

# Differential Organization of the Local Immune Response in Patients with Active Cavitory Tuberculosis or with Nonprogressive Tuberculoma

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**Background.** In 90% of all cases, *Mycobacterium tuberculosis* infection results in latency rather than active disease, with the pathogen being contained within granulomatous lesions at the site of primary infection. Failure of this containment leads to reactivation of postprimary tuberculosis (TB). The regional immune processes that sustain the delicate balance with persistent *M. tuberculosis*, however, remain unclear.

**Methods.** We compared activation statuses, biological functions, and interactions of host immune cells in human nonprogressive tuberculoma and active cavitory tuberculous lung tissue.

**Results.** Dissection of early granuloma formations revealed differential cellular distribution and activation statuses of distinct cell types in different regions relative to the central caseotic caverna or the tuberculoma in tuberculous lung tissue. In patients with tuberculoma with latent infection, distant parts of lung tissue exhibited strong vascularization and profound proliferative activity, indicating that continuous immune defense is required for mycobacterial containment, which is absent in cavitory tuberculous lung lesions.

**Conclusions.** We conclude that differential regulation of the local immune response is crucial for the containment of *M. tuberculosis* and that a continuous antigen-specific cross talk between the host immune system and *M. tuberculosis* is ensured during latency. This activation requires sufficient supply of nutrients and well-coordinated structural organization, both of which are lost during reactivation of TB.

Even in the 21st century, tuberculosis (TB) accounts for an annual death toll of ~2 million individuals. One-third of the world's population (i.e., 2 billion people) are infected with *Mycobacterium tuberculosis*. Of TB infections, 90% remain latent and do not transform into active clinical disease [1, 2]. Latency describes the absence of clinical symptoms, sputum negativity for acid-fast bacilli, and the nonprogression of x-ray-dense lung lesions [3, 4]. The pathogen persists in the face of a functioning immune system and survives within pro-

fessional phagocytes in granulomatous lesions. In recent years, dormancy of *M. tuberculosis* has received increasing attention, leading to dissection of the enzymatic apparatus required for adaptation to the changed environmental conditions. These include activation of alternative pathways in the citrate cycle to allow metabolism of lipids in the necrotic center of granulomas [5, 6], adaptation to low oxygen pressure [7], and up-regulation of genes involved in stress responses, such as sigma factors [8, 9] and PGRS [10]. Thus, dormancy of the pathogen has been characterized intensively [3]. In contrast, the question remains whether latency is caused by camouflage due to metabolic quiescence of the pathogen or whether, alternatively, latency is the result of a continuous cross talk between the host immune system and persisting pathogens that is mediated by antigen-specific T cells and executed by mononuclear phagocytes.

The tuberculin skin test used to detect patients newly infected with active TB is based on the delayed-type

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hypersensitivity (DTH) response, which is induced by activated antigen-specific T cells migrating to the site of intradermal application of antigen. A positive DTH response, as measured by a tuberculin skin test, is supposed to represent a specific immune response to mycobacterial antigens in the infected human host; however, it does not distinguish between latent infection and active TB (except for miliary TB and Landouzy sepsis, which produce a negative DTH response) or between *M. tuberculosis* infection and bacille Calmette-Guérin (BCG) vaccination or contact with environmental mycobacteria [11]. Characterization of antigen-specific reactions of lymphocytes from peripheral blood mononuclear cells (PBMCs) from patients with TB versus those from infected but healthy patients or naive individuals revealed differential T cell responses to specific cell-wall [12] and secreted antigens (e.g., early secreted antigenic target [ESAT]-6 [13, 14]). However, none of the peripheral antigen-specific T cell responses describes unequivocal correlates of protective immunity to TB. Thus, the relevance of data obtained from PBMCs to the regional immune response operative at the site of mycobacterial containment in patients with TB can be validated only by comparative analysis of PBMCs with lung-derived mononuclear cells.

The present analysis aims at defining latent versus active TB at the tissue site of primary infection in humans. To define correlates of protection within the local immune response, we investigated lung tissue from progressive and nonprogressive disease states (i.e., from patients with active cavitary TB undergoing surgery to decrease the load of *M. tuberculosis*, which is often multidrug resistant [MDR]) and compared it with tissue obtained from patients undergoing surgery for unrelated reasons, in whose lungs tuberculoma lesions were detected and removed to prevent reactivation of active TB later in life. Immunohistological analyses and in vitro immunological assays indicated a highly active state in patients with tuberculoma, detected by Ki-67 staining and follicle-like organization, and a distinct histopathological pattern in the granulomatous walls of patients with cavitary TB.

## MATERIALS AND METHODS

**Tissue specimens.** Because of extensive lung disease and prevalence of MDR isolates, patients with TB in Russia frequently undergo elective thoracic surgery. Explorative thoracic surgery is also performed to distinguish between tumors and tuberculous lesions and to determine the stage of tuberculous lung destruction. Destroyed regions are removed within the anatomical borders of lung segments from 2 different groups: (1) those with progressive disease (i.e., active cavitary TB, Ziehl-Neelsen [ZN]-positive sputum, and exhibition of all symptoms of clinically active TB) and (2) those with nonprogressive lesions (i.e., tuberculoma with distinct x-ray-detectable, circular lesions, without any sign of disease activity or access to the

bronchial system). Lung-tissue samples were obtained from 21 patients at the Department of Thoracic Surgery of the Central Tuberculosis Research Institute: 11 patients with tuberculoma (mean  $\pm$  SD age,  $38.4 \pm 13.7$  years; 5 women and 6 men) and 10 patients with cavitary TB (mean  $\pm$  SD age,  $34.8 \pm 10.0$  years; 4 women and 6 men) (table 1) [15]. Immediately after surgery, removed lung tissue was either fixed in 4% paraformaldehyde overnight for histological analysis or treated with collagenase and DNase for cell isolation (see below). All procedures involving patients were fully reviewed and approved by the appropriate ethical review boards in Moscow and Berlin. Informed consent was obtained from each patient in this study.

**Immunohistological staining of cell-surface markers, proliferating cells, and mycobacterial antigens.** After deparaffination, tissue sections were washed with H<sub>2</sub>O, incubated in 1 mmol/L EDTA buffer at 2 bars for 5 min, and then washed with 5% Tween 20 in PBS (TBS). Sections were incubated with mouse monoclonal antibodies against CD20 (1:100; CM004B; Biocarta), CD31 (1:25; CM131B; Biocarta), CD68 (1:100; CM033B; Biocarta), or Ki-67 (1:75; M7240; DAKO) for 2 h at room temperature. Ki-67 binds to the nuclei of proliferating cells without any preparatory treatment of the tissue sections. Antibody binding to surface markers and proliferation (Ki-67) were detected by use of the same secondary antibody system. After washing in TBS, sections were incubated for 1 h with goat anti-mouse polyclonal antibody labeled with alkaline phosphatase (115-055-146; Dianova).

For detection of mycobacteria, tissue sections were blocked with goat serum (1:100) for 10 min. Sections were then stained with a rabbit polyclonal antiserum against *M. bovis* BCG (pAbBCG) (1:1000; B0124; DAKO) [15].

**Photography.** All images were captured by use of a Leica DMLB microscope fitted with a Hitachi HV-C20A 3CCD video camera. To maintain comparability between slides, light parameters were optimized for ZN and pAbBCG staining and then kept constant for all subsequent slides. Images were saved by use of DISKUS (version 42034; Hilgers) and processed by use of PowerPoint software (Microsoft).

**Preparation of cells from lung tissue.** Samples of surgically removed lung tissue ( $\sim 1$ – $5$  cm<sup>3</sup>) were cut into small pieces and incubated in complete medium (RPMI 1640, 10 mmol/L HEPES buffer, 200 mmol/L L-glutamine, and 5 U/mL streptomycin-penicillin [all from Gibco-BRL]) containing collagenase D (1088866; Roche) and DNase (AMP-D1; Sigma), for 3 h at 37°C with 5% CO<sub>2</sub>. Cells were isolated from tissue debris by use of a thin mesh. After washing several times with Ca<sup>2+</sup>-free PBS, cells were purified by Ficoll-Paque (Pharmacia) density centrifugation. Viability was checked by trypan blue staining, and homogeneity of the isolated leukocytes was assessed by microscopy. PBMCs from blood samples from patients with TB were also isolated by Ficoll-Paque density centrifugation. Im-

**Table 1. Clinical and macropathological characteristics of patients with tuberculoma versus patients with active tuberculosis (TB).**

No.	Age, years	Sex	Tuberculoma diagnosis		Mycobacterial load in surgically removed lung tissue, cfu/g	ZN staining			pAbBCG staining		
			Microscopy	Culture		Tuberculoma wall	Distant lung	Pericavity tissue	Tuberculoma wall	Distant lung	Pericavity tissue
M1	36	F	–	–	$6.4 \times 10^5$	–	–	–	+	+	–
M2	28	M	–	–	$5.5 \times 10^3$	(+)	–	–	+	+	–
M4	24	M	–	–	$5 \times 10^7$	–	–	–	+	+	–
M5	NK	F	–	–	$4.4 \times 10^5$	+	+	–	+	+	–
M7	30	F	–	–	No growth	(+)	(+)	–	+	+	–
M11	63	M	–	–	No growth	–	–	–	–	–	–
M14	56	F	–	–	ND	–	–	–	+	+	–
M15	64	M	–	–	ND	–	–	–	+	+	–
M20	28	M	–	–	$2.6 \times 10^6$	–	–	–	+	–	–
M22	NK	M	–	–	$2.6 \times 10^7$	–	–	–	++	+	–
M24	NK	F	–	–	ND	–	–	–	–	–	–
			Active TB diagnosis			Cavity wall			Cavity wall		
M3	48	M	+++	+++	$3.7 \times 10^9$	–	–	–	+	+	+
M8	26	M	++	+	$7.5 \times 10^5$	–	–	–	–	+	+
M9	52	M	+++	+	$3.0 \times 10^7$	–	–	+	–	+	+
M10	25	M	+	+	$4.8 \times 10^4$	–	–	–	++	+	+
M12	35	F	++	++	ND	++	–	–	+++	++	++
M19	35	M	–	+	ND	–	–	–	+	–	–
M21	NK	M	+	+	ND	–	–	–	–	–	–
M23	30	F	–	+	$1.5 \times 10^6$	–	–	–	ND	ND	ND
M25	NK	M	+	+	ND	–	–	–	++	+	+
M26	26	F	+	+	ND	–	–	–	+	+	++

**NOTE.** Surgically removed lung tissue was assessed from patients with active TB (sputum Ziehl-Neelsen [ZN] positive) and from those with tuberculous lesions but without clinical signs of TB. Quantification of mycobacteria was performed according to the Gaffky scale. ND, not determined; NK, not known.

mature dendritic cells (iDCs) were derived from adherent cells, in accordance with standard procedures [12].

**Interferon (IFN)- $\gamma$  ELISA.** Irradiated (5000 R) iDCs (from PBMCs or lung tissue) or isolated macrophages were plated in U-bottom microtiter plate wells at 25,000 cells/well, in 0.2 mL of complete medium containing 10% human male AB serum (Sigma). Mycobacterial antigens (H37Rv sonicate and recombinant Ag85) were added at a final concentration of 20  $\mu$ g/mL. Detection was performed in accordance with standard procedures [14].

**Proliferation assays.** Irradiated (5000 R) iDCs were plated in U-bottom microtiter plate wells at 25,000 cells/well, in 0.2 mL of complete medium containing 10% human male AB serum (Sigma). Mycobacterial antigens (H37Rv sonicate) were added at a final concentration of 20  $\mu$ g/mL. Responding autologous nonadherent lymphocytes were added to a density of 50,000 cells/well. Proliferation was detected by use of [ $^3$ H]-thymidine incorporation, in accordance with standard procedures [12, 14]. Stimulation indices (SI) were calculated by the ratio of incorporated [ $^3$ H]-thymidine in the presence and absence of mycobacterial antigens. SI >3 were considered to be significant.

**Statistical analyses.** The statistical significance of the results was determined by use of the statistics program included in the

GraphPad Prism program (version 3.0; GraphPad). Mean proliferative responses were derived from triplicate experiments, and differences between the 2 patient groups were determined by use of the unpaired *t* test with data on cytokine concentrations (nanograms per milliliter). The Mann-Whitney *U* test was used to compare SI derived from proliferative responses (counts per minute) and cellular contents in tissue (percentages).

## RESULTS

**Microbiological and clinical findings.** Patients with cavitory TB presented with ZN<sup>+</sup> sputum, from which mycobacteria could be cultured. Tissue specimens from patients with tuberculoma were ZN<sup>–</sup> and TB culture negative. However, both groups had pulmonary densities of *M. tuberculosis* of  $10^3$ – $10^9$  cfu/g of lung tissue, with higher densities in patients with cavitory TB. In some cases, the centers of tuberculoma lesions presented remarkably high densities of  $10^5$ – $10^7$  cfu/g of lung tissue (table 1), with similar properties in terms of culture growth, colony morphology, and virulence as in the mouse model (data not shown). No correlation was detected between mycobacterial load in infected tissue and immune response. Within surgically removed cavitory TB or tuberculoma tissue, *M. tuber-*

**Table 2. Density of macrophages and lymphocytes in different regions of tuberculous human lungs.**

No.	TB disease	Distant lung tissue			Pericavity/tuberculoma and cavity/tuberculoma wall tissue		
		Cell density, cfu/g	Macrophages, %	Lymphocytes, %	Cell density, cfu/g	Macrophages, %	Lymphocytes, %
M5	Tuberculoma	ND	68	22	ND	52	21
M7	Tuberculoma	$5.7 \times 10^6$	73	18	$20 \times 10^6$	ND	ND
M11	Tuberculoma	$2.1 \times 10^6$	57	31	$1.0 \times 10^6$	56	27
M20	Tuberculoma	$2.6 \times 10^6$	48	25	ND	ND	ND
M22	Tuberculoma	$26 \times 10^6$	70	21	$13 \times 10^6$	60	20.5
M24	Tuberculoma	ND	70	18	ND	44	54
M19	Cavitary TB	ND	65	17	ND	50	36
M21	Cavitary TB	$21 \times 10^6$	40	54	ND	ND	ND
M23	Cavitary TB	$1.2 \times 10^6$	40	30	$1.5 \times 10^6$	42	42
M25	Cavitary TB	ND	35	49	ND	ND	ND
M26	Cavitary TB	ND	40	30	ND	42	42
M27	Cavitary TB	$30.5 \times 10^6$	44	38	$25.4 \times 10^6$	41	35
M28	Cavitary TB	$30 \times 10^6$	39	35	$20.9 \times 10^6$	31.2	47.4

**NOTE** After surgical removal, tissue was treated with collagenase, and cells were isolated and counted. Relative proportions of macrophages and lymphocytes are given in percentages of total cell counts in the 2 tuberculosis (TB) disease groups. ND, not determined.

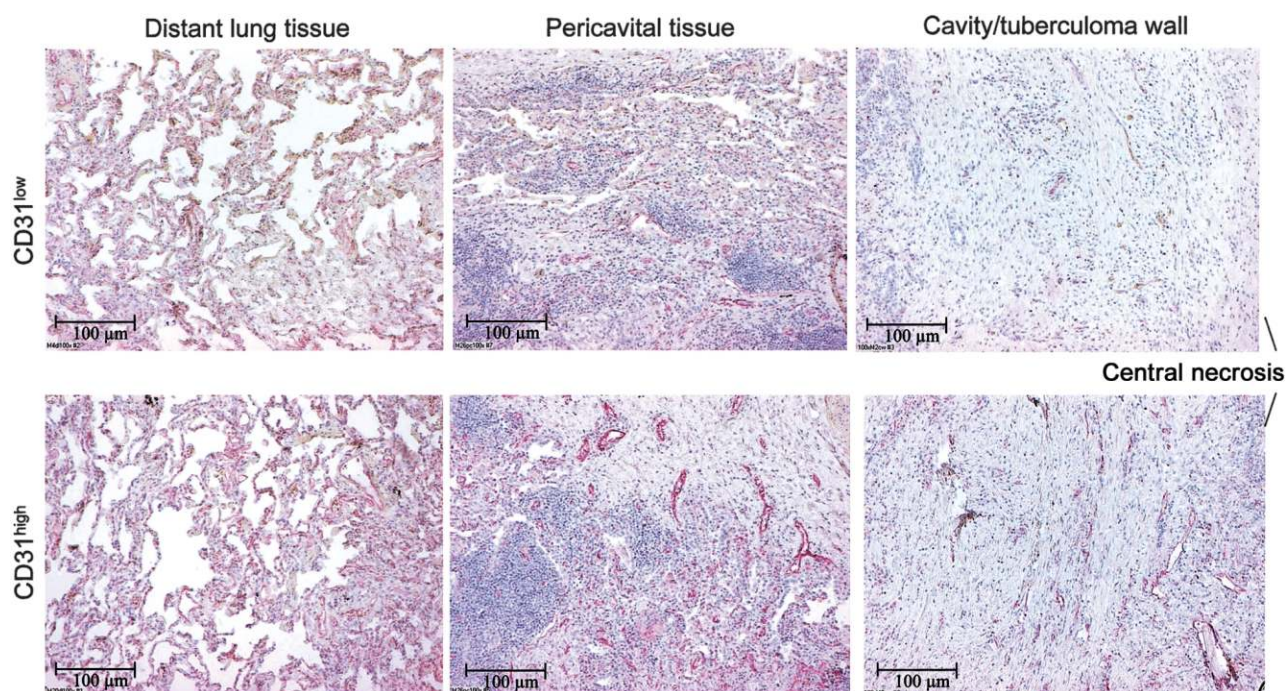
*culosis* was detected by use of colony-forming units, immunohistological staining (see below), and ZN staining. Tissue was often found to be ZN<sup>-</sup> [16], especially in latently infected tuberculoma tissue. In general, caseous cavities with access to the bronchial system contained ZN<sup>+</sup> mycobacteria in high concentrations, whereas mycobacteria-containing peripheral lung tissue was ZN<sup>-</sup> in both patient groups.

**Histopathological analysis.** Clinical and microbiological diagnoses of active or suspected latent *M. tuberculosis* infection were confirmed by macrohistopathological findings in the surgically removed tissue, and they defined the tuberculoma group by tuberculoma formation that included intact cellular infiltration and that was restricted to a single site in the lungs and the cavitary TB group by cavitary TB with 1 or more large cavity containing a caseous necrotic mass with access to the bronchial system. For this distinction, tissue was divided into 3 regions: (1) thick layer of the cavity wall, directly neighboring the caseotic mass in cavitary tuberculous tissue or forming the wall of a productive granuloma in tuberculoma tissue; (2) pericavity tissue surrounding these central lesions with dense infiltrations; and (3) superficially normal tissue far from the macroscopic lesions and harboring numerous small intact granulomas. The distant parts of the surgically removed tissue were rich in small granulomas containing *M. tuberculosis* organisms within the necrotic center or in antigen-presenting cells (APCs) in the peripheral infiltrated tissue [15] (in brief, this study by Ulrichs et al. describes the morphological correlates of granuloma formation in *M. tuberculosis*-infected human lung tissue and characterizes follicle-like active centers as the major site of host-pathogen interaction). Both patient groups were infected with an *M. tuberculosis* “Beijing W” genotype

family member, which was often MDR. Distribution of mycobacteria or intracellular mycobacterial material can be visualized by immunohistological staining using pAbBCG [16]. The pAbBCG<sup>+</sup> cells, which contained mycobacterial material, were identified in all 3 regions of tuberculous lung tissue. In cavitary TB and tuberculoma tissue material, such cells were found not only close to the necrotic centers but also in peripheral infiltrations.

**Macrophage and lymphocyte content.** To identify cellular markers of mycobacterial containment, cell densities, as well as macrophage and lymphocyte counts, were determined. Several different regions (with a minimal volume of  $\sim 5 \text{ cm}^3$ ) in distant lung tissue and pericavity tissue (according to the above-mentioned definition) from each patient were cut out and treated with collagenase (table 2). Tuberculoma tissue was significantly richer in macrophages than was cavitary tuberculous tissue ( $P = .0008$ , Mann-Whitney *U* test) and was less dense in lymphocyte infiltration in both central and peripheral regions of the tuberculous lung tissue ( $P = .0147$ , Mann-Whitney *U* test). Further analysis of the lymphocyte subpopulations by flow cytometry revealed similar percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in both study groups (data not shown).

**Differential vascularization.** Staining with CD31 and morphological identification of endothelial cells detected the relative distribution of blood vessels throughout the different tissue regions. Distant lung tissue was well vascularized in patients with tuberculoma (figure 1 and table 3), whereas the comparable region in patients with cavitary TB contained low densities of CD31<sup>+</sup> blood vessels (figure 1 and table 3). The cavity wall, as well as the tuberculoma wall, contained numerous vessels. Twenty-six different tissue samples from 14 patients



**Figure 1.** Vascularization of tuberculous lung tissue. CD31<sup>high</sup>-stained regions are predominantly found in the distant lung regions of patients with tuberculoma (*left, bottom panel*) and in the cavity walls of patients with cavitary tuberculosis (TB) (*right, bottom panel*). Distant lung tissue from patients with cavitary TB was predominantly CD31<sup>low</sup> (*left, top panel*). In total, tissue samples from 8 patients with tuberculoma and 6 patients with cavitary TB were stained for CD31. Frequencies of CD31<sup>high</sup> and CD31<sup>low</sup> patterns are summarized in table 3.

(6 patients with cavitary TB and 8 patients with tuberculoma) were assessed.

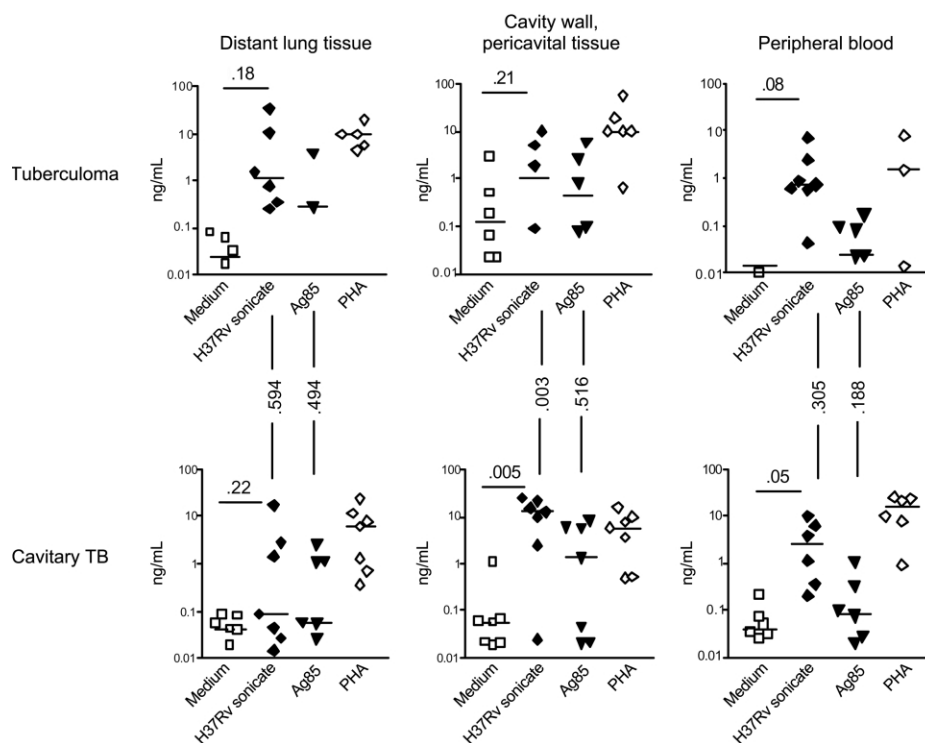
**Production of IFN- $\gamma$  in different parts of tuberculous lung tissue.** Production of IFN- $\gamma$ , the major Th1 cytokine [17], by cells isolated from tuberculous lung tissue was evaluated upon restimulation with mycobacterial antigens in vitro. Cells isolated from the cavity wall and distant lung tissue were compared with PBMCs from the same patients with TB. Overall, restimulation with H37Rv sonicate resulted in high levels of IFN- $\gamma$  responses, whereas Ag85 only elicited low levels of production of IFN- $\gamma$ . Cells from distant lung tissue and PBMCs produced comparable concentrations of IFN- $\gamma$ . In contrast, production of IFN- $\gamma$  by cells from PBMCs and distant lung tissue from both patients with cavitary TB and patients with tuberculoma were higher than was production of IFN- $\gamma$  by PBMCs from naive donors or cells isolated from lung tissue from patients with cancer (data not shown) [18, 19]. For distant lung tissue, however, production of IFN- $\gamma$  did not differ between the 2 patient groups. The most potent producers of IFN- $\gamma$  resided in wall regions of cavitary tuberculous tissue, secreting significantly more IFN- $\gamma$  than cells from comparable regions in tuberculoma tissue ( $P = .003$ ,  $t$  test) (figure 2).

**Active centers within the outer lymphocyte infiltration.** To determine the degree of immune activation in tuberculous lesions in the 2 groups, proliferating lymphocytes were identified by Ki-67 staining. Discrete spots of proliferating cells were frequently detected within peripheral lymphocyte infiltrations (figure 3). Serial sections of these regions revealed that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells surrounded the active (Ki-67<sup>+</sup>) centers and were also present within these centers, although to a lesser extent. Proliferating cells were surrounded by CD68<sup>+</sup> APCs that

**Table 3.** Frequencies of high and low densities of CD31 staining in different regions of tuberculous tissue from patients with cavitary tuberculosis (TB) and patients with tuberculoma.

Region	Cavitary TB (n = 6 patients)		Tuberculoma (n = 8 patients)	
	CD31 <sup>high</sup>	CD31 <sup>low</sup>	CD31 <sup>high</sup>	CD31 <sup>low</sup>
Cavity/tuberculoma wall	4	0	NA	NA
Pericavity/tuberculoma	6	0	5	4
Distant lung	1	5	5	2

**NOTE.** In total, 26 different tissue samples from patients with cavitary TB and patients with tuberculoma were stained and assessed for their CD31 patterns. The table presents the no. of patient samples per tissue localization (compare with figure 1). NA, not applicable.



**Figure 2.** Mycobacteria-specific production of interferon (IFN)- $\gamma$  by cells from different lung regions. Pericavity tissue and macroscopically normal tissue from distant parts of the surgically removed lung tissue were treated with collagenase and DNase. Blood samples were drawn before surgery. After isolation and purification, cells were separated by adherence into nonadherent lymphocytes and adherent antigen-presenting cells. Equal cell numbers of each fraction were stimulated with mycobacterial antigens in vitro. IFN- $\gamma$  in the supernatants was measured by ELISA. *P* values are indicated above and between the compared groups. PHA, phytohemagglutinin.

contained mycobacteria. To compare activities of the 2 groups, Ki-67 patterns were assessed in the 3 tissue regions. Unique spots of high activity (figure 3A) predominated in distant lung tissue from patients with tuberculoma, whereas corresponding regions in patients with cavitory TB were found to be negative for Ki-67. Immune activation was detected at the rim of the cavitory wall in a disseminated, nonfocused form and at a lower level than that at the active centers of the periphery (figure 3B).

**Proliferative activity in vitro.** To verify the cellular activity within parts of distant lung tissue from patients with tuberculoma, tissue-derived cells and PBMCs from the same patients were restimulated in vitro with *M. tuberculosis* antigens. Figure 4 shows significantly higher proliferative responses to *M. tuberculosis* antigens by distant lung tissue-derived lymphocytes from patients with tuberculoma than by those from patients with cavitory TB ( $P = .041$ , pericavity tissue from patients with tuberculoma vs. that from patients with cavitory TB;  $P = .093$ , Mann-Whitney *U* test). As with production of IFN- $\gamma$ , proliferative activity was highest at the rim of the cavity wall (figure 2). Proliferative activity of lymphocytes from tuberculous lung tissue was consistently higher than that of PBMCs from the same patients with TB. In contrast, in patients with cancer,

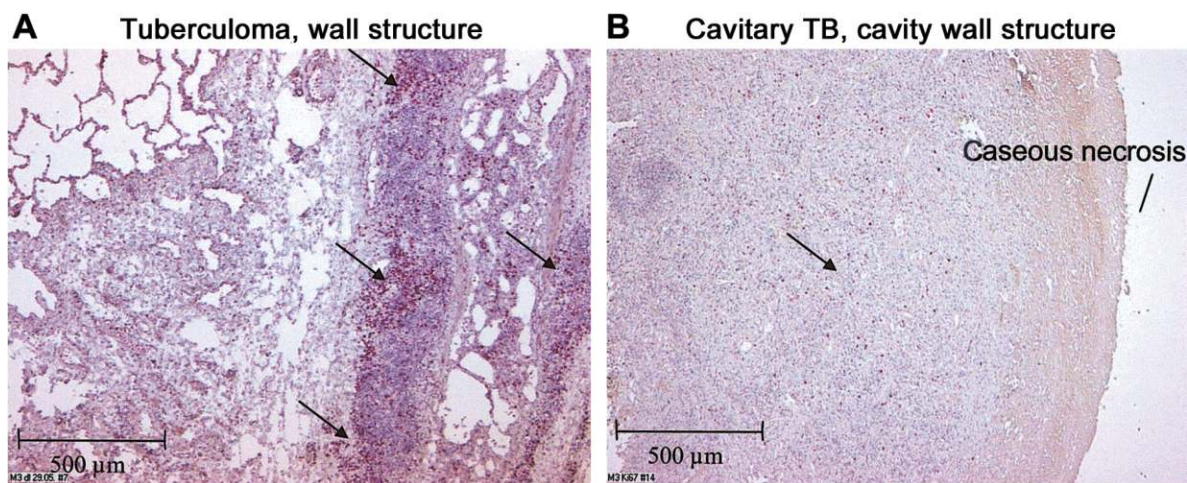
proliferative responses of lung tissue-derived cells were lower than those of PBMCs (figure 4).

## DISCUSSION

Ninety percent of all *M. tuberculosis* infections do not transform into active disease [1, 2], and infected individuals can carry the pathogen in lung lesions throughout their lifetimes without signs of active TB, since containment at the site of infection is highly effective. The functional and morphological correlates of resistance against reactivation that are operative in these highly productive tuberculomas, however, remain unclear. Principally, latency could be caused by mycobacterial camouflage or, alternatively, by continuous cross talk between the host immune system and the pathogen.

Older studies have shown that the pathogen can be completely cleared from small lesions [20, 21]. However, tuberculoma lesions used for the present study were larger, harbored culturable mycobacteria (table 1), and presented all signs of inactive, latent infection. In a comparison of these lesions to those of patients with active cavitory TB, cells harboring mycobacteria were detected in the cavity wall, pericavity tissue, and, interestingly, in peripheral, superficial, normal lymphocyte-infiltrated lung tissue





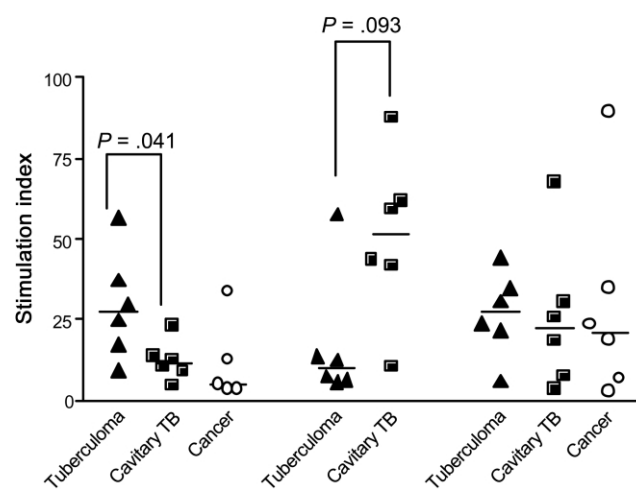
**Figure 3.** Distribution patterns of proliferating cells. Tissue samples from cavity/tuberculoma wall, pericavity/perituberculoma tissue, and distant lung tissue were stained with Ki-67 and assessed for density of Ki-67<sup>+</sup> regions. Distant lung tissue from patients with tuberculoma revealed the highest density (A), whereas distant lung tissue from patients with cavitary tuberculosis (TB) was mainly Ki-67<sup>-</sup>. The cavity walls of patients with cavitary TB presented a disseminated pattern of proliferating cells (B).

in both study groups (as has been shown earlier for the lung apices by polymerase chain reaction [22]). This abundance of mycobacteria suggests that all 3 tissue regions were recognized by antigen-specific immune cells in both patient groups. This finding already strongly argues against camouflage as a cause of mycobacterial persistence [23] and is consistent with active cross talk throughout the infiltrated tissue.

Tissue dissection revealed that distant lung parts were highly vascularized in patients with tuberculoma, whereas the comparable region in patients with cavitary TB contained only low densities of vascularization (figure 1). Although the cavity wall contained numerous vessels, we assume that the positive correlation between vascularization and outcome of TB infection (tuberculoma vs. active TB disease) points to a critical role of oxygen and nutrient supply to the affected tissue in sustaining the productive cross talk between the host immune system and *M. tuberculosis* that permits latency. Oxygen and nutrient supply may support both the immune system and growth of the pathogen, as has been shown elsewhere [7, 24].

Another marked difference between lung tissue from patients with tuberculoma and that from patients with cavitary TB was the distribution of macrophages and lymphocytes as cellular mediators and executors of mycobacterial containment, respectively [1]. Since immunohistological findings do not reflect the absolute numbers and distribution of leukocytes, cells were counted after collagenase treatment. Tuberculoma tissue was found to be enriched in macrophages and less dense in lymphocyte infiltration (table 2). Although antigen-specific T cell responses are crucial for control of TB [1, 25, 26], sufficient numbers of activated macrophages, rather than quantities of infiltrating T cells, seem to be limiting. Macrophages have re-

cently been shown to function as carriers of mycobacteria to the site of granulomatous lesions, thereby contributing to mycobacterial containment [27]. Macrophage activation by T cells is mediated by cytokines, notably IFN- $\gamma$ . As a consequence, a single T cell should be capable of activating numerous macrophages in its vicinity. It has been well documented that mac-



**Figure 4.** Proliferative responses to mycobacterial antigens of human lung-derived cells compared with peripheral blood lymphocytes in vitro. Pericavity tissue and macroscopically normal tissue from distant parts of the surgically removed lung were treated with collagenase and DNase. Blood samples were drawn before surgery. After isolation and purification, cells were separated by adherence into nonadherent lymphocytes and adherent antigen-presenting cells. Equal cell numbers of each fraction were stimulated with H37Rv sonicate in vitro (figure 2). Proliferative responses are shown in stimulation indices (SI, ratio of incorporated [<sup>3</sup>H]-thymidine in the presence and absence of mycobacterial antigens).

rophages play an important executor role in protection against *M. tuberculosis* [28, 29], and one of the pathogen's evasion strategies includes the numerical reduction of macrophages by induction of apoptosis [30]. At the same time, mycobacteria misuse macrophages as their habitat by suppressing their antibacterial capacities [31]. The precise role that mononuclear phagocytes play in active human TB remains to be determined.

Among lymphocyte subsets, B cells appeared in higher concentrations in distant lung regions than in the cavity wall and PBMCs (as analyzed by flow cytometry of cells isolated from lung tissue [data not shown]). Their numbers in bronchoalveolar fluids correlate with the state of infection [32]. The presence of B cells in active centers of follicle-like structures (see below and [15]) (figure 3) indicates contribution of B cells to antigen presentation.

Restimulation of isolated cells from the 3 tissue regions in vitro revealed profound production of IFN- $\gamma$  in the cavity wall, indicating marked lymphocyte activation at this tissue site, as has been demonstrated on the histological level elsewhere [33, 34]. A comparison of lung tissue from different patient groups, however, demonstrated that, in contrast to data obtained with PBMCs [12, 35], production of IFN- $\gamma$  did not correlate with mycobacterial containment or control of TB infection in patients with cavitary TB, regardless of which mycobacterial antigen preparation was used (high response to the sonicate and lower response to the single antigen Ag85). Since cavity walls and tuberculomas can be vascularized intensely (table 3), high concentrations of IFN- $\gamma$  in these regions do not seem to account for either inhibition of vascularization or, subsequently, the formation of a necrotic core, as has been found for inhibition of tumor growth [36, 37].

Detection of active sites in situ within the tissue, by Ki-67 staining, revealed active centers in peripheral lung tissue from patients with tuberculoma but not in patients with cavitary TB. These centers appeared well organized: APCs in the center of follicle-like cell aggregates were surrounded by T cells. In contrast, the cavity walls of patients with cavitary TB presented activated cells in a disseminated, nonfocused distribution. Such an unfocused pattern suggests loss of organization, which facilitates mycobacterial spread in patients with cavitary TB. Lower levels of activation at the rim of tuberculous lesions confirm published evidence of impaired immune responses to *M. tuberculosis* at the luminal site of the cavity wall [38]. Tuberculoma walls, in contrast, mainly presented an organized structure comprising small aggregates of highly proliferating cells (figure 3A). We conclude that peripheral foci represent active centers where cross talk between the host immune system and the pathogen is orchestrated, leading to productive protection in patients with tuberculoma.

This differential distribution of cellular activity could be confirmed by proliferation data from antigen restimulation of isolated cells from the 3 tissue regions. Proliferation was significantly

higher for lymphocytes from distant lung parts from patients with tuberculoma than for those from patients with cavitary TB, whereas no such difference could be detected in the proliferation patterns of lymphocytes from the peripheral blood (PBMCs) derived from the 2 patient groups. We conclude that, on the one hand, proliferative activities of lymphocytes from PBMCs do not mirror the activation status at the site of primary infection in human tuberculous lungs and that, on the other hand, active sites in the periphery of affected tissue represent the morphological substrate where cross talk between the host immune system and the pathogen is coordinated (figure 4).

Our results suggest that the dynamic balance between active immune response and persistent *M. tuberculosis* is crucial for long-term containment and prevention of disease [2]. We propose that the organization and continuous activation of the cell aggregates in the distant lung tissue surrounding granulomatous lesions in patients with tuberculoma represent the morphological and functional correlates of an efficacious immune response that ensures mycobacterial containment and prevents dissemination.

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