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Immunotherapy with fragmented *Mycobacterium tuberculosis* cells increases the effectiveness of chemotherapy against a chronical infection in a murine model of tuberculosis

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Abstract

Reduction of colony forming units by rifampicin-isoniazid therapy given 9–17 weeks post-infection was made more pronounced by immunotherapy with a vaccine made of fragmented *Mycobacterium tuberculosis* cells detoxified and liposomed (RUTI), given on weeks 17, 19 and 21 post-infection, in the murine model of tuberculosis in C57BL/6 and DBA/2 inbred strains. RUTI triggered a Th1/Th2 response, as demonstrated by the production of IgG1, IgG2a and IgG3 antibodies against a wide range of peptides. The histological analysis did not show neither eosinophilia nor necrosis, and granulomatous infiltration was only slightly increased in C57BL/6 mice when RUTI was administered intranasally.

Keywords: Immunotherapy; Chemotherapy; Histological analysis

1. Introduction

It has been estimated that one third of mankind harbours a latent infection with *Mycobacterium tuberculosis* [1]. Latent bacilli have to adapt to stressful conditions generated by the infected host against them by slowing down the metabolism or becoming dormant [2,3]. Since chemotherapy and immunity are mainly directed against growing bacilli [4,5], their destruction in a short period of time is difficult. It has been hypothesized the usefulness of a immunotherapeutic vaccine, but the results of some recent studies strongly recommend not to use post-infection vaccination with killed or alive BCG, as this induces strong tissue toxicity [6,7]. In the present study, we have assessed the benefit of the administration of a fragmented *M. tuberculosis* cells formulation (RUTI) to reduce the bacterial load after the treatment with chemotherapy in a murine model of chronical tuberculosis infection.

2. Material and methods

2.1. Experimental infection

Specific pathogen-free DBA/2 and C57BL/6 female mice, 6 weeks old, were provided by Charles River (L'Arbresle Cedex, France) and kept under controlled conditions in a P3 High Security Facility with sterile food and water ad libitum. *M. tuberculosis* standard strain NC007416 (H37Rv) was grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase and stored in aliquots at −70 °C.

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Mice were placed in a Middlebrook Airbone Infection Apparatus (Glas-col Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with a 6-mL suspension at 10^6 bacteria/mL in distilled water delivering 20–50 viable bacilli into the lungs. CFUs at each time point from the left lung and the spleen homogenized in 1 mL of distilled water were monitored by plating serial dilutions on nutrient Middlebrook 7H11 agar (Biomedics s.l., Madrid, Spain) and counted after 21 days incubation at 37 °C. Broncho-alveolar lavage (BAL) was obtained by gentle intratracheal injection of 1 mL phosphate-buffered saline (PBS) and lungs were immediately extracted after euthanasia by means of a halothane (Zeneca Farma, Pontevedra, Spain) overdose.

2.2. Serum samples

Blood samples were collected from the retro-orbitary veins of all the animals after being anaesthetized with halothane. Samples were kept at 4 °C for 8 h; serum was obtained by centrifugation at 2500 × *g*, aliquoted and then kept at −20 °C until needed. A pool of previous positive samples was titrated, aliquoted and kept at −20 °C to be used as controls for Western blotting.

2.3. mRNA quantification

The procedures are described elsewhere [8]. In short, total RNA from the middle right lobe was extracted with a commercial phenol–chloroform method, RNAzol (Cinna/Biotecx, Friendswood, IX, USA). After a DNase treatment with DNA-free kit (Ambion, Woodward Austin, USA), a denaturing agarose gel was used to assess the stability of RNA. Five micrograms of RNA was reverse transcribed using a Superscript RT kit (Gibco BRL, Grand Island, NY, USA) following the manufacturer's recommendations to obtain cDNA. The quantitative analysis for IFN-γ and TNF was performed using a LightCycler™ System (Roche Biochemicals, Idaho Falls, ID, USA). A real-time PCR was carried out in glass capillaries to a final volume of 10 μL in the presence of 1 μL of 10× reaction buffer (Taq Polymerase, dNTPs, $MgCl_2$, SYBRGreen, Roche Biochemicals), 1 μL of cDNA (or water as negative control, which was always included), $MgCl_2$ to a final concentration of 2–5 mM and primers to a final concentration of 0.5 μM. A single peak was obtained for each PCR product by melting curve analysis and only one band of the estimated size was observed on the agarose gel. Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression was analyzed for every target sample to normalize for efficiency in cDNA synthesis and RNA loading. A ratio based on the HPRT mRNA expression was obtained for each sample.

2.4. Histology and morphometry

The procedures have been described in previous works [8]. In short, two right lung lobes from each mouse were fixed in buffered formalin and subsequently embedded in paraffin. Each sample was stained with hematoxilin–eosin. For histometry, 5 μm thick sections from each specimen were stained with hematoxilin–eosin and photographed at 10× using a SMZ800 stereoscopic microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Three sections from two lung lobes per animal were studied. Using the appropriate software (Scion Image, Scion Corporation, Frederick, MA, USA) and Photoshop 5.0 (Adobe Systems Incorporated, San José, CA, USA), the area of each single lesion and the total tissue area were determined on photomicrographs at each time point. The sections were blindly evaluated to obtain a more objective measurement.

2.5. RUTI

RUTI is the name of a patent-pending product (P.J. Cardona and I. Amat, 31 October 2003, Spanish Patent Office). Originally, RUTI is the name of the mountain where the Hospital Universitari Germans Trias i Pujol is placed, and all the experiments were done. Nowadays, RUTI is manufactured under Good Manufacturing Procedures (GMP) by Archivel Technologies, s.l. (Mataró, Catalonia, Spain). Different batches have been tested and showed equivalent efficiency. Briefly, fragmented *M. tuberculosis* cells (FMtbC) were obtained after culturing *M. tuberculosis* H37Rv for 3 weeks in Middlebrook 7H11 agar (Biomedics s.l.) at 37 °C. Colonies were carefully removed and mechanically disrupted using silica–zirconia beads in a Bead Beater (BioSpec products Inc., OK, USA) and a PBS buffer with 4% Triton-X114 (Sigma–Aldrich Chemie, Stenheim, Germany). After centrifuging at 3000 × *g* at 4 °C for 30 min the supernatant was centrifuged twice at 27,000 × *g* at 4 °C for 60 min and washed with PBS to remove the lipidic supernatant, to be finally pasteurized at 65 °C for 40 min and lyophilized. RUTI was obtained by dissolving FMtbC in liposomes made of phosphatidyl choline (Lipoid AG, Cham, Switzerland) and sodium cholate (New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand) (20:1.4) after mixing at high speed. Analysis of RUTI with a Laser Diffraction Particle Size Analyzer PS 13320 (Beckman Coulter, Miami, FL, USA) showed particles with an average diameter of 100 nm, 99.9% of them smaller than 1 μm. RUTI showed a mixture of multilamellar and unilamellar liposomes after being examined with a transmission electron microscope (JEOL 1010, Tokyo, Japan). The peptidical composition of FMtbC was determined after comparing it with the profile obtained with *M. tuberculosis* well known products like purified protein derivative (PPD), culture filtrate proteins (CFP) and Ag85 using SDS-PAGE, and with the patterns obtained through Western blot analysis with monoclonal antibodies (mAbs) against CFP peptides [9]: IT-12, 20, 23, 43, 44, 46, 51, 56, 58, 69 and 70 (Fig. 1). All these products were kindly donated by Dr. John Belisle, from the Colorado State University according to the NIH contract no. AI-75320.

2.6. Treatments

RUTI was administered subcutaneously (SC) or intranasally (IN) on weeks 17, 19 and 21 post-infection. A volume of 50 μL containing 185 μg of FMtbC was used. Chemotherapy was administered orally 5 days a week by gavage from week 9 to 17 post-infection. The suspension of rifampicin (RIF) (Sigma) was prepared in a one-tenth volume of water with a pH 2 and made up to volume with 5% sucrose (Sigma). Once it was well dissolved isoniazid (INH) was added (Sigma). The final suspension was stored at −20 °C, and thawed at 5 °C 1 day before administration. INH was administered at 25 mg/kg and RIF at 10 mg/kg.

2.7. Humoral response

Humoral response was analyzed against a pre-established peptidic profile (6, 10, 17, 19, 27, 30, 38, 40, 47, 50, 55, 65, 70 kDa) (Fig. 1). Western blot was run as described [10] in a SDS-PAGE preparative gel (12% acrylamide NuPAGE BisTris Gel, Invitrogen), electrotransferred onto nitrocellulose sheets (Millipore, Bedford, MA, USA) using a semidry transfer method (Invitrogen, Carlsbad, CA, USA) that was finally divided into 16 lanes. The strips (0.30-cm wide) were incubated with 1:25 to 1:51,200 diluted sera, or 1:1 to 1:256 diluted BAL for 1h at room temperature. Appropriate horseradish peroxidase (HRP)-labeled secondary goat anti-mouse antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA) was used as well as chemoluminiscent substrate Super Signal West Pico (Pierce) and X-ray films Hyperfilm ECL (Amersham Biosciences, Arlington Heights, IL, USA) for developing. A molecular weight standard was run in each gel (Invitrogen).

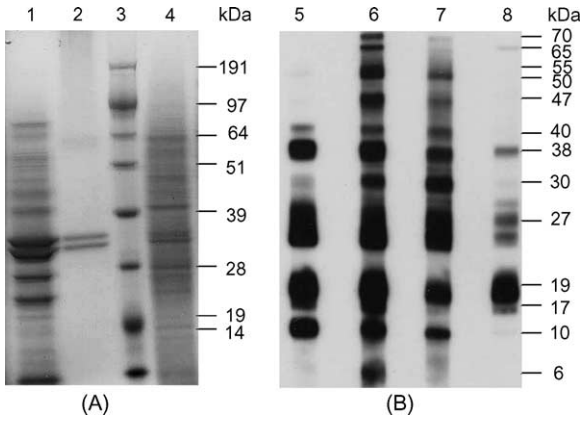

Fig. 1. Composition of RUTI. Comparison of the peptidic profiles in a 12% SDS-PAGE gel (A) of culture filtrate proteins (CFP) (1), Ag85 (2) and fragmented *M. tuberculosis* Cells (FMtbC) (4). Line 3 shows the molecular weight control of 14–198 kDa. (B) Shows a Western blot analysis of FMtbC, using the control serum of infected mice inoculated with RUTI and the kDa profile of bands previously defined with known mAbs and the SDS-PAGE pattern. Lines 5, 6, 7 and 8 are obtained against IgG1, IgG2a, IgG2b and IgG3 conjugates, respectively.

3. Results

Fig. 2 shows the bactericidal activity induced by the inoculation of RUTI IN or SC in both susceptible or resistant mice strains [8,11] after chemotherapy, which also impedes the reactivation suffered by mice treated only with chemotherapy. Besides, inoculation of RUTI without a previous chemotherapy did not affect the bacterial load although elicited a similar immunological response (data not shown). This bactericidal activity seems to be related to the Th1 response as it is demonstrated by the increase of the IFN-γ expression in the lungs after the inoculation of RUTI when compared with the group only treated with chemotherapy (Table 1). This effect was more evident in C57BL/6 mice and it was only significant in the IN inoculated. On the other hand, no significant increase on TNF expression was detected (Table 1). It must be taken into account that the expression of such cytokines are

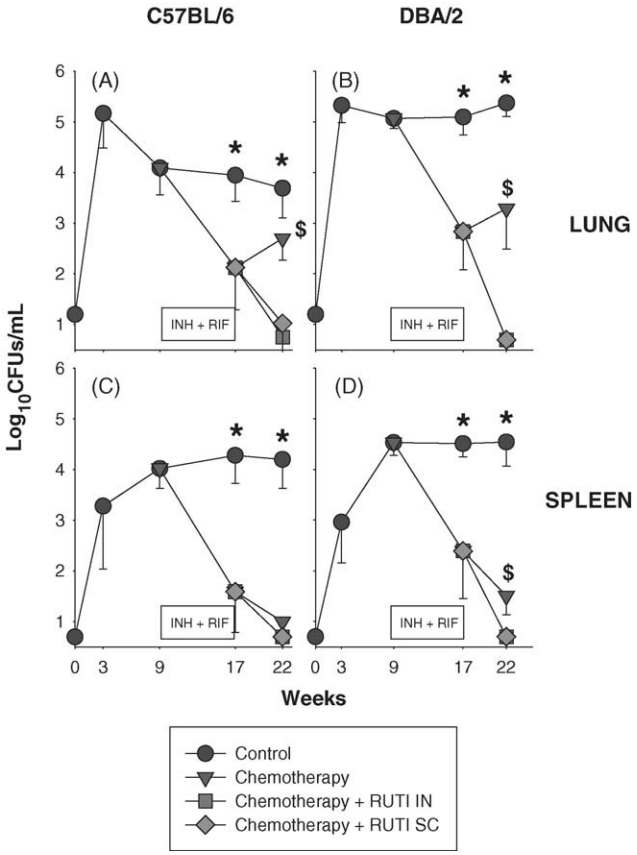

Fig. 2. Bactericidal activity of RUTI in lung (A and B) and spleen (C and D) in C57BL/6 and DBA/2 mice. The results are the mean values and the standard deviations obtained from 4 to 6 mice in each time point. Differences were considered significant when p was <0.05 and were marked with (*) among control and every other groups; and ($) among groups treated with chemotherapy alone or groups treated with chemotherapy and both IN or SC inoculation with RUTI.

increased in control mice because they harbour a high bacillary concentration [8]. The study of the humoral response (Fig. 3) gave us an indirect view on the immunity generated as Th1, Th2 and Th3 responses are related to IgG2a and IgG3, IgG1 and IgG2b isotypes, respectively [12]. In general, RUTI triggered a wide response against FMtbC peptides in both mice strains implying all the IgG isotypes (Fig. 3). Furthermore, IgA production was detected in BAL after IN or SC inoculation in DBA/2 and also in IN immunized C57BL/6 mice (Fig. 4). No IgE levels were detected in any samples. The histological analysis did not show eosinophilia or necrosis, and granulomatous infiltration was only slightly increased in C57BL/6 mice when RUTI was administered IN (Table 1).

4. Discussion

RUTI might be a useful vaccine for reducing the period of chemotherapy in *M. tuberculosis* infection at the point when it has less efficacy because of the presence of latent bacilli in the lesions [13–16]. Its mechanism of action might be related to the induction of a Th1 response against a wide range of *M. tuberculosis* antigen able to elicit a protective response against *M. tuberculosis* infection like ESAT-6, CFP-10, Ag85 or Hsp70 [4]. Also, its effectiveness is necessarily linked to a previous chemotherapy, as treatment with RUTI alone does not induce any decrease in the bacterial load. This fact can be linked to the decrease on the immunological response induced

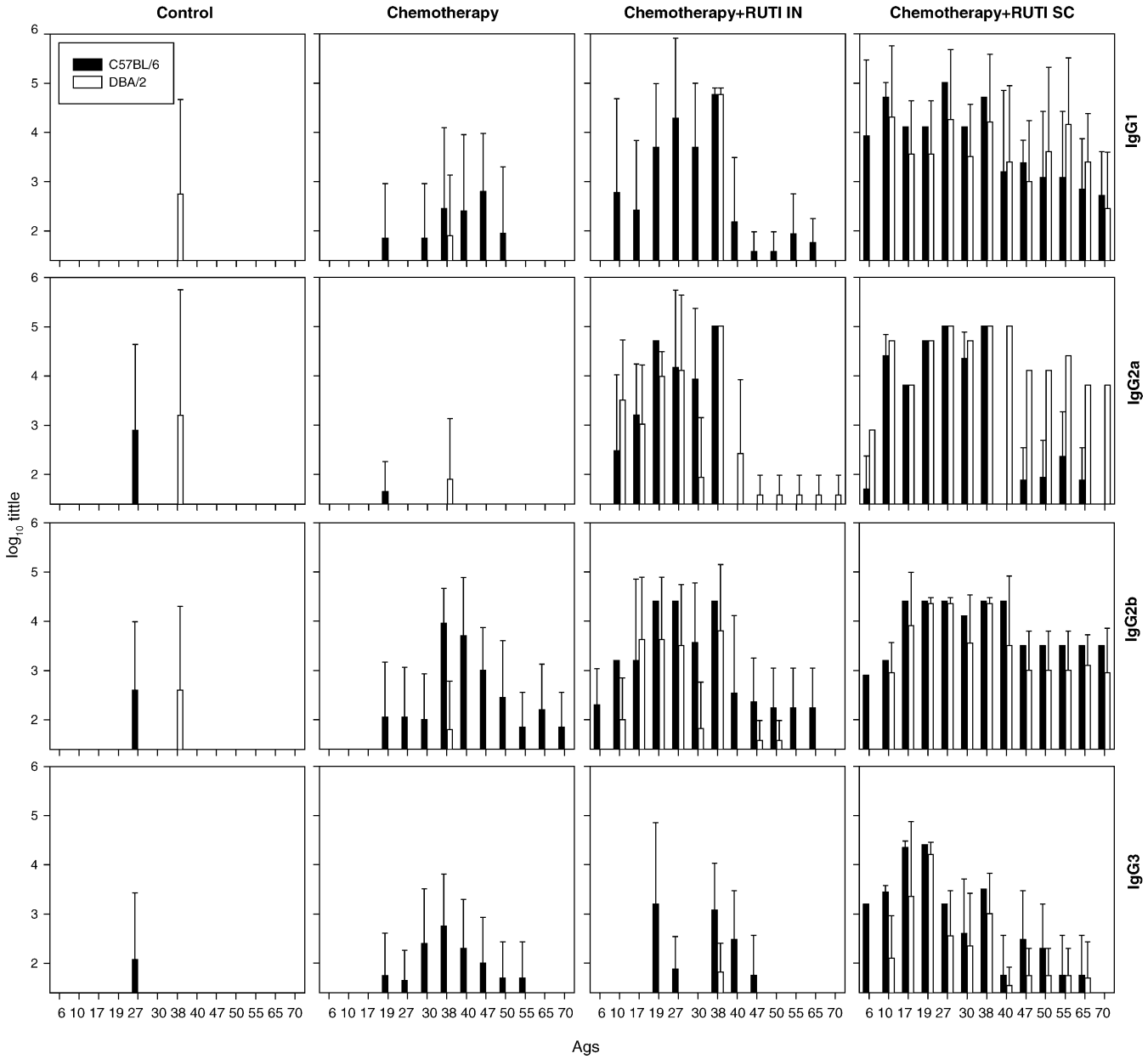

Fig. 3. Humoral IgG response to FMtbC proteins in a Western blot determination at week 22. The results are the mean values and the standard deviations of the sera obtained from 4 to 6 mice in each time point. The values in DBA/2 mice are represented with open bars and those in C57BL/6 mice with closed bars.

Table 1
Local expression of cytokines (IFN-γ and TNF) and granulomatous infiltration in the pulmonary parenchyma at week 22

	Ratio cytokine/HPRT x 10^3		Morphometry (% of infiltration)
	IFN-γ	TNF	
DBA/2			
Control	$193.5 \pm 80.32^{*}$	$313 \pm 60.61^{*}$	$11.92 \pm 1.3^{*}$
Chemotherapy	$1.16 \pm 0.45^{*}$	$0.07 \pm 0.07^{*}$	$5.01 \pm 2.26^{*}$
Chemotherapy + RUTI IN	1.88 ± 1.95	0.32 ± 0.47	3.38 ± 2.31
Chemotherapy + RUTI SC	1.31 ± 0.43	0.08 ± 0.07	1.66 ± 0.88
C57BL/6			
Control	$220.\ 3 \pm 35.75^{*}$	$198.8 \pm 96.32^{*}$	$4.22 \pm 0.7^{\$}$
Chemotherapy	$0.70 \pm 0.51^{*,**}$	$0.02 \pm 0.01^{*}$	$2.43 \pm 0.4^{\$,\#}$
Chemotherapy + RUTI IN	$4.42 \pm 2.43^{**}$	0.05 ± 0.02	$5.25 \pm 1.26^{\#,\infty}$
Chemotherapy + RUTI SC	2.45 ± 1.94	0.05 ± 0.05	$3.53 \pm 0.59^{\infty}$

Sigma Stat (SPSS Software, San Rafael, CA, USA) was used to compare the differences between groups through all pairwise multiple comparison procedures (Student–Newman–Keuls method).

* $p < 0.05$.
** $p = 0.009$.
$^{\$}$ $p = 0.012$.
$^{\#}$ $p = 0.002$.
$^{\infty}$ $p = 0.03$.

by chemotherapy, as described before [17]. In this case, RUTI would act both as a booster of the previous immunity and as an inducer of a new response against other FMtbC peptides, that will favour the bacterial destruction. On the contrary, bacilli in the group treated only with chemotherapy reactivate after ceasing the treatment because of the absence of immunological response. A threshold bacillary concentration must be achieved to trigger this immune response again [18].

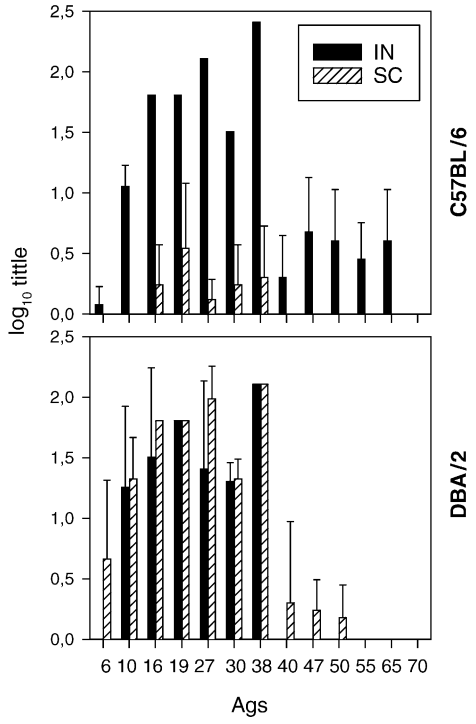

Fig. 4. IgA response to FMtbC proteins in a Western blot determination in BAL at week 22. The results are the mean values and the standard deviations of the samples obtained from 4 to 6 mice in each time point. RUTI inoculations administered intranasally or subcutaneously (IN or SC) are represented with closed or striped bars, respectively.

On the other hand, RUTI also triggers Th2 and Th3 mechanisms. Although it has been demonstrated that Th2 response tends to counterbalance the Th1 one [19,20], recent data demonstrated Th1/Th2 response induced by BCG was as efficient as the Th1 alone against *M. tuberculosis* infection [21]. Less is known about Th3 counterbalancing, although it is linked to the anti-inflammatory role of transforming growing factor-beta (TGF-β) which has been considered one of the facts that allows the chronicity in the murine model of tuberculosis [22].

Interestingly, inoculation of RUTI did not induce local or systemic toxicity. This fact has been the major point against the use of immunotherapy against *M. tuberculosis* infection since Robert Koch firstly assayed the old tuberculin [23]. In this regard, the formulation of RUTI includes the extraction of endotoxin-like molecules like lipoarabinomannan [24] with Triton-X114 [25], which could be related to the production of intragranulomatous necrosis in tuberculosis [26]. On the other hand, it seems paramount the previous treatment with chemotherapy to decrease the inflammation induced by the growing bacilli [17], and thus reducing the chance of developing a local Koch reaction that would induce necrosis [26]. Chemotherapy also removes the accumulation of foamy macrophages outside the granulomas (data not shown). These cells are potent inducers of local immunosupression [27], this is why its elimination would be also a major issue to make RUTI efficient.

Although further studies must be done to study specific and innate cellular immunity, as well as the particular role played by humoral response, specially regarding on local IgA production [28], our data suggest that RUTI would be a useful immunotherapeutic vaccine against *M. tuberculosis* infection

by triggering a wide response against a lot of peptides present both in growing and non growing bacilli.

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References

[1] World Health Organization. Global Tuberculosis Control. WHO Report 2001, Geneva, Switzerland, WHO/CDS/TB/2001.287.
[2] Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. J Bacteriol 1998;180:801–8.
[3] Michele TM, Ko C, Bishai WR. Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis sigF* gene: implications for chemotherapy against mycobacterial persistors. Antimicrob Agents Chemother 1999;43:218–25.
[4] Andersen P, Askgaard D, Ljungqvist L, Bentzon MW, Heron I. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. Infect Immun 1991;59:1558–63.
[5] Grosset J. Bacteriologic basis of short-course chemotherapy for tuberculosis. Clin Chest Med 1980;1:231–41.
[6] Moreira AL, Tsenova L, Aman MH, Bekker LG, Freeman S, Mangaliso B, et al. Mycobacterial antigens exacerbate disease manifestations in *Mycobacterium tuberculosis*-infected mice. Infect Immun 2002;70:2100–7.
[7] Turner J, Rhoades ER, Keen M, Belisle JT, Frank AA, Orme IM. Effective preexposure tuberculosis vaccines fail to protect when they are given in an immunotherapeutic mode. Infect Immun 2000;68:1706–9.
[8] Cardona PJ, Gordillo S, Diaz J, Tapia G, Amat I, Pallares A, et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*. Infect Immun 2003;71:5845–54.
[9] Sonnenberg MG, Belisle JT. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect Immun 1997;65:4515–24.
[10] Rabilloud T. Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. Electrophoresis 1990;11:785–94.
[11] Medina E, North RJ. Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype. Immunology 1998;93:270–4.
[12] Cobbold S, Waldmann H. Infectious tolerance. Curr Opin Immunol 1998;10:518–24.
[13] Lucas SB, Histopathology. In: Davies PDO, editor. Clinical tuberculosis. London: Chapman & Hall; 1998. p. 113–27.
[14] McCune RM, Feldmann FM, Lambert HP, McDermott W. Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J Exp Med 1966;123:445–68.
[15] Orme I. The latent tuberculosis bacillus (I'll let you know if I ever meet one). Int J Tuberc Lung Dis 2001;5:589–93.
[16] Scanga CA, Mohan VP, Joseph H, Yu K, Chan J, Flynn JL. Reactivation of latent tuberculosis: variations on the Cornell murine model. Infect Immun 1999;67:4531–8.
[17] Cardona PJ, Julian E, Valles X, Gordillo S, Munoz M, Luquin M, et al. Production of antibodies against glycolipids from the *Mycobacterium tuberculosis* cell wall in aerosol murine models of tuberculosis. Scand J Immunol 2002;55:639–45.
[18] Swain SL, Croft M, Dubey C, Haynes L, Rogers P, Zhang X, et al. From naive to memory T cells. Immunol Rev 1996;150:143–67.
[19] Power CA, Wei G, Bretscher PA. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. Infect Immun 1998;66:5743–50.
[20] Hernandez-Pando R, Pavon L, Arriaga K, Orozco H, Madrid-Marina V, Rook G. Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental mycobacterial saprophyte before infection. Infect Immun 1997;65:3317–27.
[21] Gruppo V, Orme IM. Dose of BCG does not influence the efficient generation of protective immunity in mice challenged with *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2002;82:267–73.
[22] Hernandez-Pando R, Orozco H, Arriaga K, Sampieri A, Larriva-Sahd J, Madrid-Marina V. Analysis of the local kinetics and localization of interleukin-1 alpha, tumour necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. Immunology 1997;90:607–17.
[23] Koch R, Weitere. Mitteilungen uber ein Heilmittel gegen Tuberkulose. Dtsch Med Wochenschr 1890;16:1029–32.
[24] Roach TIA, Barton CH, Chatterjee D, Blackwell JM. Macrophage activation: lipoarabinomannan from avirulent and virulent strains of *Mycobacterium tuberculosis* differentially induces the early genes c-fos, KC, JE and tumor necrosis factor-α. J Immunol 1993;150:1886–96.
[25] Karplus TE, Ulevitch RJ, Wilson CB. A new method for reduction of endotoxin contamination from protein solutions. J Immunol Methods 1987;105:211–20.
[26] Cardona PJ, Llatjos R, Gordillo S, Diaz J, Viñado B, Ariza A, et al. Towards a 'human-like' model of tuberculosis: intranasal inoculation of LPS induces intragranulomatous lung necrosis in mice infected aerogenically with *Mycobacterium tuberculosis*. Scand J Immunol 2001;53:65–71.
[27] Stumbles PA, McWilliam AS, Holt PG. Dendritic cells and mucosal macrophages. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. San Diego: Academic Press; 1999. p. 397–412.
[28] Williams A, Reljic R, Naylor I, Clark SO, Falero-Diaz G, Singh M, et al. Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. Immunology 2004;111:328–33.