This is a postprint of an article published in
Reljic, R., Clark, S.O., Williams, A., Falero-Diaz, G., Singh, M.,
Challacombe, S., Marsh, P.D., Ivanyi, J.
Intranasal IFNgamma extends passive IgA antibody protection of mice
against Mycobacterium tuberculosis lung infection.
Intranasal IFNγ Extends Passive IgA Antibody Protection of Mice against *Mycobacterium tuberculosis* Lung Infection

*Short title*: Protection against *M. tuberculosis* infection by IgA and IFNγ

*Key words*: IgA, TB, Interferon-γ, infection, intranasal, lungs

Rajko Reljic¹, Simon Clark², Ann Williams², Gustavo Falero-Diaz³, Mahavir Singh⁴, Stephen Challacombe¹, Philip Marsh² and Juraj Ivanyi¹*

¹ Mucosal Biology Research Group, Guy's Campus of King's College London, London, UK
² Centre for Emergency Preparedness and Response, Health Protection Agency, Salisbury, UK
³ Department of Monoclonal Antibodies, Finlay Institute, Havana, Cuba
⁴ Department of Biochemistry, Technical University, Braunschweig, Germany

*Correspondence*: Dr Juraj Ivanyi, Department for Oral Medicine and Immunology, Floor 28 Guy's Tower, Guy's Campus of King's College London, London, SE1 9RT, UK.
Tel: + 0207-1884383; e-mail: juraj.ivanyi@kcl.ac.uk
Abstract

Intranasal inoculation of mice with monoclonal IgA against the α-crystallin (acr1) antigen can diminish the tuberculous infection in the lungs (Williams et al., Immunology, 111; 328, 2004). As this effect has been observed only over a short term, we investigated if it could be extended by inoculation of IFNγ 3 days before infection, and further co-inoculations with IgA, at 2 h before and 2 and 7 days after aerosol infection with *M. tuberculosis* H37Rv. This treatment reduced the lung infection at 4 weeks more than either IgA or IFNγ alone (i.e.17-fold, from $4.2 \times 10^7$ to $2.5 \times 10^6$ CFU, p = 0.006), accompanied also by lower granulomatous infiltration of the lungs. IFNγ added prior to infection of mouse peritoneal macrophages with IgA-opsonised bacilli resulted in a synergistic increase of nitric oxide and TNFα production and a 2-3 fold decrease in bacterial counts. Our improved results suggest, that combined treatment with IFNγ and IgA could be developed towards prophylactic treatment of AIDS patients, or as an adjunct to chemotherapy.
INTRODUCTION

Tuberculosis (TB) is causing approximately 2 million deaths annually. This major health problem is aggravated further by the association of the disease with the HIV epidemic and with the emergence of multidrug drug-resistance (MDR). Chemotherapy can be effective, but poor patient compliance, due to the long (6-9 months) duration of the treatment leads to clinical relapse and to the emergence of MDR strains of *Mycobacterium tuberculosis*. Therefore, new treatment strategies are required, that could be employed for MDR tuberculosis, or as preventive treatment of AIDS patients at high risk of developing TB.

The efficacy of passive antibody treatment for various intracellular bacterial infections, including tuberculosis has been a subject of recent debate [1, 2]. Unlike the variable, and sometimes contradictory outcomes of early studies with polyclonal antisera, recent application of monoclonal antibodies (mAb) showed consistently protective activity against a number of intracellular pathogens, such as *Cryptococcus neoformans* [3]. *Listeria monocytogenes* [4] and *Ehrlichia chaffensis* [5]. Notably, these studies demonstrated that mAbs acted intracellularly against *L. monocytogenes* or revealed an extracellular phase of the *E. chaffensis* infection. Intratracheal infection with anti-arabinomannan IgG3 opsonised Mtb was reported to prolong the survival of mice, but enhanced the granulomatous infiltration of the lungs, while the bacterial load was not reduced [6]. In contrast, Hamasur et al [7] reported a substantial decrease in the lung bacterial load, in addition to prolonged long-term survival, in mice inoculated intravenously with a lipoarabinomannan (LAM) specific IgG1 mAb. Opsonization of *M.
*bovis* with an IgG1 mAb against the MPB83 surface glycoprotein also prolonged the survival of mice and changed the morphology of lung granulomas [8].

Recently, we reported that intranasal (i.n.) application of an IgA mAb directed against the \(\alpha\)-crystallin (acr1) antigen of *M. tuberculosis* was protective against early tuberculous pulmonary infection of mice [9]. This protective effect of IgA mAb was shown to be both epitope and isotype specific, since another IgA mAb, directed against 38 kDa antigen, as well as an IgG1 against \(\alpha\)-crystallin, were much less effective. Inoculation of the antibody both before and after the aerosol challenge was required for optimal reduction of lung colony forming units (CFU), but this effect was not significant beyond 9 days post infection. Aiming to prolong the protection, we hypothesised that IgA-opsonised bacilli could be killed more efficiently by IFN\(\gamma\) activated alveolar macrophages. This was based on our observation, that IgA and IFN\(\gamma\) synergistically inhibit the growth of J774 mouse macrophage cells lines and induce TNF\(\alpha\) synthesis and apoptosis in mouse peritoneal exudate macrophages [10]. In this study, we investigated whether intranasal co-administration of IFN\(\gamma\) and the IgA anti-acr1 mAb could impart greater and longer lasting protection against *M. tuberculosis* pulmonary infection *in vivo*.

**MATERIALS AND METHODS**

**MAbs used in this study**

TBA61 IgA mAb against surface expressed \(\alpha\)-crystallin homologue antigen (acr1, hsp16.3, hspX or 16 kDa antigen) [11] was purified from hybridoma supernatants (CL-
1000 cell culture device, Integra Biosciences, Letchworth, UK), using an acr1-Affigel 15 (Bio-Rad, Hemel Hempstead, UK) affinity column. Following affinity chromatography, the antibody preparation was passed through a Polymyxin B agarose column (Sigma, Poole, UK), in order to remove any contaminating LPS. Purified antibody was stored at 4°C as a 2 mg/ml solution in PBS. MOPC315 (Sigma, Poole, UK) myeloma antibody, with specificity for nitrophenylated proteins, was used as non-specific IgA control in the experiments presented in Figures 4 and 5.

Passive protection and aerosol infection studies

The passive protection experiments were carried out as previously described [9]. Briefly, Balb/c mice (8-10 weeks old females, 8 mice per group) were inoculated i.n. (in 25 µl volume) with 1 µg mouse IFNγ (10,000 U/mg, Serotec Oxford, UK) 3 days before aerosol challenge with chemostat grown Mycobacterium tuberculosis H37Rv. Co-inoculation of 1 µg IFNγ and 50 µg TBA61 mAb i.n. was also made at 2h before and again at 2 and 7 days after aerosol challenge with M. tuberculosis (see the scheme of inoculations in Figure 1), Mice were infected by aerosol with suspensions of M. tuberculosis H37Rv bacilli, using a Henderson apparatus and a Collison 3-jet nebulizer. The aerosol was delivered directly to the snouts (an estimated dose of 100 CFUs), at a flow rate of 55 l/min, for 5 minutes. Lungs and spleens were harvested at 9, 21 and 28 days following the challenge, and 1 ml homogenates in 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates, and incubated for three weeks at 37°C to determine organ CFU.
**Histopathology of lungs: morphometric analysis**

Lungs harvested 4 weeks after H37Rv infection were fixed in 10% neutral buffered formalin, processed on a Tissue-Tek VIP 150 and embedded into wax. Lung cross-sections of 5 µm were cut using a Leica RM2035, stained on Varistain 24-3 with haematoxylin & eosin and mounted using the DPX mountant. 3-5 sections from each organ were scanned on an Olympus BX51 microscope, linked with a ColourView camera and digitised images were analysed using the AnalySIS software (ver 3.2) of the Soft Imaging System. The mm² values for each granulomatous lesion were totalled for the entire section area examined. After subtracting the areas occupied by bronchiolar spaces, the percentage of the granulomatously infiltrated area was calculated.

**In vitro infection studies**

MH-S cells (mouse alveolar macrophages, obtained from European Collection of Cell Cultures, Porton Down), were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS). Cells were seeded in 24-well plates with or without IFNγ (10 ng/ml) at high density (10⁶ cell/well), which eliminated the inhibitory effect of IgA and IFNγ on the growth of cells at low density [10]. After 24 hours of incubation, macrophages were infected (10 bacteria per 1 cell for 2 h at 37°C) with luciferase tagged H37Rv *M. tuberculosis* [12]) which had been pre-treated for 1 h at 37°C with 50 μg/ ml of TBA61 or MOPC315 control IgA. After washing and 2 h treatment with amikacin
(200 µg/ml) to kill the extracellular bacteria, cultures were either lysed in 0.1 % Triton X-100, to determine bacterial uptake, or further incubated for 4 days, prior to CFU and chemiluminescence assays. The medium was replaced after 2 days with RPMI 1640 containing only 2 % FBS (to minimise cell death in confluent cultures).

Macrophages from peritoneal exudates of Balb/c mice in RPMI 1640 medium with 10 % FBS, were allowed to adhere in 24 well culture plates overnight (5 x 10^5 cells/well), prior to addition of IFNγ (10 ng/ml). Following 2 day incubation, the cells were infected for 2 h with either IgA-opsonised, or non-opsonised bacilli, as described for MH-S cells. The amikacin treated, infected cells were returned to culture for a further 4 days. Finally, cells were washed with PBS, lysed in 0.1 % Triton X-100 and dilutions of the lysate were used for enumeration of the infection by luminescence and by the standard CFU assay.

**Detection of nitric oxide (NO) and TNFα**

Culture supernatants obtained 24 h following the infection of peritoneal macrophages, were filter-sterilised. NO was measured using the Griess reagent (Sigma, Poole, UK), and the concentration determined from a standard curve obtained with sodium nitrite solution standards. TNFα was determined using the Bioscience (Middlesex, UK) detection kit, according to the manufacturer's instructions.

**Statistical analysis**

The effects of antibody on the degree of infection was analysed by Student's t-test and by the ANOVA test with multiple comparisons of means using Fisher’s method.
RESULTS

The effect of combined inoculation of TBA61-IgA and IFNγ

The results of CFU counts in the lungs of experimental and control groups of BALB/c mice, harvested at three different time intervals are presented in Figure 2. Inoculation of mice with TBA61-IgA resulted in a significant decrease of lung CFUs compared to untreated controls (p = 0.027), 9 days after the aerosol M. tuberculosis challenge. IFNγ was not protective alone, nor did it enhance the protective effect of IgA at this time point after the infection. However, a synergistic protective effect of IgA and IFNγ was found for the lungs harvested 3 and 4 weeks after H37Rv infection, while neither component alone was protective. The observed inhibition obtained by the co-inoculation schedule was 10-fold at three weeks (p = 0.001) and 17-fold at four weeks, representing a reduction from 4.2 x 10^7 CFU in controls to 2.5 x 10^6 CFU in the IgA+ IFNγ co-injected mice (p = 0.006). However, no significant differences in CFU counts were found in the spleens of infected mice, at 4 weeks after challenge (results not shown).

Pulmonary granuloma formation

Since a significant decrease in the lung bacterial load at the latest time point (4 weeks) after H37Rv infection was observed only for IgA/IFNγ treated group, we examined the extent of granulomatous lesions in lung sections of these mice, and compared them with the untreated controls. Representative sections are shown in Figure 3 A and B. Quantitative planimetric evaluation of the mm² area of the lungs affected with these lesions showed that the mean values (n=5) of the relative representation of the
granulomatous areas were significantly smaller (p = 0.038) in IgA/IFNγ treated (3.03 mm² +/- 2.12 % ) as compared with the control infected mice (10.73 mm² +/- 2.22 % ) (Figure 3.C). In fact, four out of the five IgA/IFNγ treated mice had only marginal or no lesions (n = 0.91 mm² +/- 0.34), while only one mouse had lesions of similar extent as the mean control value. The anomaly in that mouse could have been due to defective delivery (i.e. sneezing or swallowing) of IgA/IFNγ.

Effect of IgA and IFNγ on the infection of macrophages in vitro

Following infection of MH-S cells, no difference could be observed in the 2 h bacterial uptake between TBA61 or control IgA treated luciferase-H37Rv, by either luminescence or CFU method (Fig.4). IFNγ pre-treatment of cells also did not diminish the uptake of bacilli. However, following 4 days of incubation, the TBA61/IFNγ treated cultures showed diminished infection, compared to those treated with either reagent alone, or to untreated cultures (Figure 4). This reduction of infection was anti-acr antigen specific, since control IgA did not synergize with IFNγ.

We also ascertained the levels of macrophage activation markers following the culture of quiescent peritoneal macrophages for 2 days in the presence of IFNγ, prior to the infection with TBA61-IgA opsonised or non-opsonised tubercle bacilli. After 24 h incubation, culture supernatants were tested for the levels of nitric oxide and TNFα, which are important indicators of the activation status of macrophages and are known to contribute to the mycobactericidal activity of these cells. The results showed that both TBA61-IgA and IFNγ stimulated NO production above background level but the
combined treatment resulted in a further, synergistic increase in NO concentration (Figure 5a). A similar synergistic action of TBA-61 IgA but not MOPC315, and IFNγ, was also observed for TNFα production, although neither agent was stimulatory alone (Figure 5b). These results suggested that the pre-treatment of macrophages with IFNγ and subsequent infection with IgA-opsonised bacilli enhanced the activation of these cells. As TNFα and NO are thought to be involved in the regulatory/effector mechanisms of killing of intracellular mycobacteria, we examined the levels of mycobacteria in macrophages 4 days after the infection (Figure 5c and 5d). A statistically significant 2-3 fold decrease of the infection after 4 days in cultures that had been treated with TBA61-IgA + IFNγ, when compared to un-stimulated cultures, was observed by both the luminescence method (p = 0.006) and CFU counts (p = 0.0001). The decrease in infection in the IgA/IFNγ treated cultures was statistically significant also when compared to IgA alone (p = 0.001 and 0.002, respectively) and IFNγ alone, by CFU method (p = 0.024).

**DISCUSSION**

The employed schedule of combined intranasal administration of anti-acrl IgA mAb (TBA61) and IFNγ significantly reduced the lung bacillary counts and the extent of granulomatous infiltration, 4 weeks after the aerosol challenge of mice with *M. tuberculosis*. This outcome could have involved the following possible mechanisms. The exclusion of infection by antibodies [7] has been discounted on the grounds that the 24 h harvest following aerosol challenge did not result in a reduction of lung CFUs [9] and that TBA61 on its own did not inhibit infection of peritoneal macrophages or MH-S cells
Moreover, infection of MH-S lung macrophages did not result in diminished 2 h uptake of IgA-opsonised bacilli, regardless whether they were pre-stimulated with IFNγ or not (Figure 4). The tubercle bacilli have not been pre-incubated with TBA61 prior to aerosol infection and therefore subsequent in vivo agglutination of bacilli by the antibody prior to uptake by macrophages is unlikely. Bacterial agglutination in the lungs seems also improbable, given the very small number of bacilli in the inoculum (100 CFUs), and the relatively large surface/volume of the lungs. On the other hand, the essential requirement for inoculating the mice with mAb before challenge suggests that their extracellular encounter with the bacteria is mandatory for protection.

Most importantly, the observation of synergy between IFNγ and IgA mAb, renders the "blocking of infection’ mechanism unlikely. Instead, it further supports the interpretation, that IgA mAb alters the intracellular fate of the opsonised tubercle bacilli, involving both Fcα-receptor and IFNγ mediated macrophage functions. These cellular events could affect the nature and distribution of granulomas, being one of the key determinants for the outcome of the infection. However, the molecular nature of the involved IgA surface receptor in mouse macrophages, which do not express the human CD89 receptor, is yet unknown.

The acr1 specificity of the used IgA mAb could be significant. The other studies reporting protection, utilised mAbs to the glycolipid constituent, arabinomannan [6] or LAM [1, 7], and against the strongly glycosylated MPB83 lipoglycoprotein [8] or the heparin-binding hemagglutinin (HBHA) glycoprotein [13]. LAM is known to be
involved in the attachment of tubercle bacilli to macrophages [14], while HBHA can influence the interaction with epithelial cells and extrapulmonary, e.g. splenic dissemination of the infection [13], and therefore, antibodies could interfere with any of these functions. The synergistic effect of the anti-acr IgA and IFNγ in our studies appears to be confined to the lungs, since we could see no evidence for either increased or diminished dissemination of the infection to the spleen. Although the precise role of the acr1 antigen in the pathogenesis of TB is not known, there is evidence that its expression is required for bacterial growth inside macrophages [15, 16]. In that case, targeting of the acr1 antigen with TBA61 could possibly interfere with intracellular fate of the bacilli. One possibility for this to happen would be via interaction involving Gal-3. Gal-3, an intracellular β-galactoside-binding lectin, was shown to be an important membrane constituent of the mycobacterial phagosome [17]. As we have demonstrated previously that IgA can interact with intracellular Gal-3 [18], it is plausible that IgA binding to Gal-3 may interfere with mycobacterial interaction with the phagosomal membrane, and thus may also affect bacterial survival/replication in the phagosome.

IFNγ in synergy with IgA-opsonisation of M. tuberculosis inhibited its replication in infected macrophages only to a limited extent. Thus, IFNγ combined with antibody had a more pronounced protective efficacy in vivo than in vitro. This could be due to the need for recruited uninfected monocytes (lacking in vitro) to eliminate the infection. Our data are similar to the results relating to another intracellular pathogen, Brucella melitensis, where synergy between IFNγ and antibody also increased NO production, but inhibited
bacterial replication in infected mouse peritoneal macrophages \textit{in vitro} only to a limited extent [19].

In conclusion, our results lend further support to a growing body of evidence that mAbs can modify various aspects of mycobacterial infections. Their effectiveness can be further increased by IFN\(\gamma\), which is a major pleiotropic stimulator of macrophage functions and considered essential for the host resistance against tuberculosis. The optimal delivery of IFN\(\gamma\) needs further development, considering the diminished IFN\(\gamma\) inducible gene expression in \textit{M. tuberculosis} infected macrophages [20-22] and the disappointing therapeutic effect of recombinant IFN\(\gamma\) [23-26] in patients with tuberculosis. Further work to improve the protective co-administration of TBA61-IgA and IFN\(\gamma\) is justified because of the potential for prophylactic treatment of AIDS patients at high risk of developing TB as well as a possible adjunct to the chemotherapy of tuberculosis.

**ACKNOWLEDGEMENTS**

Funding was obtained from The Dunhill Medical Foundation and from the Department of Health, UK. The views expressed in the publication are those of the authors and not necessarily those of the funding bodies. We are grateful for the technical assistance of staff from the Biological Investigations Group and Histology Department of HPA, Porton Down and for the help with the morphometry of histological sections to Professor Eddy Odell at the Guy’s Campus of Kings College London.
REFERENCES


Figure legends

Figure 1: Schematic representation of the inoculation protocol of Balb/c mice infected with *M. tuberculosis* (100 CFU/mouse) aerosol. Note: Inoculations at 7 days after challenge were not carried out for the 9 day harvest group; d = days, h = hours.

Figure 2: Inhibition of *M. tuberculosis* infection of the lungs by intranasal co-inoculation of TBA61-IgA and IFNγ. Balb/c mice were inoculated as shown in Figure 1 and harvested at 9, 21, or 28 days after the aerosol challenge. Mean lung CFU values from 8 mice and standard errors. Statistical significance (indicated by asterisks) of the differences with the PBS control: TBA61: p = 0.027 at 9d; TBA61 + IFNγ: = 0.02 at 9d, p = 0.001 at 21d and p = 0.006 at 28d harvests.

Figure 3: Morphometric analysis of granulomatous infiltration of the lungs in mice. Representative haematoxylin and eosin stained sections of lungs, harvested at 28 days after aerosol challenge with *M. tuberculosis* H37Rv, for control group (phosphate buffered saline) (A) or for IgA/IFNγ treated mice (B). The arrow indicates the area of granulomatous infiltration. Individual values of the relative proportion of lung areas with granulomatous lesions from 5 mice are shown, together with mean values (C). The reduction of the granulomatous area in the lungs of mice treated with IgA/IFNγ, compared to untreated mice, was statistically significant (p = 0.038).
**Figure 4:** The effect of TBA61-IgA and IFNγ on the infection of MH-S alveolar macrophages. Luminescence (A) and CFU values (B) were tested 2 h (open columns) and 4 days (full columns) after infection (n=3, means and SE bars). Asterisks indicate statistical significance ($p < 0.05$). Notably, the difference between TBA61/IFNγ and IFNγ alone cultures was significant at $p = 0.043$ by luminescence and $p=0.0001$ by CFU), while the control IgA (MOPC315) did not synergize with IFNγ. The columns represent mean values from triplicate cultures, + SE.

**Figure 5:** The effect of TBA61-IgA and IFNγ on the activation and infection of mouse peritoneal macrophages. BALB/c peritoneal macrophages pre-incubated with IFNγ for 2 days were infected for 2 h with luciferase tagged *M. tuberculosis* at 1:10 ratio, in the presence or absence of 50 µg/ml purified TBA61-IgA or MOPC315. The cells were washed, treated with amikacin for 2 h and incubated for further 4 days in complete medium with 10% FBS. 24 h after the infection, filter-sterilised aliquots of culture supernatants were assayed for NO production (A) and TNFα secretion (B). Whole cell lysates were tested for luminescence (C) or CFU counts (D). The values for each assay represent arithmetic means from triplicate cultures. The differences were significant (asterisk) for IgA + IFNγ compared with PBS control at $p = 0.006$ for luminescence and $p = 0.0001$ for CFU and also compared to IgA or IFNγ alone (see Results section) treated cultures.
Mouse IFN$_\gamma$, 1µg (10,000 U), i.n.

TBA61-IgA, 50 µg i.n.

Aerosol H37Rv

Harvest of lungs, CFU assay

Time (days)

- 3  - 2h  0  2  7  9  21  28
Reljic et al., Fig. 2

Log$_{10}$ CFU/lung

9 days

3 weeks

4 weeks

PBS  TBA61-IqA  IFNγ  TBA61 + IFNγ
Reljic et al., Fig. 3

A) Control

B) IgA/IFNγ

C) % Granuloma

<table>
<thead>
<tr>
<th></th>
<th>Mean: 10.73</th>
<th>Mean: 3.03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA/IFNγ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Reljic et al., Fig. 4

A) Luminescence (RLU)

B) CFU x 10^3

PBS  MOPC 315  TBA61-IgA  IFN  MOPC 315 + IFN  TBA61 + IFN

*
Reljic et al., Fig. 5

A) Nitric oxide (uM)

B) TNF-α (pg/ml)

C) Luminescence (RLU)

D) CFU x 10^3

Groups:
- PBS
- MOPC 315
- TBA61
- IFN
- MOPC 315 + IFN
- TBA61 + IFN

* denotes significant difference.
This is a postprint of an article published in
Reljic, R., Clark, S.O., Williams, A., Falero-Diaz, G., Singh, M.,
Challacombe, S., Marsh, P.D., Ivanyi, J.
Intranasal IFNgamma extends passive IgA antibody protection of mice
against Mycobacterium tuberculosis lung infection.