated by innate immunity in resistant individuals. Nevertheless, the efforts of many research groups for the last decades have not yet resulted in the identification of the mechanisms responsible for a protective immunity against tuberculosis.

Phagocytes (macrophages and neutrophils) are prominent among the cellular components of innate immunity because they remove invading microorganisms. Alveolar macrophages, one of the main cellular targets of *M. tuberculosis* infection, play a dual role: they aim to kill the bacilli and they modulate the immune microenvironment through the secretion of cytokines and chemokines. Alveolar macrophages are considered a key mediator of inflammatory control in the tuberculous granuloma because they are the cells that interact most frequently with the bacillus and the other granuloma cells[4]. It is, however, remarkable that the *in vitro* infection of

human macrophages usually results in uncontrolled intracellular multiplication of the mycobacterium, as it is difficult to activate these cells[5].

Epidaemiological studies have lead to the identification of a number of predisposing factors that increase susceptibility to tuberculosis, including congenital immunodeficiencies, protein-calorie malnutrition, haematological malignancies, diabetes mellitus, or local lung damage due to smoking[6]. In low-prevalence countries, a major susceptibility group is the elderly people. The trend of tuberculosis into aged individuals appears to be explained by the ageing of the population[7]. Nevertheless, we have recently reported that there might be a cellular basis for this susceptibility because *M. tuberculosis* multiplies faster within macrophages from the elderly than from adults[8]. Therefore, older individuals comprise a suitable group for analysing tuberculosis susceptibility. To test the hypothesis that the *in vitro* monocyte response to *M. tuberculosis* infection is different in resistant (infected individuals, as determined by an interferon-gamma release assay, who had not developed tuberculosis) and susceptible (tuberculosis patients) elderly people, we have performed protein array and gene microarray experiments to analyse differential expression. In this way, we identified a number of candidate genes for tuberculosis susceptibility. The confirmation of their relationship with susceptibility will provide new targets for tuberculosis immunotherapy.

Materials and methods

Patients and controls

Peripheral blood was collected from volunteers following consent and approval of the protocol by the Hospital of León Clinical Research Ethics Board. The total number of volunteers included in the study was 74. Samples from 49 individuals were analysed in gene and protein expression studies: tuberculosis patients (11 pulmonary, 5 ganglionar

and 3 pleural) were classified as either “elders” (n = 11, 77–95 years old, average 81.6) or “adults” (n = 8, 28–56 years old, average 43.5); non-tuberculous controls were also classified as “elders” (n = 17, 75–89 years old, average 81.6) or “adults” (n = 13, 20–45 years old, average 28.1). In the gene microarray study, seven pulmonary tuberculosis patients (three men and four women, 77–95 years old, average 82.7) with different clinical conditions were included: one had psoriasis, one a previous heart failure, two had arterial hypertension, one had bronchial asthma, two had chronic obstructive pulmonary disease, and one had prostate cancer. The eight non-tuberculous controls (six men and two women, 76–89 years old, average 81.1) had scored a positive result in the QuantiFERON-TB Gold in-tube test (Cellestis, Carnegie, Victoria, Australia) . For the analysis of whole blood antimycobacterial activity, we used samples from 25 non-tuberculous individuals belonging to both age groups (20–90 years old, average 61.4).

Cellular isolation, cellular line and bacterial strain

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 (Miltenyi Biotec, Pozuelo de Alarcón, Madrid, Spain). $CD14^-$ cells were considered lymphocytes. Neutrophils were purified using dextran and Ficoll-Paque Plus density gradients. Cells were cultivated, within 4 hours from blood collection, in RPMI-1640 /10% autologous serum, at 37ºC in 5% CO_2. The A549 lung epithelial cell line was a kind gift of Antonio Fernández Medarde and was grown in RPMI-1640 medium with 10 % foetal bovine serum at 37ºC in 5% CO_2. *Mycobacterium tuberculosis* HL186T, a clinical strain isolated at the Hospital de León was grown on 7H11 agar that were frozen as described elsewhere[8]. This strain has been characterised by spoligotyping and the pattern has been compared with the Institut Pasteur de la Guadeloupe database[9]. HL186T has the spoligo

international type (SIT) 58 pattern (T5_Madrid2 family), with the octal code 777777557760771. This genotype is included in the Euro-American lineage[10] and in single nucleotide polymorphism cluster group (SCG) 6a[11].

Antibody array analysis

Monocytes were cultivated in 24 well plates (4×10^5 monocytes/well), infected with 4×10^5 bacteria (Multiplicity of Infection, MOI = 1.0) in 500 µl medium, and incubated for 24 hours. The supernatants obtained from two patients (1 and 2, 83 and 95 years old, respectively) and controls (1 and 2, 84 and 89 years old, respectively) were centrifuged for 5 min at 10.000 × g at room temperature in ultrafree-MC filter units (Millipore) of 0.45 µm to remove bacteria and then frozen at –80ºC. The supernatants were diluted ¼ in blocking buffer supplied by the manufacturer of the Ray Bio® Human Inflammation Antibody Array 3 (RayBiotech, GA, USA), which detects 40 cytokines and chemokines. Proteins were detected by chemiluminescence and developed using a Chemidoc-XRS image analyser (Bio-Rad).

Microarray analysis

Total RNA from cells infected as described above was prepared using Speedtools Total RNA Extraction Kit (Biotools B&M Labs, Madrid, Spain) and concentrated with centrifugal filter units: AMICON-0.5 ml-30K (Millipore IRELAND, Cork IRL). Microarrays were performed at the HCUV-IECSCYL. The evaluation of the quantity and quality was performed by spectrometry (NanoDrop ND1000, Thermo Fisher Scientific, DE, USA) and by the RNA Experion Bioanalyzer (BioRad) assay. According to the 'One-Color Microarray-Based Gene Expression Analysis' protocol Version 5.7 (Agilent p/n 4140-90040) from Agilent (Agilent Technologies, Santa Clara, CA, USA), 100 ng of purified total RNA was used to produce Cyanine 3-CTP-labeled cRNA using the Quick Amp Labeling kit (Agilent p/n 5190-0442). The cRNA was purified with the

RNeasy MinElute Cleanup kit (QIAGEN Iberia, Las Matas, Madrid, Spain) and eluted with 30 µl of RNase-free H_2O. Then, 1.65 ng of labeled cRNA was hybridised with Whole Human Genome Microarray 4×44K v2 (Design ID: 026652) containing 41,000+ unique human genes and transcripts. The arrays were scanned using an Agilent Microarray Scanner (Agilent G2565BA) according to the manufacturer's protocol, and the data were extracted using Agilent Feature Extraction Software 10.7.1.1 following the Agilent protocol GE1-107_Sep09. The microarrays data analysis was performed by using GeneSpring GX 11.0 software. The original data were cleansed and normalised using an algorithm consisting of three steps: background correction, p75 normalisation and expression calculation. Subsequent to log2 transformation, baseline transformation of the data was performed using the median of the control samples. Before the statistical analyses, all microarrays were subjected to quality analysis, as assessed by Principal Component analysis (PCA) plots and BoxWhisker Plots and filtering criteria. Entities were filtered based on their flag values and their signal intensity values. Student´s un paired *t* test was used to identify genes that were differentially expressed between the different groups at the level of significance $p < 0.05$ level of significance, applying the Westfall and Young permutation multiple testing corrections post-hoc method. The microarray data were submitted to the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57736).

Quantitative polymerase chain reaction (qPCR)

Total RNA from monocytes infected for 24 hours as described above was reverse transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences, MD, USA). Real-time PCR was performed with a Bio-Rad iCycler system (Hercules, CA, USA) using SYBR-Green (Molecular Probes, Invitrogen, Barcelona, Spain). The threshold cycle (Ct) values for each of the target genes were normalised to the Ct of the

reference gene *HPRT1* (hypoxanthine phosphoribosyltransferase 1). The $\Delta\Delta C_t$ method was used for quantification of the relative gene expression as the following ratio[12]: $(E_{target})^{\Delta Ct,\ target\ (calibrator\text{-}test)}/(E_{ref})^{\Delta Ct,\ ref\ (calibrator\ -\ test)}$, where target gene is the gene of interest, reference gene is *HPRT1*, calibrator is RNA from infected monocytes from one of the elders without tuberculosis, and test is RNA from the rest of the volunteers. The primers sequences used for qPCR are shown in Table 1.

Cytokine quantification

Purified monocytes were infected, and supernatants were recovered and microfiltered after 24 hours of incubation, as already indicated. Interleukin 26 from either cellular supernatants or sera was quantified by the Human Interleukin 26 (IL-26) ELISA kit (CUSABIO, Hubei, P. R. China).

Whole blood was diluted with RPMI-1640 without serum (1:1), and two 500-µl aliquots were infected with 5×10^5 bacteria. After the addition of IL-26 (25 ng/ml, R&D, MN, USA) to one of the aliquots, both were incubated at 37ºC in a tube rotator. After 4 days the infected blood was centrifuged at 14.000 × g for 5 min, and the supernatant was recovered. To remove bacteria the samples were microfiltered as indicated above and frozen at –80ºC. Cytokine determination was performed by flow cytometry using a FACSCan instrument (Becton Dickinson) with the BD Cytometric Bead Array system (Becton Dickinson) and the Human Th1/Th2/Th17 cytokine kit, which measures the amounts of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A. The concentration of IL-12p40 was measured using the Human IL-12p40 ELISA kit (Diaclone, Besançon, France).

Whole blood antimycobacterial activity

Diluted whole blood was infected as already indicated. For neutralisation experiments 2.5 µg of anti-IL26 IgG rabbit polyclonal antibody (orb6198, Biorbyt; Cambridge, UK)

was added. As a mock control 2.5 μg of a purified rabbit polyclonal anti-glutathione S-transferase (GST) antibody (obtained in our laboratory) was used. After 4 days, 10 μl was diluted in water (390 μl). After adding 50 μl of Middlebrook enrichment ADC and 50 μl of concentrated 7H9 medium (× 10) with 2% glycerol, serial dilutions were inoculated into 96-well plates and incubated at 37ºC for 10 days. CFU (Colony Forming Units) were counted under an inverted microscope at × 100 magnification[13].

Statistical analysis

Two groups comparisons of normally distributed data (the Shapiro-Wilk test) were performed by Student's *t*-test or paired Student's *t*-test (variances were homogeneous according to the Levene test). Non-normally distributed data were analysed by the Mann-Whitney *U* test and for paired data by the Wilcoxon signed-rank test. A p value < 0.05 was considered significant. The analysis was performed with PASW Statistics 18 for Windows (IBM Corporation, Somers, NY, USA).

Results

M. tuberculosis-infected monocytes from either elderly tuberculosis patients or controls exhibit a similar pattern of cytokine production

We decided to work with a clinical isolate in our *in vitro* infection studies, rather than with a laboratory strain because we wanted to reproduce as closely as possible the interaction between immune cells and a virulent strain that had recently caused disease. To accomplish this purpose, we used the HL186T strain in the 6th to 8th passage from the initial isolation at our hospital.

Monocytes become alveolar macrophages in the lung, where they play a major role in shaping the immune microenvironment, largely depending on their cytokine production. We investigated whether infected monocytes from elderly tuberculosis patients exhibit a different pattern of cytokine production compared with monocytes

from control elders. We chose patients with low Charlson indexes[14], which classify prognostic comorbidities. Tuberculosis patient 1 was a 95-year-old male with chronic obstructive pulmonary disease (Charlson index = 1) and tuberculosis patient 2 was an 83-year-old female with bronchial asthma (Charlson index = 0). Control 1 was an 84-year-old male who had suffered a heart failure and ischaemic cardiopathology (Charlson index = 2), and control 2 was an 89-year-old female who was an Alzheimer patient who had suffered heart failure (Charlson index = 2). The controls did not suffer from any infectious disease. Using an array that includes a number of inflammatory cytokines we observed that the most abundant proteins secreted were IL-6, IL-8, MCP-1, and MIP-1β (Fig. 1). The cytokines and amounts detected in each array were very similar, though the monocytes from control 1 secreted some TNFα but not control 2 or the patients. Therefore, by analysing the proteins included in the array, no specific pattern could be associated with any of the studied groups.

Differential gene expression between *M. tuberculosis*-infected monocytes from elderly tuberculosis patients and controls

We have hypothesised that susceptibility to tuberculosis may in some cases be based on subtle gene expression differences in infected monocytes. To identify these differences, we performed a whole-genome microarray analysis comparing the aforementioned groups of *M. tuberculosis*-infected monocytes from tuberculous and control elders (the individuals who were analysed in the protein array study were also included in this experiment) and detected 78 transcripts. Four of the 78 transcripts corresponded to pseudogenes (LOC390998, LOC646214, FKSG2 and RPS10P7) and six to uncharacterised transcripts with Agilent probe names A_33_P3312564, A_24_P366457, A_33_P3266609, A_33_P3295543, A_33_P3359984 and A_24_P255384. A list of the remaining 68 genes, classified by gene function categories, is shown in Table 2.

To confirm these results, we performed quantitative PCR experiments for selected genes, analysing a portion of the same samples used in the microarrays and also new ones. For further characterisation, we also included samples from younger tuberculosis patients and controls, indicated as "adults" (Fig. 2). Regarding the elderly, although the expression trend was the same as in the microarray analysis, the differences were not statistically significant for two of the genes (*Axl* and *IL-26*). For the adult samples the trend of the expression was reversed for IL-26, though the differences were again not significant, likely because the expression of these genes exhibited large variability. We observed in the quantitative PCR experiments that the expression of both *Axl* and *IL-26* was low, and as a consequence of the limitations of the technique, the variability was higher. When we compared *IL-26* expression in the samples that were analysed in both the microarray and the quantitative PCR experiments we found important differences in their variability (the interquartile range for the microarray data was 1.69 and for the quantitative PCR data was 6.78). Hence, to obtain meaningful results from the quantitative PCR experiments we would need larger sample sizes. Nonetheless, when the differences showed statistical significance (*Rab5C* and *Tyk2*), they followed the same pattern in both the elderly and adult groups. Therefore, monocytes from either elders or adults appeared to respond similarly to *M. tuberculosis* infection.

IL-26 protein expression

Our initial interest was in cytokines and chemokines, and it was a surprise to find that IL-26 was the only cytokine in the microarray gene list (Table 2). as infected monocytes are induced to produce several cytokines and chemokines (Fig. 1). The lack of statistical significance in the quantitative PCR experiment caused us to speculate about the biological relevance of the differential expression detected by microarrays. Thus, to

obtain further evidence, we analysed the amount of IL-26 in cellular supernatants and sera. In both elders and adults, the amount of protein was lower in the infected monocyte supernatants from tuberculosis patients than from controls, though the differences were significant only for the adult samples (Fig. 3A). A remarkable finding was, however, the high concentration of IL-26 observed in the supernatant of the non-infected monocytes from the elderly controls, which was greatly diminished after *M. tuberculosis* infection (Fig. 3B). This result indicates that IL-26 is constitutively expressed but is strongly downregulated after *M. tuberculosis* infection. Finally, the amount of IL-26 was significantly lower in the sera from tuberculosis patients in the adult group, but not in the elderly group (Fig. 3C). One of the adults, a 26-year-old female, had a very large serum concentration of IL-26 (1488 pg/ml), but she was healthy and apparently immunocompetent; no distinct feature that could justify this large cytokine concentration was found.

IL-26 biological activity

The IL-26 receptor is composed by two subunits: IL10 receptor 2 (IL10R2) and IL20 receptor 1 (IL20R1). It has already been described that both subunits are expressed in some, but not all, epithelial cells, namely, colon carcinoma cells and keratinocytes, but that monocytes do not express *IL20R1*[15]. We confirmed these results in monocytes and added A549, a human pulmonary cell line frequently used in tuberculosis studies, to the list of epithelial cells that express the receptor (Fig. 4). Furthermore, as in monocytes, no *IL20R1* expression was detected in neutrophils. We wondered whether any haematopoietic cell could be activated with the cytokines produced by monocytes and found that lymphocytes did express *IL20R1.* We also analysed the expression of *IL10R1*, which together with *IL10R2* constitute the *IL10R*, as a control but found no

differences in mycobacterial intracellular multiplication in A549 cells when IL-26 was added to the culture (data not shown).

The whole blood model is appropriate for measuring antimycobacterial activity because it shows killing activities depending on the bacterial strain and the donor[16], however, given the complexity of this model, interpretation of the results is difficult. Although our clinical isolate (HL186T) was killed by the blood components from most donors, this killing activity was inhibited in the presence of IL-26 (Fig. 5A). As IL-26 is constitutively produced by monocytes, we analysed whether its neutralisation with antibodies would have any influence; we observed that the basal antimycobacterial activity was significantly enhanced in the presence of an anti-IL26 antibody, but was unaffected by a negative control (rabbit IgG). IL-26 also influenced the amount of a cytokine in the infected blood. Using a CBA kit for seven cytokines, we barely detected five of them (< 3 pg/ml) and did not observe any influence of IL-26 on the amount of TNFα. The amount of IL-2 was lower, however, in infected blood incubated with IL-26. As another important cytokine in the immune response to tuberculosis is IL-12, we measured the IL-12p40 subunit by ELISA. Similarly to TNFα, no changes were detected in IL-12 production in infected cells activated with IL-26 (Fig. 5B).

Discussion

Susceptibility to tuberculosis is influenced by a number of variables, including genetic factors[6].We hypothesised that some of these factors may impair the ability of macrophages to control *M. tuberculosis*, resulting in the disease progression. As a useful model to test this hypothesis, we chose the population of elderly individuals, acknowledged as a susceptibility tuberculosis group. We based our experiments on the *in vitro* infections of monocytes obtained from volunteer donors, as these cells are the main targets of mycobacteria.

Microarray experiments have been widely used to analyse expression differences between different RNA populations. When we chose a whole-genome microarray approach, we were aware that the populations to be compared, namely, infected monocytes from elders (tuberculosis patients versus control donors), were most likely too similar to show a large number of differentially expressed genes. Nevertheless, our aim was to search for variations in the regulation of single relevant genes. In fact, when we applied a Bonferroni post-hoc test in the statistical analysis, no positives were detected; thus we utilised the Westfall and Young permutation test, which also adequately controls the family-wise error, to select just 78 transcripts. We consider these positives to be candidate genes for tuberculosis susceptibility that deserve to be analysed individually.

We detected only a few genes classically associated with the immune response. Notably, two of the categorised functional groups were vesicle processing and ubiquitination. Because it is an intracellular pathogen, vesicle processing plays a fundamental role in the control of mycobacterial multiplication[17]. One of the genes associated with this group corresponded to Rab5C, one of the three isoforms of Rab 5, together with Rab5A and Rab5B. We validated that Rab5C is more highly expressed in infected monocytes from tuberculosis patients than in controls. It has been shown that Rab5 accumulates in the *M. tuberculosis* phagosome, but that Rab7 is excluded, inducing a maturation arrest and *M. tuberculosis* survival[18]. Although there is little knowledge about the specific functional roles of each isoform, it is known that they are not redundant[19]. In addition, the importance of protein ubiquitination to macrophage antimicrobial activity has recently emerged[20]. As a last example, we also validated the increased gene expression of Tyk2, a non-receptor tyrosine kinase that has been demonstrated to be one of the genes involved in mendelian susceptibility to

mycobacterial diseases. This kinase is activated by the binding of cytokines such as IFN α/β, IL-10, and IL-12 to their receptors[21]. There are several reports that show that patients with mutations in *Tyk2* suffer from mycobacterial diseases[22,23,24], and it has been postulated that the interruption of IL-12 signalling downstream of the ligand-receptor interaction is the main mechanism related to susceptibility to mycobacterial diseases[21].

Our initial interest was the influence of the cytokines produced by macrophages in the granuloma immunological environment[4], but we did not find differential cytokine expression between tuberculosis patients and controls by protein array. In line with this result, we identified only one cytokine in the microarray analysis, IL-26, which belongs to the IL-10 cytokine family comprising IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26; however, there is little information regarding the immunological role of IL-26[25]. Here we present evidence that monocytes strongly downregulate *IL-26* expression after *M. tuberculosis* infection. We performed *in vitro* infections of whole blood to gain further insight in the influence of this cytokine on the immune response to tuberculosis and found that it exhibited an inhibitory effect, as has been described for IL-10, the prototype cytokine of the family[26], which is linked to the ability of *M. tuberculosis* to evade immune responses and mediate long-term infections in the lung[27]. The IL-10 receptor is expressed in monocytes and inhibits some antimycobacterial activities through the blockade of phagosomal maturation or the enhancement of mycobacterial survival[28]. Nevertheless, we have confirmed that the gene encoding the IL20R1 subunit of the IL26R is not expressed in monocytes[15]. In addition, we have found that this gene is also not expressed in neutrophils either. Intrigued by the nature of cells stimulated by IL-26, we studied lymphocytes and found that they expressed the genes encoding both IL20R1 and IL10R2. This result had been already reported by Nagalakshmi *et al.*[29] who

showed in a graphic the minimal expression of *IL20RI* in resting PBMCs. In fact, expression was so low that the authors concluded that haematopoietic cells did not express the gene. Consequently, unlike IL-10, the effect of IL-26 on blood antimycobacterial activity may be exclusively mediated by the inhibitory interaction between lymphocytes and phagocytes. Nevertheless, there is also the possibility that the biological activity of IL-26 is not mediated by its receptor, as has been described for viral infection of epithelial cells[30]. In any case, we did not detect differences in the intracellular multiplication of the bacterium in monocytes in the presence of IL-26 (data not shown). As mentioned above, IL-10 is an important inhibitory cytokine and has been intensively studied in tuberculosis. Although it was not one of the genes identified in the microarray experiment, we cannot rule out the possibility that we would detect differential expression of *IL-10* in differentiated macrophages, as opposed to monocytes. Interestingly, the IL-10R2 subunit of the IL-26 receptor signals through Tyk2[31], indicated above as one of the kinases identified in the microarray analysis presented in this report.

In contrast with some of the functional characteristics that are shared by both IL-26 and IL-10, their protein production patterns differ greatly. In unstimulated monocytes, IL-10 production is very low and is enhanced after *M. tuberculosis* infection[32]. In contrast, we found that IL-26 expression is constitutively high and is strongly decreased after infection. Additionally, IL-10 is more abundant in sera from tuberculosis patients than from healthy controls[33], but we observed that the serum IL-26 concentration was somehow lower in tuberculous than in non-tuberculous younger adults, but not in elders. It may be speculated that the immune system attempts to fight *M. tuberculosis* infection by downregulating the constitutive production of the inhibitory cytokine IL-26, but fails to reach a level that curtails disease development.

Two other members of the family, IL-22 and IL-24, stimulate cellular antimycobacterial activity. IL-22 produced from either NK cells or $IL22^{+}$ $CD4^{+}$ T cells inhibits intracellular replication in macrophages in human and rhesus macaque models, respectively[34,35], whereas in the mouse model, IL-22 is dispensable for the development of immunity against *Mycobacterium avium*[36]. Additionally, the administration of IL-24 has a positive effect against the bacterium in the mouse model and, as would be expected, the serum concentration of IL-24 is decreased in tuberculosis patients[37]. The lower concentration of IL-2 in the presence of IL-26 may also affect the immune response against *M. tuberculosis*. It has been postulated that an elevated IL-2/IFNγ ratio may be a marker for the successful elimination of *M. tuberculosis* infection[38] and that IL-2 confers resistance to severe tuberculosis in the macaque model[39]. Given the influence of IL-26 on the antimycobacterial activity observed in blood, the absence of IFNγ production was unexpected, even though some of the samples were from QuantiFERON-TB-positive volunteers. It is possible that the amount of bacteria inoculated was insufficient to elicit the lymphocyte response that may be observed in the QuantiFERON-TB test, which uses purified recombinant mycobacterial antigens. TNFα and IL-12 are also critical in the immune response to tuberculosis, but IL-26 did not influence their production by infected cells.

The patterns of both the IL-26 gene and protein expression are puzzling. The array data showed significantly lower *IL-26* expression in elderly tuberculosis patients compared to elderly control patients (latently infected). As shown in Figure 2, *IL-26* was only lower in elderly tuberculosis patients compared to elderly controls, whereas the result was the opposite in adult tuberculosis patients vs. adult controls. Differences in IL-26 at the protein level are lower in TB adults vs. control adults (opposite to the qPCR data), and no differences were observed in elderly tuberculosis patients vs.

controls (Figure 3A and C). We do not know the reasons for these conflicting results, but they are likely a consequence of the small sample sizes and large variability. Nevertheless, several arguments support the consideration of *IL-26* as a tuberculosis susceptibility gene candidate. First, the log2-fold change in the microarray experiment was one of the largest found (-3.47). Second, IL-26 protein production was lower in monocytes from tuberculosis patients than from controls for both the elderly and adult groups (Figure 3A). Third, there was a dramatic inhibition in IL-26 production in infected cells (Figure 3B). Fourth, recombinant IL-26 exerts a significant inhibitory influence on the antimycobacterial activity detected in whole blood. It is counterintuitive that an inhibitory protein is less expressed in monocytes from tuberculosis patients than from non-tuberculous controls, but a similar observation was also reported for Tyk2. As discussed above, *Tyk2* is a demonstrated susceptibility gene for mycobacterial diseases. Although mutations in this gene cause susceptibility, in our microarray experiment, we observed a higher expression in infected monocytes from tuberculosis patients than in those from controls.

In conclusion, our study has identified IL-26, a member of the IL-10 family, as a candidate gene for tuberculosis susceptibility. Although IL-26 also displays inhibitory properties, its behaviour is unlike that of other members of the IL-10 family. Furthermore, this gene is absent in mice[40], which makes it tempting to speculate that it is one the causes of the vast difference between the murine and human tuberculosis models. A deeper knowledge of the biological function of IL-26 is needed to understand its clinical importance in tuberculosis. Furthermore, the identification of IL-26 as a previously unrecognised factor encourages the analysis of each of the genes scored in the microarray experiment as candidates for tuberculosis susceptibility.

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Disclosures

The authors declare no conflict of interest.

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Table 1. Primers used in real time polymerase chain reaction

Gene name (GeneBank no.)	Sequence
AXL (NM_021913)	TTCCTCCTCTATTCCCGGCT TCAGCATGCAGTTCCTGGC
IL26 (NM_018402)	ATATCAAAGCAGCATGGCTCAAA TGACCAAAAACGTCTTCCATGA
RAB5C (NM_201434)	GTCTGCGGTAGGCAAATCCA TCCAGCTGTGTCCCAGATCTC
TYK2 (NM_003331)	ACCAGCCAGTGTCTGACCTATGA GCTTGTGGAAAACCGTAGGGT
IL10R1 (NM_001558)	GCCTGGGTAGCTGAATCTTC CACCAACACCCGCTTCTC
IL10R2 (NM_000628)	GTCTTCTTGTAAACGCACCAC GAATGGAGTGAGCCTGTCTG
IL20R1 (NM_014432)	ATGATTTTAGCCTTGAACTCTGATG CGAACACTCTTTACTGCGTACA
HPRT1 (NM_000194)	GGCCATCTGCTTAGTAGAGCTTTT TTAAACAACAATCCGCCCAAA

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	Eotax.	Eotax.2	G CSF	GM CSF	ICAM1	IFNγ	I309	IL1α
2	IL1β	IL2	IL3	IL4	IL6	IL6sR	IL7	IL8	IL10	IL11	IL12 p40	IL12 p70
3	IL13	IL15	IL16	IL17	IP10	MCP1	MCP2	M CSF	MIG	MIP 1α	MIP 1β	MIP 1δ
4	Rantes	TGF β2	TNF α	TNF β	sTNF RI	sTNF RII	PDGF B	TIMP 2	BLANK	BLANK	NEG	POS

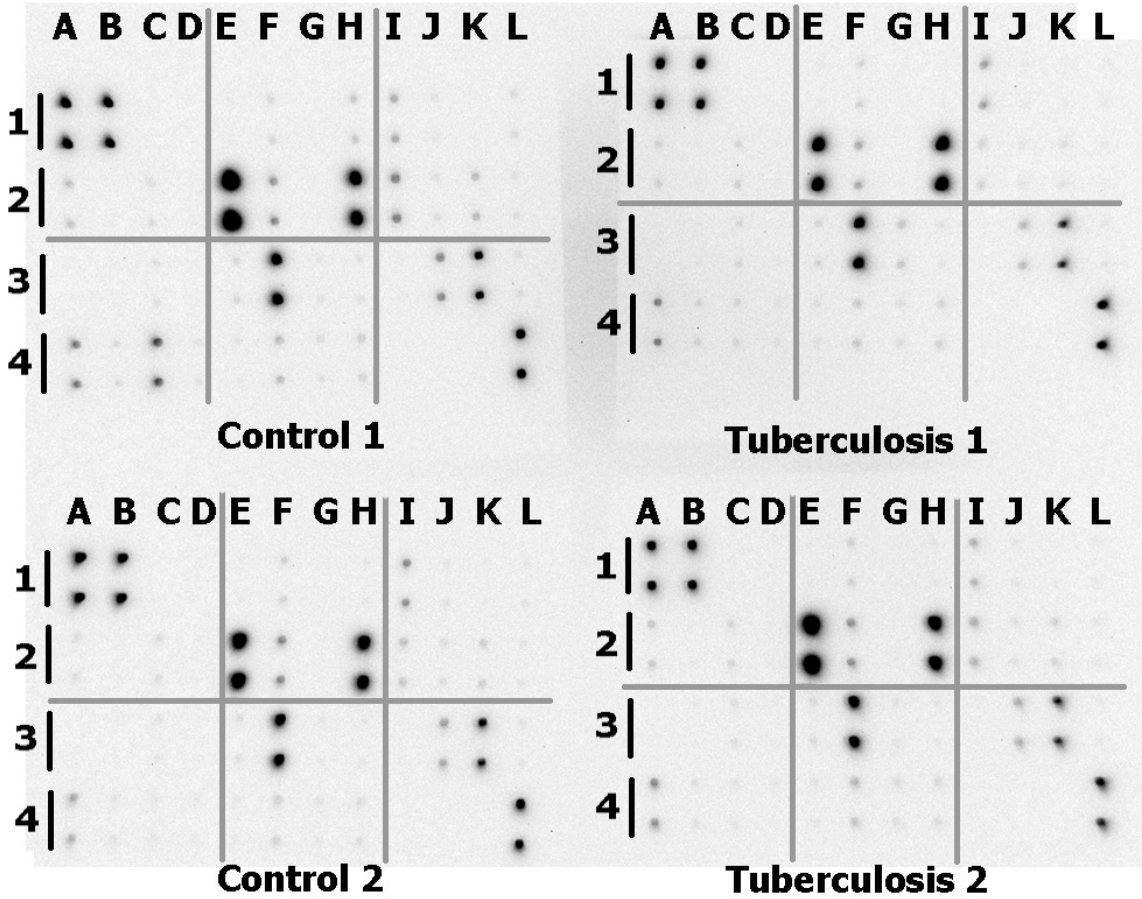

Figure 1. Cytokine production patterns in supernatants from *M. tuberculosis*-infected monocytes. Monocytes were infected with *M. tuberculosis* (MOI = 1) for 24 hours in RPMI/10% autologous serum. Antibodies on the membrane are dotted in duplicates, and the names of the proteins that they recognise are indicated. Positive (POS) and negative (NEG) array controls are included. The left panels correspond to infected monocytes from two different elderly donors without infectious diseases and the right panels to infected monocytes from two different elderly tuberculosis patients.

Table 2. Differential gene expression of *Mycobacterium tuberculosis* infected monoytes from elderly tuberculosis patients versus elderly Quantiferon positive controls

GeneBank no.[a]	Name	Log2 FC[b]	GeneBank no.[a]	Name	Log2 FC[b]
SIGNAL TRANSDUCTION			**SOLUTE CARRIERS**		
NM_003331	TYK2	+ 2.76	NM_003562	SLC25A11	+ 1.98
NM_021913	AXL	+ 2.15	NM_017877	SLC35F6	+ 1.78
NM_001136029	DEPDC5	+ 2.02	**UBIQUITINATION**		
NM_001087	AAMP	+ 1.98	NM_015710	GLTSCR2	+ 2.46
NM_014216	ITPK1	+ 1.84	NM_015528	RNF167	+ 1.97
NM_006148	LASP1	+ 1.68	NM_181575	AUP1	+ 1.91
NM_024776	SGK269	- 1.49	NM_006677	USP19	+ 1.78
NM_016038	SBDS	- 1.65	NM_020429	SMURF1	- 1.42
NM_015656	KIF26A	- 3.18	**OTHER FUNCTIONS**		
VESICLE PROCESSING			NM_001402	EEF1A	+ 2.59
NM_032389	ARFGAP2	+ 2.44		Translation elongation factor	
NM_014063	DBNL	+ 2.42	NM_006816	LMAN2	+ 2.41
NM_033198	PIGS	+ 2.30		Lectin	
NM_176812	CHMP4B	+ 1.83	NM_130395	WRNIP1	+ 1.84
NM_201434	RAB5C	+ 1.71		DNA synthesis	
NM_184231	NCKIPSD	+ 1.66	NM_022830	TUT1	+ 1.81
NM_031899	GORASP1	+ 1.56		Terminal uridylyl transferase	
NM_013306	SNX15	+ 1.47	NM_032116	KATNAL1	- 2.23
NM_014328	RUSC1	+ 1.35		Microtubule dynamics	
NM_044472	CDC42	- 1.64	NM_018402	IL26	- 3.47
NM_153235	TXLNB	- 3.87		Cytokine	

TRANSCRIPTION		
NM_032204	ASCC2	+ 2.31
NM_001950	E2F4	+ 2.15
NM_032377	ELOF1	+ 1.88
NM_001002878	THOC5	+ 1.86
NM_001350	DAXX	+ 1.63
NM_014815	MED24	+ 1.60
NM_001002259	CAPRIN2	- 1.70
NM_006454	MXD4	- 1.95
NM_015208	ANKRD12	- 2.10
NM_04381	ATF6B	- 3.17
METABOLISM		
NM_002629	PGAM1	+ 2.89
NM_212461	PRKAG1	+ 2.25
NM_000116	TAZ	+ 2.19
NM_002631	PGD	+ 1.89
NM_138387	G6PC3	+ 1.75
NM_025233	COASY	+ 1.65
NM_012239	SIRTUIN3	+ 1.64
NM_021100	NFS1	+ 1.38
NM_000353	TAT	- 4.76
NM_007185	TNRC4	- 3.74
	RNA processing	
UNKNOWN FUNCTION		
NM_015926	TEX264	+ 2.37
NM_033296	MRFAP1	+ 2.32
NM_016732	RALY	+ 2.16
NM_015680	CNPPD1	+ 2.08
NM_001033088	NGRN	+ 2.04
NM_024589	ROGDI	+ 2.03
NM_021943	ZFAND3	+ 1.84
NM_025078	PQLC1	+ 1.82
NM_001006109	DC12	+ 1.81
NM_015609	SZRD1	+ 1.64
NM_144611	CYB5D2	+ 1.62
NM_057161	KLHDC3	+ 1.46
NM_018259	TTC17	+ 1.36
NM_001145450	MORN2	- 1.88
NM_080764	ZNF280B	- 2.84

NOTE Genes are organized by gene function categories

[a] Genebank accesión number

[b] Log2 Fold Change

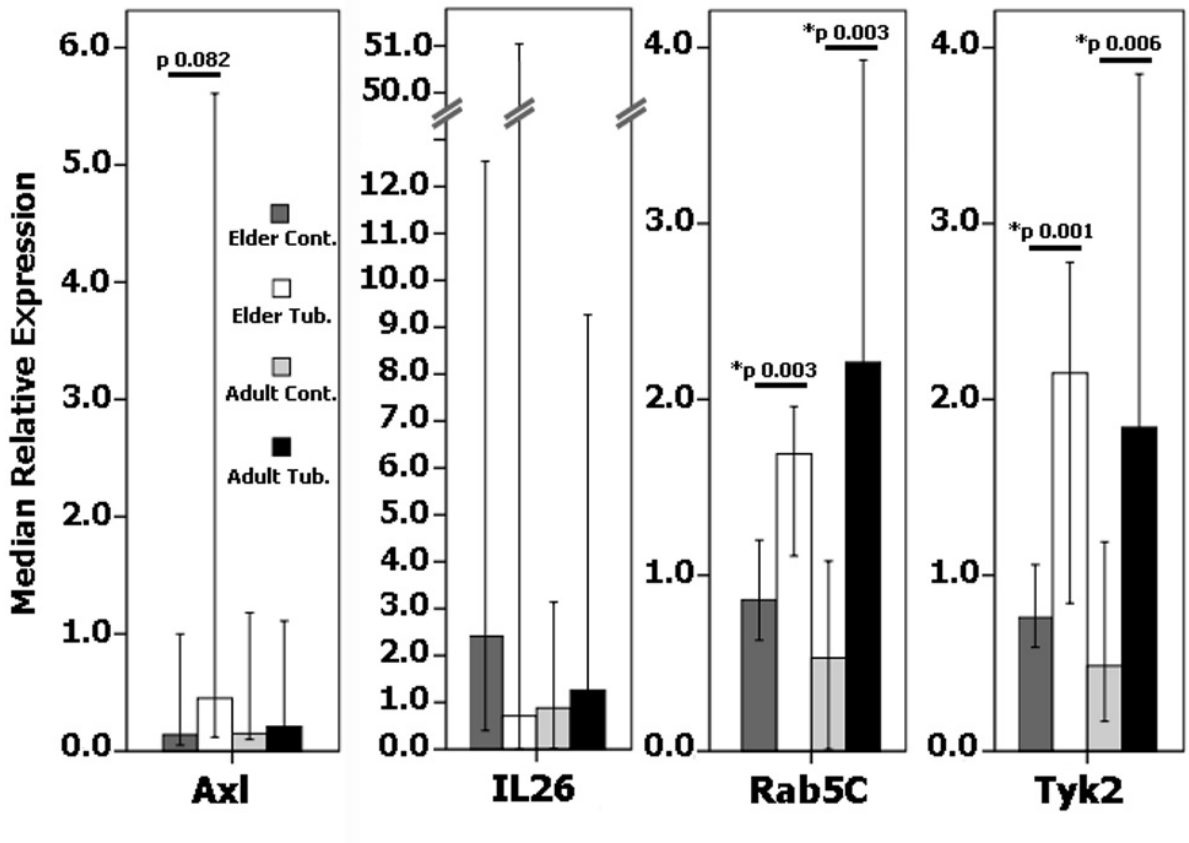

Figure 2. Relative expression of selected genes in *M. tuberculosis*-infected monocytes measured by qPCR. Monocytes were infected with *M. tuberculosis* (MOI = 1) for 24 hours in RPMI/10% autologous serum. Expression in the monocytes from one of the control elders was given the value 1 and was used for comparison to the other samples using the ΔΔCt method, normalising to *HPRT1* expression. Monocytes were obtained from four different groups: control elders (without infectious diseases, n = 16, dark grey bars), elders with tuberculosis (n = 7, white bars), control adults (n = 7, light grey bars) and adults with tuberculosis (n = 5, black bars). Group comparisons were performed between the monocytes from tuberculosis patients and from controls without infectious diseases using the Mann-Whitney *U* test. The results were considered significant when $P < 0.05$.

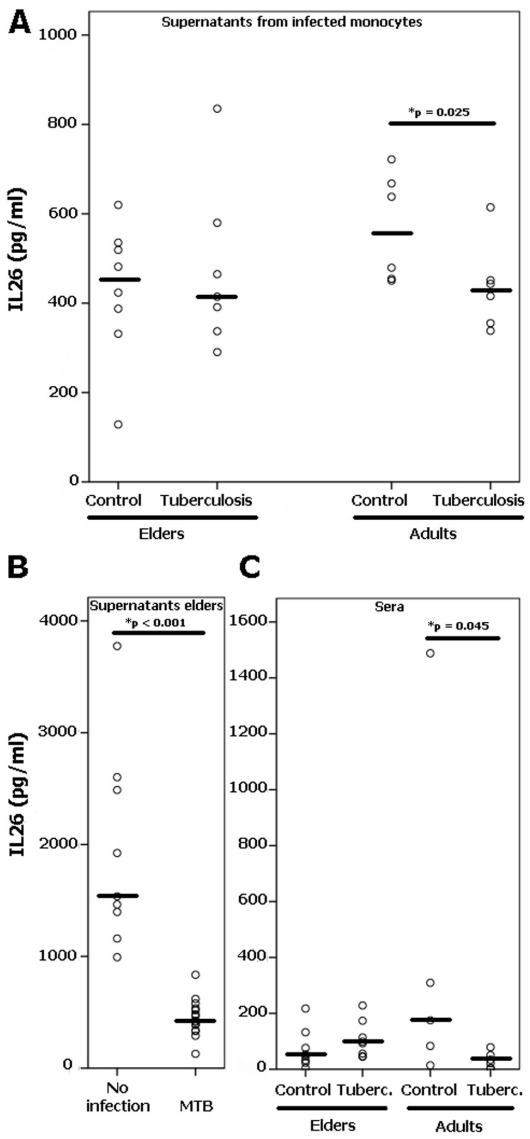

Figure 3. IL-26 protein expression in monocyte supernatants and sera. Monocytes were cultured for 24 hours in RPMI/10% autologous serum. *A*, comparison of IL-26 expression in infected monocytes from different groups. Monocytes were purified from four groups: control elders (without infectious diseases, n = 8), elders with tuberculosis (n = 7), control adults (n = 6) and adults with tuberculosis (n = 6) and were infected with *M. tuberculosis* (MOI = 1). *B*, diminished production of IL-26 after infection with *M. tuberculosis*. Monocytes were purified from a single group (elders) and were not infected (n = 8) or were infected with *M. tuberculosis* (MOI = 1, n = 15). *C*, comparison of IL-26 concentrations in sera from different groups. Sera were obtained from four groups: control elders (n = 8), elders with tuberculosis (n = 7), control adults (n = 5), and adults with tuberculosis (n = 6). Group comparisons were performed with the Mann-Whitney *U* test. The results were considered significant when $P < 0.05$.

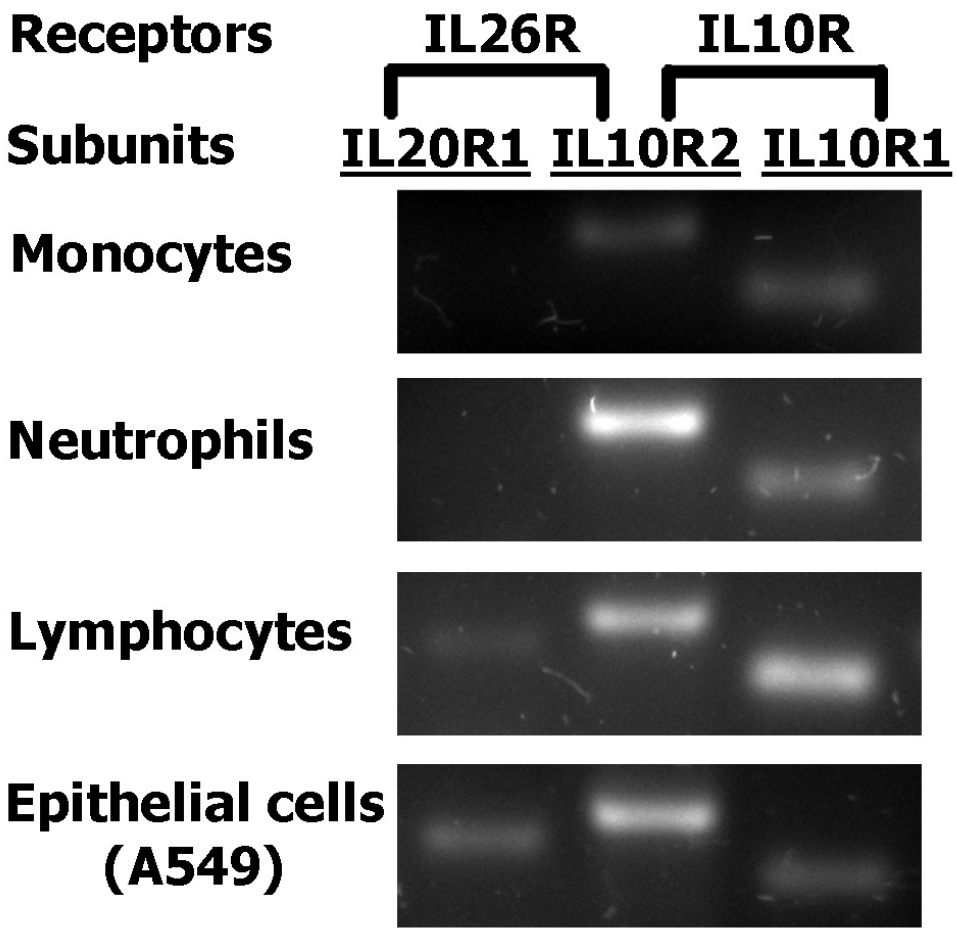

Figure 4. Gene expression of the IL-26 receptor subunits. Monocytes, neutrophils and lymphocytes cDNA from three elders and cDNA from the A549 cell line were analysed by PCR using primers specific for *IL20R1* (PCR band 127 base pairs), *IL10R2* (149 base pairs), and *IL10R1* (104 base pairs). The expression patterns were the same for all elders; a representative one is shown. Reactions were visualized in a 2% agarose gel stained with ethidium bromide.

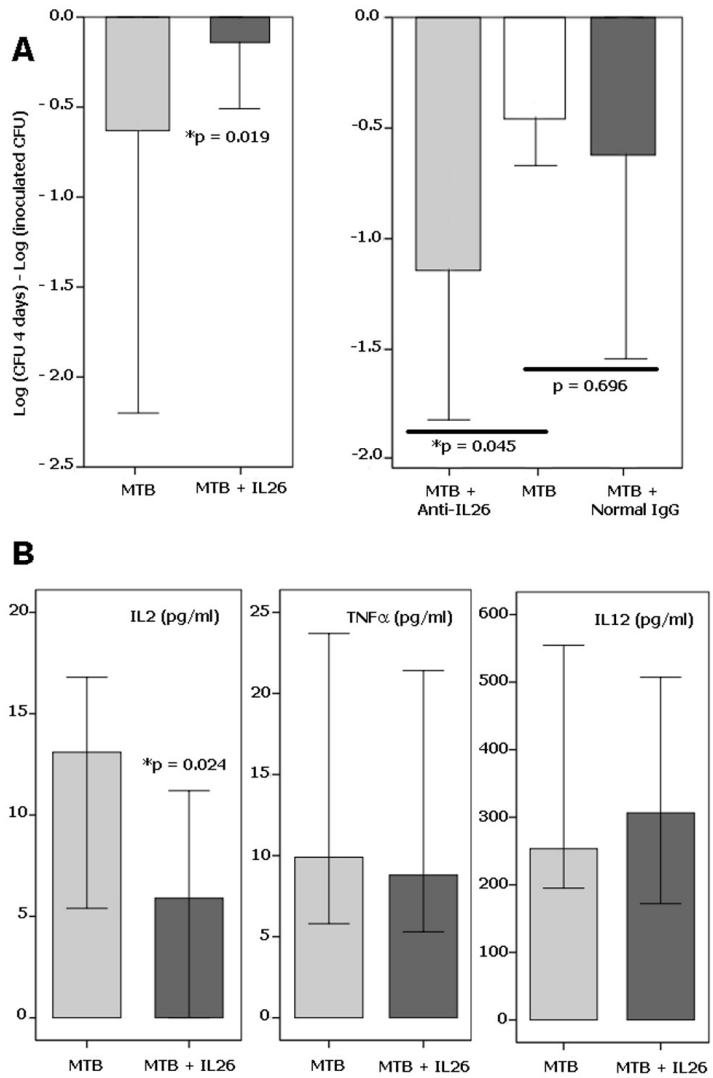

Figure 5. IL-26 biological activity. Diluted blood (500 µl) from donors without tuberculosis were infected with 5×10^5 bacteria and incubated for 4 days with or without IL-26 (25 ng/ml). *A*, antimicrobial activity was measured as the number of bacteria that survived after an incubation of 4 days. The data are represented as the mean difference between log10 (CFU after 4 days) and log10 (inoculated CFU) ± S.D. A paired Student's *t*-test was performed to compare the antimicrobial activity in the presence or absence of IL-26 (n = 25) or an anti-IL26 neutralising antibody (n = 6). A mock IgG rabbit polyclonal antibody was used as a negative control. *B*, amount of cytokines (IL-2 and TNFα) measured by the Cytometric Bead Array. The data represent the median of the amount of each cytokine in pg/ml, and the bars correspond to the 95% confidence interval (n = 25). Paired comparisons were performed by the Wilcoxon signed-rank test. A $P < 0.05$ was considered significant.