

RESEARCH ARTICLE

Open Access



HIV-1 Tat protein vaccination in mice infected with *Mycobacterium tuberculosis* is safe, immunogenic and reduces bacterial lung pathology

Aurelio Cafaro^{1*}, Giovanni Piccaro², Giuseppe Altavilla³, Vincenzo Gigantino^{4,5}, Giuseppe Matarese^{6,7}, Erika Olivieri¹, Flavia Ferrantelli¹, Barbara Ensoli¹ and Carla Palma^{2*}

Abstract

Background: The therapeutic HIV-1 Tat protein vaccine is in advanced clinical development. Tuberculosis, the main AIDS co-infection, is highly endemic in areas where AIDS prevention through vaccination is needed. However, safety and immunogenicity of Tat vaccination in the course of *Mycobacterium tuberculosis* (*Mtb*) infection is still unknown and it prevents the possibility to administer the vaccine to *Mtb*-infected individuals. We addressed the interplay and effects of Tat vaccination on *Mtb* infection in immunocompetent mice.

Methods: C57BL/6 mice were vaccinated or not with Bacillus Calmette-Guerin (BCG), the current tuberculosis vaccine, and after 5 weeks were infected with *Mtb* by intravenous route. The Tat protein was injected intradermally at 1, 2 and 4 weeks after *Mtb* challenge. Eight weeks after *Mtb* infection, all mice were sacrificed, and both the degree of pathology and immune responses to *Mtb* and Tat were evaluated. As additional control, some mice were either vaccinated or not with BCG, were not challenged with *Mtb*, but received the Tat protein. Statistical significances were evaluated by one-way or two-way ANOVA and Tukey's multiple comparisons post-test.

Results: In the lungs of *Mtb*-infected mice, Tat-vaccine did not favour *Mtb* replication and indeed reduced both area of cellular infiltration and protein levels of Interferon- γ , Chemokine (C-C motif) ligand-4 and Interleukin-1 β , pathological events triggered by *Mtb*-infection. Moreover, the protection against *Mtb* infection conferred by BCG remained good after Tat protein treatment. In spleen cells of *Mtb*-infected mice, Tat vaccination enhanced *Mtb*-specific Interferon- γ and Interleukin-17 responses, which may have a protective role. Of note, *Mtb* infection reduced, but did not suppress, the development of anti-Tat antibodies, required for Tat vaccine efficacy and the titer of anti-Tat IgG was potentiated by BCG vaccination in *Mtb*-free mice. In general, Tat treatment was well tolerated in both *Mtb*-infected and *Mtb*-free mice.

Conclusions: Tat protein vaccine, administered in *Mtb*-infected mice with a protocol resembling that used in the clinical trials, was safe, immunogenic, limited the lung *Mtb*-associated immunopathology and did not abrogate the protective efficacy of BCG. These data provide preliminary evidence for a safe use of Tat vaccine in people vaccinated with BCG and/or suffering from tuberculosis.

Keywords: Tat vaccination, *M. tuberculosis* infection, Cytokines, T cell responses, Antibodies, Rodent

* Correspondence: aurelio.cafaro@iss.it; carla.palma@iss.it
Giuseppe Altavilla is retired.

¹National AIDS Center, Istituto Superiore di Sanità, Viale Regina Elena, Rome 299 00161, Italy

²Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, Rome 299 00161, Italy

Full list of author information is available at the end of the article



Background

The HIV regulatory Tat protein is crucial in AIDS pathogenesis and is a promising vaccine candidate in advanced clinical development. Tat is the transactivator of HIV gene expression and it is essential for viral replication, establishment of infection and virus reactivation [1, 2]. Tat is expressed by proviral DNA prior to virus integration into the host genome [3], and it is commonly found extracellularly both during acute infection and at the time of virus reactivation [4, 5], even in patients on effective antiretroviral therapy [6]. Extracellular Tat protein concurs to cell-to-cell virus transmission, disease progression [4, 7] and immune dysregulation [8], contributing to the chronic immune hyperactivation and dysfunction observed in HIV infection [3, 9].

Approaches employing biologically active Tat protein have been shown to contain virus replication, preventing disease onset and/or progression in monkey models [10, 11], (<http://www.hiv1tat-vaccines.info>). The Tat-based vaccine has then been advanced to clinical testing in preventative phase I, and therapeutic phase I and II trials showing safety and immunogenicity [12–17]. Moreover, two different trials indicated that Tat vaccine contributed to HIV-1 containment in patients on effective HAART [14, 18], (ISS T-003, *ClinicalTrial.gov identifier: NCT01513135*). In particular, results from an open-label randomized exploratory therapeutic phase II trial in 168 patients on effective HAART showed that vaccination induced a durable and significant restoration of T, B, natural killer cells, and CD4⁺ and CD8⁺ central memory subsets, as well as up-regulation of the expression of HLA-DR⁺ on CD8⁺ killer T cells, a phenotype found to be increased in elite controllers [16] and to contribute to HIV-1 containment [17]. Moreover, the Tat vaccine induced a significant reduction of blood proviral DNA which was significantly associated with anti-Tat immunoglobulin (Ig)M and IgG antibody titers and neutralization of Tat-mediated entry of oligomeric Env in dendritic cells [14, 18]. More recently, a confirmatory phase II, randomized, double-blind, placebo-controlled trial in HIV-infected, anti-Tat antibody negative, antiretroviral-treated adult volunteers [18], (ISS T-003, *ClinicalTrial.gov identifier: NCT01513135*) has been completed in South Africa, an endemic region in which approximately 28 % of individuals living with HIV and tuberculosis (TB) reside [19]. Infections caused by *Mtb* are the main and most dangerous co-infections in HIV/AIDS patients. It is estimated that one-third to one-half of the over 30 million AIDS death can be ascribed to TB. Especially in the endemic regions, *Mtb* and HIV co-infection hampers control of both diseases. Thus, it is of relevance to verify whether vaccines or immunotherapies against HIV infections can be safely administered to individuals

infected by *Mtb*. In fact, the lack of this information has prevented enrollment of HIV-1 infected individuals with active TB in the Tat vaccine therapeutic trial conducted in South Africa [18], (ISS T-003, *ClinicalTrial.gov identifier: NCT01513135*).

Thus, to start addressing the interplay and reciprocal effect of Tat vaccination on *Mtb* infection, we investigated the effects of Tat vaccination on the outcome of active *Mtb* infection and on the protective efficacy of Bacillus Calmette-Guerin (BCG), the current TB vaccine, in a murine TB model. The immunogenicity of the Tat vaccine in these contexts was also assessed.

Methods

Microorganisms

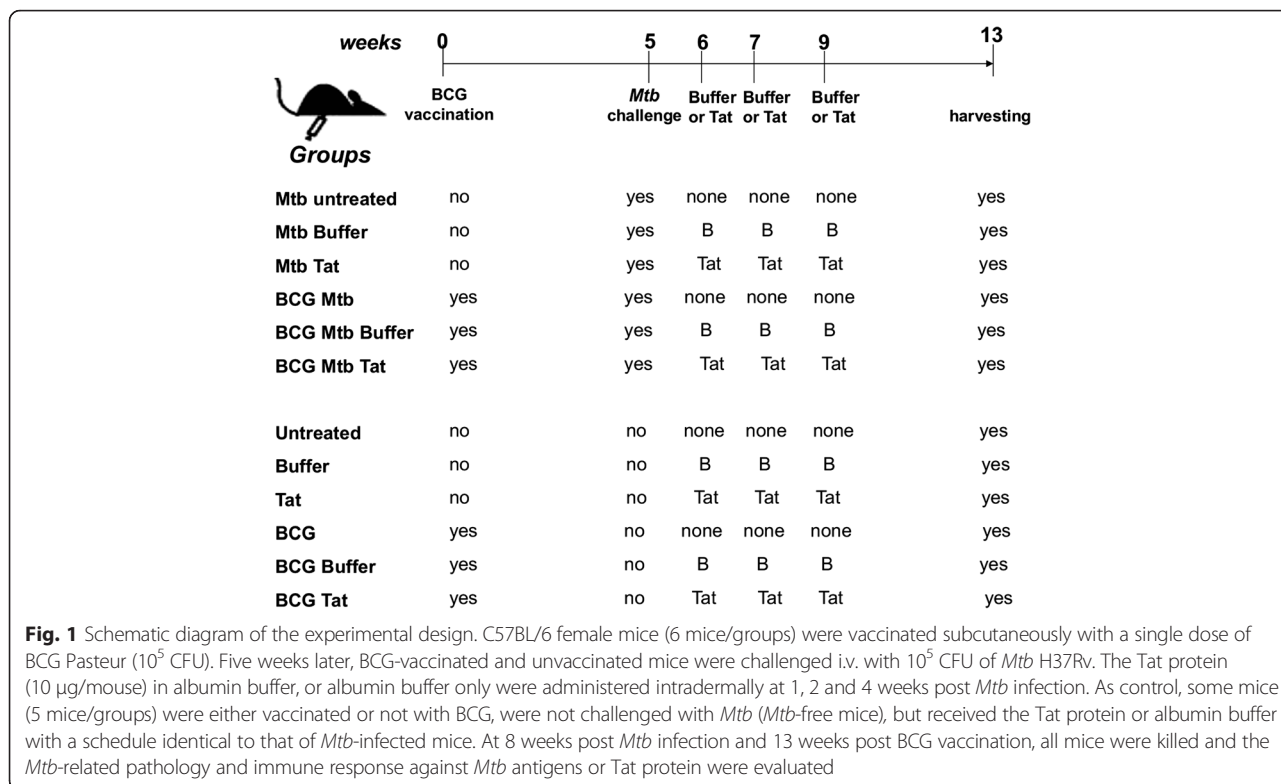
M. tuberculosis H37Rv (ATCC 27294) and *M. bovis* BCG strain Pasteur (ATCC 27291) were grown at 37 °C in Middlebrook 7H9 medium supplemented with albumin-destrose-catalase enrichment, under agitation (120 rpm), up to mid-exponential phase. Aliquoted stocks were stored at -70 °C until use.

Reagents

HIV-1 Tat protein from IIB-BH-10 (subtype B) strain was produced in *Escherichia coli* and prepared as previously reported [20]. The lipopolysaccharide content of this preparation was measured by a *Lymulus* amebocyte lysate test and shown to be <0.06 EU/μg of protein. The recombinant (r)Ag85B protein was prepared as previously reported [21]. The lipopolysaccharide content of this preparation was measured by a *Lymulus* amebocyte lysate test and shown to be below 4.3 EU/μg of protein. All these reagents were purchased from Diatheva, (Fano, PU, Italy). Purified protein derivative (PPD) was purchased from Statens Serum Institute, (Copenhagen, Denmark).

Experimental design

C57BL/6 female mice were supplied as specific pathogen-free mice by Charles River (Calco, Lecco, Italy) and were maintained in specific-pathogen-free conditions. Food and water were available ad libitum. According to the experimental design drawn in Fig. 1, 4 weeks old mice were immunized with a single dose of BCG (10⁵ CFU) injected subcutaneously. After five weeks, BCG-vaccinated and unvaccinated mice were challenged intravenously (i.v.) in a lateral tail vein with 10⁵ CFU of *Mtb* H37Rv. Infection studies were performed in a biosafety level 3 facility; mice were housed in microisolator cages and fed with autoclaved food and water at libitum. Tat protein (10 μg/100 μL of a buffer containing 0.5 % albumin) was injected intradermally at 1, 2 and 5 weeks after the *Mtb* challenge. Mice treated only with buffer served as control.



BCG and Tat protein were also administered, with identical timing, to *Mtb*-free mice, as controls. Thirteen weeks after BCG vaccination, corresponding to 8 weeks post *Mtb*-infection and 4 weeks after the last Tat administration, all uninfected and *Mtb*-infected mice were sacrificed by cervical dislocation, according to the ethics requirements. The degree of pathology and levels of specific anti-*Mtb* or anti-Tat immune responses were evaluated. Five or six mice were used for each experimental mouse group. In total we used 66 mice.

Histology and morphometry

All right lung lobes from each mouse were fixed in buffered formalin and subsequently embedded in paraffin; the tissue was then cut in 5- μ m sections. For morphometry, 5- μ m sections of entire paraffin-embedded lung lobes were stained with hematoxylin-eosin, were magnified at 2 and 40 \times by use of a Leica DM LB microscope, and were photographed by an HV-C20A camera (Hitachi). The histopathological parameters were evaluated and granuloma formation was scored by estimating the area occupied in the lung section. Appropriate software (Scion Image; Scion) was used to measure both the total and the infiltrated area across the whole lobe of the lung. All

sections were evaluated in a blinded fashion by 2 investigators.

Lung homogenates

Lung homogenates were prepared by mechanical homogenization of the left lobe of lungs in distilled water containing 0.01 % Tween 80 and protease inhibitors (complete Mini, Roche Diagnostic, Indianapolis, IN, USA) using a Gentle Macs dissociator (Miltenyi Biotec Srl, Calderara di Reno, Italy). Lung homogenates were used freshly for CFU assay; the homogenate was spun free from any debris by centrifugation and the supernatant filtered before being assayed for cytokine detection.

Spleen cell preparation

Single cell suspension from spleens were prepared in 2 ml of PBS by mechanic dissociation (Gentle Macs dissociator); 200 μ l were used freshly for CFU assay, the left was applied to Falcon 2360 cell strainers (BD Discovery Labware, San Diego, CA), centrifuged, separated into aliquots and frozen for use in immunological assays.

CFU assay

The number of bacteria in lung homogenates or in spleen cell suspensions after lysis with saponin 0.1 % in

distilled water was enumerated by plating 10-fold dilutions, prepared in distilled water, on Middlebrook 7H10 agar. The colonies were counted visually after 21 days of incubation.

Cell cultures

Spleen cells from both uninfected and *Mtb*-infected mice were cultured in 96-well plates (4×10^5 cells/well) in RPMI-1640 supplemented with 10 % heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES buffer, 50 μ M 2-mercaptoethanol. Fifty U/ml penicillin and 50 μ g/ml streptomycin were added to cultures of cells from uninfected mice. Cells were stimulated with 5 μ g/ml rAg85B protein, 2 μ g/ml PPD for 96 h, before detection of Interferon (IFN)- γ and Interleukin (IL)-17 production in culture supernatants.

Cytokine detection

Supernatants of spleen cell cultures and lung homogenates were assayed for cytokine/chemokines detection by quantitative sandwich ELISA specific for IFN- γ , IL-17, IL-1 β , IL-22 and Chemokine (C-C motif) ligand-4 (CCL-4) (mouse Quantikine, R&D System, Inc.), in accordance with manufacturer's instructions.

Antibody measurement

The levels of total anti-Tat IgG antibodies in the sera collected by retro-orbital bleeding were determined by ELISA, as previously described [22]. Briefly, polyvinyl microtiter plates (Nunc) were coated overnight at 4 °C with 100 ng of Tat/well in 200 μ L of 0.05 mol/L carbonate buffer (pH 9.6). Plates were blocked with PBS containing 1 % bovine serum albumin (BSA) and 0.05 % Tween 20 or milk 1 % and several dilutions of mouse sera were then incubated for 90 min at 37 °C. Horseradish peroxidase-conjugated goat μ -Mouse IgG (Sigma) and then ABTS solution (Roche Diagnostic SpA) were used for anti-Tat IgG detection. The mean absorbance of 1/100-diluted, naïve, uninfected mouse sera plus 3 standard deviations was adopted as the cutoff absorbance for determining antibody titers.

Statistical analysis

GraphPad Prism 6 Software was used to perform one-way or two-way ANOVA and Tukey's multiple comparisons post-test. A *P*-value of < 0.05 was considered significant.

Results

Tat protein vaccination is well tolerated in both *Mtb*-infected and *Mtb*-free mice

Mice were infected with virulent *Mtb* by i.v. route 5 weeks after BCG vaccination. Tat protein was injected intradermally at 1, 2 and 4 weeks after *Mtb*

challenge with a protocol of administration resembling that used in the clinical trials [12–15]. The scheme of the experimental design is illustrated in Fig. 1.

Vaccination with Tat protein was well tolerated in both *Mtb*-infected and *Mtb*-free mice, irrespective of BCG vaccination. No evidence of toxicity, as judged by mortality, body weight, food consumption, behavioral and gross examination of vital organs at the time of sacrifice was observed in Tat-treated *Mtb*-free mice (data not shown). In *Mtb*-infected mice, all those parameters were comparable among Tat-treated, buffer-treated and untreated groups (data not shown).

Administration of Tat protein to *Mtb*-infected mice reduces lung immunopathology without affecting bacterial load

Tat vaccination did not affect the bacterial load in both lungs and spleens of *Mtb*-infected mice (Fig. 2a, b). The Tat-treated group showed only a slight and non-significant reduction in CFU levels compared to untreated or buffer-treated, *Mtb*-infected mice, (Fig. 2a, b). Of note, administration of Tat protein reduced *Mtb*-driven lung immunopathology. When lung sections were examined microscopically, untreated mice were found to develop larger areas of inflammatory infiltration than Tat-treated mice (Fig. 2c); in particular, the cellular infiltrates in the total area of lung sections, which was 31 ± 2.6 % in untreated mice, almost halved (18.7 ± 3.2 %) in Tat-treated mice (Fig. 2d). Lungs of *Mtb*-infected mice, irrespective of Tat-treatment showed a nodular and diffuse infiltration in the peribronchial, perivascular and septal interstitial space, with the infiltration also present in the alveolar spaces. Also the cell composition of granulomatous lesions was similar among the groups of *Mtb*-infected mice. It consisted mainly (60-70 %) of epithelioid macrophages sometimes with eosinophilic cytoplasm, sometimes with clear vesicular degeneration and foamy appearance (Fig. 2c), and the remaining 30-40 % represented by lymphocytes and rare neutrophils. No necrotic core or destruction of the lung parenchyma was present.

In agreement with histological examination, Tat vaccination lowered the protein levels of IFN- γ , CCL-4 and IL-1 β , in the lungs of *Mtb*-infected mice (Fig. 3a-c). Elevated pulmonary amounts of these *Mtb*-induced cytokines/chemokines correlate with a more severe outcome during the chronic phase of *Mtb* infection [23, 24]. In contrast, *Mtb* infection did not induce expression of IL-17 and IL-22 whose low levels remained unaltered in the lungs upon Tat administration (Fig. 3d, e).

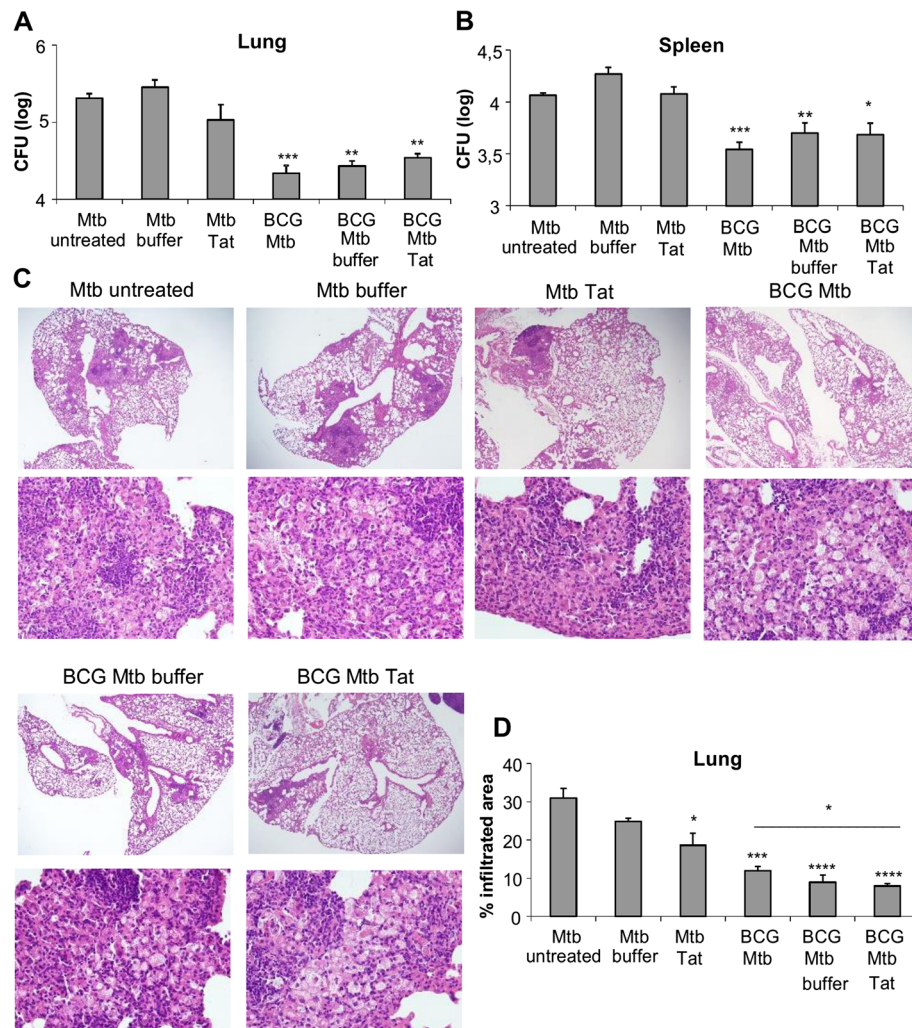


Fig. 2 Tat vaccine administered to *Mtb*-infected mice reduces pulmonary infiltration and does not abrogate the protection conferred by BCG vaccination. Eight weeks post *Mtb* infection, bacteria in the lung (**a**) and spleen (**b**) were enumerated by CFU assay in mice treated as shown in Fig. 1. **c** Comparison of percentages of infiltrated area of sections of lung right lobes from the different mice groups. All data are expressed as mean of 6 individual mice/group \pm SE. Statistical analysis was done by one-way ANOVA and Tukey's multiple comparisons post-test *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, differences respect to untreated *Mtb*-infected group. In panel C, the difference between BCG and BCG Tat-treated group is indicated. **d** Hematoxylin-eosin-stained sections of paraffin-embedded sections of lungs from *Mtb*-infected mice killed at the 8 weeks post infection (upper panel) (magnification, $\times 2$) and (lower panel) (magnification, $\times 40$)

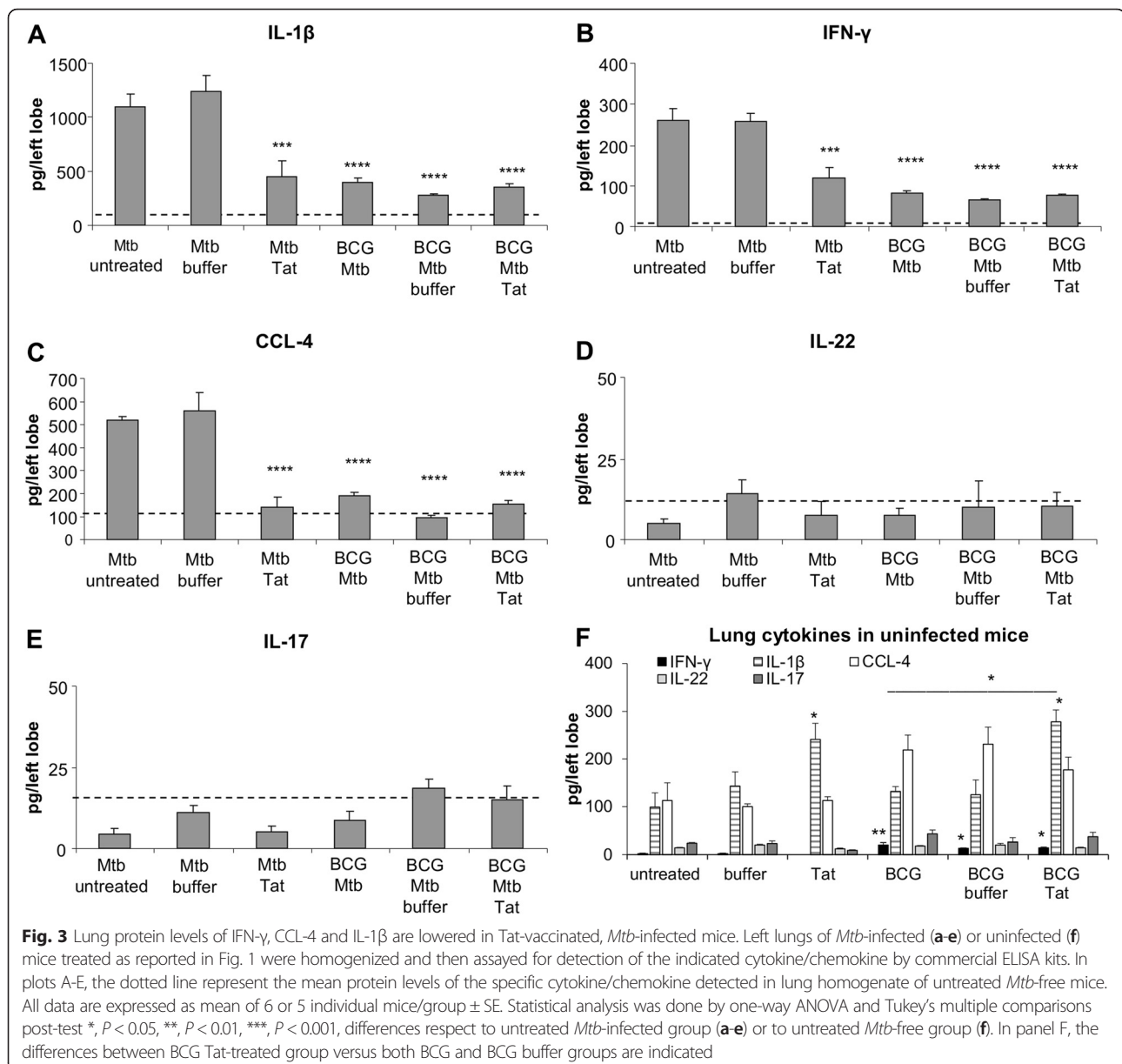
Protection against *Mtb* infection conferred by the BCG vaccine remains good in mice receiving Tat protein vaccine

We evaluated whether the administration of Tat vaccine could alter the protection conferred by BCG, the gold standard TB vaccine. Respect to unvaccinated mice, BCG vaccination significantly reduced the bacterial load in both lungs and spleen (Fig. 2a, b), decreased areas of inflammatory infiltration (Fig. 2c, d), and lowered the lung levels of IFN- γ , IL-1 β and CCL-4 (Fig. 3a-c). The BCG-mediated protective effects were maintained in mice receiving Tat vaccination post *Mtb*-infection. No significant differences were

observed among the three BCG-vaccinated, *Mtb*-infected groups in terms of bacterial loads (Fig. 2a, b) and cytokine/chemokine expression in the lungs (Fig. 3a-e). Of note, administration of Tat protein further reduced the area of inflammatory infiltration, suggesting a beneficial effect on lung pathology development (Fig. 2c, d).

Administration of Tat vaccine enhances the level of IL-1 β in the lungs of both unvaccinated and BCG-vaccinated, *Mtb*-free mice

Lungs of Tat-treated, *Mtb*-free mice showed a normal tissue architecture and the absence of notable inflammatory



infiltrates by histological examination (data not shown). The protein levels of IFN- γ , CCL-4, IL-17 and IL-22 were comparable among Tat-treated, buffer-treated and untreated, *Mtb*-free mice (Fig. 3f). Instead, the expression of IL-1 β , a pro-inflammatory cytokine contributing to host immune defense against *Mtb* [23, 25], was significantly up-regulated by Tat vaccine administration.

In the lungs of *Mtb*-free mice, BCG vaccination induced the expression of IFN- γ (Fig. 3f) and the appearance of small inflammatory infiltrates consisting of lymphocytes (the prevalent cell type) and macrophages (data not shown). Tat vaccination did not affect the expression of IFN- γ and the mild inflammation triggered by BCG, while increased the protein level of IL-1 β

(Fig. 3f), as found in unvaccinated, *Mtb*-free mice (Fig. 3f).

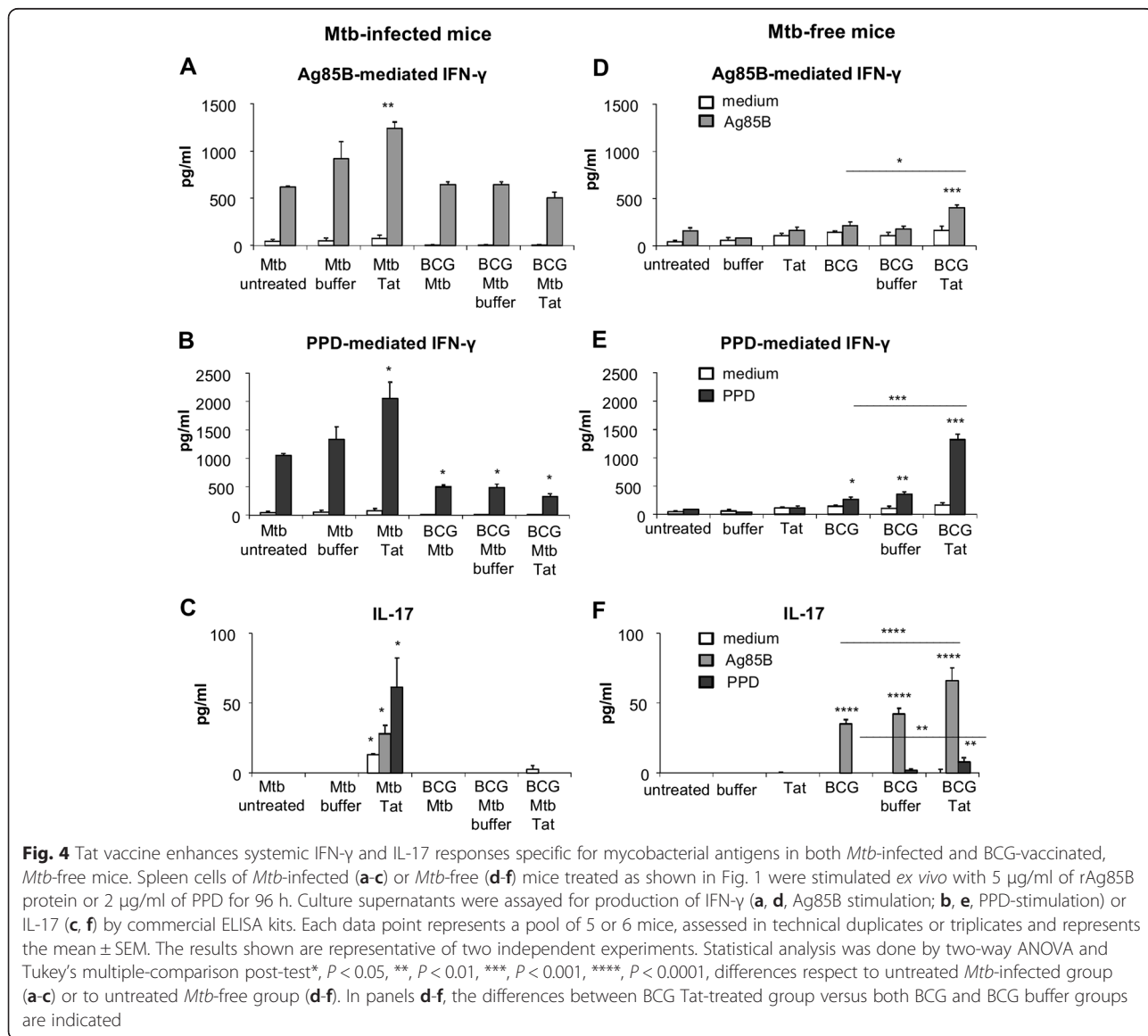
Administration of Tat vaccine enhances mycobacterial-specific IFN- γ and IL-17 responses in mice infected with *Mtb* and in *Mtb*-free mice vaccinated with BCG

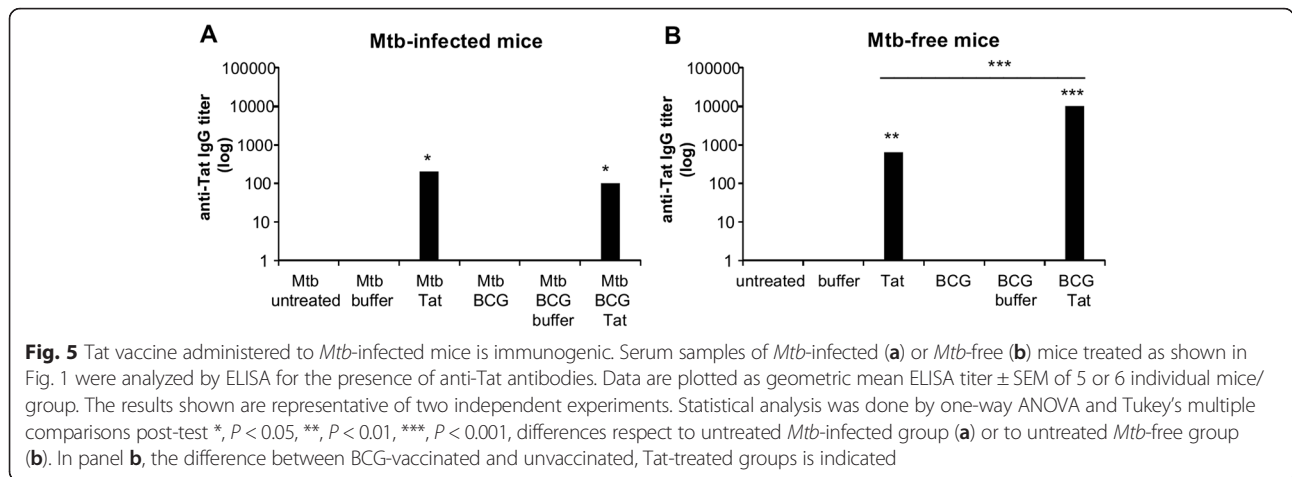
We evaluated the effect of Tat vaccination on the development of systemic *Mtb*-specific immune responses, focusing on IFN- γ , which is essential for control of *Mtb* infection [26, 27] and IL-17, which appears to play a protective role in the early phase of *Mtb* infection [28]. Spleen cells recovered from both *Mtb*-infected and *Mtb*-free mice were stimulated *ex vivo* with Ag85B, an immunodominant antigen with a role in TB protection [21, 29–31], or PPD, the

mixture of antigens released by *Mtb* during in vitro growth. Splenocytes of all groups of mice infected with *Mtb* produced significant amounts of IFN- γ , but not IL-17, in response to both Ag85B and PPD stimulation (Fig. 4a, b). Tat vaccine administration to *Mtb*-infected mice enhanced antigen-specific IFN- γ responses triggered by infection and even induced the production of IL-17 (Fig. 4a-c). However, the Tat-mediated immunomodulatory effects were not observed in BCG-vaccinated, *Mtb*-infected mice (Fig. 4a-c). Irrespective of Tat administration, no IFN- γ or IL-17 responses specific for mycobacterial antigens were detected in unvaccinated *Mtb*-free mice (Fig. 4d-f). Instead, Tat protein administration potentiated the low mycobacterial-specific IFN- γ and IL-17 responses elicited by BCG vaccination in *Mtb*-free mice (Fig. 4d-f).

The infection with *Mtb* reduces but does not suppress the production of anti-Tat antibodies which even is increased by BCG-vaccination in *Mtb*-free mice

Finally, we determined whether mice infected with *Mtb* and vaccinated with Tat developed anti-Tat antibodies, a key parameter to evaluate the immunogenicity of the Tat vaccine and a *bona fide* correlate of efficacy [8, 14, 18, 32]. Mice receiving Tat vaccine post-*Mtb* infection were still able to mount a specific humoral response against Tat (Fig. 5a). The titers of anti-Tat IgG detected in sera of Tat-treated, infected mice (Fig. 5a), were only slightly lower than those measured in Tat-treated, *Mtb*-free mice (Fig. 5b). In Tat-treated, *Mtb*-infected mice, BCG vaccination did not affect the production of anti-Tat antibodies (Fig. 5a). On the other hand, in *Mtb*-free mice, a BCG priming before administration of Tat vaccine (the BCG





vaccinated, Tat-treated group) potentiated the production of IgG specific for Tat respect to unvaccinated, Tat-treated mice (Fig. 5b).

Discussion

The HIV-1 Tat protein-based vaccine, a promising therapeutic vaccine for AIDS patients, is in advanced clinical development [18, 19], (ISS T-003, *ClinicalTrial.gov identifier: NCT01513135*). Here we show that the administration of Tat vaccine to mice infected with *Mtb*, the main coinfection of AIDS, was well-tolerated, immunogenic, did not abrogate the protective efficacy of BCG, the current TB vaccine, and, overall, reduced the lung immunopathology caused by *Mtb*.

Tat protein vaccine did not affect bacterial growth in mice infected with *Mtb*. The scarce influence of Tat vaccine on bacterial load was in line with the results obtained in mice primed at 7 weeks post-*Mtb* infection with a MultiHIV DNA/protein vaccine encoding several HIV-1 products, including Tat [33] and with the outcome of *Mtb* H37Rv infection of Tat-transgenic mice, showing a lung bacterial burden comparable to that found in non-transgenic littermates [34]. However, *Mtb* growth is just one of the events that determine TB pathology. The immunocompetent C57BL/6 mice used in this study are able to contain *Mtb* replication in the lungs upon the initial exponential *Mtb* growth but cannot control the immuno-mediated lung pathology, which progressively worsens and causes death [35]. In this context, the *Mtb*-triggered expression of IFN- γ , IL-1 β and CCL-4 in the lungs [23] plays a dual, opposite role. In the early stage of infection, these pro-inflammatory cytokines/chemokines trigger protective mechanisms [23], while their persistence in the late stages, indicates the inability of controlling/clearing the infection and represents a major marker of progressing TB [24]. Unexpectedly, we found that Tat protein vaccination, when compared to untreated mice, limited *Mtb* infection-

driven lung immunopathology by reducing both the protein levels of IFN- γ , IL-1 β and CCL-4 and the area of cellular infiltration. When the *Mtb*/Tat-treated group was compared to the *Mtb*/buffer group differences were less evident with regard to the infiltrated area, while differences remained significant with respect to the reduction of cytokine/chemokine levels, confirming the ability of Tat vaccine to improve lung immunopathology.

Since containment of lung damage occurred independently from bacterial load control, it is conceivable that Tat-treatment affected immune/inflammatory mechanisms driven by the infection but not closely related to *Mtb* active replication. Of note, incapacity to control progression of lung disease due to an unstoppable inflammation despite containment of *Mtb* load is a key feature of TB pathology in these mice [35]. We know little about the sustainability and mechanisms involved in Tat mediated-improvement of lung pathology. However, the Tat-mediated protective/immunosuppressive effects persisted up to study completion, that is 4 weeks after the last Tat dose, and were independent of IL-17/IL-22 axis and lung neutrophil influx, which cause the high susceptibility to *Mtb* infection in mice [36]. The containment of excessive *Mtb*-triggered inflammatory responses is a novel, and thus far unreported, feature of Tat-mediated immune-modulatory capabilities, which may have important implications in public health. Control of excessive inflammation to prevent lung damage is relevant in human pulmonary TB [37] and correlates with a better prognosis and efficacy of chemotherapy in TB patients [38].

In sharp contrast with our findings, Tat has been reported to favor TB progression [34]. In particular, an accelerated mortality rate, poorly organized lung granuloma-lesions and increased IFN- γ and TNF- α expression as signs of the worsening outcome have been reported in Tat transgenic mice [34]. However, differences between our model of Tat protein vaccination and the Tat transgenic mouse model

are substantial, and may explain the discrepancy of results. In Tat-transgenic mice, Tat is permanently expressed in large amounts in all tissues, and can be found both intra- and extra-cellularly. More importantly, Tat-transgenic mice are tolerant to Tat and do not mount an anti-Tat immune response. To the opposite, in our vaccine model tiny amounts of Tat protein were administered a few times in mice, which mounted an antibody response to the protein. Thus, in our model, exposure to Tat was much lower and shorter, and further reduced by the ensuing antibody response, as compared to Honda's transgenic one [34].

In Tat-vaccinated, *Mtb*-infected mice, the reduction of both bacterial load and lung immunopathology conferred by BCG was maintained, indicating that Tat treatment did not abrogate the vaccine efficacy of BCG. Our data provide preliminary evidence for the use of Tat vaccine in those countries with high HIV/TB co-endemicity and where BCG vaccination is mandatory [39]. BCG, given to over 3 billion individuals, in the setting of routine newborn immunization in high endemic TB countries, is effective in controlling the severe forms of pediatric TB [39], although unable to control pulmonary TB in adult patients [40].

Tat vaccination in *Mtb*-infected mice potentiated systemic IFN- γ and IL-17 responses specific for mycobacterial antigens, including Ag85B. The ability of Tat to activate antigen unrelated Th1 responses is well known, and several of the involved mechanisms have been described [20, 41–44]. Tat protein alters lymphocyte signaling (e.g. activation of NF- κ B), modulates dendritic cell maturation, enhances antigen processing and presentation and promotes the induction and effector functions of memory T cells [20, 41–44]. Instead, little is known about the Tat-mediated modulation of IL-17 response. Recently, it has been reported that Tat increases IL-17 production by T cells, via activation of the vascular endothelial growth factor receptor 2 [45]. Since Ag85B-specific CD4⁺ T cells are induced to produce IL-17 through antigen-unrelated activation of dendritic cells, particularly of the CD8 α ⁻ subtype [46], we cannot exclude that, in our model, Tat promoted IL-17 response via its known ability to activate dendritic cells [20, 41]. Whether the Tat-mediated modulation of mycobacterial-specific IFN- γ and IL-17 responses might have contributed to lowering lung immunopathology remains to be investigated. It is known that protection from *Mtb* infection is dependent on robust IFN- γ secretion by antigen-specific CD4⁺ T cells [26] and, to a lesser extent, on Th17 responses [28]. In particular, activation of IFN- γ response specific for Ag85B, a leading TB vaccine candidate [29], (<http://www.clinicaltrials.gov/show/NCT01049282>), results in a better control of *Mtb* infection in numerous experimental animal models [21, 30, 31].

Curiously, the Tat-mediated enhancement of immune responses was observed in unvaccinated, *Mtb*-infected mice and BCG-vaccinated uninfected mice, but not in BCG-vaccinated, *Mtb*-infected mice. This finding was unexpected, and we do not have a definitive explanation for it. It is conceivable that this latter condition, a recall response to BCG triggered by an acute *Mtb* infection, markedly differs, with respect to either BCG vaccination (untriggered memory) or *Mtb* infection alone (primary response), in terms of antigen load, inflammatory environment and quality of adaptive and/or innate immune responses.

In addition to the modulation of the adaptive response, Tat may improve the control of *Mtb*-mediated lung immunopathology by affecting innate immunity. In the lungs of *Mtb*-free animals vaccinated with Tat, a significant increase of IL-1 β was found 4 weeks after the last Tat dose. Interleukin-1 β is important for host immune defense against *Mtb* [23, 25], likely increasing the mycobactericidal activity of macrophages [47], the main cell type harboring the pathogen.

Finally, we evaluated the impact of *Mtb* infection and/or BCG vaccination on the development of anti-Tat antibodies, a key parameter to evaluate immunogenicity of the Tat vaccine and a *bona fide* correlate of HIV vaccine efficacy [8, 14, 18, 32]. *Mtb* infection only slightly reduced the production of specific anti-Tat IgG. Similar results were observed in *Mtb*-infected mice vaccinated with a MultiHIV DNA/protein vaccine, which included Tat [33]. The moderately high titer of anti-Tat serum IgG elicited by this vaccine was not altered by the concurrent *Mtb* infection, despite an impairment in both the magnitude and quality of antibodies against other vaccine components (p24 Gag and gp160 Env) [33]. Altogether, these data indicate that *Mtb*-infected hosts are still able to mount a humoral response against the Tat vaccine, a mandatory step for vaccine efficacy. However, it remains to be determined whether the anti-Tat antibodies elicited in the presence of *Mtb*-infection are effective at neutralizing the biological activities of Tat, as required for Tat vaccine efficacy [14, 32].

In Tat-vaccinated, *Mtb*-free mice, a priming with BCG increased the production of anti-Tat antibodies. Likely, this effect was due to the “adjuvant” capabilities of this live non-pathogenic vaccine. Indeed, BCG has been used to treat non-muscle-invasive bladder cancer for more than 30 years [48], being one of the most successful biotherapies for cancer in use [49]. BCG triggers the secretion of pro-inflammatory cytokines [50], modulates the activation of granulocytes, macrophages, and dendritic cells [51, 52] and promotes bystander activation of CD4⁺ and CD8⁺ lymphocytes [53]. In line with those findings, we observed signs of a very mild inflammation in the lungs of BCG vaccinated, *Mtb*-free mice, highlighted

by the presence of small foci of infiltrates and by the expression of IFN- γ as late as 13 weeks after the subcutaneous BCG inoculum. The concurrent *Mtb* infection subverted the BCG-mediated increase of anti-Tat antibodies. It is conceivable that the inflammatory/immune responses triggered by the virulent *Mtb*, and mitigated only in part by BCG vaccination, were predominant, and abrogated the BCG-mediated “adjuvant” effects.

Although the route of *Mtb* infection used in our studies is unnatural, reports from several studies indicate that *Mtb*-driven pulmonary pathological events, such as granuloma formation and expression of cytokines/chemokines as well as vaccine efficacy, are comparable in mice infected with *Mtb* either by i.v. or by aerosol route [54–56]. Accordingly, although confirmation of our results in mice challenged by the aerosol route is warranted, we are confident that the i.v. route did not invalidate the significance of our findings. Moreover, given the high proportion of individuals with active TB in countries where HIV-1 is highly prevalent, it will be of importance to assess in future studies whether Tat vaccination is beneficial also during anti-TB chemotherapy.

Conclusions

The anti-HIV Tat protein vaccination administered to mice infected i.v. with *Mtb*: 1) did not favour *Mtb* replication; 2) limited the pathogen-driven immuno-mediated lung injury for at least 4 weeks after the last Tat dose; 3) did not abrogate the protective efficacy of BCG, the current TB vaccine; 4) enhanced early systemic *Mtb*-specific IFN- γ responses, essential for control of *Mtb* infection; 5) was immunogenic, as indicated by the production of anti-Tat antibodies; 6) was well tolerated. In addition, BCG vaccination enhanced the anti-Tat specific humoral response in Tat-vaccinated, *Mtb*-free mice. Our findings provide preliminary evidence of the feasibility, in terms of safety and immunogenicity, of administering the Tat vaccine in BCG-vaccinated and/or *Mtb*-infected subjects. These results are of clinical relevance considering that people who mostly need vaccines against HIV/AIDS live in geographical areas where TB is highly prevalent.

Abbreviations

BCG, Bacillus Calmette-Guerin; CCL-4, Chemokine (C-C motif) ligand-4; i.v., intravenous; IFN, Interferon; Ig, immunoglobulin; IL, Interleukin; *Mtb*, *Mycobacterium tuberculosis*; PPD, purified protein derivative; TB, tuberculosis

Acknowledgments

We thank Guendalina Fornari Luswergh and Silvia Tobelli (NAC, ISS, Rome, Italy) for editorial and administrative assistance, respectively.

Funding

This work was supported by the Italian Ministry of Health, grant 3H/16 to CP. GM is supported by grants from the EU Ideas Programme, ERC-StG “menTORingTregs” n. 310496 and CNR-Medicina.

Availability of data and materials

All the data supporting our findings is contained within the manuscript.

Authors' contributions

AC participated in the design and interpretation of the study and writing of the manuscript; GP carried out the in vivo mouse experiments; GA carried out and interpreted histological studies; VG carried out and interpreted histological studies; GM analyzed immunological and histological data; EO carried out and interpreted immunoassays; FF carried out and interpreted immunoassays; BE participated in conception, design and interpretation of the study; CP conceived, designed and coordinated the study, carried out in vivo mouse experiments and immunological studies; performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This article conforms to the relevant ethical guidelines for animal research. The research was approved by the institutional animal care committee of the Italian Ministry of Health (N° 220/2010-B) and the handling of mice was conducted in compliance with European Community Directive 86/609 and the U.S. Association for Laboratory Animal Care recommendations for the care and use of laboratory animals.

Author details

¹National AIDS Center, Istituto Superiore di Sanità, Viale Regina Elena, Rome 299 00161, Italy. ²Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, Rome 299 00161, Italy. ³Institute of Pathology, University of Padua, Padua, Italy. ⁴Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche (IEOS-CNR) c/o Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli Federico II, Naples, Italy. ⁵Dipartimento di Patologia Fondazione G. Pascale IRCCS, Naples, Italy. ⁶Dipartimento di Medicina e Chirurgia, Università di Salerno, Salerno, Italy. ⁷MultiMedica IRCCS, Milan, Italy.

Received: 21 October 2015 Accepted: 19 July 2016

Published online: 22 August 2016

References

- Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, et al. The *trans*-activator gene of HTLV-III is essential for virus replication. *Nature*. 1986;320:367–71.
- Chang HK, Gendelman R, Lisiewicz J, Gallo RC, Ensoli B. Block of HIV-1 infection by a combination of antisense tat RNA and TAR decoys: a strategy for control of HIV-1. *Gene Ther*. 1994;1:208–16.
- Wu Y, Marsh JW. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science*. 2001;293:1503–6.
- Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature*. 1990;345:84–6.
- Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, et al. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature*. 1995;375:497–500.
- Mediouni S, Darque A, Baillat G, Ravaux I, Dhiver C, Tissot-Dupont H, et al. Antiretroviral therapy does not block the secretion of the human immunodeficiency virus Tat protein. *Infect Disord Drug Targets*. 2012;12:81–6.
- Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, et al. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature*. 1994;371:674–80.
- Cafaro A, Tripiciano A, Sgadari C, Bellino S, Picconi O, Longo O, et al. Development of a novel AIDS vaccine: the HIV-1 transactivator of transcription protein vaccine. *Expert Opin Biol Ther* 2015;1–17. doi:10.1517/14712598.2015.1021328.
- Ott M, Emiliani S, Van Lint C, Herbein G, Lovett J, Chirmule N, et al. Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway. *Science*. 1997;275:1481–5.

10. Cafaro A, Caputo A, Fracasso C, Maggiorella MT, Goletti D, Baroncelli S, et al. Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat Med*. 1999;5:643–50.
11. Cafaro A, Bellino S, Titti F, Maggiorella MT, Semicola L, Wiseman RW, et al. Impact of viral dose and major histocompatibility complex class IB haplotype on viral outcome in mauritian cynomolgus monkeys vaccinated with Tat upon challenge with simian/human immunodeficiency virus SHIV89.6P. *J Virol*. 2010;84:8953–8.
12. Ensolì B, Fiorelli V, Ensolì F, Lazzarin A, Visintini R, Narciso P, et al. The therapeutic phase I trial of the recombinant native HIV-1 Tat protein. *AIDS*. 2008;22:2207–9.
13. Ensolì B, Fiorelli V, Ensolì F, Lazzarin A, Visintini R, Narciso P, et al. The preventive phase I trial with the HIV-1 Tat-based vaccine. *Vaccine*. 2009;28:371–8.
14. Ensolì B, Bellino S, Tripiciano A, Longo O, Francavilla V, Marcotullio S, et al. Therapeutic immunization with HIV-1 Tat reduces immune activation and loss of regulatory T-cells and improves immune function in subjects on HAART. *PLoS One*. 2010;5, e13540. doi:10.1371/journal.pone.0013540.
15. Longo O, Tripiciano A, Fiorelli V, Bellino S, Scoglio A, Colacchi B, et al. Phase I therapeutic trial of the HIV-1 Tat protein and long term follow-up. *Vaccine*. 2009;27:3306–12.
16. Sáez-Cirión A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection *ex vivo* and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci USA*. 2007;104:6776–81.
17. Sáez-Cirión A, Sinet M, Shin SY, Urrutia A, Lacabaratz C, Versmisse P, et al. Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. *J Immunol*. 2009;182:7828–37.
18. Ensolì F, Cafaro A, Casabianca A, Tripiciano A, Bellino S, Longo O, et al. HIV-1 Tat immunization restores immune homeostasis and attacks the HAART-resistant blood HIV DNA: results of a randomized phase II exploratory clinical trial. *Retrovirology*. 2015;12:33. doi:10.1186/s12977-015-0151-y.
19. Middelkoop K, Bekker LG, Mathema B, Shashkina E, Kurepina N, Whitelaw A, et al. Molecular epidemiology of *Mycobacterium tuberculosis* in South African community with high HIV prevalence. *J Infect Dis*. 2009;200:1207–11.
20. Fanales-Belasio E, Moretti S, Nappi F, Barillari G, Micheletti F, Cafaro A, Ensolì B. Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. *J Immunol*. 2002;168:197–206.
21. Palma C, Iona E, Giannoni F, Pardini M, Brunori L, Orefici G, et al. The Ag85B protein of *Mycobacterium tuberculosis* may turn a protective immune response induced by Ag85B-DNA vaccine into a potent but non-protective Th1 immune response in mice. *Cell Microbiol*. 2007;9:1455–65.
22. Buttò S, Fiorelli V, Tripiciano A, Ruiz-Alvarez MJ, Scoglio A, Ensolì F, et al. Sequence conservation and antibody cross-recognition of clade B human immunodeficiency virus (HIV) type 1 Tat protein in HIV-1-infected Italians, Ugandans, and South Africans. *J Infect Dis*. 2003;188:1171–80.
23. Juffermans NP, Florquin S, Camoglio L, Verbon A, Kolk AH, Speelman P, et al. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis*. 2000;182:902–8.
24. Mishra BB, Rathinam VA, Martens GW, Martinot AJ, Kornfeld H, Fitzgerald KA, Sasseti CM. Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1 β . *Nat Immunol*. 2013;14:52–60.
25. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Caspase-1 independent IL-1 β production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling *in vivo*. *J Immunol*. 2010;184:3326–30.
26. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med*. 1993;178:2249–54.
27. Dorman SE, Picard C, Lammass D, Heyne K, van Dissel JT, Baretto R, et al. Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies. *Lancet*. 2004;364:2113–21.
28. Khader SA, Bell K, Pearl JE, Fountain JJ, Rangel-Moreno J, Cille GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4⁺ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol*. 2007;8:369–77.
29. van Dissel JT, Soonawala D, Joosten SA, Prins C, Arend SM, Bang P, Tingskov PN, et al. Ag85B-ESAT-6 adjuvanted with IC31[®] promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine*. 2011;29:2100–9.
30. Weinrich Olsen A, van Pinxteren LA, Meng Okkels L, Birk Rasmussen P, Andersen P. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and esat-6. *Infect Immun*. 2001;69:2773–8.
31. Palma C, Iona E, Giannoni F, Pardini M, Brunori L, Fattorini L, et al. The LTK63 adjuvant improves protection conferred by Ag85B DNA-protein prime-boosting vaccination against *Mycobacterium tuberculosis* infection by dampening IFN- γ response. *Vaccine*. 2008;26:4237–43.
32. Monini P, Cafaro A, Srivastava IK, Moretti S, Sharma VA, Andreini C, et al. HIV-1 tat promotes integrin-mediated HIV transmission to dendritic cells by binding Env spikes and competes neutralization by anti-HIV antibodies. *PLoS One*. 2012;7, e48781. doi:10.1371/journal.pone.0048781.
33. Ignatowicz L, Mazurek J, Leepiyasakulchai C, Sköld M, Hinkula J, Källenius G, Pawlowski A. *Mycobacterium tuberculosis* infection interferes with HIV vaccination in mice. *PLoS One*. 2012;7, e41205. doi:10.1371/journal.pone.0041205.
34. Honda JR, Shang S, Shanley CA, Caraway ML, Henao-Tamayo M, Chan ED, et al. Immune responses of HIV-1 tat transgenic mice to *Mycobacterium tuberculosis* W-Beijing SA161. *Open AIDS J*. 2011;5:86–95.
35. North RJ, Jung YJ. Immunity to Tuberculosis. *Annu Rev Immunol*. 2004;22:599–623.
36. Kozakiewicz L, Chen Y, Xu J, Wang Y, Dunussi-Joannopoulos K, Ou Q, et al. B cells regulate neutrophilia during *Mycobacterium tuberculosis* infection and BCG vaccination by modulating the interleukin-17 response. *PLoS Pathog*. 2013;9, e1003472. doi:10.1371/journal.ppat.1003472.
37. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 2010;137:122–8.
38. Ribeiro-Rodrigues R, Resende T, Johnson JL, Ribeiro F, Palaci M, Sá RT, et al. Sputum cytokine levels in patients with pulmonary tuberculosis as early markers of mycobacterial clearance. *Clin Diagn Lab Immunol*. 2002;9:818–23.
39. Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against *Mycobacterium tuberculosis* infection in children: systematic review and meta-analysis. *BMJ*. 2014;349:g4643. doi:10.1136/bmj.g4643.
40. Lawn SD, Zumla AI. Tuberculosis. *Lancet*. 2011;378:57–72.
41. Fanales-Belasio E, Moretti S, Fiorelli V, Tripiciano A, Pavone Cossut MR, et al. HIV-1 Tat addresses dendritic cells to induce a predominant Th1-type adaptive immune response that appears prevalent in the asymptomatic stage of infection. *J Immunol*. 2009;182:2888–97.
42. Gavioli R, Gallerani E, Fortini C, Fabris M, Bottoni A, Canella A, et al. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. *J Immunol*. 2004;173:3838–43.
43. Nicoli F, Finessi V, Sicurella M, Rizzotto L, Gallerani E, Destro F, et al. The HIV-1 Tat protein induces the activation of CD8⁺ T cells and affects *in vivo* the magnitude and kinetics of antiviral responses. *PLoS One*. 2013;8, e77746. doi:10.1371/journal.pone.0077746.
44. Sforza F, Nicoli F, Gallerani E, Finessi V, Reali E, Cafaro A, et al. HIV-1 Tat affects the programming and functionality of human CD8⁺ T cells by modulating the expression of T-box transcription factors. *AIDS*. 2014;28:1729–38.
45. Johnson TP, Patel K, Johnson KR, Maric D, Calabresi PA, Hasbun R, Nath A. Induction of IL-17 and nonclassical T-cell activation by HIV-Tat protein. *Proc Natl Acad Sci USA*. 2013;110:13588–93.
46. Palma C, Schiavoni G, Abalsamo L, Mattei F, Piccaro G, Sanchez M, et al. *Mycobacterium tuberculosis* PstS1 amplifies IFN- γ and induces IL-17/IL-22 responses by unrelated memory CD4⁺ T cells via dendritic cell activation. *Eur J Immunol*. 2013;43:2386–97.
47. Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG, Behar SM. IL-1 β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J Immunol*. 2013;190:4196–204.
48. Brandau S, Suttman H. Thirty years of BCG immunotherapy for non-muscle invasive bladder cancer: a success story with room for improvement. *Biomed Pharmacother*. 2007;61:299–305.
49. Hall MC, Chang SS, Dalbagni G, Pruthi RS, Seigne JD, Skinner EC, et al. Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. *J Urol*. 2007;178:2314–30.

50. Liu X, Dowell AC, Patel P, Viney RP, Foster MC, Porfiri E, et al. Cytokines as effectors and predictors of responses in the treatment of bladder cancer by bacillus Calmette-Guérin. *Future Oncol.* 2014;10:1443–56.
51. Böhle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD. Effects of local bacillus Calmette-Guérin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. *J Urol.* 1990;144:53–8.
52. Simons MP, O'Donnell MA, Griffith TS. Role of neutrophils in BCG immunotherapy for bladder cancer. *Urol Oncol.* 2008;26:341–5.
53. Pryor K, Goddard J, Goldstein D, Russell P, Golovsky D, Penny R. Bacillus Calmette-Guérin enhances monocyte- and lymphocyte-mediated bladder tumour cell killing. *Br J Cancer.* 1995;71:801–7.
54. Algood HM, Flynn JL. CCR5-deficient mice control *Mycobacterium tuberculosis* infection despite increased pulmonary lymphocytic infiltration. *J Immunol.* 2004;173:3287–96.
55. De Groot MA, Gilliland JC, Wells CL, Brooks EJ, Woolhiser LK, Gruppo V, et al. Comparative studies evaluating mouse models used for efficacy testing of experimental drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 2011;55:1237–47.
56. Copenhaver RH, Sepulveda E, Armitage LY, Actor JK, Wanger A, Norris SJ, et al. A mutant of *Mycobacterium tuberculosis* H37Rv that lacks expression of antigen 85A is attenuated in mice but retains vaccino-genic potential. *Infect Immun.* 2004;72:7084–95.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

