Immunopathology and Infectious Diseases

Compartmentalization of Immune Responses in Human Tuberculosis

Few CD8+ Effector T Cells but Elevated Levels of FoxP3+ Regulatory T Cells in the Granulomatous Lesions

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Immune responses were assessed at the single-cell level in lymph nodes from children with tuberculous lymphadenitis. Tuberculosis infection was associated with tissue remodeling of lymph nodes as well as altered cellular composition. Granulomas were significantly enriched with CD68+ macrophages expressing the *M. tuberculosis* complex-specific protein antigen MPT64 and inducible nitric oxide synthase. There was a significant increase in CD8+ cytolytic T cells surrounding the granuloma; however, CD8+ T cells expressed low levels of the cytolytic and antimicrobial effector molecules perforin and granulysin in the granulomatous lesions. Quantitative real-time mRNA analysis revealed that interferon- γ , tumor necrosis factor- α , and interleukin-17 were not up-regulated in infected lymph nodes, but there was a significant induction of both transforming growth factor- β and interleukin-13. In addition, granulomas contained an increased number of CD4+FoxP3+ T cells co-expressing the immunoregulatory cytotoxic T-lymphocyte antigen-4 and glucocorticoid-induced tumor necrosis factor receptor molecules. Low numbers of CD8+ T cells in the lesions correlated with

high levels of transforming growth factor- β and FoxP3+ regulatory T cells, suggesting active immunosuppression at the local infection site. Compartmentalization and skewing of the immune response toward a regulatory phenotype may result in an uncoordinated effector T-cell response that reduces granule-mediated killing of *M. tuberculosis*-infected cells and subsequent disease control. (*Am J Pathol* 2009, 174:2211–2224; DOI: 10.2353/ajpath.2009.080941)

Infection with *Mycobacterium tuberculosis* (Mtb) is a major cause of morbidity and mortality in large parts of the world and considered one of the most important global health problems. Protective immunity to tuberculosis (TB) in humans depends on both CD4⁺ and CD8⁺ T cells; however cell-mediated immune responses rarely result in complete eradication of infection. On antigen-specific T cell activation, effector cytokines are produced that promote macrophage activation and control of Mtb growth, partly through the production of reactive oxygen and nitrogen intermediates, including nitric oxide (NO). However, Mtb-infected macrophages that fail to eradicate the bacteria promote the generation of chronic inflammation and formation of granulomas.¹ Mtb-infected macrophages residing in the granuloma subsequently recruit T cells to the area of infection, in an attempt to organize and contain the infection.^{2,3} Despite vigorous immune reactivity at the site of infection, Mtb has evolved strategies to survive in the granulomas, resulting in infection that persists for extended periods of time. This may

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be partly explained by the finding that the adaptive immune response to human TB is delayed compared with most other infections or immunizations, allowing the initial bacterial population to expand markedly in host macrophages that organizes the granulomatous response before induction of cell-mediated immunity.⁴

Rapid onset of a Th1 cytokine response, including primarily interferon (IFN)- γ ⁵ and tumor necrosis factor (TNF)- α ,⁶ has been shown to be instrumental in the development of protective TB immunity. More recently it has also been proposed that Th17 cells, which produce interleukin (IL)-17 and IL-23, may contribute to inflammation,⁷ induction of antimicrobial peptides and recruitment of Th1 cytokine producing CD4⁺ T cells, resulting in restricted Mtb growth in the lungs.⁸ Thus, induction and kinetics of the Th17 response may be critical for the triggering of Th1 cells and subsequent macrophage and effector T cell activation at the primary site of TB infection. In contrast to a Th1/Th17 response, a Th2 or anti-inflammatory cytokine profile characterized by production of IL-4/IL-5/IL-13⁹ or IL-10/transforming growth factor (TGF)- β ¹⁰ respectively, has been associated with loss of immune control and increased dissemination of Mtb.¹¹ Delayed or inappropriate T cell activation, leading to inadequate production of inflammatory cytokines, may therefore result in the immunopathogenesis characteristic of clinical TB.

A Th1 cytokine response promotes the activation of cytolytic T cells (CTLs) that express different cytolytic effector molecules inside cytoplasmic granules. It has been demonstrated that the granule-associated antimicrobial molecule granulysin can kill intracellular Mtb bacilli through osmotic lysis in cooperation with the cytolytic protein perforin.^{12,13} Indeed, granulysin and perforin have been shown to be co-expressed in human CD8⁺ CTLs after exposure to Mtb-infected macrophages, suggesting that these molecules constitute a multifunctional unit of the T cell response with the capacity to attract and kill TB-infected target cells.¹⁴ The coordinated expression of granulysin and IFN- γ correlates with clinical improvement of TB disease,^{15,16} providing additional evidence that multiple effector functions are crucial in protective immunity to TB. Therefore, a selective dysfunction in the expression of Th1 cells and subsequent CTL function could alter the host's ability to generate sterilizing TB immunity. Accumulation and activation of regulatory T (Treg) cells at the site of TB infection may prevent the development of polyfunctional T cell responses.¹⁷ Natural or induced Treg cells are a heterogeneous population of CD4⁺ T cells and some Treg cell subsets co-express activation markers such as CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR), while the most rigorous marker for these cells is the transcription factor forkhead box p3 (FoxP3). Treg cells are known to suppress excess immune activation and thus prevent the development of immunopathology, including inhibition of Mtb-induced production of IFN- γ in CD4⁺ T cells^{18–20} and the cytolytic function of CD8⁺ CTLs,^{21–24} which could lead to chronic infection instead of pathogen clearance.

In this report, we have examined if clinical TB infection in treatment naïve children was associated with an inadequate Th1 response and low expression of cytolytic and antimicrobial molecules. Spatial assessment including tissue morphology, cellular composition and distribution of inflammatory and immunosuppressive markers was performed at the single-cell level in lymphoid tissue of children with local TB-lymphadenitis. Moreover, compartmentalization of the CTL response was determined in the local environment of the TB granuloma. Our aim was to study potential alterations in the expression of cytokines and cytolytic effector molecules in TB infected lymph nodes and determine whether induction of Treg cells could play a role in establishment of disease. Here, we present the first evidence demonstrating a deficiency in CD8⁺ T cells and cytolytic effector molecules, perforin and granulysin, at the site of infection in human TB lesions. Reduced numbers of CTLs expressing low levels of perforin and granulysin, correlated with an elevated frequency of FoxP3⁺ Treg cells inside the granulomas. These results suggest that an imbalance in the proportion of effector T cells to Treg cells, present at the site of infection, may contribute to establishment of TB infection.

Materials and Methods

Patients

A one year prospective study was performed and included 21 HIV-negative children, 3 to 10 years old, with a regional or local lymphadenopathy suggestive of TB. This included patients with a persistent (>3 to 8 weeks) enlargement of a non-tender, palpable lymph node in the cervical or submandibular areas of the neck as determined by physical examination by an experienced clinician. Common clinical symptoms were fever, cough, sweating, and anorexia. Lymph nodes in the neck region were surgically removed from children with persistent swelling of one or multiple lymph nodes. Chest X-ray was normal (ie, no signs of pulmonary lesions) in all enrolled study subjects, suggesting a local lymphadenopathy without involvement of a pulmonary infection. Accordingly, cases of systemic or pulmonary infection were excluded from the study as were children with previous or ongoing treatment with anti-TB drugs. Children included in the study were recruited at the Department of Pediatrics and Child Health, Tikur Anbessa Hospital, Addis Ababa, Ethiopia, with parent's or guardian's approval and signed informed consent. All children were previously vaccinated with Bacille Calmette Guérin (BCG) and had an average body mass index of 14.3 (compared with 16 among Swedish age-matched controls). At surgery, a lymph node biopsy and a blood sample (5 to 10 ml) was obtained from the study subjects. Serum was used for viral screening for HIV, cytomegalovirus, Epstein-Barr virus, rubella, and adenovirus by PCR. Stool and urine samples showed no signs of an invasive parasitic disorder. Patients were divided into two groups; TB-positive(+) lymphadenitis ($n = 11$) and TB-negative(–) non-specific lymphadenitis ($n = 10$) (Table 1). A definite TB

Table 1. Clinical and Microbiological Demography of Children with a Local Lymphadenitis*

Patient No.	Sex [†]	Age (years, months)	BMI [‡]	TB-culture [§]	Histology [¶]	PCR	Antibiotic treatment**
#1	M	3 y	16.5	pos (+)	TB	Mtb	cured
#2	M	7 y	14.3	pos (+)	TB	Mtb	improved
#3	F	4 y	15.4	pos (+)	TB	Mtb	nd
#4	F	6 y	12.7	pos (+)	TB	Mtb	cured
#5	F	9 y	13.7	pos (+)	TB	Nd	cured
#6	F	4 y 6 m	15.8	pos (+)	TB	Mtb	cured
#7	F	6 y 6 m	11.5	pos (+)	TB	Mtb	cured
#8	F	7 y 2 m	12.7	pos (+)	TB	Mtb	cured
#9	F	3 y 7 m	16.3	pos (+)	TB	Mtb	cured
#10	M	7 y	13.2	neg (–)	TB	Mtb	cured
#11	F	4 y 11 m	15.3	neg (–)	TB	Mtb	cured
#12	F	10 y	12.8	neg (–)	not TB	neg (–)	cured
#13	F	8 y	14	neg (–)	not TB	neg (–)	improved
#14	F	4 y	16.9	neg (–)	not TB	neg (–)	cured
#15	F	3 y 2 m	14.2	neg (–)	not TB	neg (–)	cured
#16	M	4 y 9 m	14.8	neg (–)	not TB	neg (–)	cured
#17	F	4 y 8 m	14.3	neg (–)	not TB	neg (–)	improved
#18	M	6 y 7 m	15.4	neg (–)	not TB	neg (–)	cured
#19	M	9 y 3 m	13	neg (–)	not TB	neg (–)	improved
#20	M	6 y 11 m	13.1	neg (–)	not TB	neg (–)	cured
#21	M	4 y 5 m	14.7	neg (–)	not TB	neg (–)	cured

*All children were in the age of 3 to 10 years and had clinical symptoms of a local TB-lymphadenitis including chronic (>3 to 8 weeks) non-tender swelling of lymph nodes in the neck region as well as a normal pulmonary X-ray. Biopsy and blood samples were collected from the children before initiation of antibiotic treatment.

TB diagnosis was based on clinical symptoms, X-ray, TB culture, histology and PCR.

[†]Sex; M = male; F = female.

[‡]BMI = body mass index.

[§]Tissue homogenate from biopsy were used for TB culture. TB positive culture = pos(+); TB negative culture = neg(–).

[¶]Histopathological analysis of formalin-fixed lymph node biopsies was performed by a pathologist and determined as either a granulomatous reaction consistent with tuberculosis (TB) or as a reactive follicular hyperplasia consistent with chronic non-specific lymphadenitis (not TB).

^{||}TB-specific PCR on DNA obtained from tissue biopsy. *Mycobacterium tuberculosis* = Mtb, not determined = nd, negative = neg(–).

**Patients #1 to #11 were treated with conventional anti-TB drugs: rifampicin, isoniazid, pyrazinamid and streptomycin, whereas patients #12 to #21 were treated with the broad-spectrum antibiotic amoxicillin. Not determined = nd.

diagnosis was based on a positive TB-culture of tissue homogenate and/or histopathological evidence of a granulomatous reaction as well as amplification of TB-DNA by PCR. Lymph node biopsies obtained from the children were divided into three parts; one for TB-culture and PCR, one for histology, and one (snap-frozen and stored at –85°C) for future immunological analysis. TB-culture was performed using the established Løwenstein-Jensen methodology whereas typing was done on DNA extracts from heat killed isolates. Tissue homogenate from lymph node biopsies were decontaminated in 1% NaOH containing 3% SDS, and neutralized in 0.1% sulfuric acid containing 0.0008% bromocresol-purple. After centrifugation, tissue pellets were resuspended in 1 ml of 7H9 media that were inoculated in Løwenstein-Jensen tubes, one tube containing glycerol and another tube containing pyruvate. The culture media were incubated at 37°C up to 8 weeks with weekly observation for growth. Isolates were confirmed as Mtb using PCR-based deletion analysis including specific primers for RD4, RD9, and RD10 according to established procedures.^{25,26} H&E staining was used for histopathological tissue analysis of the lymph node samples performed by a specialized pathologist at the Armauer Hansen Research Institute. TB-pos(+) specimens ($n = 11$) revealed a granulomatous reaction with multinucleated giant cells, epithelioid cell clusters and tissue necrosis consistent with TB (Table 1). The histology of TB-neg(–) lymph node samples ($n = 10$) typically demonstrated a reactive

follicular hyperplasia characteristic of chronic non-specific inflammation (Table 1). After surgery, children with culture-confirmed TB were treated with standard anti-TB drugs (rifampicin, isoniazid, pyrazinamide, and streptomycin) whereas children with non-specific lymphadenitis were treated with broad-spectrum antibiotics (amoxicillin), which normally cured their lymphadenitis (Table 1).

Lymphoid tissue (tonsils) from age-matched children ($n = 10$; M/F: 5/5; age: 3 to 10 years) undergoing tonsillectomy due to non-infectious tonsil hyperplasia were used as controls for immunocytochemical staining. The uninfected controls were recruited at the Karolinska University Hospital, Huddinge, Sweden. Ethical permission for this study was obtained in both Ethiopia and Sweden and children were only recruited into the study after parent's or guardian's approval and signed informed consent.

Immunohistochemistry and Confocal Analysis of Frozen Tissue Sections

Cryopreserved lymphoid tissue biopsies were embedded in OCT-compound (Tissue-TEK, Sakura) and cut into 8 μ m thick sections, 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 as previously described.²⁷ Positive staining was developed using a diaminobenzidine substrate

(Vector Laboratories, Burlingame, CA) while hematoxylin was used for nuclear counterstaining. We used acquired computerized image analysis to quantify immunohistochemical staining *in situ* by transferring digital images of the stained tissue samples from a DMR-X microscope to a computerized Quantimet 5501W image analyzer (Leica Microsystems, Germany).²⁸ Positive immunostaining was quantified at the single-cell level in 10 to 50 high-power fields using a Qwin 550 software program (Leica Imaging Systems, Germany).²⁹ Protein expression was determined as the percent positive area of 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. The complete tissue section scanned had a mean size of $4.5 \times 10^6 \mu\text{m}^2$. Immunohistochemistry slides were coded and each staining was assessed independently by two individuals in a blinded fashion, generally resulting in <10% intra-assay variation. For image analysis of TB-pos(+) lymph nodes, positive immunostaining determined from total tissue sections was compared with image analysis of the granulomatous lesions (granulomas) only. Here the same tissue section was assessed twice; once for immunohistochemical analysis of total lymph node tissue and once again for analysis including tuberculous granulomas only. Differentiation between total and granulomatous tissue was performed using the tissue excluder function of the soft ware. Specific granulomas were counted and individually assessed using the *in situ* soft ware and visual identification of granulomas. On average, TB-pos(+) lymph node sections contained 10 to 20 granulomas with a mean size of $2 \times 10^6 \mu\text{m}^2$ included in the image analysis. Tissue sections stained with secondary antibodies only were used as negative controls. The specificity of the primary antibodies used had previously been tested, particularly in human lymphoid tissue.^{27,29–32} Two-color staining was performed using indirect immunofluorescence and analysis performed using a filter-free spectral confocal microscope (Leica TCS SP2 AOBs).

Antibodies

Primary antibodies were CD3, CD4, and CD8 (BD), elastase/neutrophil, CD56, CD68, MAC387, CD45RA, CD45RO, CD20, polyclonal *Mycobacterium bovis* (pAb-BCG) (Dako, Glostrup, Denmark), collagen type I (Abcam, Cambridge, UK), iNOS (BD/Transduction Laboratories, San Jose, CA) and nitrotyrosine (n-tyr) (Upstate, Lake Placid, NY), DC-SIGN, granzyme A (clone CB9), CTLA-4 (BD/Pharmingen, San Diego, CA), perforin (clone p16-17) (Mabtech, Stockholm, Sweden), GITR (R&D systems, Abingdon, UK), FoxP3 (Novus Biologicals, Littleton, CO), and TGF- β (Santa Cruz Biotechnology Inc., Santa Cruz, CA) Affinity purified human granulysin was kindly provided by Dr. Alan Krensky and Dr. Carol Clayberger, Stanford University, CA. An affinity purified rabbit polyclonal antibody directed against the secreted Mtb-protein MPT64 was helpfully provided by Prof. Harald Wiker and Prof. Lisbet Svinland, Bergen University, Norway.

iNOS was used as an indirect marker for NO production whereas NO metabolism was detected using n-tyr. FoxP3 and co-expression of CTLA-4 and GITR were used as markers to detect Treg cells. MPT64 detects an Mtb-specific antigen³³ while the use of cross-reactive pAb-BCG for detection of Mtb-antigens in Ziehl-Neelsen negative tissue samples has recently been described.³⁴ CD1a (Dako, Glostrup, Denmark) and DC-SIGN (BD/Pharmingen, San Diego, CA) were used to distinguish the dendritic cell population from CD4⁺ T cells in the lymph nodes. Furthermore, double-staining with CD4 and CD3, revealed that most CD4⁺ cells in the lymph node were T cells.

Biotinylated secondary antibodies, goat anti-mouse IgG, rabbit anti-goat IgG and swine anti-rabbit F(ab')₂, were purchased from Dako. For dual staining, tissues were stained with rat anti-human CD8 or CD4 (Serotec, Oxford, UK) and mouse anti-human CD68, granzyme A, perforin, FoxP3, GITR, and CTLA-4, as well as rabbit anti-human granulysin and rabbit polyclonal MPT64 followed by the appropriate Alexa Fluor-conjugated secondary Ab (Molecular Probes, Eugene, Oregon).

mRNA Extraction and Real-Time PCR of Frozen Sections from Human Lymphoid Tissue

RNA was extracted from frozen tissue sections ($2 \times 50 \mu\text{m}$) using the Ambion RiboPure extraction kit according to the manufacturers instructions. RNA was reverse transcribed using superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexanucleotide primers (Roche, Mannheim, Germany). Amplification of ubiquitin C, CD4, IFN- γ , TNF- α , IL-17A, CD8, granzyme A, perforin, granulysin, FoxP3, TGF- β , IL-10, and IL-13 cDNA was performed using the ABI PRISM 7700 sequence detection system and commercial FAM dye-labeled TaqMan MGB probes and primers (Applied Biosystems, Foster City, CA). Ubiquitin C was tested together with a panel of commonly used house-keeping genes and was selected as our calibrator as the expression was shown to be constitutive and stable in both test and control samples. Hence, Ct values for the different mRNAs were normalized to ubiquitin C and relative expression was determined using the Livak method.³⁵ The Ct values obtained for TB-pos(+) and TB-neg(–) lymphadenitis were compared with that of control tonsil tissue and data are presented as fold change of mRNA in the infected groups compared to controls.

Statistical Analysis

Due to the small sample size in each group ($n = 10$ to 11), the data are mostly presented as median $\pm$ interquartile range (IQR). Values from 2 individual experiments are shown. Non-parametric analyses used to calculate indicated *P* values, included a Mann Whitney test (when comparing two unmatched samples), a Wilcoxon signed rank test (when comparing two matched samples) or a Kruskal-Wallis test (when comparing more than two groups). A *P* value <0.001 was considered extremely

significant (***), a *P* value between 0.001 and 0.01 was considered very significant (**), a *P* value between 0.01 and 0.05 was considered significant (*) whereas a *P* value >0.05 was considered not significant (ns). Statistical analyses were performed in GraphPad Prism-4.

Results

TB Infection is Associated with Extensive Tissue Remodeling and Altered Cellular Composition in Infected Lymph Nodes

All patients (age 3 to 10 years) included in this study had clinical symptoms of a local subacute TB-lymphadenitis. Based on TB diagnosis, the patients were divided into two groups: TB-positive(+) and TB-negative(-) cases (Table 1). TB-pos(+) children had culture and/or histology/PCR proven TB and responded to treatment with conventional anti-TB drugs. In contrast, TB-neg(-) patients were considered to suffer from a reactive non-specific lymph node

inflammation ie, non-specific lymphadenitis. This group was dominated by cases of lymphadenitis caused by bacteria or possibly parasites, since all biopsy samples were negative for a panel of viral pathogens that are most commonly associated with pediatric lymphadenopathy (HIV, cytomegalovirus, Epstein-Barr virus, rubella, and adenovirus). Lymphadenopathy among TB-neg(-) children was cured or improved after treatment with the broad-spectrum antibiotic, amoxicillin. Immunological analysis was performed on frozen lymph nodes biopsies obtained from TB-pos(+) and TB-neg(-) lymphadenitis cases before treatment, as well as from one group of age-matched Swedish control children with tonsil hyperplasia.

Microscopic analysis revealed that follicular and parafollicular areas of the TB-pos(+) tissue were disturbed by the expansion of confluent granulomas, whereas normal lymphoid structure and cellular architecture was maintained in the TB-neg(-) and control tonsil group (Figure 1A). Despite extensive tissue remodeling associated with TB infection, the presence of CD3⁺ T cells was easily detected within the tissue (Figure 1A). Furthermore, granulomatous lesions in

Figure 1. Induction of tissue remodeling and altered cellular composition in TB-infected lymph nodes. **A:** The images demonstrate tissue morphology and CD3 immunohistochemical staining in TB-pos(+) and TB-neg(-) lymph nodes as well as uninfected control tonsil. Magnification = original $\times 50$. **B:** Expression and distribution of CD68, CD8 and collagen type I in TB-pos(+) lymph nodes compared with uninfected control tonsil. Granulomatous lesions in TB-pos(+) lymphadenitis are marked with a solid line. Magnification = original $\times 125$. GC indicates germinal centers or follicles in lymphoid tissue. Staining was performed using immunohistochemistry and positive cells are shown in brown (diaminobenzidine) whereas negative cells are counterstained blue with hematoxylin. **C:** Mean cellularity ($\pm$ SD) of TB-pos(+) and TB-neg(-) lymphadenitis compared with uninfected control, respectively, was estimated in the analysis. Data are presented as % cellularity of lymphoid tissue, in a box and whisker plot. **D:** *In situ* computerized image analysis was used to assess median expression ($\pm$ IQR) of the indicated APC markers and Mtb-specific antigen MPT64 or **(E)** T cell markers in TB-pos(+) compared with TB-neg(-) lymphadenitis as well as uninfected control tonsils. Data are presented as % positive area of the total cell area. Statistical significance of differences in tissue cellularity and protein expression was determined by a non-parametric Kruskal-Wallis test, TB-pos(+) lymphadenitis versus TB-neg(-) lymphadenitis versus uninfected control. The statistical significance of the indicated *P* values was determined as: *P < 0.05, **P < 0.01, ***P < 0.001 and *P* > 0.05 ns (not significant).

TB-pos(+) lymph nodes were enriched with CD68+ macrophages and CD4⁺ T cells, while CD8+ T cells were predominantly located in the T cell rich peri-granulomatous areas (Figure 1B). Granulomatous inflammation induced by Mtb was also associated with tissue fibrosis involving widespread collagen deposition (TB-pos(+): 20.8%; Control tonsil: 2.5%) (Figure 1B).

To determine whether TB infection involved altered cellular composition of lymph node tissue, *in situ* image analysis was performed at the single cell level. As illustrated in Figure 1C, the median cellularity of TB-pos(+) lymph node tissue was significantly lower ($P < 0.001$) compared with TB-neg(-) lymphadenitis and control tonsil tissue (Figure 1C). The reduction in cellularity was primarily due to a decreased proportion of CD20+ B cells in TB infected lymph nodes (Figure 1D). In contrast, we observed increased frequencies of DC-SIGN+ dendritic cells, CD68+ macrophages and MAC387+ monocytes and neutrophils in TB-pos(+) lymph nodes compared with both TB-neg(-) ($P < 0.05$) and control ($P < 0.001$) tissue (Figure 1D). Expression of mycobacterial protein antigens in lymphoid tissue including cross-reactive *M.bovis* (data not shown) and the Mtb-specific MPT64 protein, was highly significant ($P < 0.001$) in TB-pos(+) cases (Figure 1D). No TB antigen was evident in the other groups. Importantly, the proportion of CD3+ and CD4⁺ T cells was unaltered in TB-pos(+) compared with uninfected control tissue whereas the proportion of CD8+ T cells was increased ($P < 0.05$) (Figure 1E). TB-pos(+) lymph nodes also had a significantly ($P < 0.05$) higher number of memory CD45RO+ T cells compared with control samples, while the number of naïve CD45RA+ T cells was similar in the three groups compared (Figure 1E). Thus, lymph node TB was associated with extensive tissue remodeling and altered cellular composition involving reduced B cell numbers but increased proportions of macrophages, dendritic cells and relatively stable T cell numbers. These observations encouraged us to perform a more detailed spatial analysis of the T cell response associated with persistent TB-lymphadenitis.

Weak Induction of Inflammatory Cytokines and Cytolytic Effector Molecules in TB Infected Lymph Nodes

Quantitative real-time PCR analysis was performed to investigate the cytokine profile and expression of T cell-associated cytolytic effector molecules in TB infected lymph nodes. Relative change in mRNA extracted from lymphadenitis cases were compared with uninfected control tonsil. Consistent with *in situ* protein analysis, mRNA levels of CD4 remained unchanged while CD8 mRNA was significantly up-regulated in TB-pos(+) and TB-neg(-) lymphadenitis cases compared with control tonsil tissue (Figure 2A). Interestingly, induction of the important anti-TB cytokines IFN- γ and TNF- α was poor and IL-17 was significantly lower in TB-pos(+) lymphadenitis cases compared with controls (Figure 2A). Moreover, whereas mRNA expression of the granule-associated effector molecule granzyme A was significantly

increased, both perforin and granulysin remained low in TB-pos(+) lymphadenitis (Figure 2A). In contrast, all cytolytic effector molecules were significantly higher in TB-neg(-) lymphadenitis (Figure 2A).

Next, we aimed to determine tissue expression of cytolytic and antimicrobial effector molecules at the protein level. Since *in situ* assessment of IFN- γ , TNF- α , and IL-17 protein in lymphoid tissue resulted in too few positive cells (<0.5%) to perform computerized image analysis, we focused on protein analysis of granzyme A, perforin, granulysin, and iNOS. Quantitative *in situ* image analysis revealed a significant increase in granzyme A ($P < 0.001$) and perforin ($P < 0.01$) but not granulysin in TB-pos(+) lymph nodes compared with control tonsils (Figure 2B). These T cell-associated effector molecules were also up-regulated in TB-neg(-) lymphadenitis (grzA: $P < 0.05$; pfn: $P < 0.01$; grs: $P < 0.01$) compared with uninfected controls (Figure 2B). In contrast, induction of iNOS ($P < 0.001$) was evident only in TB-pos(+) lymphadenitis (Figure 2B). Despite a significant increase in perforin protein expression in TB-pos(+) lymph nodes, the ratio of perforin- and granulysin expressing cells to total CD8+ T cells remained low at both protein and mRNA level (data not shown). In contrast, granzyme A expression was significantly increased in the CD8+ T cells (mRNA: $P < 0.04$, protein: $P < 0.05$), providing evidence of T cell activation since this molecule is not expressed in naïve T cells. The ratio of granzyme A, perforin and granulysin expressing cells to total CD8+ T cells was low in both TB-neg(-) lymphadenitis and in controls.

Microscopic analysis of TB-pos(+) lymph nodes revealed that the macrophage-associated effector molecule iNOS was primarily produced inside the granulomas, whereas granzyme A was expressed in granulomatous as well as non-granulomatous areas (Figure 2C). Instead, perforin and granulysin were strictly expressed in the non-granulomatous areas outside the TB lesions (Figure 2C). Confocal microscopy revealed that co-expression of granzyme A, perforin and granulysin was evident in CD8+ T cells primarily located in the parafollicular areas of TB-pos(+) lymph node tissue (Figure 2D). While perforin and granulysin expression was restricted to CD8+ T cells, double-staining showed that expression of granzyme A was also evident in a limited number of CD4⁺ T cells (below 15% of all positive cells). Together, these findings suggests that the spatial organization of the cytolytic T cell response in TB-pos(+) lymph nodes are suboptimal.

Low Levels of CD8+ T cells and T cell-associated Cytolytic Effector Molecules in MPT64-positive Lymph Node Granulomas

To determine in more detail the tissue distribution of T cell-associated cytolytic effector molecules within TB-pos(+) lymph nodes, we performed comprehensive microscopic analyses as outlined in Figure 3A. Expression of the MPT64 protein, which is a 26-kDa secreted Mtb-specific protein, was strictly localized to the granulomatous lesions within infected lymph nodes (Figure 3A). To study functional immune responses in close proximity to infected cells, *in situ*

Figure 2. Activated T cells produce suboptimal levels of inflammatory cytokines and cytolytic effector molecules in TB infected lymph nodes. **A:** Cytokine profile and expression of T cell-associated cytolytic effector molecules in lymphoid tissue was determined using quantitative real-time PCR. Fold change of CD4 mRNA and pro-inflammatory cytokines IFN- γ , TNF- α , and IL-17, as well as CD8 mRNA and cytolytic effector molecules granzyme A, perforin, granulysin to ubiquitin C mRNA in TB-pos(+) lymph nodes was compared with that of TB-neg(-) lymph nodes and uninfected control tissue. Data are presented as fold change of mRNA in a box and whisker plot and the dotted line represents a relative difference of 1. **B:** *In situ* computerized image analysis was used to assess median expression ($\pm$ IQR) of cytolytic and antimicrobial effector molecules in TB-pos(+) compared with TB-neg(-) lymphadenitis as well as uninfected control tonsils. Data are presented as % positive area of the total cell area. **C:** The expression and distribution of granzyme A, perforin, granulysin and iNOS in TB-pos(+) lymph nodes was compared with uninfected control tonsil. Staining was performed using immunohistochemistry and **arrows** indicate positive (brown staining) cells whereas negatively stained cells are counterstained with hematoxylin. Granulomatous lesions in TB-pos(+) lymphadenitis are marked with a solid line. GC indicates germinal centers or follicles in the tonsil tissue. Magnification = original $\times$ 125. **D:** Immunofluorescent staining and confocal microscopy showed local distribution and co-expression of CD8⁺ T cells (red; Alexa-594), granzyme A, perforin and granulysin (green; Alexa-488) in the para-follicular area of a TB-pos(+) lymph node. **Arrows** indicate double-positive cells in yellow. Magnification = original $\times$ 300. Statistical significance of differences in mRNA and protein expression was determined by a non-parametric Kruskal-Wallis test (TB-pos(+) lymphadenitis versus TB-neg(-) lymphadenitis versus uninfected control). The statistical significance of the indicated *P* values was determined as: *P < 0.05, **P < 0.01, ***P < 0.001 and *P* > 0.05 ns (not significant).

image analysis was performed on the granulomatous lesions and compared with total lymph node tissue (Figure 3A). Assessment of cellularity in lymph node granulomas revealed that the median cell density was 50%, which was similar to total lymph node tissue (data not shown). The morphology of granulomas found in the lymph node biopsies, varied from smaller cellular clusters to large granulomas containing a necrotic core. Statistically significant differences in tissue expression of different markers were generally representative for all granulomas in a patient and also comparing different patients within a group. Granulomatous lesions were associated with a higher proportion of CD68⁺ macrophages (*P* = 0.001) expressing functional iNOS (*P* = 0.002) as determined by the expression of the NO metabolite nitro-tyrosine (*P* = 0.002) (Figure 3B). In addition, granulomatous lesions expressed significantly higher levels of *M. bovis*-specific protein antigens (*P* = 0.001) as well as the *Mtb*-specific antigen MPT64 (*P* = 0.008) (Figure 3B), which co-localized to CD68⁺ macrophages (Figure 3C). In contrast, MPT64 and CD8 showed

no evidence for overlapping or proximate expression (Figure 3C), suggesting that *Mtb*-antigen expressing cells and CD8⁺ T cells were segregated from one another in the tissue. As a consequence, profoundly reduced numbers (*P* = 0.001) of CD8⁺ and CD56⁺ T and NK cells expressing the cytolytic effector molecules, perforin and granulysin, was found in granulomatous lesions compared with total lymph node tissue (Figure 3D). Interestingly, granzyme A was maintained at comparable levels at both sites (Figure 3D). As a result, the ratio of granzyme A to total CD3⁺ T cells was significantly increased in both total lymph node tissue (*P* < 0.01) and granulomatous lesions (*P* < 0.001) compared with TB-neg(-) lymphadenitis (data not shown) and uninfected control tonsil (Figure 3E). However, the ratio of perforin- and granulysin-expressing cells to total CD3⁺ T cells was unaltered in granulomas compared with uninfected controls, indicating that these effector molecules were specifically down-regulated inside the granuloma (Figure 3E). Accordingly, immunofluorescence and confocal microscopy analysis revealed co-expression of perforin and

Figure 3. Expression and distribution of APCs, mycobacterial antigens, T cells and cytolytic effector molecules in total TB-pos(+) lymph node tissue compared with granulomatous lesions. **A:** The Mtb-specific protein antigen, MPT64, was predominantly expressed inside tuberculous granulomas. An excluder function of the image analysis software program was used to determine protein expression in total TB-pos(+) lymph node tissue compared with the granulomatous lesions (solid line). **B:** *In situ* computerized image analysis was used to assess median expression ($\pm$ IQR) of CD68+ macrophages, iNOS, and n-tyr, and mycobacterial antigens, BCG and MPT64, in total lymph node tissue as compared with protein expression in the granulomatous lesions. **C:** Confocal images reveal double-staining of CD68 or CD8 (red; Alexa 594) with Mtb-antigen MPT64 (green; Alexa-488). **Arrows** indicate double-positive cells in yellow and single-positive cells in red and green. Magnification = original $\times$ 200. **D:** *In situ* imaging was used to determine median expression ($\pm$ IQR) of CD8+ and CD56+ T and NK cells, granzyme A, perforin and granulysin in total lymph node tissue compared with protein expression in the granulomatous lesions. The data are presented as % positive area of total cell area. **E:** Computerized image analysis was used to determine the relative expression of effectors and total CD3+ T cells in lymphoid tissue. The ratios of granzyme A (grzA), perforin (pfn) and granulysin (grs) expression to total CD3+ T cell expression in total TB-pos(+) lymph node tissue compared with granulomatous lesions and uninfected control are presented. The median values of paired expression of effectors and CD3+ T cells from all individual patients are shown. **F:** Confocal microscopy illustrate distribution and co-expression of perforin (green; Alexa-488) and granulysin (red; Alexa-594) in the granulomatous lesions and T cell rich areas of a TB-pos(+) lymph node. **Arrows** indicate single-positive cells inside the granuloma but also double-positive cells in yellow outside the granuloma. Note the granular and polarized co-expression of the cytolytic effector molecules in cells located outside the lesion. Magnification = original $\times$ 200 and $\times$ 600. Statistical significance of differences in protein expression was determined by a non-parametric Wilcoxon signed rank test (total TB-pos(+) lymph node tissue versus granulomatous lesions). The statistical significance of the indicated *P* values was determined as: **P < 0.01, ***P < 0.001 and *P* > 0.05 ns (not significant).

granulysin primarily in non-granulomatous areas outside Mtb granulomas while mainly a few single-expressing cells were present in the granulomatous lesions (Figure 3F). Therefore, the CTL deficiency in the lesions was characterized by reduced numbers of CD8+ and CD56+ cells and a low expression of perforin and granulysin (Figure 3D) including little co-expression of these cytolytic effectors in CTLs (Figure 3F). Instead the granuloma was enriched with CD68+ macrophages expressing the Mtb-protein antigen MPT64 (Figure 3C). Collectively, these results demonstrate a selective down-modulation of CTLs at the site of infection

in the granulomas as compared with CTLs present in non-granulomatous areas.

Low CD8+ T cell Numbers Correlate with an Increased Expression of FoxP3 and TGF- β in MPT64-Positive Lymph Node Granulomas

Our results indicated that CTL-mediated anti-microbial activity was mainly associated to the parafollicular area of TB-pos(+) lymphnodes, whereas the granulomatous le-

Figure 4. Expression and distribution of FoxP3 and TGF- β in total TB-pos(+) lymph node tissue compared with granulomatous lesions. **A:** Expression of immunoregulatory molecules and anti-inflammatory as well as Th2 cytokines in lymphoid tissue was determined using quantitative real-time PCR. Fold change of FoxP3 mRNA and cytokines TGF- β , IL-10, IL-13 to ubiquitin C mRNA in TB-pos(+) lymph nodes was compared with that of TB-neg(-) lymph nodes and uninfected control tissue. Data are presented as fold change of mRNA in a box and whisker plot and the dotted line represents a relative difference of 1. **B:** Immunohistochemical analysis of MPT64, CD8, FoxP3, and TGF- β expression in a TB-pos(+) lymph node; both granulomatous lesion (solid line) and non-granulomatous areas outside the granuloma are shown. **Arrows** indicate positive (brown staining) cells whereas negatively stained cells are counterstained with hematoxylin. Magnification = original $\times 125$. **C:** Confocal analysis of Treg cell expression in a granuloma. Upper panel shows co-expression of CD4 (red; Alexa-594) and FoxP3 (green; Alexa-488) at a high ($\times 125$) and low ($\times 600$) magnification. Note the nuclear staining pattern of FoxP3, whereas CD4 is located at the surface of positive cells. Lower panel shows co-expression of CTLA-4 (red; Alexa-594) and GITR (green; Alexa-488) as well as CD4 (red; Alexa-594) and CTLA-4 (green; Alexa-488) inside a granuloma. **Arrows** indicate double-positive cells. Magnification = original $\times 200$ and $\times 600$. **(D)** Computerized image analysis was used to determine the median expression ($\pm$ IQR) of CD4⁺ T cells, FoxP3 Treg cells, CTLA-4, GITR, and TGF- β in total lymph node tissue compared with protein expression in the granulomatous lesions. The data are presented as % positive area of total cell area. **E:** Computerized image analysis was used to determine the relative expression of CD8⁺ T cells and FoxP3⁺ Treg cells in lymphoid tissue. The ratios of total CD8⁺ T cells to total FoxP3⁺ Treg cells expressed in total TB-pos(+) lymph node tissue compared with granulomatous lesions as well as TB-neg(-) lymphadenitis and uninfected control are presented. The median values of paired expression of CD8⁺ T cells and FoxP3⁺ Treg cells from all individual patients are shown. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

sions contained few CTLs producing low levels of cytolytic effector molecules. Therefore we investigated whether an enrichment of Treg cells at the site of infection in the granuloma, could help to explain the failure to induce CTLs producing perforin and granulysin. Quantitative real-time PCR analysis revealed that mRNA for FoxP3 as well as TGF- β and IL-13, was significantly up-regulated in the TB infected tissue, whereas there was no change in mRNA expression of IL-10 (Figure 4A) and IL-4 (data not shown). *In situ* imaging demonstrated that opposed to CD8⁺ T cells, the numbers of FoxP3⁺ and TGF- β ⁺ cells were increased at the site of infection in the granuloma (Figure 4B). Hence, the expression of CD8⁺ T cells was inversely correlated to FoxP3⁺ Treg cells. Furthermore, two-color staining and confocal microscopy confirmed that FoxP3⁺ cells mainly belonged to the CD4⁺ T cell subset located in the granulomas (Figure 4C). Additionally, there was an enrichment of CTLA-4 and GITR double-positive cells inside the granuloma compared with the surroundings areas and CTLA-4 was mainly expressed on CD4⁺ T cells (Figure 4C).

In situ image analysis confirmed that granulomatous lesions contained similar numbers of CD4⁺ T cells and significantly higher levels of FoxP3⁺ Treg cells ($P = 0.002$) compared with total lymph node tissue (Figure 4D). Expression of CD45RA was significantly reduced ($P = 0.001$) whereas CD45RO expression was unchanged in granulomas compared with total lymph node tissue, suggesting that there were few naïve T cells in the lesions (data not shown). CTLA-4 and GITR were also expressed at significantly higher levels ($P = 0.001$; $P = 0.007$) at the site of infection in the granuloma (Figure 4D). Furthermore, TGF- β was expressed at a fivefold higher level in the granulomas and was significantly ($P = 0.008$) up-regulated as compared with total lymph node tissue (Figure 4D). In accordance with the mRNA analysis, TGF- β levels in TB-pos(+) lymph nodes (5,62%) were significantly higher ($P < 0.001$) compared with TB-neg(-) lymphadenitis (1,95%) and uninfected controls (0,09%). Interestingly, the ratio of CD8⁺ T cells to FoxP3⁺ Treg cells was significantly ($P = 0.003$) lower in granulomatous lesions compared with total lymph node

tissue, TB-neg(–) lymphadenitis and uninfected controls (Figure 4E). In addition, the mRNA and protein ratio of IFN- γ /FoxP3 and IL-17/FoxP3 was significantly lower in TB-pos(+) lymphadenitis compared with uninfected controls (data not shown). Together, these results demonstrate an imbalance in the proportion of effector T cells to Treg cells present at the site of infection in TB-lymphadenitis.

Discussion

The immunological mechanisms involved in the pathogenesis of human TB are poorly understood. In this study, we explored immune cell effector functions at the site of infection in treatment naïve children with a recent diagnosis of local TB-lymphadenitis. We demonstrated that TB infection was accompanied by considerable remodeling of lymphoid tissue, including granuloma formation and enhanced deposition of collagen type I. Despite this tissue remodeling, the level of CD4⁺ and CD8⁺ T cells remained stable whereas the B cell compartment was significantly reduced. In addition, our results suggest a weak induction of pro-inflammatory cytokines IFN- γ , TNF- α , and IL-17, as well as cytolytic granule-associated effector molecules, perforin and granulysin, in TB infected lymph node tissue. Interestingly, there was a compartmentalization of the immune response resulting in surprisingly low numbers of CD8⁺ T cells expressing low levels of perforin and granulysin in the granulomatous lesions that were enriched in macrophages expressing iNOS and the Mtb-specific protein antigen MPT64. Besides, CD8⁺ T cells present in the TB lesions had little co-expression of cytolytic effector molecules. A weak induction of inflammatory cytokines and important anti-TB effector molecules correlated with up-regulated numbers of FoxP3⁺ Treg cells and elevated expression of TGF- β and IL-13, in patients with persistent TB-lymphadenitis. There was also a relative increase in TGF- β as well as FoxP3⁺ Treg cells co-expressing CTLA-4 and GITR inside Mtb granulomas. Our present findings support the hypothesis that Treg cells induced or accumulated in response to TB infection may act locally to suppress immune activation at the site of bacterial replication.

It is generally believed that NO is an important first line of defense that limits or prevents early intracellular growth of mycobacteria. However, even though NO has been determined to be important for TB control in the mouse,^{36,37} the clinical relevance of NO produced in human TB infection^{38,39} has not been properly evaluated. Importantly, oxidative stress and/or NO produced on chronic inflammatory conditions may inhibit T cell activation and expansion.⁴⁰ It has been shown that murine CD8⁺ T cells are significantly more sensitive to APC-derived NO as compared with CD4⁺ T cells⁴¹ or CD4⁺ CD25⁺ Treg cells.⁴² Accordingly, we demonstrate that CD8⁺ T cells were mostly found in the parafollicular areas outside the granuloma and were not co-localized with MPT64-positive macrophages, suggesting that CTLs cannot mediate killing of TB infected cells inside the lesions. Possibly, high local concentrations of NO inside the granulomas may restrict bacterial growth but simul-

taneously prevent important CTL responses. This type of immune suppression of pathogen-specific effector T cells may have severe clinical consequences on disease progression and requires further investigation.

Previous studies suggest that functional CD8⁺ T cells are required in sterilizing immunity to TB^{43–45} and that TB infected cells can be lysed by CTLs expressing perforin⁴⁶ and granulysin.⁴⁷ Adults¹⁶ and children⁴⁸ with active TB had significantly lower plasma granulysin levels compared with controls, suggesting that granulysin is important in eradication of TB infected cells. In a recent study, it was also reported that granulysin and perforin, but not FasL, contribute to the capacity of human peptide-specific CD4⁺ T cell clones to lyse infected target cells and to inhibit intracellular mycobacterial growth.⁴⁹ Interestingly, patients with mutations in the gene encoding perforin possess deficient lymphocyte cytotoxicity due to a severely reduced capacity to degranulate and lyse infected cells, which results in an inability to induce target cell killing.⁵⁰ Accordingly, it has been determined that both acute and chronic HIV infection in adults is associated with deficient expression of perforin in HIV-specific CD8⁺ effector T cells.^{29,51} Inadequate differentiation of CD8⁺ T cells and activation of dendritic cells due to lack of CD4⁺ T cell help may partly explain this insufficient HIV response.⁵² Similarly, active immunosuppression induced in TB may inhibit cytolytic activity and bacterial clearance due to functional inactivation and a subsequent inability of CD4⁺ T cells to provide necessary help to CTLs.

Although it is known that CD4⁺ T cells producing IFN- γ are essential for protective immunity in TB, it is very likely that antigen-specific polyfunctional T cells characterized by the coordinated expression of multiple effector functions, including other inflammatory cytokines, chemokines and effector molecules contribute to achieve full protection against TB.^{53,54} Recently it was described that a subset of Mtb-specific multifunctional CD4⁺ effector memory T cells co-expressing granulocyte macrophage colony-stimulating factor (GM-CSF), IFN- γ , and TNF- α were increased in children with latent but not active TB.⁵³ A coordinated T cell expression of perforin and granulysin with IFN- γ ^{55,56} or CCL5¹⁴ has also been shown to be required for control of mycobacterial growth and curative host responses in patients with TB.¹⁶ Interestingly, depletion of IL-17 during Mtb infection in mice reduced chemokine expression and subsequent accumulation of IFN- γ producing CD4⁺ T cells in the lung, suggesting that Th17 cells regulate infiltration of CD4⁺ T cells with anti-mycobacterial properties at the site of infection.⁸ More than 20% of cytokine-producing CD4⁺ T cells in peripheral blood of healthy, mycobacteria-exposed adults expressed IL-17.⁵⁷ In addition, Mtb-specific memory T cells have been found to produce substantial amounts of both IFN- γ and IL-17.⁵⁸ Thus, the quality and magnitude of multiple T cell effector functions most certainly serves as an immune correlate of disease protection and is therefore important in the immunopathogenesis of TB.

Previously, we have demonstrated that perforin and granulysin are deficient in pathological lung biopsies

from adult patients with active pulmonary TB.³⁹ Interestingly, despite an elevated infiltration of T cells, perforin and granzysin expression was selectively low in the TB lesions compared with distal lung parenchyma. In TB lymphadenitis, T cells co-expressing both perforin and granzysin were mostly found in the parafollicular areas whereas granulomas primarily contained low levels of single-positive cells. Thus, in both TB infected lung³⁹ and lymph nodes it seems as if this CTL defect is compartmentalized, being locally restricted to the granulomatous lesions. Granzyme A was abundantly expressed in both lung³⁹ and lymph node granulomas, indicating that the impairment of perforin and granzysin in CD8+ T cells is selective. Although it has previously been described that CD8+ T cells are mainly found in the peripheral regions of the granuloma in human,^{3,59} murine⁶⁰ and bovine⁶¹ TB, this phenomenon has never been properly investigated. An outer mantle of activated CD8+ T cells may be enough to restrain granuloma advancement but may be insufficient to mediate contact-dependent killing of infected cells and eradication of infection.

TB is a pathogen associated with delayed type hypersensitivity (DTH) and chronic inflammation, which often results in extensive fibrosis and tissue destruction. Th2 and anti-inflammatory cytokines such as IL-4, IL-13, IL-10, and TGF- β , have the important function of preventing severe immunopathology in TB infection, but if produced in excess before CTL activation, these cytokines efficiently antagonize Th1 induced TB-immunity.⁶² In this study, we found a significant induction of TGF- β and also IL-13, with a less pronounced induction of IL-10 and no change in IL-4 mRNA levels compared with the control. Premature induction of an immunosuppressive response may blunt important CTL activity and instead enhance pathological alterations in TB infected tissue. Here it has been shown that high levels of TGF- β correlate with massive fibrosis and deposition of collagen type I in the lymphatic tissue of SIV infected rhesus macaques.⁶³ It is well-established that IL-4 and IL-13 can subvert Th1-mediated immunity and promote inappropriate activation of macrophages.⁹ Here, stimulation of peripheral blood CD4+ T cells from BCG-vaccinated cattle enhanced transcription of perforin, granzysin and IFN- γ but also IL-4 and IL-13.⁶⁴ In addition, Mtb granulomas in the human lung that were positive for IL-4 were always positive for IFN- γ .⁶⁵ Novel findings also provide evidence that IL-4 and IL-13 abrogated IFN- γ induced autophagy and autophagy-mediated killing of intracellular mycobacteria in murine and human macrophages.⁶⁶ These results suggest that a Th1 response is mounted simultaneously with a Th2 response, which may prevent full protection provided by Th1-induced immunity. Thus, long-term control of TB infection may require a coordinated Th1 response together with inhibition of a Th2/immunoregulatory response.

In murine TB, Treg cells accumulate in high numbers in the lung⁶⁷ including all sites where CD4+ T cells are found, specifically perivascular/peribronchiolar regions and within lymphoid aggregates of pulmonary granulomas.¹⁷ Several recent studies report that CD4+ CD25+FoxP3+ natural Treg cells are increased in the blood and at disease sites in human TB.⁶⁸⁻⁷⁰ Recently, it

was also shown that antigen-specific induction of FoxP3 was predictive for active versus latent TB infection in humans.⁷¹ Treg cell-mediated suppression of CTL activity in viral infections^{21-24,72} and cancer^{73,74} has previously been described to involve impaired proliferation, degranulation and expression of perforin and granzymes in dysfunctional CD8+ T cells. Human Treg cells from hepatitis C patients, could suppress proliferation and the intracellular expression of perforin in activated CD8+ T cells, which may explain the low frequencies and retarded maturation state of virus-specific CTLs.²³ Functional impairment of retrovirus-specific CD8+ T cells have been shown to be associated with an expansion of CD25+FoxP3+ T cells *in vivo*.⁷² Accordingly, *in vivo* depletion of CD25+ Treg cells significantly enhanced CD8+ T cell responses to virus-transformed cells.^{24,75} In this study, a significantly decreased ratio of CD8+ T cells to FoxP3+ Treg cells was found in the granulomatous lesions of TB infected lymph nodes. Interestingly, patients with a progressive HIV infection have been shown to have significantly increased levels of FoxP3 mRNA⁷⁶ but low levels of perforin in CD8+ CTLs⁵¹ as compared with HIV non-progressors. Accumulation of FoxP3+ Tregs at the site of viral replication seems to involve a redistribution of Treg cells from blood to lymphoid tissue.³² Kinter et al have demonstrated that CD25+FoxP3+ Treg cells isolated from both lymph nodes and peripheral blood significantly suppress HIV-specific CTL function.^{22,77} Importantly, the suppressive activity by tissue-associated Treg cells at the site of infection in the lymph nodes was particularly potent, especially in patients with high levels of plasma viremia.²² An early Treg cell response during acute SIV infection may contribute to viral persistence by prematurely limiting the CTL response before the infection is cleared.⁷⁸ In addition it has been shown that increased numbers of intratumoral Treg cells correlated with low numbers of CD8+ T cells in biopsy specimens from patients with B-cell non-Hodgkin's lymphoma.⁷⁴ This supports functional findings *in vitro* showing that intratumoral Treg cells inhibit granule production in CD8+ T cells, thus making lymphoma B cells resistant to CTL-mediated apoptosis. Similar findings in clinical TB, suggest that immunosuppression observed in patients with active TB is associated with naturally occurring Treg cells expressing high levels of mRNA for FoxP3, TGF- β , and IL-4.⁷⁹ One function of TGF- β could be to repress IL-23R expression and subsequent Th17 cell differentiation, instead favoring the development of FoxP3+ Treg cells.⁸⁰ Numerous studies also provide evidence that Treg cells could suppress antigen-specific IFN- γ production by human T cells, by which mechanism they would limit immunopathology but also down-regulate cellular immunity.^{18-19,20,81} Thus, local CD4+ T cell responses could also be inhibited, which may result in a failure to recruit CD8+ effector T cells to the granulomatous lesions in TB. Altogether, these data propose a function of Treg cells in dampening the magnitude of the CTL response at the site of infection, suggesting that Treg cells might play a key role in the control of cellular immune responses during persistent TB.

In conclusion, this study provided evidence that the adaptive immune response in establishment of clinical TB was skewed toward a suppressive or regulatory phenotype that may inhibited proper immune activation and down-regulated the host response at the local site of infection. This Th2/Treg immune response may have antagonized a Th1/Th17 response and simultaneously prevented the action of CTLs, especially during later stages of TB infection. These results suggest that proper anti-TB immunity was not present in the granuloma which is the main site of bacterial replication and containment. Compartmentalization of the immune response in human TB could be part of the reason why Mtb is never completely eradicated but instead develops into a chronic infection. These important findings merit further investigation, as a potential CTL dysfunction may lead to bacterial escape, therefore representing a novel and disease-relevant mechanism by which Mtb evades cellular immunity. New immunotherapies may involve targeting of certain subpopulations of Th2/Treg cells to enhance cell-mediated immune responses that are down-regulated in patients with TB.

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