Identification of Human T-Cell Responses to *Mycobacterium tuberculosis* Resuscitation-Promoting Factors in Long-Term Latently Infected Individuals[▽][†]

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The *Mycobacterium bovis* BCG vaccine is the only tuberculosis (TB) vaccine available, yet it provides limited protection against pulmonary TB in adults and fails to protect against TB reactivation. We hypothesized that immunity against *Mycobacterium tuberculosis* "resuscitation-promoting factors" (Rpfs), which are small bacterial proteins that promote proliferation of dormant mycobacteria, may be relevant in the human immune response to *M. tuberculosis*. In previous unpublished work, we found that Rpfs Rv0867c and Rv2389c induced gamma interferon (IFN-γ) production in the blood of TB patients' healthy household contacts in several different African populations. Here we examine these two dominant Rpf antigens in more detail and define the nature of the responding T-cell subsets. Multiparameter cytokine profiling showed that Rv2389c and, to a lesser extent, Rv0867c were recognized by mycobacterium-responsive healthy Dutch individuals; peptide-scanning revealed several epitopes, including a single immunodominant epitope in Rv2389c. Rv0867c and, to a lesser extent, Rv2389c Rpf-specific T-cell responses were maintained for decades in long-term *M. tuberculosis* nonprogressors. Prominent Rv0867c-specific double- and single-cytokine-producing CD8⁺ T-cell subset responses were found, including a large population of CD8⁺ effector memory and effector T-cell subsets. We conclude that *M. tuberculosis* Rpf antigens are important targets in the human immune response to *M. tuberculosis* and represent interesting TB vaccine candidate antigens.

It is estimated that over 2 billion people are latently infected with *Mycobacterium tuberculosis* and that 5 to 10% of these individuals will develop active tuberculosis (TB) at one point in their lifetime, whereas the remainder are able to contain infection long term without developing clinical symptoms (28). During latency, the bacteria are thought to be in a dormant or slowly replicating state (16). The vast reservoir of individuals with latent infection is a major source of new TB cases due to reactivation and resuscitation of dormant bacilli (8, 18).

The term "dormancy" was first introduced by Joseph Warwick Bigger, who discovered that a culture of *Staphylococcus pyogenes* could not be sterilized after penicillin treatment since there was a small group of antibiotic-resistant bacteria that could be regrown from such cultures. Bigger proposed that these bacteria were dormant, nonreplicating, and thus insensitive to antimicrobials targeting bacterial metabolic pathways (5).

It is assumed that environmental factors are involved in inducing bacterial dormancy (40). *M. tuberculosis* enters a state of nonreplicating or slowly replicating persistence when grown under gradual oxygen depletion, which is thought to be one of the stress factors that *M. tuberculosis* encounters upon infection (39). Not only oxygen deprivation, but also low pH, NO, nutrient deprivation, and host immune pressure are stress factors *M. tuberculosis* is subjected to in the lung granulomatous lesions. In response to these stress factors, *M. tuberculosis* decreases its metabolic activity and alters its gene expression pattern (4, 38, 40). This adaptation results in increased resistance to environmental stress, by means of entering the nonreplicating or slowly replicating persisting state (35).

While several studies have addressed the bacterial transition from the replicating to the nonreplicating, slowly replicating, or dormant state, little is known regarding the cues that induce bacteria to reactivate and resume growth from dormancy. Mukamolova et al. were the first to discover a resuscitation-promoting factor (Rpf), a hormone-like protein secreted by *Micrococcus luteus*. Addition of this Rpf protein to dormant *M. luteus* resulted in resuscitation of *M. luteus* bacteria (6, 25). *rpf* genes were then found to be conserved throughout high-G+C Gram-positive bacteria, including *M. tuberculosis*. Five such genes were identified in the *M. tuberculosis* genome, notably *Rv0867c* (*rpfA*), *Rv1009* (*rpfB*), *Rv1884c* (*rpfC*), *Rv2389c* (*rpfD*), and *Rv2450c* (*rpfE*). Each *M. tuberculosis* Rpf protein contains an ∼70-amino-acid Rpf-like domain similar to the *M. luteus rpf*-encoded protein (26). *M. tuberculosis* Rpfs showed similar properties to *M. luteus* Rpf, including their ability to resuscitate dormant mycobacteria (20, 26). *M. tuberculosis* Rpf

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protein expression was observed *in vitro* in actively growing *M. tuberculosis* and in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Importantly, *M. tuberculosis rpf* gene expression was detected in infected murine and human tissue. Moreover, the presence of Rpf-like proteins was shown in *M. tuberculosis*-infected human tissue (11, 26, 30, 36). Recently, *rpf* gene expression was analyzed in *M. tuberculosis* cells grown under different physiological stress conditions and growth factors. All five *rpf* genes were expressed during actively replicating early log-phase growth of *M. tuberculosis*, confirming previous findings. Of note, *M. tuberculosis rpf* genes displayed differential expression patterns when analyzed in cultures grown under hypoxia, nutrient starvation, and acidic conditions and stationary-, nonculturable-, and resuscitation-phase-like conditions. These differential adaptive *rpf* expression profiles indicate that *M. tuberculosis* Rpfs likely play different roles (18).

While *M. luteus* Rpf is essential for *M. luteus* growth, individual *M. tuberculosis* Rpfs were found to be redundant for growth *in vitro* and *in vivo* in single-gene-knockout mutants (12, 36). However, when multiple *rpf* deletions were introduced simultaneously in *M. tuberculosis* (Δ*Rv0867c* Δ*Rv1009* Δ*Rv1884c* and Δ*Rv0867c* Δ*Rv1009* Δ*Rv2389c*), a significant loss in the ability of *M. tuberculosis* to resuscitate was found, accompanied by *in vitro* growth attenuation (13).

BCG vaccination is widely used and affords protection from severe forms of TB in children, but it provides only limited and highly variable protection against pulmonary TB in adults and does not protect against reactivation. Better TB vaccines are clearly needed (14). As the *M. tuberculosis* Rpf proteins are associated with resuscitation of mycobacteria, we hypothesized that immunity directed against these proteins may play a role in sensing actively replicating *M. tuberculosis* organisms and possibly play a role in host immune control of reactivating bacteria. Only two reports have investigated the immunogenicity of the *M. tuberculosis* Rpf antigens. One study with mice showed that Rv0867c, Rv1009, Rv2389c, and Rv2450c were immunogenic (42). Recently we identified the first human *M. tuberculosis* Rpf-specific T-cell responses against a subset of the five *M. tuberculosis* Rpfs in a larger antigen T-cell screening (33). Gamma interferon (IFN-γ) production was detected in tuberculin skin test (TST)-positive individuals in response to Rv1009, Rv1884c, Rv2450c, and, to a lesser extent, to Rv0867c, whereas limited to no IFN-γ production was found in TST-negative individuals. Rv2389c, however, was not included in this study (33).

These two studies indicate a possible role for T-cell responses in detecting *M. tuberculosis* Rpf antigen during *M. tuberculosis* infection. We have, therefore, performed a more detailed analysis studying the immunogenicity of the Rv0867c and Rv2389c *M. tuberculosis* Rpf proteins in several groups of mycobacterium-exposed individuals, including long-term nonprogressors. These two antigens were selected based on the highest recognition of all 5 Rpfs in a cross-sectional cohort study of HIV-negative, TST- and/or ESAT6/CFP10-positive household contacts from the Gambia, Uganda, and South Africa (BMGF GCGH GC6#74 Biomarkers for TB Consortium; http://www.biomarkers-for-tb.net/; unpublished results). Of interest, both Rv0867c and Rv2389c are predicted to be secreted proteins (SignalP and TMMHM server, Technical University

of Denmark) (17), which may enhance availability of the antigen to the innate immune system. Indeed, Rv0867c protein is present in *M. tuberculosis* culture filtrate (24). *Rv0867c* and *Rv2389c* are both expressed in early log-phase-grown *M. tuberculosis*, but *Rv2389c* expression is induced in the stationary and noncultivatable phases of *M. tuberculosis* growth and during acidic conditions, whereas *Rv0867c* expression appears to be higher in nutrient-starved *M. tuberculosis* culture. Both genes are also highly induced during early resuscitation (18). In the work reported here, we (i) identify frequent and significant human T-cell responses against Rv0867c and Rv2389c; (ii) identify a series of novel *M. tuberculosis* Rpf epitopes, including a single dominant peptide epitope in Rv2389c; and (iii) describe *M. tuberculosis* Rpf-specific polyfunctional memory CD4+ and particularly CD8+ T-cell memory responses to *M. tuberculosis* Rv0867c and Rv2389c Rpf proteins in long-term naturally protected *M. tuberculosis* nonprogressors. Based on these data, we propose that Rpf antigens are potentially interesting new TB vaccine candidates.

MATERIALS AND METHODS

Study subjects. Blood samples were collected by venipuncture from a group of Dutch individuals, including 9 tuberculosis (TB) patients, 10 tuberculin skin test (TST)-positive individuals (indurations of ≥10 mm), 10 BCG-vaccinated individuals, and 10 non-BCG-vaccinated, TST-negative, healthy individuals, as well as 12 Norwegian TST-positive individuals. The Norwegian donor group consisted of elderly people (average age, 70 years) exposed to TB transmission several decades ago without developing any clinical disease and without receiving any treatment. Previously recorded TST indurations associated with natural conversion ranged from 12 to 60 mm (average of 18 mm). Recent IFN-γ release assay (IGRA) testing with the Quantiferon Gold in-tube test showed that 9 of 12 donors in this category were positive. All donors gave written consent before blood donation. The study protocol (P027/99) was approved by the Institutional Review Board of the Leiden University Medical Center and the Regional Committees for Medical and Health Research Ethics in Norway. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll density gradient centrifugation and stored in liquid nitrogen until further use.

Recombinant proteins. Recombinant proteins were produced as previously described (15). Briefly, *M. tuberculosis* genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway technology (Invitrogen, Carlsbad, CA) in a bacterial expression vector containing a histidine tag at the N terminus. Vectors were overexpressed in *Escherichia coli* BL21(DE3) and purified. The size and purity of recombinant proteins were analyzed by gel electrophoresis and Western blotting with an anti-His antibody (Invitrogen, Carlsbad, CA) and an anti-*E. coli* polyclonal antibody (a kind gift from the Statens Serum Institute [SSI]). Endotoxin contents were below 50 IU/mg recombinant protein, as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). All proteins were tested in lymphocyte stimulation assays in order to exclude antigen-nonspecific T-cell stimulation and cellular toxicity by using PBMCs of *in vitro* purified protein derivative (PPD)-negative healthy Dutch donors (22). PPD of *M. tuberculosis* was purchased from SSI, Copenhagen, Denmark.

Synthetic peptides. Peptides (20-mers overlapping 10 amino acids) were produced at the Leiden University Medical Center (LUMC) facility by simultaneous multiple-peptide synthesis as described previously (19). Homogeneity and purity were confirmed by analytical reversed-phase high-pressure liquid chromatography, and mass spectrometry showed the expected masses. Peptide purity was ≥75%.

Lymphocyte stimulation assay. PBMCs (1.5×10^5/well) were cultured in triplicate in 96-well round-bottom plates (Nunc, Roskilde, Denmark) and incubated with or without protein (10 μg/ml) in AIM-V medium (Invitrogen, Breda, Netherlands) at 37°C in 5% CO_2. After 6 days, supernatants were harvested and used for cytokine and chemokine profiling.

Cytokine and chemokine profiling. Levels of cytokines (IFN-γ, interleukin-1β [IL-1β], IL-6, and tumor necrosis factor alpha [TNF-α]) and chemokines (IFN-γ-induced protein, 10 kDa [IP-10]; and macrophage inflammatory protein 1β [MIP-1β]) were analyzed with the Lincoplex kit (Millipore) according to the
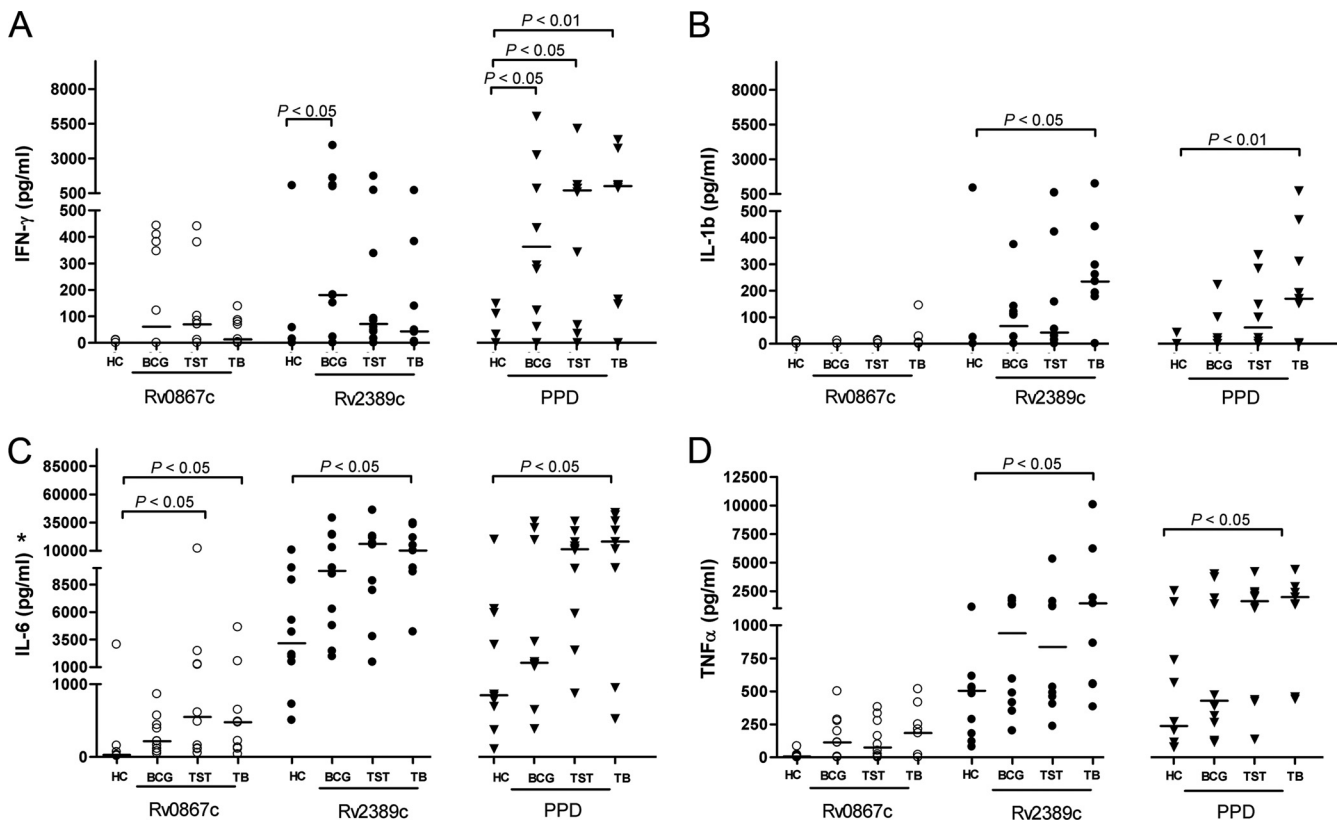
FIG. 1. Cytokine and chemokine profiling. PBMCs from healthy individuals (HC; $n = 10$), BCG-vaccinated individuals (BCG; $n = 10$), TST-positive individuals (TST; $n = 10$), and TB patients (TB; $n = 9$) were stimulated with Rv0867c and Rv2389c proteins and PPD for 6 days. Levels of IFN-γ (A), IL-1β (B), IL-6 (C), and TNF-α (D) were measured and corrected for background levels. Horizontal bars represent median cytokine production levels. Antigen stimuli: $\bigcirc$, Rv0867c; $\bullet$, Rv2389c; and $\blacktriangledown$, PPD. *, background not corrected.

Milliplex Map protocol. Plates were analyzed with a BioPlex array reader with Bio-Plex software (Bio-Rad Laboratories, Veenendaal, Netherlands).

Generation of antigen-specific T-cell lines. T-cell lines were generated as previously described (29). PBMCs (2×10^6 cells/well) from cured TB patients or TST-positive or BCG-vaccinated individuals were cultured in 24-well plates in the presence of protein (2 to 10 μg/ml) in IMDM medium (Gibco, Paisley, United Kingdom) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco, Paisley, United Kingdom), and 10% pooled human serum at 37°C in 5% CO_2. At day 6, recombinant IL-2 (rIL-2) (Cetus, Emeryville, CA) was added to the cell cultures in a final concentration of 25 U/ml. Cultures were maintained for an additional 2 to 3 weeks in the presence of rIL-2. T cells were harvested and stored in liquid nitrogen until further use.

T-cell proliferation of antigen-specific T-cell lines. Thawed T-cell lines were cultured in triplicate in 96-well flat-bottom plates (1.5×10^4/well) together with HLA-DR-matched irradiated (2,000 rads) PBMCs (5×10^4/well) with or without protein (10 μg/ml) or peptide (10 μg/ml) at 37°C in 5% CO_2. After 3 days, supernatants were harvested and stored at −20°C. Cells were pulsed for an additional 18 h with [^{3}H]thymidine (0.5 μCi/well), harvested, and counted on a Microbetaplate counter (Wallac, Turku, Finland) (3). A stimulation index (SI) of ≥3 was considered positive.

IFN-γ ELISA. The concentration of IFN-γ in supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (U-CyTech, Utrecht, Netherlands) according to the manufacturer's instructions. The detection limit of the assay was 20 pg/ml IFN-γ. Samples were tested in duplicate. An IFN-γ response of ≥100 pg/ml was considered positive.

Flow cytometric analysis. PBMCs were thawed and rested. After 24 h, PBMCs were stimulated for 16 h with protein (10 μg/ml) in the presence of costimulatory antibodies anti-CD28 and anti-CD49d (Sanquin, Netherlands, and BD Biosciences, respectively). Brefeldin A (3 μg/ml; Sigma) was added after the first 4 to 6 h. Cells were stained for 30 min at 4°C with the following surface markers: anti-CD3-Pacific blue (PB), anti-CD4-peridinin chlorophyll protein (PerCP)-Cy5.5, anti-CD8-AmCyan, anti-CD25-allophycocyanin (APC)-Cy5, anti-CD45RA-

phycoerythrin (PE)-Cy5, and anti-CCR7-PE-Cy7, and intracellular staining was performed with anti-IFN-γ–Alexa 700, anti-TNF-α–APC, anti-IL-2–PE, and anti-CD69–fluorescein isothiocyanate (FITC) (BD Biosciences) by using the Intrastain kit (Dako Cytomation, Denmark). Samples were acquired on an LSR II flow cytometer and analyzed with SPICE (software provided by M. Roederer, National Institute of Allergy and Infectious Disease) and FlowJo software (Tree-star, Inc., Ashland, OR). Cell populations should contain at least 100 events.

Statistical analyses. Differences between groups were analyzed with the non-parametrical Kruskal-Wallis test in GraphPad Prism (version 4). P values were corrected for multiple comparisons. The statistical significance level used was $P < 0.05$.

RESULTS

Recognition of *M. tuberculosis* Rpf antigens by T cells from *M. tuberculosis*-responsive individuals. To determine the immunogenicities of both Rv0867c and Rv2389c, we investigated whether these two *M. tuberculosis* Rpf proteins were recognized by PBMCs of mycobacterium-exposed individuals. PBMCs from four groups of Dutch individuals were tested: (i) HIV-negative, treated tuberculosis patients (TB; $n = 9$); (ii) tuberculin skin test (TST)-positive individuals (TST; $n = 10$); (iii) BCG-vaccinated individuals (BCG; $n = 10$); and (iv) non-BCG-vaccinated, TST-negative and *in vitro* purified-protein derivative (PPD)-negative healthy controls (HCs; $n = 10$). The secretion of four cytokines (IFN-γ, IL-1β, IL-6, and TNF-α) was analyzed as a multiparameter readout of antigen-specific responses.

As expected, IFN-γ production in response to PPD was low in healthy individuals, whereas the TST⁺ individuals and TB patients showed high responses to PPD, by both IFN-γ and the other cytokines analyzed. The BCG-vaccinated individuals showed intermediate levels of responses (Fig. 1A to D).

While minor IFN-γ levels were detectable in the three my-cobacterium-exposed groups in response to Rv0867c, higher levels were found in response to Rv2389c (HCs and BCG; $P < 0.05$). The healthy controls did not respond to Rv0867c and Rv2389c (Fig. 1A). No IL-1β was produced upon Rv0867c stimulation in any of the four groups analyzed; however, Rv2389c stimulation induced production of IL-1β in all myco-bacterium-exposed groups (HCs and TB; $P < 0.05$), but not in the healthy controls (Fig. 1B). Rv0867c stimulation also induced IL-6 in all three mycobacterium-exposed groups, but not in the healthy individuals (HCs, BCG, and TB; $P < 0.05$). In contrast to Rv0867c, Rv2389c induced high levels of IL-6 in the HC group but much higher levels in the mycobacterium-exposed individuals (HCs and TB; $P < 0.05$) (Fig. 1C). Rv2389c stimulation induced also high levels of TNF-α, especially within the mycobacterium-exposed groups (HCs and TB; $P < 0.05$), while Rv0867c induced only limited levels of TNF-α in all four groups (Fig. 1D).
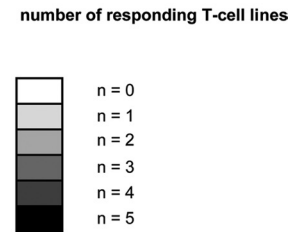
Overall, the mycobacterium-exposed individuals produced the highest levels of cytokines upon Rv0867c and Rv2389c stimulation, and TB patients were often the highest responders to Rv2389 (HCs and TB; $P < 0.05$ for IL-1β, IL-6, and TNF-α). Rv2389c was more strongly recognized than Rv0867c, which induced no or low levels of cytokines.

Identification of immunogenic peptides of *M. tuberculosis* Rpf proteins. To further characterize the Rpf-specific responses observed above, five CD4⁺ T-cell lines specific for Rv0867c and five CD4⁺ T-cell lines specific for Rv2389c were generated. These T-cell lines were tested for recognition of all individual 20-mer peptides (overlapping by 10 amino acids) (see Table S1A and B in the supplemental material), and both IFN-γ production and T-cell proliferation were measured. Table 1 shows the recognition pattern of the Rpf peptides by the Rv0867c and Rv2389c antigen-specific T-cell lines examined. All T-cell lines responded to the corresponding Rpf protein and to one or more of the proteins' corresponding individual peptides. The Rv0867c-specific T-cell lines recognized many different peptides ranging from 1/5 to 4/5 of the donors. Four out of the five T-cell lines recognized peptides P5₄₁₋₆₀ and P10₉₁₋₁₁₀, and three of the five lines recognized peptide P7₆₁₋₈₀. In contrast to Rv0867c-specific T cells, all Rv2389c-specific T-cell lines recognized only a single peptide, P12₁₁₁₋₁₃₀.

Characterization of *M. tuberculosis* Rpf-specific polyfunctional T cells in long-term *M. tuberculosis* nonprogressors. We next investigated T-cell responses to Rv0867c and Rv2389c antigens in a cohort of long-term *M. tuberculosis* nonprogressors which had been infected several decades ago but had never developed any signs of active TB. We analyzed responsive CD4⁺ and CD8⁺ T-cell subsets producing IFN-γ, TNF-α, and/or IL-2 by multiparameter flow cytometry. Polyfunctional T cells have been associated with protection following vaccination (10), but the situation in human infection is more complex, as polyfunctional T cells are also found in active TB patients (8). In any case, the memory T-cell subsets responding to Rv0867c or Rv2389c were analyzed with PBMCs from long-term nonprogressors ($n = 12$) and PPD-negative HCs ($n = 11$).

Figure 2A shows that the Rv0867c protein induced polyfunctional T cells with different cytokine profiles (triple-positive [IFN-γ⁺ TNF-α⁺ IL-2⁺] or double-positive [IFN-γ⁺ TNF-α⁺, TNF-α⁺ IL-2⁺, and IFN-γ⁺ IL-2⁺] cells). Such polyfunctional T cells were detected in 9 out of the 12 long-term latently infected individuals. Strikingly, higher frequencies of polyfunctional CD8⁺ T cells (ranging between 0.20 and 9.72%) were observed, exceeding those of CD4⁺ T cells (ranging between 0.21 and 1.29%). Only 2 out of 12 donors showed polyfunctional CD8⁺ T cells recognizing Rv2389c, typically with a lower frequency (ranging between 0.24 and 0.82%) (Fig. 2C). Single-cytokine-producing

TABLE 1. CD4⁺ antigen-specific T-cell responses to single peptides of *M. tuberculosis* Rpf proteins[a]

peptide	Rv0867c	Rv2389c
p1 1-20		
p2 11-30		
p3 21-40		
p4 31-50		
p5 41-60	n=4	
p6 51-70	n=2	
p7 61-80	n=3	
p8 71-90		
p9 81-100		
p10 91-110	n=3	
p11 101-120		
p12 111-130	n=2	n=5
p13 121-140		
p14 131-150		
p15 141-160	n=1	
p16 151-170	n=1	
p17 161-180		
p18 171-190		
p19 181-200		
p20 191-210		
p21 201-220		
p22 211-230		
p23 221-240		
p24 231-250		
p25 241-260	n=2	
p26 251-270	n=1	
p27 261-280		
p28 271-290	n=1	
p29 281-300	n=1	
p30 291-310	n=1	
p31 301-320		
p32 311-330	n=1	
p33 321-340	n=3	
p34 331-350		
p35 341-360		
p36 351-370		
p37 361-380		
p38 371-390		
p39 381-400	n=1	
p40 388-407		

number of responding T-cell lines: n = 0, n = 1, n = 2, n = 3, n = 4, n = 5

[a] Peptide responses with a proliferation SI of ≥3 and IFN-γ responses of ≥100 pg/ml were considered positive.
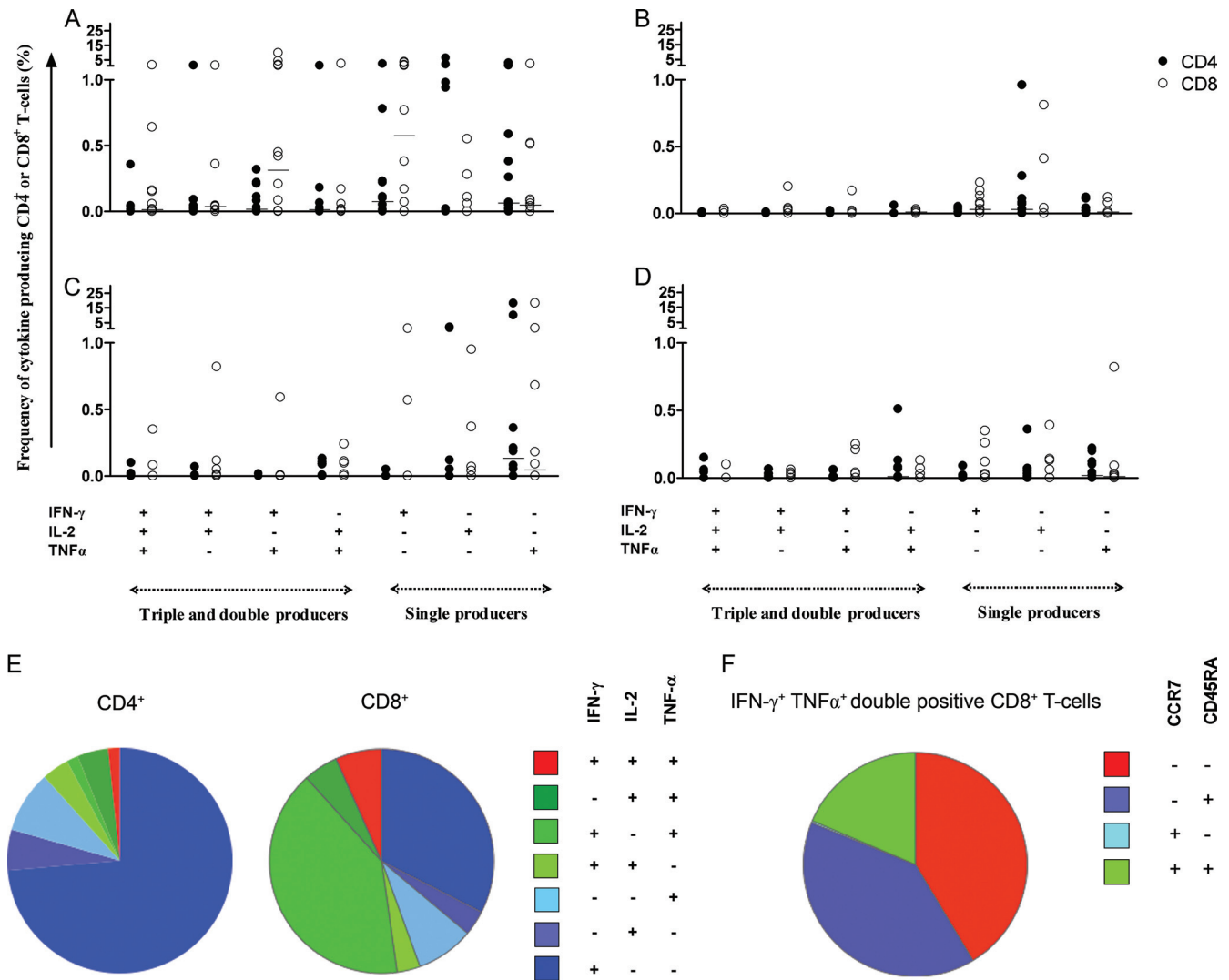
FIG. 2. Frequency of antigen-specific polyfunctional T cells in long-term *M. tuberculosis* nonprogressors. Shown is the frequency of antigen-specific CD4$^+$ and CD8$^+$ T cells in long-term latently infected elderly subjects ($n = 12$) producing combinations of IFN-γ, TNF-α, and IL-2 after stimulation for 16 h with Rv0867c (A) or Rv2389c (C) protein. Healthy individuals were also analyzed for their polyfunctional responses to Rv0867c (B) and Rv2389c (D). CD4$^+$ T cells are indicated as closed circles and CD8$^+$ T cells as open circles. Horizontal bars represent the median frequency of antigen-specific CD4$^+$ and CD8$^+$ T cells. Slices in the pie chart represent the fraction of single (blue)-, double (green)-, or triple (red)-positive CD4$^+$ and CD8$^+$ T cells for Rv0867c (E). Expression of T-cell memory markers CCR7 and CD45RA was analyzed and is shown for the largest T-cell population identified: Rv0867c-specific IFN-γ and TNF-α double-positive CD8$^+$ T cells (F). Effector memory T cells are CCR7$^-$ and CD45RA$^-$, central memory T cells are CCR7$^+$ and CD45RA$^-$, naive T cells are CCR7$^+$ and CD45RA$^+$, and effector T cells are CCR7$^-$ and CD45RA$^+$.

CD4$^+$ and CD8$^+$ T cells were observed for both Rv0867c and Rv2389c, where IFN-γ^+ CD4$^+$ T cells are the most prominent T-cell subset of all single-cytokine-producing T cells. No polyfunctional T cells were induced within the negative healthy control population upon Rv0867c stimulation, although for unknown reasons, low numbers of IL-2$^+$ single-positive cells were seen (Fig. 2B). Some low frequencies of polyfunctional and single-positive T cells were observed upon Rv2389c stimulation within the healthy control group (Fig. 2D), but lower than those in the latently infected group.

Figure 2E shows the proportions of polyfunctional and single-positive CD4$^+$ and CD8$^+$ T cells for Rv0867c, as this

antigen was the best recognized in this cohort. IFN-γ^+ TNF-α^+ double-cytokine-producing CD8$^+$ polyfunctional T cells were the most prominent polyfunctional T-cell subset identified.

In addition, we analyzed the expression of the T-cell memory markers CCR7 and CD45RA in these IFN-γ^+ TNF-α^+ double-cytokine-producing CD8$^+$ T cells in the Rv0867c responders. After dividing the responding T cells into central memory (Tcm) and effector memory (Tem) T-cell subsets (according to Seder et al. [31, 34]), we found that Rv0867c-responding IFN-γ^+ TNF-α^+ CD8$^+$ T cells consisted mostly of effector memory (CCR7$^-$ and CD45RA$^-$) and effector (Teff) (CCR7$^-$ and CD45RA$^+$) T-cell subsets (Fig. 2F). These data
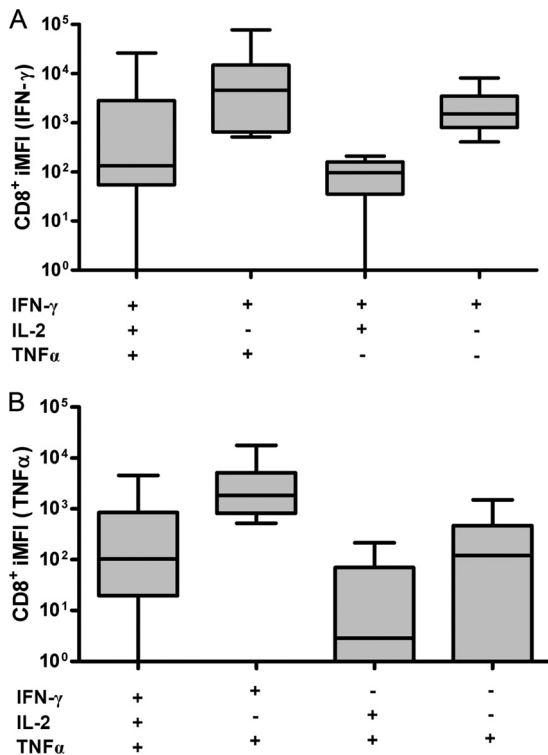
FIG. 3. Integrated mean fluorescence intensity (iMFI) of IFN-γ-, TNF-α-, and/or IL-2-producing CD8$^+$ T cells. Shown is a box and whisker plot representing the iMFI values of CD8$^+$ T cells from long-term nonprogressors ($n = 8$) in response to Rv0867c protein. iMFI is the product value of multiplication of the frequency of CD8$^+$ T cells with the indicated (poly- or mono-) cytokine profiles with the MFI of IFN-γ (A) and TNF-α (B) produced by these T-cell subsets. The horizontal line represents the median, the lower boundary of the box represents the 25th percentile, and the upper boundary represents the 75th percentile. Whiskers extend from the box to the highest and lowest values.

show the presence of Rv0867c-specific CD8$^+$ memory and effector T cells in long-term *M. tuberculosis* nonprogressors.

Besides the quantity of the T-cell response, the quality of the T-cell response plays an important role in protection. We therefore also measured the median fluorescence intensity (MFI) of each cytokine produced by the Rv0867c-responsive T cells. Multiplication of the frequency by the MFI results in an integrated MFI (iMFI) value, which was introduced previously as a quantitative parameter of the overall functionality of the T-cell response analyzed (10). The MFI values showed that the triple-positive T cells had the highest MFI values, followed by the double-cytokine-producing T cells, whereas single-positive T cells showed only minor MFI values (data not shown). However, when iMFI values were analyzed, the IFN-γ iMFI was the highest intensity within the IFN-γ$^+$ TNF-α$^+$ double-positive CD8$^+$ T-cell population, followed by IFN-γ$^+$ single-cytokine-producing T cells (Fig. 3A). This was also observed for TNF-α iMFI (Fig. 3B). Taken together, these results indicate that double- and single-positive T cells contribute quantitatively more to the *M. tuberculosis* Rpf Rv0867c antigen response in long-term nonprogressors than triple-positive T cells.

DISCUSSION

New TB vaccines are urgently needed to help control the TB pandemic. Both prophylactic and postexposure vaccines are considered important (2), but the antigens that can best be incorporated into such vaccines have been identified incompletely at best. Previously we have shown that dormancy-related or DosR regulon-encoded *M. tuberculosis* antigens, which are expressed during hypoxia and nitric oxide stress, were preferentially recognized by TST-positive individuals, suggesting an association with control of infection (7, 21, 33). Unexpectedly, BCG vaccination failed to induce responses to these antigens in humans and mice, even though BCG is able to express the DosR regulon under hypoxia conditions *in vitro* (22). In contrast to the DosR regulon-encoded antigens, *rpf* genes *Rv0867c* and *Rv2389c* are expressed by actively replicating early log-phase-grown *M. tuberculosis* (18). Rv0867c and Rv2389c share the *M. luteus* Rpf protein's property of being able to resuscitate dormant mycobacteria. We therefore hypothesized that immunity directed against these proteins may play a role in sensing and detecting actively resuscitating and replicating *M. tuberculosis* organisms and therefore possibly also in host immune control of reactivating bacteria.

We observed significant differences in levels of Rv0867c and Rv2389c recognition between mycobacterium-exposed versus PPD-negative individuals, yet some Rv2389c and, to a lesser extent, Rv0867c responses were found in the healthy PPD-nonresponding group. We have previously reported a similar recognition pattern for some *M. tuberculosis* DosR regulon-encoded antigens by PPD-nonresponding donors. We explained these results by cross-reactive immunity to nontuberculous mycobacteria (NTM) which share large parts of the DosR regulon with *M. tuberculosis* and *M. bovis* BCG (23). Antigen contaminants (*E. coli* products) are less likely to be involved, since our recombinant proteins are standard quality control (QC) tested on cells from 4 or 5 PPD-negative donors and released only when no IFN-γ is produced. A BLAST search (http://www.ncbi.nlm.nih.gov/blast/) of the *M. tuberculosis* Rv0867c and Rv2389c Rpf protein sequences indeed showed protein sequence identity in several NTM and nonmycobacterial environmental bacteria (Table 2). Part of the immunogenic peptides identified in our current study are indeed conserved in the identified protein sequences of the species shown in Table 2 (P5$_{41-60}$ of Rv0867c, CE*GGNW*INT; and P12$_{111-130}$ of Rv2389c, TQG*GAWP*C), compatible with cross-reactivity at the polyclonal T-cell level.

To more precisely investigate the function and phenotype of Rv0867c- and Rv2389c-specific T-cell subsets, we analyzed the presence of mono- and polyfunctional CD4$^+$ and CD8$^+$ T cells in PBMCs of latently infected individuals who had been infected decades ago without developing TB. In the Dutch cohort above, we had identified Rv2389c as the best-recognized antigen, whereas in the long-term *M. tuberculosis* nonprogressors, Rv0867c induced higher frequencies of polyfunctional and single-positive T cells. This difference might be attributable to the type and/or longevity of the infection as *M. tuberculosis* is more likely to be chronic than NTM infections, and *M. tuberculosis* might be a stronger immunogen than NTM.

Among the polyfunctional antigen-specific T cells present in the long-term nonprogressors, predominantly Rv0867c-specific

TABLE 2. *M. tuberculosis* Rpf protein sequence identity in NTM and nonmycobacterial environmental bacteria

Strain	% protein sequence identity to *M. tuberculosis* Rpf antigen[a]:	
	Rv0867c	Rv2389c
NTM		
Mycobacterium marinum M	88	55
Mycobacterium ulcerans Agy99	88	54
Mycobacterium avium subsp. paratuberculosis K10	84	49
Mycobacterium sp. strain MCS	76	55
Mycobacterium vanbaalenii PYR-1	76	52
Mycobacterium smegmatis MC2 155	78	57
Mycobacterium avium 104	83	59
Mycobacterium bovis BCG	100	100
Nonmycobacterial environmental bacteria		
Nocardia farcinica IFM 10152	62	58
Rhodococcus jostii RHA1	61	56
Streptomyces avermitilis MA-4680	56	45
Streptomyces coelicolor A3(2)	70	43
Corynebacterium jeikeium K411	54	52
Corynebacterium diphtheriae NCTC 13129	55	45
Corynebacterium glutamicum ATCC 13032	55	54

[a] H37Rv Rpf protein sequences were compared to nontuberculous mycobacteria and nonmycobacterial environmental bacterial strains by BLAST searches (http://www.ncbi.nlm.nih.gov/blast/). The percentage of protein sequence identity is given per antigen and species. For analysis, a cutoff value of 40% was used.

CD8$^+$ T cells were identified, particularly double-producing IFN-γ^+ TNF-α^+ Tem and T effector (Teff) cells with high iMFI. This suggests that these two Rpf-specific CD8$^+$ T-cell subsets may play a significant role in TB infection, next to triple-positive T cells. Of interest, IFN-γ^+ TNF-α^+ double-positive CD8$^+$ T cells were also found following vaccination with AERAS-402 vaccine, containing Ag85A, Ag85B, and TB10.4 antigens as a BCG boost, and these cells persisted over time (1). Overall, CD8$^+$ T cells are important in controlling *M. tuberculosis* infection, and the identification of prominent populations of Rv0867c-specific CD8$^+$ T cells supports this notion. Nevertheless, relatively little is known about these antigen-specific polyfunctional CD8$^+$ T cells and their role in protection (9).

Rv0867c-specific, IFN-γ^+ TNF-α^+ double-cytokine-producing CD8$^+$ T cells were mainly Tem and Teff cells. These high CD8$^+$ Tem and Teff subset responses to Rv0867c in the long-term *M. tuberculosis* nonprogressors do not seem to correspond to the hypothesis that after clearance of infection Tem populations gradually wane, resulting mainly in long-lived Tcm cells (41). A recent study showed that reexposure to antigens increased the number of CD8$^+$ Tem cells. Our latently infected individuals may be continuously reexposed to Rpf antigens expressed by either endogenous uncleared *M. tuberculosis* organisms or by other environmental bacteria expressing cross-reactive Rpf-like antigens, which boost Rv0867c-specific Tem cells (37). Regardless, Rv0867c *M. tuberculosis* Rpf antigen is recognized in long-term *M. tuberculosis* nonprogressors, indicating that Rpf-specific T-cell responses are maintained for decades after the initial *M. tuberculosis* infection. Of note, the immune system changes with aging. Fewer naïve T cells will be

available during aging as a result of thymic involution. However, CD8$^+$ Tcm, Tem, and Teff cells accumulate with age (27, 32). These data are in agreement with our Tem and Teff findings in our elderly population (average age, 70 years).

Besides CD8$^+$ T cells, also a minor proportion of polyfunctional CD4$^+$ T cells was observed (Fig. 2E). Although previous results have indicated that triple-positive polyfunctional CD4$^+$ T cells can be induced by BCG vaccination, they are also clearly present in TB patients, such that they do not necessarily correlate with protection against developing disease (8). Minor but significant proportions of triple-positive CD4$^+$ polyfunctional T cells were identified in our work reported here.

We conclude that *M. tuberculosis* Rv0867c and Rv2389c Rpf antigens are immunogenic in humans, as evidenced by antigen-specific cytokine production and high frequencies and iMFI values. Both single- and double-cytokine-producing Rv0867c-specific CD4$^+$ and CD8$^+$ T cells were identified in long-term latently infected individuals who did not develop TB. By directing T-cell responses to *M. tuberculosis* Rpf antigens, it may be possible to enhance immune surveillance of reactivating and resuscitating *M. tuberculosis* bacilli and thereby help to control TB reactivation, which is the major complication in latent TB that keeps fueling the TB pandemic.

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