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Enhanced Gamma Interferon Responses of Mouse Spleen Cells following Immunotherapy for Tuberculosis Relapse▽

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Gamma interferon responses of spleen cells in mice were examined during postchemotherapy relapse of intraperitoneally induced latent tuberculous infection. The mycobacterial extract RUTI, which prevented the relapse, significantly enhanced the immune responses to secreted and structural recombinant mycobacterial antigens, suggesting that RUTI-mediated protection was mediated by activated T cells.

The aim of this study was to assess the mechanisms of the vaccine RUTI as an adjunct to chemotherapy, in a latent tuberculosis experimental model based on the intraperitoneal (IP) infection of mice (6).

RUTI has already demonstrated a protective effect in a low-dose aerosol model, inducing a large and fast immune response against antigens secreted by actively growing *Mycobacterium tuberculosis* bacilli (2, 4). Given the long period required to test this therapeutic approach using the aerosol model, the IP model could be a reliable one to test new immunotherapeutic candidates.

Specific-pathogen-free, 7-week-old C57BL/6 female mice were treated by using procedures approved and supervised by the Animal Care Committee of the Germans Trias i Pujol University Hospital. *M. tuberculosis* strain H37Rv Pasteur was grown in Proskauer Beck medium containing 0.01% Tween 80 (9) to mid-log phase and stored at −70°C in 2-ml aliquots. Mice were vaccinated subcutaneously twice, at weeks 9 and 11, with RUTI, which consists of liposome-based detoxified fragments of *M. tuberculosis* cells, cultured under stress conditions and under good manufacturing procedure quality control by Archivel Farma (Badalona, Catalonia, Spain) (2).

Mice were infected with 1×10^5 CFU *M. tuberculosis* by IP injection and divided into three groups: the control group, infected but not treated; the chemotherapy-treated group (receiving 25 mg/kg of body weight isoniazid plus 10 mg/kg rifapentin once a week from week 3 to week 9 postinfection); and the vaccinated group, which received two RUTI inoculations after the chemotherapy, at weeks 9 and 11 after infection.

The animals were euthanized with an overdose of isofluorane at week 13 postinfection, and the spleens were harvested. Viable bacteria (CFU) were counted four weeks after the spleen homogenates were plated on 7H11 Middlebrook agar (Biomedics s.l., Madrid, Spain) and incubated at 37°C. Data were recorded as the $\log_{10}$ of the mean number of bacteria recovered per organ. The antigen-stimulated numbers of gamma interferon (IFN-γ)-secreting cells (in spot forming units [SFU] per $1 \cdot 10^6$ splenocytes; enzyme-linked immunospot assay [ELISPOT]) and levels of IFN-γ production (in pg/ml; enzyme-linked immunosorbent assay [ELISA]) were determined after the splenocytes were stimulated for 18 and 96 h with 15 different recombinant antigens, purified protein derivative (10g/ml), or *Mycobacterium bovis* BCG (10^6 CFU), both purchased at SSI, Denmark. The *M. tuberculosis* antigens, comprising ESAT-6 (Rv3875), CFP-10 (Rv3874), MPT64 (Rv1980c), Ag 85B (Rv1886c), Ag 16kDa (Rv2031c), Ag 19kDa(Rv3763), Ag 38kDa (Rv0934), Ag 40kDa (Rv2780), and Hsp65 (Rv0440) (Lionex Therapeutics & Diagnostics Ltd., Braunsweig, Germany), were present in the spleen cell cultures at a concentration of 5 μg/ml. The ELISPOT assay was performed after the culture was incubated for 16 h by using a BD murine IFN-γ ELISPOT kit (BD Bioscience, San Diego, CA) according to the manufacturer's recommendations, and the SFUs counted using an ELISPOT reader. For the ELISA assay, the supernatants of the 72-h cell cultures were harvested and frozen at −80°C to be tested for IFN-γ with a murine double-sandwich ELISA kit (Diaclone, Besançon, France) according to the manufacturer's recommendations. Both techniques' results were corrected for background values. All results were evaluated for statistical significance (the Student *t* test and one-way analysis of variance), and the differences were considered significant when the *P* value was <0.05.

The chemotherapy treatment reduced the bacillary load in the spleens to 2.84 ± 0.49 (mean ± standard deviation) $\log_{10}$ CFU/ml at week 9 postinfection, while the untreated control group had a bacillary load of 4.88 ± 0.62 $\log_{10}$ CFU/ml. After four weeks, at week 13 postinfection at the end of the experiment, the group treated with chemotherapy alone had relapsed to 3.92 ± 0.25 $\log_{10}$ CFU/ml, while the chemotherapy-and-RUTI-treated group showed a significantly lower bacterial load (3.2 ± 0.24 $\log_{10}$ CFU/ml). The infected but untreated control group had 5.16 ± 0.32 $\log_{10}$ CFU/ml (Fig. 1).

Antigen-stimulated IFN-γ secretion by the spleen cell cul-

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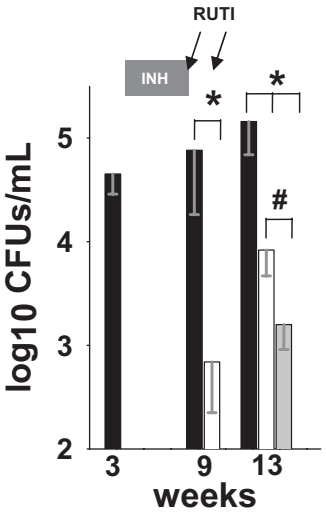

FIG. 1. Bacillary loads in the spleens of IP-infected mice. Chemotherapy treatment was administered once a week from weeks 3 to 9, and the RUTI vaccine was subcutaneously injected at weeks 9 and 11 (arrows). Black, white, and gray bars refer to control, chemotherapy-treated, and chemotherapy-plus-RUTI-treated groups, respectively. Error bars show standard deviations. Significant differences ($P < 0.05$) are marked as follows: *, control group; #, chemotherapy-treated group. INH, isoniazid.

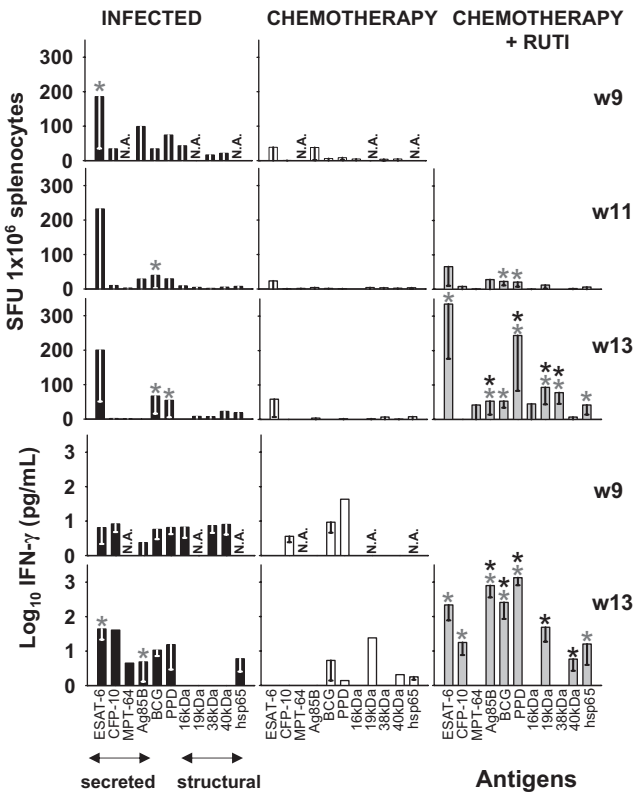

FIG. 2. Enhanced IFN-γ response of C57BL/6 mouse splenocytes against *M. tuberculosis* antigens following RUTI vaccination. Mean values and standard deviations of results of ELISPOT (A) and ELISA of culture supernatants (B) following the stimulation of spleen cells with different stimuli are shown. The week at which each assay was performed is shown at the right; the antigens used are listed below the graphs. Significant differences ($P < 0.05$) are marked with asterisks. Black and gray asterisks refer to control and chemotherapy-treated groups, respectively. N.A., not assayed.

tures of RUTI-vaccinated animals was found to be higher than the levels in the spleen cell cultures of the other groups by both ELISPOT and ELISA (Fig. 2). This immune response was polyantigenic, i.e., in respect to a number of both secreted and structural antigens. This finding is plausible, considering that RUTI, which is produced from *M. tuberculosis* cultures grown under stress conditions, contains a wide range of *M. tuberculosis* antigens that are able to trigger or boost immune responses.

Previously reported data (1, 2) show the immunotherapeutic effect of RUTI in aerosol-infected mice. In the present study, IP-infected mice were used. The results from experiments using the aerosol-infected mouse model couldn't demonstrate a high level of response to structural *M. tuberculosis* antigens as clearly as do the results from this IP experiment (4).

One of the limitations of working with the *M. tuberculosis*-infected mouse model is the good tolerance of these animals to the infection, as well as the absence of intragranulomatous necrosis. Therefore, even though it is the most used to study the immunopathology of the tuberculous infection, the mouse model doesn't mimic what really happens in humans (5, 7), making the extrapolation of the results obtained difficult.

In the present study, we tested the vaccine RUTI as an adjunct to chemotherapy in a latent tuberculosis model based on the IP infection of mice, as an experimental mouse model that seems to better resemble the latent infection in humans (6, 8). The vials to be used to infect the animals, regardless of the route, were previously frozen, and there was a reduction of 1 $\log_{10}$ in the viable counts because of this process. This is why in using the IP route to infect the animals, a large amount of dead bacilli was injected, reaching the spleen much more easily than in the aerosol model. We consider this to be the cause of the mice developing immunity against structural antigens.

Regarding the labels below the graphs in Fig. 2, it must be clarified that although the 19- and 38-kDa lipoproteins are classified as structural, they can be also found in their nonacylated form in the culture supernatant; i.e., they are akin to secreted antigens in many ways (1).

The findings of the present study show that the proposed therapeutic regimen enhanced the recognition of a wide range of mycobacterial antigens, as well as reducing the relapse of latent tuberculosis infection in IP-infected mice. It boosts the immune response, including structural antigens, a fact that was not demonstrated by previous studies using an aerosol as the route of infection. This fact could be important in helping to diminish that relapse.

On the other hand, the scenario found in the IP model used in the present study resembles human tuberculosis, where intragranulomatous necrosis is quickly induced both in lungs and lymph nodes (3), and thus, a high concentration of extracellular dead bacilli is generated. For that reason we consider that the results shown here will also help to show IP treatment to be a good option as a mouse model mimicking human tuberculosis.

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P.-J.C. is a coinventor holding the patent on RUTI as a therapeutic vaccine. Regulatory approval and development is being undertaken by a spin-out biopharma company (Archivel Farma, s.l.) in collaboration with the Institut Germans Trias i Pujol. P.-J.C. is the scientific director for this development.

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