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Pulmonary tuberculosis patients with a vitamin D deficiency demonstrate low local expression of the antimicrobial peptide LL-37 but enhanced FoxP3$^+$ regulatory T cells and IgG-secreting cells

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Abstract Control of human tuberculosis (TB) requires induction and maintenance of both macrophage and T cell effector functions. We demonstrate that pulmonary TB patients with a vitamin D deficiency had significantly reduced local levels of the vitamin D-inducible antimicrobial peptide LL-37 in granulomatous lesions compared to distal parenchyma from the infected lung. Instead, TB lesions were abundant in CD3$^+$ T cells and FoxP3$^+$ regulatory T cells as well as IgG-secreting CD20$^+$ B cells, particularly in sputum-smear positive patients with cavitary TB. Mycobacteria-specific serum IgG titers were also elevated in patients with active TB. An up-regulation of the B cell stimulatory cytokine IL-21 correlated with mRNA expression of CD20, total IgG and also IL-10 in the TB lesions. Altogether, vitamin D-deficient TB patients expressed a weak antimicrobial response but an IL-21 associated expansion of IgG-secreting B cells combined with a rise in FoxP3$^+$ regulatory T cells at the local site of infection.
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Abbreviations: TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; NO, nitric oxide; CTL, cytolytic T cell; Th, T helper; Treg, regulatory T cell; Breg, regulatory B cell; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; Ig, immunoglobulin; HIV, human immunodeficiency virus; iNOS, inducible nitric oxide synthase; Ct, cycle threshold; ACIA, acquired computerized image analysis; BCG, Bacillus Calmette Guerin; PBS, phosphate buffered saline; FCS, fetal calf serum; OD, optical density; IQR, interquartile range; LA, lymphoid aggregates.

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1. Introduction

Tuberculosis (TB) remains a major global health problem and thus an improved understanding of the pathogenic mechanisms involved in the progression of infection and disease is required to develop new therapeutic strategies. Protective host immunity in *Mycobacterium tuberculosis* (Mtb) infection is dependent on both innate [1] and adaptive [2] immune responses. This includes important antimicrobial responses of both activated macrophages and T cells to produce potent bactericidal compounds such as reactive oxygen and nitrogen intermediates, antimicrobial peptides and granule-associated cytolytic effector molecules, respectively [3,4]. Macrophages kill intracellular Mtb bacilli primarily through production of nitric oxide (NO) [5] as well as the cationic antimicrobial peptide human cathelicidin, LL-37 [6]. The importance of LL-37 in human TB has been revealed partly through studies on the immunomodulatory effects of vitamin D, which is known to promote LL-37 expression and intracellular killing of Mtb [7,8]. Importantly, low levels of vitamin D have been shown to be associated with an enhanced susceptibility to develop active TB [7]. LL-37 disrupts bacterial membrane integrity and also induces autophagy, which is a physiological process known to enhance intracellular degradation of mycobacteria [9]. Cytolytic T cells (CTLs) are also important in TB immune control and execute killing of Mtb-infected cells by the coordinated release of the pore-forming protein perforin and the antimicrobial peptide granulysin [10,11]. We have previously demonstrated defective expression of perforin and granulysin in CD8+ CTLs at the site of Mtb infection in lung [12] and lymph nodes [13] from patients with progressive TB. Impaired CTL responses may be the consequence of excess regulatory T cell (Treg) responses including local expansion and infiltration of $FoxP3^+$ Treg cells [13,14] that have been reported to suppress immune responses in TB [14,15]. Although Treg cells could reduce local immunopathology in chronic infections such as TB, a high abundance of Treg cells could also prevent protective Th1 responses and result in a failure to control the infection [16].

Whereas protective immunity in human TB requires proper infiltration and activation of $CD4^+$ Th1 cells and $CD8^+$ CTLs [3,4], the need for B cells and humoral immune responses remains controversial [17]. Since Mtb is an intracellular pathogen, antibody-mediated immune responses may not confer efficient protection. Instead, Mtb-specific antibody responses may be useful for diagnostic purposes or as biomarkers of active disease and/or progression of disease [18,19]. However, B cells can also act as antigen-presenting cells that may have a role to enhance activation of local T cell responses in Mtb-infected tissues [17]. Recently, it has become evident that a subset of regulatory B cells (Breg cells) control inflammation and autoimmunity in both mice [20] and humans [21]. There may also be a functional link between certain rare subsets of Breg cell and Treg cells that may contribute to the immunological balance in different human diseases [22].

A variety of inflammatory and immunoregulatory cytokines are produced during active TB infection that could influence the outcome of disease at the local site of infection. Typically, Th1 (IFN-γ, TNF-α) and Th17 (IL-17) cytokines promote bactericidal functions in macrophages and T cells and also regulate granuloma formation in TB, while Th2 cytokines (IL-4, IL-13) and anti-inflammatory cytokines (IL-10, TGF-β) counteract Th1 responses and promote humoral responses [4,23]. While IL-17 has been shown to regulate the production of antimicrobial peptides [24] as well as the activation of IFN-γ expressing T cells at mucosal sites [25], the potential role of IL-21 in mycobacterial infections has been less investigated. Interestingly, IL-21 has been found to be highly potent to promote activation and differentiation of human B cells [26] including antibody-secretion and Ig-class switch [27].

In this study, we aimed to discover the nature of the unfavorable immune responses present at the local site of infection in patients with chronic pulmonary TB. For the first time we demonstrate that active TB patients with low serum levels of vitamin D had significantly reduced expression of LL-37 in pulmonary lesions where mycobacterial antigens were accumulated. Instead, lymphoid aggregates consisting of both $CD3^+$ T and $FoxP3^+$ T cells as well as $CD20^+$ B cells and IgG-secreting cells were significantly increased in the TB lesions compared to distal lung parenchyma of the Mtb-infected lungs. We also detected elevated mycobacteria-specific IgG titers in serum samples from active TB patients compared to healthy controls. Interestingly, we found a significant up-regulation of IL-21 as well as IL-10 in the granulomatous TB lesions, which may promote humoral and regulatory immune responses at the local site of Mtb infection. The findings from this study may be used to describe novel immune response profiles that are associated with progression of active TB disease in humans.

2. Materials and methods

2.1. Patients

Lung tissue biopsies were obtained from patients with active pulmonary TB (n = 19) who underwent surgical treatment for chronic TB at the Department of Thoracic Surgery at St Petersburg State City TB Hospital, Russia, as previously described [12]. Inclusion criteria: HIV-negative patients >18 years with a TB diagnosis based on TB contact and clinical symptoms (severe weight loss, >8 weeks of productive cough and hemoptysis), typical chest x-ray findings, positive sputum-smear microscopy (11/19), positive Mtb culture of lung tissue homogenate (6/19), histopathology consistent with TB (19/19) and/or a positive PCR (19/19). Despite extensive chemotherapy including ≥ 5 first- and second-line drugs for more than 6 months, patients with a confirmed TB diagnosis failed to respond to conventional treatment and thus surgical treatment was performed on all study subjects to limit mycobacterial load and disease progression. From the resected lung segments, tissue biopsies were collected from the granulomatous TB lesion (pathological site) and also from a distal, macroscopically normal area of the lung parenchyma (unaffected site). Thus, we compared paired observations obtained from different sites of the Mtb-infected lung of the same individual. The discrimination between pathological TB lesion and macroscopically normal lung parenchyma was made by the surgeon and a TB specialist based on visual examination of the resected lung segments. Tissue biopsies were immediately frozen and stored at -85 °C for immunological analysis. Blood samples were also collected from the patients at the

time of surgery, from which serum was obtained and stored at −85 °C for molecular analysis. Serum samples were also obtained from n = 10 Swedish healthy controls recruited at the Karolinska University Hospital Huddinge. Patients were recruited into the study after informed consent and ethical approval in both Russia and Sweden.

2.2. Quantitative real-time PCR of lung tissue sections

Cryopreserved lung tissues were embedded in OCT-compound (Tissue-TEK, Sakura, Alphen aan den rijn, the Netherlands) and sectioned in 2×50 μm thick sections that were used for RNA extraction (Ambion RiboPure extraction kit, Invitrogen, Carlsbad, CA). Reverse transcription of RNA was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Amplification of 18S, CD68, CD3, CD20, FoxP3, iNOS, LL-37, granzyme A, perforin, granulysin, total IgG, TNF-α, IFN-γ, IL-17A, IL-21, IL-4, IL-13, IL-10 and TGF-β cDNA was performed using the ABI PRISM 7700 sequence detection system and commercial 6-carboxyfluorescein dye-labeled TaqMan MGB probes and primers (Applied Biosystems). Cycle threshold (Ct) values for the target genes were normalized to the Ct value for the housekeeping gene 18S. The relative expression of the target genes was calculated by comparing the Ct value for the TB lesion to that for the distal lung tissue from the same patient [28]. Data are presented as fold change of mRNA in the TB lesions compared to the mRNA expression in the distal lung parenchyma.

2.3. Immunohistochemistry and *in situ* computerized image analysis of lung tissue sections

Immunohistology and *in situ* computerized image analysis were used to study tissue morphology and cellular phenotypes and to quantify the functional expression of effector molecules in TB infected tissues. OCT-embedded lung tissues were sectioned in 8 μm thick sections and mounted on HTC microscope slides (Histolabs, Gothenburg, Sweden) and fixed in 4% formaldehyde (Sigma, Stockholm, Sweden) for 15 minutes. Immunohistochemistry was performed according to the ABC-method [12]. Primary antibodies were CD68, MAC387, CD3 (BD, San Diego, CA), neutrophil elastase, polyclonal *Mycobacterium bovis* bacille Calmette-Guérin (pAb-BCG) (Dako, Glostrup, Denmark), CD20 and FoxP3 (Abcam, Cambridge, UK), IgG and IL-21 (Jackson ImmunoResearch Laboratories, West grove, PA), IL-10 (Nordic Biosite, Stockholm, Sweden) as well as iNOS (BD/Transduction Laboratories, San Jose, CA). LL-37 was kindly provided by Dr. Andreas Cederlund at the Department of Medical Biochemistry and Biophysics (MBB) at Karolinska Institutet. Biotinylated secondary antibodies included goat anti-mouse IgG, rabbit anti-goat IgG and swine anti-rabbit F(ab´)$_2$ from Dako. Tissue sections stained with secondary antibodies only were used as negative controls. Positive immunostaining (brown) was developed using a diaminobenzidine substrate (Vector Laboratories, Burlingame, CA) and hematoxylin was used for nuclear counterstaining (blue). Immunohistochemical stainings were analysed by acquired computerized image analysis (ACIA) using a DMR-X microscope and a digital computerized Quantimet 5501 W image analyser (Leica Microsystems, Wetzlar, Germany) [29]. Single-cell protein expression was assessed in 20 to 50 high-power fields using a Qwin 550 software program (Leica Imaging Systems, Germany). Protein expression was determined in the total relevant cell area (fibrotic and necrotic tissue areas were excluded) where the total cell area was defined as the nucleated and cytoplasmic area within the tissue biopsy. Data are presented as ACIA values, calculated as the percentage of the positively stained area in the cell area multiplied by the total mean intensity of positive staining. Tissues included in the software analysis had a mean size of 5.0×10^6 μm^2. Two-color staining was performed using indirect immunofluorescence and analysis performed using a Nikon A1R spectral detector confocal microscope (Nikon Instruments, Amstrelveen, Netherlands). For dual staining, tissues were stained with mouse anti-human CD138 and CD20 (Abcam) or rabbit anti-human IgG (Jackson ImmunoResearch Laboratories) followed by the appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, Oregon).

2.4. BCG-specific IgG ELISA

The release of mycobacteria-specific IgG antibodies in serum samples obtained from the study subjects was quantified using a Bacillus Calmette Guerin (BCG)-specific ELISA [18]. Briefly, a BCG vaccine (Japan BCG Laboratories, Tokyo, Japan) was used to coat Maxisorb plates (Nunc, Roskilde, Denmark) overnight at 4 °C. The plates were washed with PBS–0.05%Tween-20 (Sigma) and blocked with PBS + 10%FCS. Serum samples from patients and controls were added (100 μl/well, diluted 1:1800) and incubated for 2 h at 37 °C before washing and addition of a rabbit anti-human IgG horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Dilution buffer (PBS + 10%FCS) was used as negative control. The enzyme-substrate reaction was developed after 20 minutes using O-phenylenediamine (OPD) (Sigma) substrate solution. BCG-specific IgG titers were expressed as optical density (OD) measured at 492 nm multiplied by the dilution factor.

2.5. Vitamin D levels in serum samples

Concentrations of 25-hydroxyvitamin D2 and D3 in serum samples obtained from the study subjects were measured using the DiaSorin assay performed at the Chemical Laboratory, Karolinska University Hospital Solna. Serum samples were collected from TB patients and uninfected controls during the first six calendar months of the year (January-July).

2.6. Statistical analysis

Data that passed a normal distribution test (D´Agostino and Pearson omnibus normality test) were analyzed using a parametric test. Statistical significance of differences in serum vitamin D levels in sputum-smear positive (n = 11) and sputum-smear negative (n = 8) TB patients compared to controls (n = 10) was determined by a non-parametric Kruskal-Wallis test. Fold changes of mRNA determined as the ratio of mRNA expression in the TB lesions compared to distal lung parenchyma (n = 19) were analyzed using a

Table 1 Demographics of pulmonary TB patients [a].

Chest X-ray findings [b]	Number	Gender (M/F) [c]	Age (yr) (mean)	Sputum positive [a]	Culture positive [a]	PCR positive [a]	Histology positive [a]
Cavitary TB	10	6/4	31	8	5	10	10
Non-cavitary TB	9	8/1	46	3	1	9	9

[a] All patients had clinical symptoms of active pulmonary TB at the time of surgery despite prior treatment with ≥5 first- and second-line anti-TB drugs. TB diagnosis was based on clinical history, sputum-smear microscopy, Mtb culture of tissue homogenate, PCR, histopathology of tissue sections and chest X-ray data.
[b] The clinical forms of pulmonary TB were diagnosed using chest X-ray as either cavitary TB (formed cavern >3 cm, with a fibrotic capsule) or non-cavitary TB (pulmonary infiltrates or multiple nodular densities with various forms and sizes). Cavitary TB was considered to include extensive tissue destruction with formation of a fibrotic cavern whereas non-cavitary TB included limited fibrotic processes.
[c] M, male; F, female.

paired *t* test and the data are presented as a box and whisker plot (median and range). ACIA values in the TB lesions compared to distal lung parenchyma (n = 19) were analyzed using a paired *t* test and the data are presented in bar graphs (mean +/− SE). Values from two individual experiments are shown. ACIA values determined in the TB lesions from sputum-smear positive (n = 11) compared to sputum-smear negative (n = 8) TB patients was analyzed using a non-parametric Mann–Whitney test and the data are presented in bar graphs (median +/− IQR). Spearman's correlation test was used for the correlation analyses. Differences between groups were considered to be statistically significant at $p < 0.05$. The statistical analyses were performed in GraphPad Prism-5.

3. Results

3.1. Decreased serum levels of vitamin D is associated with low expression of LL-37 in TB lesions that have a high content of mycobacterial antigens

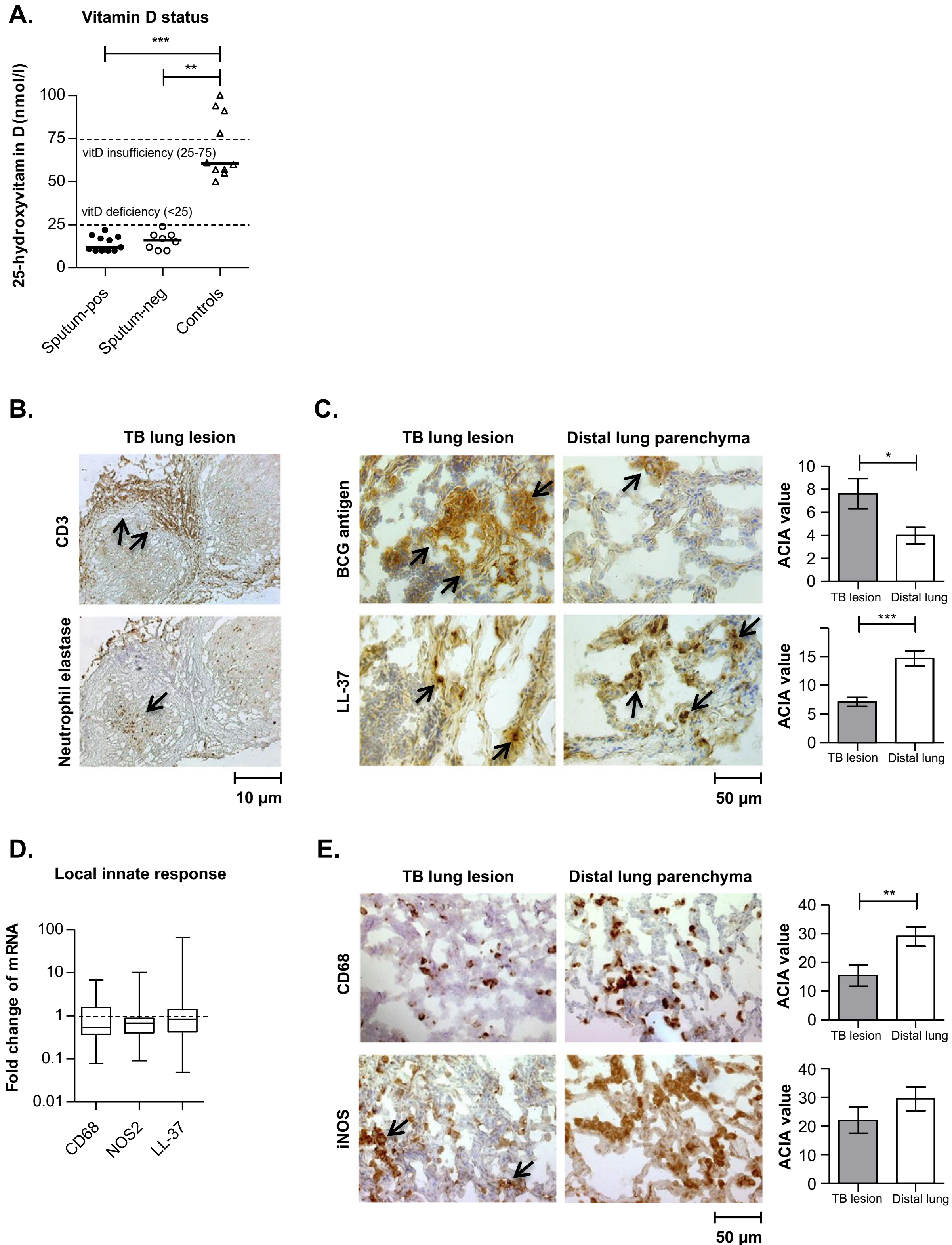
In this study, lung tissue biopsies and serum samples were collected from 19 patients with cavitary or non-cavitary forms of chronic pulmonary TB (Table 1) [12]. First, we assessed serum levels of 25-hydroxyvitamin D in samples obtained from the TB patients and healthy controls (n = 10). Vitamin D is an immunomodulatory molecule that regulates both innate and adaptive immune responses and low levels of vitamin D have been shown to be associated with an enhanced susceptibility to develop active TB [7]. Accordingly, we found that all TB patients were vitamin D deficient (<25 nmol/l), and both sputum-positive and sputum-negative patients expressed vitamin D levels that were significantly lower compared to the control cohort ($p < 0.001$ and $p < 0.01$, respectively) (Fig. 1A). Next, we assessed local immune responses including distribution and functional expression of myeloid cells and lymphocyte subsets in cryopreserved tissue sections from the Mtb-infected lungs using quantitative mRNA and *in situ* computerized image analysis. Microscopic examination of gross TB lesions at a lower magnification revealed large necrotic granulomas, with $CD3^+$ T cells in the mantel area and neutrophils in the core region (Fig. 1B). Immunostaining using a polyclonal *M. bovis* BCG antibody with documented cross-reactivity to Mtb-antigens [30], revealed a significantly ($p < 0.01$) higher antigen expression in pulmonary TB lesions compared to the distal lung parenchyma (Fig. 1C), which suggest persistence of mycobacteria primarily at the local site of infection. Contrary, significantly ($p < 0.0001$) lower levels of the antimicrobial peptide LL-37 were detected in the TB lesions compared to the distal sites (Fig. 1B), which may suggest a specific down-regulation of LL-37 at the site of mycobacterial infection. Quantitative real-time PCR demonstrated that mRNA levels of innate immune cells and effector molecules including markers of macrophages (CD68), inducible nitric oxide synthase (iNOS/NOS2) and LL-37, were all expressed at similar levels in the TB lesions compared to distal lung parenchyma (Fig. 1D). Despite comparable mRNA levels of CD68, *in situ* image analysis revealed that protein expression of CD68 was significantly ($p = 0.003$) lower in the TB lesions compared to the distal

Figure 1 Pulmonary TB patients with a vitamin D deficiency express low local levels of the vitamin D-inducible antimicrobial peptide LL-37 in granulomatous lesions from the Mtb-infected lung. (A) S-vitamin D levels (median and range) in sputum-smear positive (sputum-pos; n = 11) and sputum-smear negative (sputum-neg; n = 8) TB patients compared to healthy Swedish controls (n = 10). The dotted lines mark the threshold for vitamin D deficiency (<25 nmol/l) and insufficiency (25–75 nmol/l), respectively. Representative immunohistochemical images demonstrate expression of (B) $CD3^+$ T cells and neutrophil elastase in granulomatous TB lesions (magnification ×25) and (C) BCG antigens and LL-37-expressing cells in the Mtb-infected lung (magnification ×125). *In situ* computerized image analysis was used to determine the expression (mean ± SE) of BCG antigens and LL-37 in the TB lesions compared to distal lung (n = 19). (D) Relative mRNA expression (median and range) of CD68, iNOS and LL-37 in the TB lesions compared to distal lung parenchyma (n = 19) was quantified using real-time PCR. The dotted line represents a relative difference of 1. (E) Immunohistochemical images illustrates $CD68^+$ macrophages and iNOS-expressing cells in the Mtb-infected lung (magnification ×125) while *in situ* imaging was used to determine the expression (mean ± SE) of CD68 and iNOS in the TB lesions compared to distal lung (n = 19). Arrows in the images indicate positive cells (brown) whereas negative cells (blue) were counterstained with hematoxylin. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

sites (Fig. 1E). In addition, MAC387, which is a protein found on monocytes, reactive macrophages, neutrophils and mucosal epithelia, were also significantly (p = 0.01) lower in the TB lesions (data not shown); while iNOS expression was similar in the TB lesions and distal lung parenchyma (Fig. 1E).

3.2. Elevated levels of FoxP3$^+$ Treg cells and IgG-secreting cells in pulmonary TB lesions

We have previously demonstrated an impaired expression of perforin and granulysin in pulmonary TB lesions despite



elevated levels of CD3$^+$ T cells at this site [12,13]. Accordingly, mRNA expression of CD3 and the granule-associated effector molecule granzyme A were significantly ($p = 0.03$ and $p = 0.008$, respectively) higher in TB lung lesions compared to distal lung parenchyma, while the relative expression of perforin and granulysin were similar at the two sites (Fig. 2A). Instead, mRNA expression of the Treg marker FoxP3 was significantly ($p < 0.0001$) increased in the TB lesions (Fig. 2A). *In situ* image analysis demonstrated that the T cells were mostly organized in inflammatory infiltrates or lymphoid aggregates (LA) in the granulomatous TB lesions, while no such structures were observed in the distal lung parenchyma (Fig. 2B). Corresponding to mRNA data, acquired computerized image analysis revealed significantly ($p < 0.0001$) elevated levels of both CD3$^+$ T cells and FoxP3$^+$ Treg cells in the TB lesions as compared to the distal sites (Fig. 2B).

While diminished antimicrobial effector functions in myeloid cells and T cells result in reduced immune control in TB, local induction of B cell responses may represent adverse immune reactions in the progression of clinical TB. Here, we found a significant ($p < 0.0001$) induction of both CD20 and total IgG mRNA at the TB lesion site (Fig. 3A). Similar to CD3$^+$ T cells, both CD20$^+$ B cells and IgG-secreting cells were significantly elevated in the TB lesions compared to the distal sites and mostly enriched in the lymphoid aggregates in the lesions (Fig. 3B). We could also detect a significantly increased expression of the B cell markers CD19 ($p = 0.006$) and CD79a ($p = 0.0006$) in the TB lesions, while there was no difference in the expression of IgA-secreting cells between the lesions and the distal lung (data not shown). Double-staining of CD20 and CD138 as well as CD138 and IgG revealed the presence of double-positive plasma cells in the TB lung lesions (Fig. 3C). We also examined the

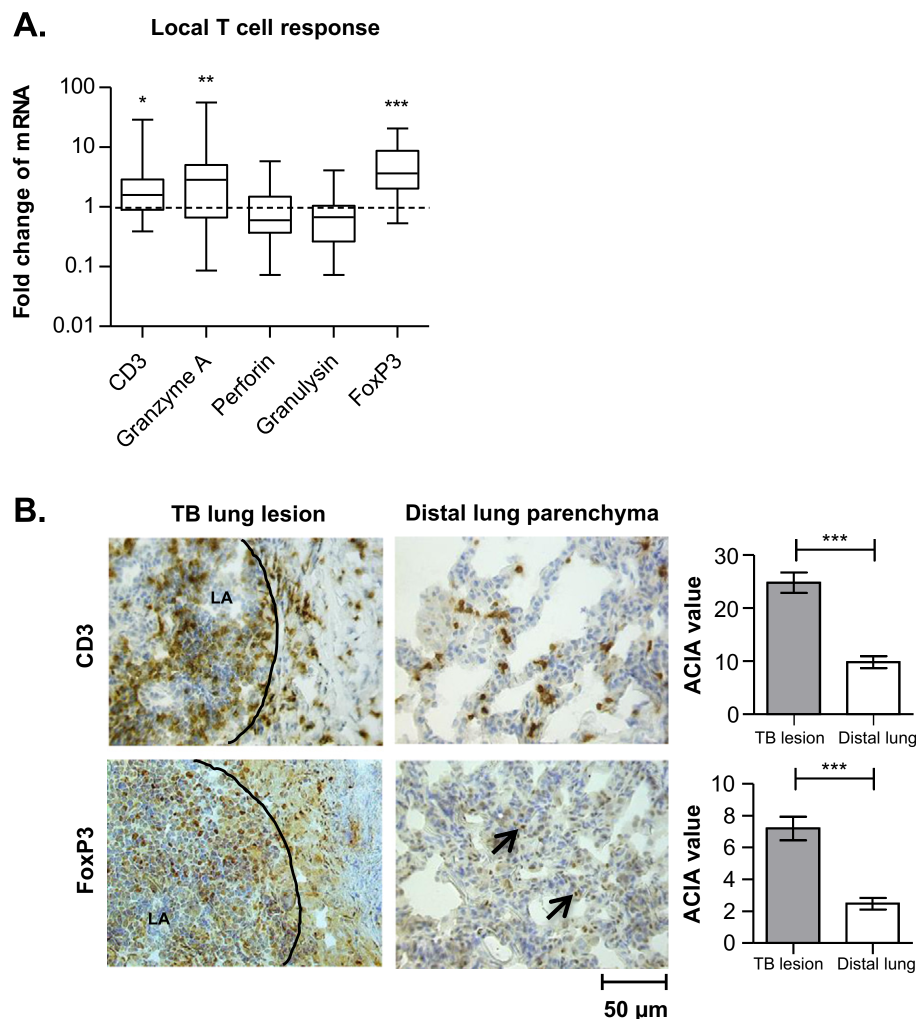


Figure 2 Elevated levels of CD3$^+$ T cells and FoxP3$^+$ Treg cells in TB lung lesions compared to distal lung parenchyma. (A) Relative mRNA expression (median and range) of CD3, granzyme A, perforin, granulysin and FoxP3 in the TB lesions compared to distal lung parenchyma ($n = 19$) was quantified using real-time PCR. The dotted line represents a relative difference of 1. (B) Representative immunohistochemical images demonstrate the expression and distribution of CD3$^+$ T cells and FoxP3$^+$ Treg cells in the Mtb-infected lung (magnification ×125). Lymphoid aggregates (LA) in the TB lung lesions are marked with a solid line. Arrows indicate positive cells (brown). *In situ* computerized image analysis was used to determine CD3 and FoxP3 expression (mean ± SE) in the TB lesions compared to distal lung ($n = 19$). $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

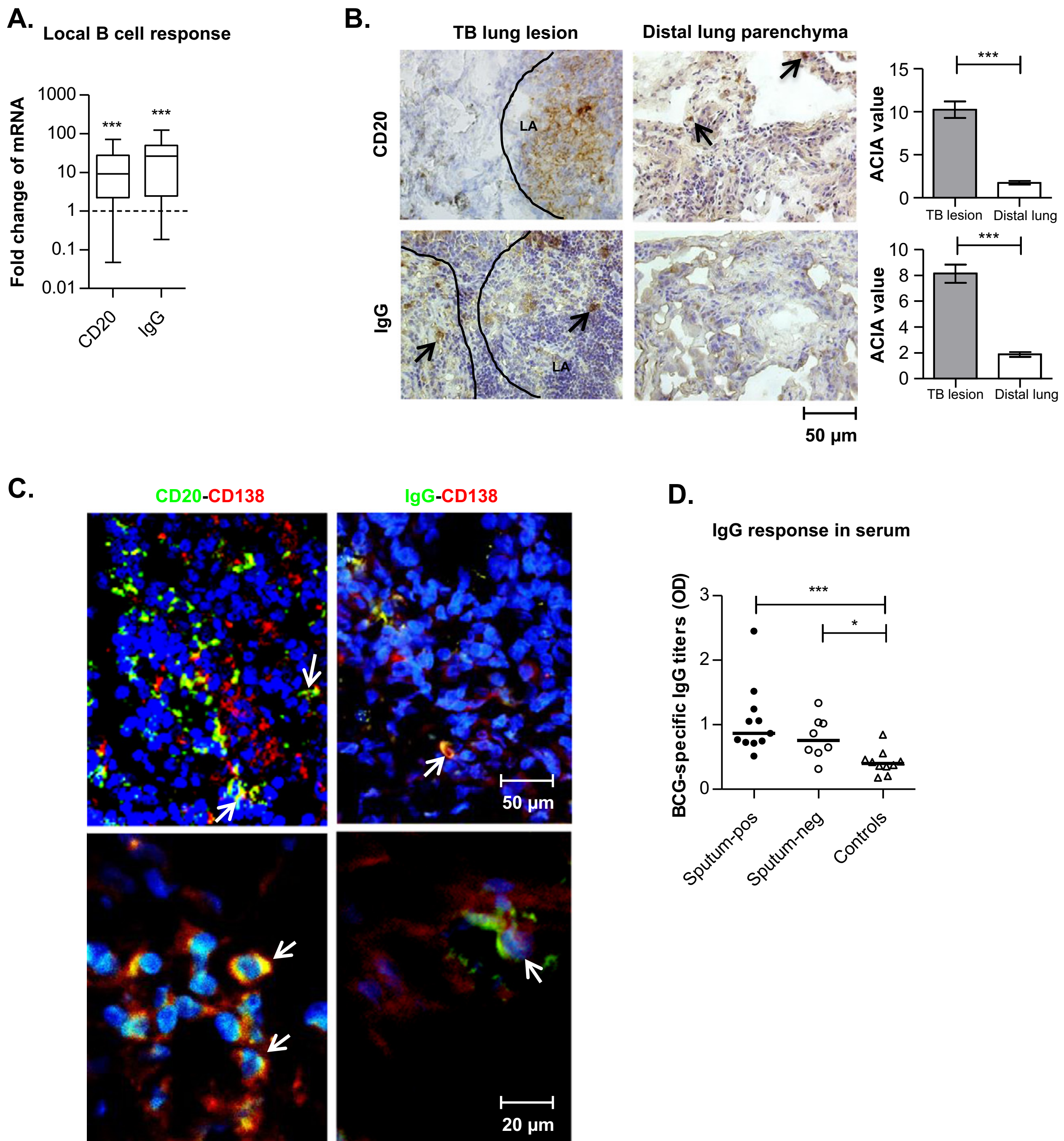


Figure 3 Elevated levels of CD20$^+$ B cells and IgG-secreting cells in TB lung lesions compared to distal lung parenchyma. (A) Relative mRNA expression (median and range) of CD20 and total IgG antibodies in the TB lesions compared to distal lung parenchyma (n = 19) was quantified using real-time PCR. The dotted line represents a relative difference of 1. (B) Representative immunohistochemical images demonstrates the expression and distribution of CD20$^+$ B cells and IgG-secreting cells in the lymphoid aggregates (LA; solid line) in the Mtb-infected lung (magnification ×125). Arrows indicate positive cells (brown) whereas negative cells (blue) were counterstained with hematoxylin. *In situ* computerized analysis determined protein expression (mean ± SE) of CD20 and IgG in the TB lesions compared to distal lung (n = 19). (C) Immunofluorescent staining (red Alexa-594; green Alexa-488) and confocal microscopy showed local distribution of CD20$^+$ CD138$^+$ B cells (left panel) as well as CD138$^+$ IgG$^+$ B cells (right panel) in the TB lesion site (magnification ×125 and ×300, respectively). Arrows indicate double-positive cells in yellow. (D) BCG-specific IgG titers (median and range) in serum samples from sputum-smear positive (sputum-pos; n = 11) and sputum-smear negative (sputum-neg; n = 8) TB patients compared to healthy Swedish controls (n = 10). $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

induction of antibody responses in the circulation of the TB patients and found that BCG-specific IgG titers were significantly higher in both sputum-smear positive ($p < 0.001$) and sputum-smear negative ($p < 0.05$) TB patients compared to healthy controls (Fig. 3D).

3.3. Increased expression of IL-21 and IL-10 in pulmonary TB lesions

Growing evidence suggest that a balance in the local cytokine environment is crucial to control progression of

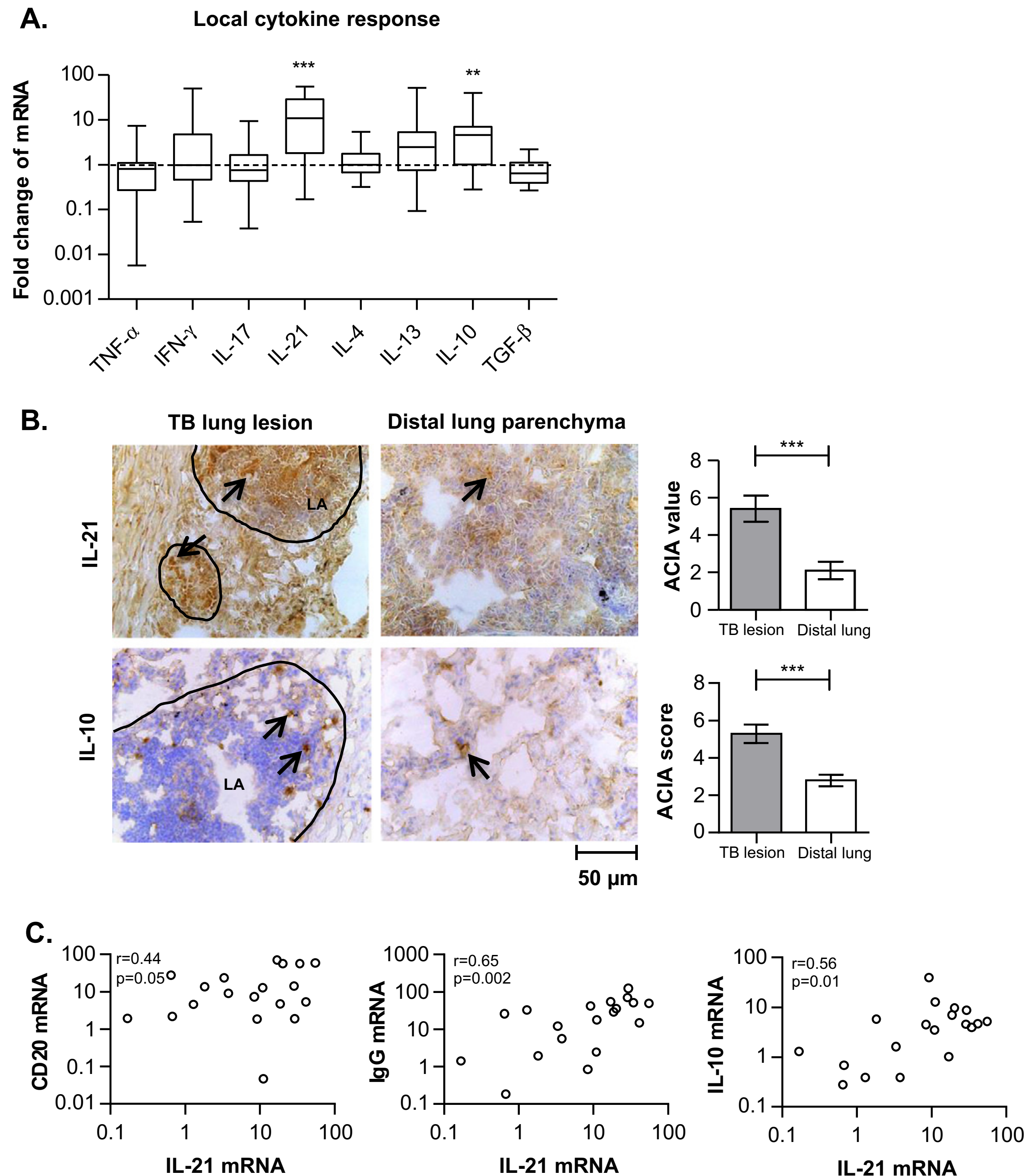


Figure 4 Increased expression of IL-21 and IL-10 in TB lung lesions compared to distal lung parenchyma. (A) Relative mRNA expression (median and range) of TNF-α, IFN-γ, IL-17, IL-21, IL-4, IL-13, IL-10 and TGF-β in the TB lesions compared to distal lung parenchyma (n = 19) were quantified using real-time PCR. The dotted line represents a relative difference of 1. (B) Representative immunohistochemical images show the expression of IL-21 and IL-10 in the Mtb-infected lung (magnification ×125). Lymphoid aggregates (LA) in the TB lung lesions are marked with a solid line. Arrows indicate positive cells (brown) whereas negative cells (blue) were counterstained with hematoxylin. *In situ* imaging was used to determine cytokine expression (mean ± SE) at the TB lesion site compared to distal lung (n = 19). (C) Correlation analyses were performed in order to assess the associations between IL-21 mRNA and CD20 mRNA, total IgG or IL-10 mRNA in the TB lung lesions. A value of r = 1 for the correlation coefficient (r_s) indicates a perfect correlation. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

active TB disease. Quantitative mRNA analysis was used to screen the expression profiles of classical Th1, Th17, Th2 and anti-inflammatory cytokine responses in the Mtb-infected lung (Fig. 4A). Interestingly, we could detect a significant up-regulation, particularly of the B cell stimulatory cytokine IL-21 ($p < 0.0001$), but also of anti-inflammatory IL-10 ($p = 0.002$) in the TB lesions compared to the distal lung parenchyma (Fig. 4A). Consistent with the mRNA analysis, *in situ* protein levels of both IL-21 and IL-10 were significantly ($p < 0.0004$) elevated in the TB lung lesions compared to the unaffected distal sites (Fig. 4B). Cytokine expression was mostly confined to the lymphoid aggregates in the TB lesions. Although the correlation between IL-21 protein and the B cells markers was not significant (data not shown), there was a significant correlation between IL-21 mRNA and CD20 mRNA ($r = 0.44$; $p = 0.05$), total IgG mRNA ($r = 0.65$; $p = 0.002$) and IL-10 mRNA ($r = 0.56$; 0.01) in the TB lung lesions (Fig. 4C).

3.4. Lower expression of LL-37 but higher expression of FoxP3⁺ Treg cells and IgG-secreting cells in pulmonary TB lesions from sputum-positive TB patients

Since TB patients with a more destructive form of cavitary TB may release more mycobacteria and bacterial antigens in the respiratory tract compared to non-cavitary TB, mycobacteria are more often detected in sputum samples from these patients. In line with this, the sputum-smear microscopy was positive in 11 out of the 19 TB patients and most (8/11) of these patients had cavitary TB (Table 1). Correspondingly, the expression of mycobacterial BCG-antigen was significantly ($p = 0.02$) higher in TB lesions from sputum-positive compared to sputum-negative patients (data not shown). While the expression of $CD68^+$ macrophages were similar in TB lesions from sputum-positive and sputum-negative TB patients, both $CD3^+$ T cells and $CD20^+$ B cells were detected at significantly ($p = 0.003$ and $p = 0.006$, respectively) higher levels in the sputum-positive group (Fig. 5A). Moreover, the expression of LL-37 was significantly ($p = 0.009$) higher in TB lesions from sputum-negative patients, whereas both $FoxP3^+$ Treg cells and IgG-secreting cells were significantly ($p = 0.01$ and $p = 0.003$, respectively) higher in lesions from sputum-positive patients (Fig. 5A). Statistical analyses revealed a significant correlation between $CD20^+$ B cells and IgG-secreting cells ($r = 0.61$; $p = 0.005$), as well as between $CD20^+$ B cells and $FoxP3^+$ Treg cells ($r = 0.51$; $p = 0.02$) in the TB lesions (Fig. 5B). These analyses also revealed a significant ($r = -0.67$; $p = 0.001$) inverse correlation between the cellular and humoral effector molecules, LL-37 and IgG (Fig. 5B), at the site of Mtb infection.

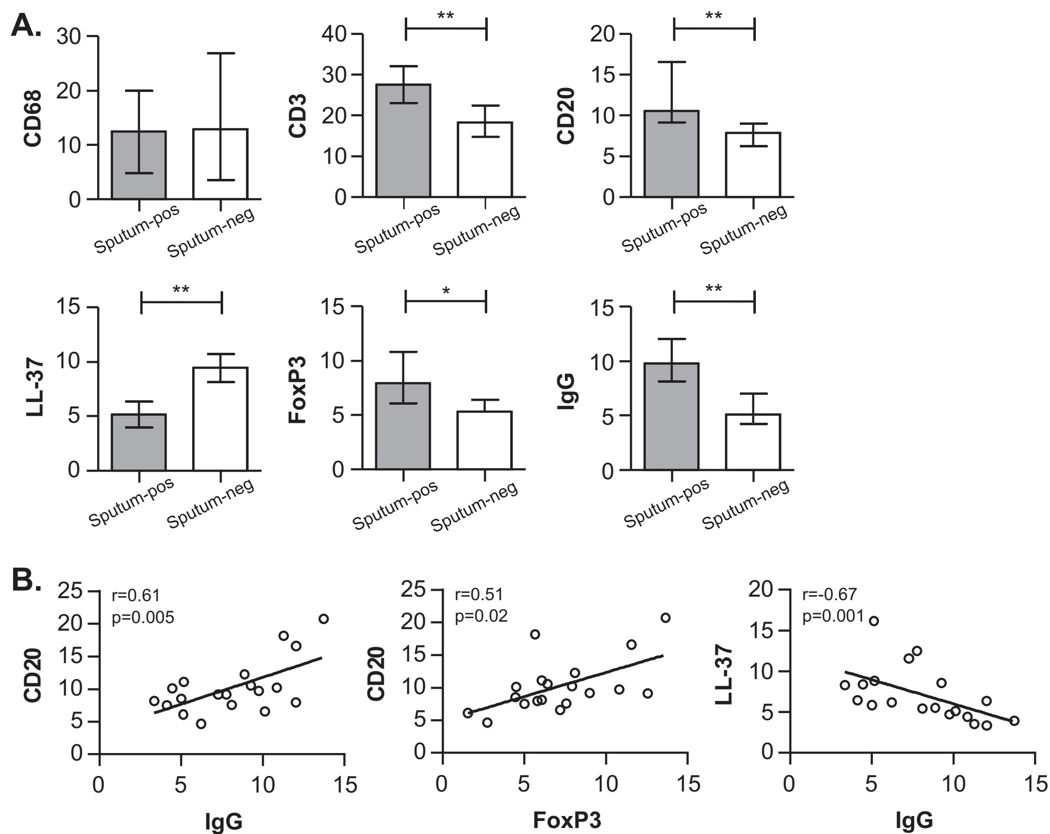


Figure 5 Lower expression of LL-37 but higher expression of $FoxP3^+$ Treg cells and IgG-secreting cells in pulmonary TB lesions from sputum-positive TB patients. (A) ACIA values (median ± IQR) for CD68, CD3, CD20, LL-37, FoxP3 and IgG in TB lesions from patients that were sputum-smear positive (sputum-pos; $n = 11$) compared to patients that were sputum-smear negative (sputum-neg; $n = 8$). (B) Correlation analyses were performed in order to assess the associations between: CD20 and IgG-secreting cells or $FoxP3^+$ Treg cells; LL-37 and IgG-secreting cells in the TB lung lesions (ACIA values). A value of $r = 1$ for the correlation coefficient (r_s) indicates a perfect correlation whereas $r = -1$ indicates a perfect negative or inverse correlation. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

4. Discussion

This study was performed to explore the nature of the unfavorable immune responses present at the local site of Mtb infection in the lung of patients with a chronic incurable TB disease. For the first time we demonstrate that pulmonary TB patients with a vitamin D deficiency expressed low local levels of the vitamin D-inducible antimicrobial peptide LL-37 in TB lesions from the Mtb-infected lung. While mycobacterial antigens were accumulated in the TB lesions, CD68^{+} and MAC387^{+} innate immune cells were reduced compared to distal lung parenchyma although the expression of iNOS was similar in the lesion and distal sites. Impaired expression of LL-37 as well as granulysin in pulmonary TB lesions, may suggest a selective down-regulation of antimicrobial peptides at the site of bacterial persistence. Instead, lymphoid aggregates in the granulomatous TB lesions were enriched with CD3^{+} T cells and FoxP3^{+} Treg as well as CD20^{+} B cells and IgG-secreting cells. In addition, we found elevated levels of mycobacteria-specific IgG titers in serum samples from active TB patients compared to healthy controls. While we have previously found an impairment of perforin and granulysin expression in local CD8^{+} CTLs in the Mtb-infected lung [12], elevated levels of FoxP3^{+} Treg cells in the TB lesions may suggest that the ratio of effector T cells to Treg cells are significantly decreased at the Mtb-infected site. Moreover, TB lesions contained high levels of the B cell stimulatory cytokine IL-21 that correlated to mRNA expression of both CD20 and total IgG levels in the tissue. In comparison to sputum-negative patients with non-cavitary TB, sputum-smear positive TB patients who mostly suffered from cavitary TB, expressed relatively lower levels of LL-37 in the TB lesions, whereas FoxP3^{+} Treg cells and IgG secreting B cells were highly abundant at this site. Collectively, these results may suggest that impaired antimicrobial effector cell responses may be associated with an IL-21 driven expansion or infiltration of IgG-secreting B cells and a concomitant rise in FoxP3^{+} Treg cells at the local site of Mtb infection that may contribute to a persistent TB infection.

In chronic infections such as TB, it is of significant relevance to study host-pathogen interactions in the infected tissue, since immune cells are recruited to and accumulate at the local site of bacterial replication [23]. Here, granuloma formation including organized clusters of macrophages and lymphocytes in Mtb-infected tissue is a typical feature of human TB disease [13,23,30,31]. We observed a lower expression of macrophages in the TB lesions compared to the distal sites, which suggests that Mtb infection results in a depletion of infected host cells at the site of infection, especially in patients with advanced TB disease. Accordingly, the cellularity of necrotic granulomas in the lesions was lower compared to non-necrotic granulomas. The presence of lymphoid aggregates in close proximity to TB granulomas have been described both in mice [32,33] and human pulmonary tissue [30,31], which indicates that such secondary lymphoid structures play a role in the control of local host-pathogen interactions. We previously demonstrated that FoxP3^{+} Treg cells and TGF-β are enriched in human lymph node granulomas [13]. Similarly, the walls of cavitary TB lesions from human lungs have also been shown to contain particularly high numbers of FoxP3^{+} Treg cells that may contribute to the inability of the immune system to eradicate TB [34]. The presence of CD4^{+} FoxP3^{+} Treg cells [35] as well as CD20^{+} B cells and antibodies [36] was recently demonstrated in the granulomas of Mtb-infected non-human primates, which support the notion that these cell subsets may modulate the host-pathogen interactions at the local site of infection.

Our findings suggest that loss of cellular immune control is related to an enhanced activation of humoral immune responses in human TB. Several studies suggest that B cells and antibodies are required to control mycobacterial infections [32,37,38], while other studies fail to show any protective effects of B cells [39,40]. Possibly, B cell responses may play a role in early protection and the induction of adaptive immunity in TB [40,41]; however, in the chronic phase of TB infection, enhanced antibody-responses may instead be a consequence of exacerbated disease. Humoral immunity is driven by soluble Mtb-antigens that are released and spread from destructive lesions present at the site of Mtb infection, especially in patients with extensive pulmonary TB including cavitary TB [42]. Accordingly, high levels of total and Mtb-specific serum antibodies have previously been shown in patients with advanced TB disease [43,44]. In addition, we recently demonstrated that elevated levels of mycobacteria-specific IgG-secreting cells in the peripheral circulation of patients with active TB, were associated with reduced Mtb-specific IFN-γ production and more severe forms of TB disease [18]. Similar to TB, *Mycobacterium leprae*-specific serum IgG1 antibodies in patients with leprosy show a direct correlation with bacterial load [45]. Importantly, antibody responses and antibody-secreting CD138^{+} plasma B cells are also elevated at the site of *M. leprae* infection in skin lesions from patients with advanced disseminated forms of disease [46], which suggest that humoral immunity in mycobacterial infections may not be associated with protection but rather to disease progression.

B cells are pleiotropic cells that can mediate multiple functions including antigen presentation, antibody production but also regulatory functions. Here, it was recently demonstrated that CD19^{+}CD24^{hi}CD38^{hi} Breg cells isolated from peripheral blood of healthy individuals, have the ability to suppress T cell functions including the differentiation of IFN-γ and TNF-α producing Th1 cells but also IL-17 producing Th17 cells [47,48]. Breg cells have also been shown to suppress the production of TNF-α by macrophages [21], while the effect on other macrophage functions such as the expression of LL-37 is currently unknown. Interestingly, immune suppression mediated by Breg cells seems to be primarily dependent on IL-10 [20,21,47,48]. Importantly, CD19^{+}CD24^{hi}CD38^{hi} Breg cells promote the expansion of FoxP3^{+} Treg cells with suppressive functions while Breg cells from patients with autoimmune diseases such as systemic lupus erythematosus (SLE) [47] or rheumatoid arthritis (RA) [48] fail to sustain their suppressive capacity and to maintain FoxP3^{+} Treg cells. RA patients with active disease also had reduced numbers of CD19^{+}CD24^{hi}CD38^{hi} B cells in peripheral blood compared to control groups [48]. In this regard, we recently demonstrated that the proportion of circulating CD19^{+}CD27^{hi}CD38^{hi} plasmablasts expressing cell-surface IgG was significantly higher in peripheral blood from patients with active TB compared with asymptomatic and healthy controls [18]. TB patients have also been found

to have elevated levels of a functionally suppressive $CD19^+CD1d^+CD5^+$ B cell subset in peripheral blood [49]. Likewise, Breg cells could promote pulmonary infiltration of $FoxP3^+$ Treg cells that prevent allergic inflammation in worm-infected mice [50]. Thus, while reduced numbers of Breg cells may fail to limit inflammatory responses in patients with autoimmune diseases, excess numbers of Breg cells may prevent imperative antimicrobial effector responses in human infectious diseases such as TB, partly by local expansion of $FoxP3^+$ Treg cells.

Our present findings revealed that the B cell stimulatory cytokine IL-21 was significantly elevated in the TB lesions compared to the distal lung parenchyma. Recent research supports the notion that IL-21 is the major T cell-derived cytokine that drives differentiation of human plasma B cells into antibody-secreting cells [51]. It has been determined that IL-21 regulates the function and persistence of germinal center B cells [52] and is a potent inducer of IgG-secreting plasma cells [26]. Accordingly, IL-21R-deficient mice exhibited a severe defect in IgG1 production following antigen priming [53], demonstrating the importance of IL-21 in plasma B cell differentiation. Interestingly, it was recently shown that *in vivo* development of functional IL-10-producing Breg cells are dependent on the production of IL-21 [54]. Thus, although IL-10 could potentially be produced by a number of cell types including T and B cells as well as macrophages, it is tempting to speculate that IL-10 is primarily produced by Breg cells present in local TB lung lesions. Moreover, increased levels of IL-21 have been shown to facilitate Treg-mediated inhibition of perforin expression in T cells and to enhance viral replication in the spleen of Coxsackievirus infected mice [55]. Further *in vivo* and *in vitro* experiments are required to investigate the specific role of IL-21 in the regulation of immune responses in TB.

5. Conclusions

In summary, our findings suggest that the progression of active pulmonary TB is associated with vitamin D deficiency and low local levels of LL-37 as well as a concomitant enrichment of $FoxP3^+$ Treg cells and IgG-secreting $CD20^+$ B cells in the granulomatous TB lesions. Elevated levels of $FoxP3^+$ Treg cells may alter the immunological balance and inhibit crucial antimicrobial effector cell responses including local production of LL-37 and granulysin in the Mtb-infected lung. In addition, IL-21 may promote the accumulation of IgG-secreting B cells, which could be the consequence of an impaired cellular immune response that contributes to the persistence of TB infection. Future studies determining the functional link between different B cells subsets and local Treg cells in human TB, would therefore be helpful.

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Conflict of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest.

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