Lung macrophages from bacille Calmette–Guérin-vaccinated guinea pigs suppress T cell proliferation but restrict intracellular growth of *M. tuberculosis* after recombinant guinea pig interferon-γ activation

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Summary

The guinea pig model of low-dose pulmonary tuberculosis has been used to study the pathogenesis of infection as well as the mechanisms of bacille Calmette–Guérin (BCG) vaccine-induced resistance. We investigated the function of lung cells from naive and BCG-vaccinated guinea pigs after enzymatic digestion of lung tissue with collagenase and DNase I. The total lung digest cells proliferated poorly to purified protein derivative (PPD) but comparatively better to ConA as assessed by [³H]-thymidine uptake. However, the non-adherent population obtained after plastic adherence of lung digests showed an enhanced response to concanavalin A (ConA) and PPD. Therefore, proliferation to ConA and PPD of nylon wool-purified T cells co-cultured with peritoneal (PMøs), alveolar (AMøs) or lung macrophages (LMøs) was assessed. Co-cultures of lung T cells and PMøs showed maximum proliferation to PPD, whereas proliferation was suppressed significantly by the addition of AMøs or LMøs. The response of T cells to ConA was unaffected in co-cultures. Incubation of co-cultures with recombinant guinea pig interferon-γ (rgpIFN-γ) did not reverse the suppression. In contrast, rgpIFN-γ-treated plastic adherent LMøs that were non-specific esterase-positive were capable of reducing the intracellular growth of *Mycobacterium tuberculosis*. Similarly, total, non-adherent and adherent lung digest cells from BCG-vaccinated guinea pigs showed IFN-γ and tumour necrosis factor (TNF)-α mRNA expression in response to ConA, lipopolysaccharide or PPD by reverse transcription–polymerase chain reaction followed by release of TNF protein but not IFN. These studies indicate that rgp-IFN-γ-treated lung tissue macrophages from BCG-vaccinated guinea pigs are defective for inducing antigen-specific proliferation in T cells, but control the intracellular accumulation of virulent *M. tuberculosis*.

Keywords: BCG, cytokine mRNA, guinea pig lung digest cells, rgpIFN-γ, T cell proliferation

Introduction

Tuberculosis is a global health problem and it is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* [1]. It is also known that ~10% of infected individuals develop active disease after *M. tuberculosis* infection, resulting in the death of 2 million cases annually [2]. The spread of HIV/AIDS infection as well as the emergence of multi-drug-resistant strains of *M. tuberculosis* have increased the risk of tuberculosis [3]. The disease is transmitted by inhalation of droplet nuclei carrying *M. tuberculosis* where the lung is the primary target of infection [4]. The tubercle bacilli that reach the alveoli are engulfed by alveolar macrophages and those that survive multiply within the macrophages and are carried into the parenchyma of the lung and, ultimately, to the lymph nodes that drain the site of infection [5]. *M. bovis* BCG is the only vaccine currently available for the prevention of tuberculosis. BCG has been shown to induce protective immunity against infectious challenge in many animal models of tuberculosis [6], and protects against tuberculosis in children [7]. However, the efficacy of BCG vaccine in adults has been highly variable [8] in clinical trials.

Effector mechanisms in anti-mycobacterial immunity involve macrophages and lymphocytes that act in concert

with the help of co-stimulatory molecules and molecular mediators such as cytokines and chemokines [9]. Macrophages act as the primary effector cells for the early clearance of mycobacteria. Both CD4^{+} and CD8^{+} T cells are known to mediate the immune response against *M. tuberculosis* but other cell types, including natural killer (NK) cells, are also involved [10–12]. Both interferon (IFN)-γ and tumour necrosis factor (TNF)-α have been identified as important cytokines for the effector functions against mycobacteria [13,14]. The formation and maintenance of the granuloma, a defensive reaction on the part of the host, is mediated by TNF-α which, along with IFN-γ, activates macrophages to produce reactive oxygen and nitrogen intermediates [14,15]. Several other cytokines such as interleukin (IL)-12 and IL-23 contribute to the host-response to mycobacteria by enhancing the development of T helper 1 (Th1) immunity [16,17]. The importance of IFN-γ as a potent activator of macrophages in the effective growth restriction and clearance of mycobacteria *in vitro* has been well documented [18]. Treatment of mice with anti-IFN-γ antibody or disruption of the mouse IFN-γ or the IFN-γ receptor gene resulted in an exacerbation of disease after *M. tuberculosis* or *M. bovis* infection [13,19]. Furthermore, mutations in the IFN-γ receptor or IFN-γ receptor signal-transducing chain predisposed humans to develop disseminated mycobacterial infections [20].

Pulmonary T cell responses are regulated by alveolar macrophages [21], which have the capacity to suppress T cell responses *in vitro* in humans and in rodents [22]. T cell proliferation in peripheral blood mononuclear cells cultured with concalavalin A (ConA), house-dust mite or tetanus toxoid was inhibited in the presence of alveolar macrophages [23]. These studies also demonstrated that early steps in T cell activation, such as CD3 down-modulation, up-regulation of IL-2 receptor expression and IL-2 production were unaffected by human, rat and mouse alveolar macrophages. In other reports, the immunosuppressive activity of alveolar macrophages was inhibited by pretreatment with granulocyte–macrophage colony-stimulating factor (GM-CSF) [24]. Similarly, immunosuppression by alveolar macrophages of ConA-induced T cell proliferation has been documented in murine models [25,26]. Guinea pig alveolar macrophages significantly suppressed the lymphoproliferation of splenic T cells to ConA [27].

Earlier studies from our laboratory focused on the cellular and molecular responses induced by BCG vaccination in phagocytic cells from various anatomical sites exposed to attenuated and virulent strains of *M. tuberculosis in vitro* [28–33]. Recently, we have also purified and characterized the biological activities of several recombinant guinea pig cytokines [34–37]. Guinea pig recombinant IFN-γ (rgpIFN-γ) was found to up-regulate major histocompatibility complex (MHC) class II expression, and to activate macrophages to produce H_2O_2 and restrict the intracellular growth of *M. tuberculosis* [34]. However, the cellular responses in the lungs of guinea pigs, the primary site of *M. tuberculosis* infection, are not well understood. Thus, the purpose of the present study was to characterize the effect of vaccination on lung digest cells by assessing the functional activity of T cells and macrophages obtained after enzyme digestion.

Materials and methods

Animals and BCG vaccination

Random-bred Hartley strain guinea pigs weighing 200–300 g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA). The animals were housed individually in polycarbonate cages in a temperature- and humidity-controlled environment; ambient lighting was controlled automatically to provide 12-h light–12-h dark cycles. Animals were given commercial chow (Ralston Purina, St Louis, MO, USA) and tap water *ad libitum*. All procedures were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee. Guinea pigs were vaccinated once intradermally with 0·1 ml (10³ viable units) of *M. bovis* BCG (Danish 1331 strain; Statens Seruminstitut, Copenhagen, Denmark) in the left and right inguinal regions. The lyophilized vaccine was reconstituted with Sauton's medium (Statens Seruminstitut) just before injection.

Preparation of lung digest cells

The lung digest cells were prepared using the protocols modified from those published earlier for mice [38,39]. The naive and BCG-vaccinated guinea pigs at 6–8 weeks after vaccination were euthanized by the injection of 3 ml sodium pentobarbital (Sleepaway; Fort Dodge Laboratories, Inc.) either intraperitoneally or intramuscularly. All the lung lobes were collected either before or after bronchoalveolar lavage (BAL). The lung tissue was cut into small pieces with scissors in a Petri dish and dispensed in 15 ml RPMI-1640 (Irvine Scientific, Santa Ana, CA, USA) medium supplemented with 2 μM glutamine (Irvine Scientific), 0·01 mM 2-mercaptoethanol (2-ME; Sigma, St Louis, MO, USA), 100 U/ml of penicillin (Irvine Scientific), 100 μg/ml of streptomycin (Irvine Scientific) and 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) containing freshly made collagenase (150 U/ml, Sigma) and DNAse I (100 U/ml, Sigma). Another 30 ml of complete medium was added to this preparation and incubated for 2 h at 37°C. Single cell suspensions were obtained by vigorous pipetting and passing the homogenate first through a 100-μm nylon Falcon cell strainer (BD Biosciences, Bedford, MA, USA) to remove large pieces of tissue, and then through a 40-μm cell strainer (BD Biosciences) to remove smaller debris. The volume was brought to 50 ml in complete RPMI-1640 medium and the cells were centrifuged at 440 *g* for 10 min. The pellet was resuspended in ammonium

chloride potassium bicarbonate (ACK) lysis buffer [0·14 M NH$_4$Cl, 1·0 mM KHCO$_3$, 0·1 mM Na$_2$ ethylenediamine tetraacetic acid (EDTA) (pH 7·2–7·4)], washed three times in RPMI-1640 medium by centrifuging for 10 min at 320 **g**, and viable cells were counted by the trypan blue exclusion method. The viability of lung digest cells was more than 95% as determined by the trypan blue staining method.

Preparation of lung mononuclear cells for flow cytometry

Lung mononuclear cells obtained by digesting the lung tissue were stained with monoclonal antibodies (mAb) against guinea pig MHC class II cells, pan T (CT5), CD4 (CT7) and CD8$^-$ T cell (CT6) phenotypic markers (Biosource International, Camarillo, CA, USA). For each mAb or control, 5–10 × 10^5 cells were incubated with 10 μl of (1 mg/ml) normal goat IgG (Sigma) for 10 min to block FcR binding. This was followed by the addition of 50 μl of mouse anti-guinea pig MHC class II (1 : 10) antibody, anti-guinea pig T cell antibody (1 : 500), anti-CD4 (1 : 500) or anti-CD8 antibody (1 : 1000) and incubating the cells in ice on a shaker. After washing, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, CA, USA) and fixed in Hank's balanced salt solution (HBSS) containing 1% paraformaldehyde. The proportions of positive cells were determined with a fluorescence activated cell sorter (FACS)Calibur flow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Cytospin preparations of the lung digests were also performed and the cells were stained by the Diff-Quik staining method (American Scientific Products, McGraw Park, IL, USA).

Harvesting of resident peritoneal, alveolar and lung macrophages

The resident peritoneal and alveolar macrophages from naive and BCG-vaccinated guinea pigs were harvested using methods published earlier [33,40]. Following euthanasia, the cells from the peritoneal cavity were harvested by flushing the cavity three times with 20 ml of cold RPMI-1640 (Irvine Scientific). ACK lysing buffer was used to deplete the erythrocytes. The cells were washed in RPMI-1640 medium and the viable cells were counted by the trypan blue exclusion method. The cells were suspended at 5 × 10^6 cells/ml in RPMI-1640 medium supplemented with glutamine, 2-ME, penicillin/streptomycin and 10% heat-inactivated FBS (Atlanta Biologicals). Peritoneal, alveolar and lung cells (2 × 10^6/ml) were incubated in 96-well microtitre plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for 2–3 h, and non-adherent cells were removed. The monolayers were comprised predominantly of macrophages (> 95%) as determined by non-specific esterase staining [41].

The resident alveolar macrophages were isolated by BAL by cannulating the trachea and washing the lungs three to four times with 10 ml RPMI-1640 containing 2% FBS and 12 mM lidocaine. The cells were then centrifuged at 4°C for 10 min at 400 g in complete RPMI-1640 medium containing glutamine, 2-ME, penicillin/streptomycin and 10% FBS. The cells were washed three more times in the same medium after lysing the red blood cells, if any, with ACK lysis buffer. The cells were counted and 2 × 10^6 cells/ml were plated in 96-well microtitre plates. The non-adherent cells were removed after 2 h of incubation at 37°C and the monolayers were used for different assays.

The macrophages from lung digest cells were enriched by adherence for 2 h on either 24-well or 96-well plates as described above. At the end of the incubation, the non-adherent cells were removed by washing three times with warm HBSS (with Ca^{2+} and Mg^{2+}) containing 25 mM HEPES, 2 mM EDTA (disodium) and 2% FBS without the antibiotics. The adherent cells were detached by adding warm phosphate-buffered saline (PBS) (without Ca and Mg) containing only 10 mM HEPES, 2 mM EDTA and no antibiotics and incubating the plate at 37°C for 30 min. The cells were detached by vigorous pipetting and then collected in tubes kept on ice. The cells were centrifuged (200 **g** for 8 min at 4°C) and the cell pellet suspended in RPMI-1640 containing 2% FBS, 10 mM HEPES, 2 μM glutamine and 0·01 mM 2-mercaptoethanol without antibiotics. The cells were counted and cytospins were prepared and stained for non-specific esterase according to the manufacturer's protocol [41].

Purification of T cells

The non-adherent cells from the digested lung tissue of BCG-vaccinated guinea pigs were enriched for T lymphocytes on nylon wool columns using our standard protocol [31]. The column was prepared by packing 0·5 g, scrubbed and combed ready for use, nylon wool fibre (Polysciences, Inc., Warrington, PA, USA) into a 10-ml syringe barrel and autoclaving for 15 min. The column was washed with RPMI-1640 containing 10% FCS and left wet with no air bubbles. The prepared column was then incubated at 37°C for 1 h and loaded with 1–2 × 10^8 viable cells in a volume of 2 ml. The loaded column was incubated for 1 h at 37°C and the non-adherent cells were collected using 50 ml warm RPMI-1640 medium. The collected cells were centrifuged at 320 **g** for 10 min and the cell pellet was resuspended in RPMI-1640 medium containing 10% FCS and counted by the trypan blue exclusion method.

Proliferation of total lung digest cells and co-cultures of lung T cells and macrophages

The ability of lung digest cells to proliferate in response to a mitogen or specific antigen was assessed in total lung cells,

nylon wool-purified T cells, or in co-cultures of nylon wool-purified T cells and macrophages obtained from various anatomical sites. The total lung digest cells (4×10^5/well) were plated onto 96-well plates with or without concanavalin A (ConA, 10 µg/ml; Sigma) or purified protein derivative (PPD, 12·5 and 25 µg/ml; Statens Seruminstitut) and cultured for 4 days using our standard protocols [42]. For the final 6 h of culture, [^{3}H]-thymidine was added at a concentration of 1 µCi/well. The cells were harvested using a FilterMate harvester, and the [^{3}H]-thymidine uptake was measured in a scintillation counter (Beckman LS-1801). The stimulation index (SI) was calculated by dividing the counts per minute (cpm) of stimulated cells by the cpm of unstimulated cells.

For the co-culture experiments, only BCG-vaccinated animals were used. The T cells from lung digests obtained after nylon wool purification were mixed at a ratio of 3 : 1 with macrophages obtained from lung digests, peritoneal or alveolar lavages. The co-cultures of T cells (3×10^5) and macrophages (1×10^5) were plated onto 96-well plates and stimulated with either ConA or PPD for 96 h using the protocols described above. Nylon wool-purified T cells from the lung digests (4×10^6/ml) were also cultured in the same manner. At the initiation of the culture period, some cells were stimulated with 200 or 500 ng/ml rgpIFN-γ, doses which have been shown to up-regulate MHC class II expression, enhance H_2O_2 production and reduce the intracellular growth of *M. tuberculosis* in peritoneal macrophages [34].

Effect of rgpIFN-γ on intracellular growth of mycobacteria in lung macrophages

The ability of macrophages obtained from lung digests to control the intracellular growth of mycobacteria was determined by the incorporation of [^{3}H]-uracil [40]. Lung digest macrophages from BCG-vaccinated guinea pigs were plated (3×10^5 cells/well) in 96-well microtitre plates in complete RPMI-1640 medium containing 10% FBS and the non-adherent cells were removed after 3 h of incubation at 37°C. The cells were treated with varying concentrations of rgpIFN-γ (50–1000 ng/ml) for 24 h, at the end of which the medium was removed and the cells washed once in antibiotic-free RPMI-1640 medium. Macrophages were then infected with *M. tuberculosis* H37Rv (multiplicity of infection [MOI] 1 : 1) for 3 h and the extracellular bacteria were removed by washing two times with antibiotic-free medium. In some experiments, rgpIFN-γ was added both before and after phagocytosis and, in the latter experiments, recombinant protein was present throughout the 7-day culture period. Infected macrophages were then incubated in medium containing gentamycin (50 µg/ml) to inhibit the extracellular mycobacteria for 7 days at 37°C. The macrophages were pulsed with 1 µCi of [^{3}H]-uracil for 24 h before harvesting. The mycobacteria were killed by incubating the

plate at 80°C for 30 min, harvested using a FilterMate harvester, and the [^{3}H]-uracil uptake was measured in a scintillation counter (Beckman LS-1801); the results are expressed as cpm.

Total RNA isolation and real-time polymerase chain reaction (PCR)

Total unseparated lung digest cells, adherent or non-adherent subpopulations from BCG-vaccinated guinea pigs were stimulated with ConA (10 µg/ml), lipopolysaccharide (LPS) (1 µg/ml) or PPD (25 µg/ml) for 3, 12 or 24 h. At the end of the incubation period, the supernatant was removed by centrifugation and the cells were lysed with RNA lysis buffer (RLT) buffer (Qiagen, Valencia, CA, USA); the lysates were kept frozen at −80°C until RNA extraction. Total RNA was isolated using the RNeasy columns (Qiagen). Reverse transcription was performed with *Taq*Man reverse transcription reagents and real-time PCR using SYBR Green I double-stranded DNA binding dye (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 7700 sequence detector according to the protocols published from our laboratory [2,11]. Real-time primers for IFN-γ, gpTNF-α and HPRT were designed with Primer Express software (Biosystems) based upon sequences that we have published previously [2,11]. Fold induction of mRNA was calculated from the threshold cycle values (Ct) normalized to HPRT Ct values and then to unstimulated cultures.
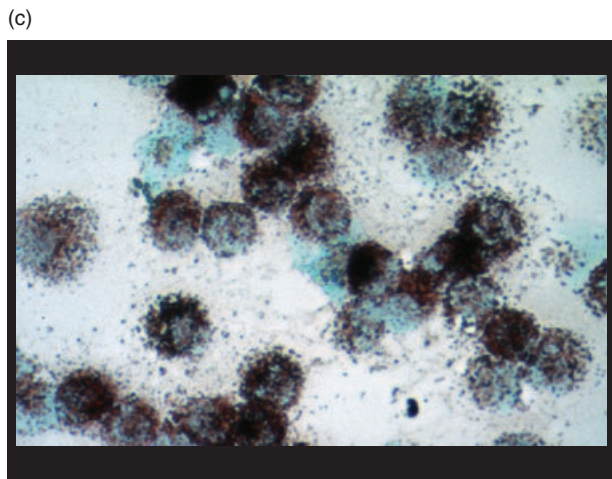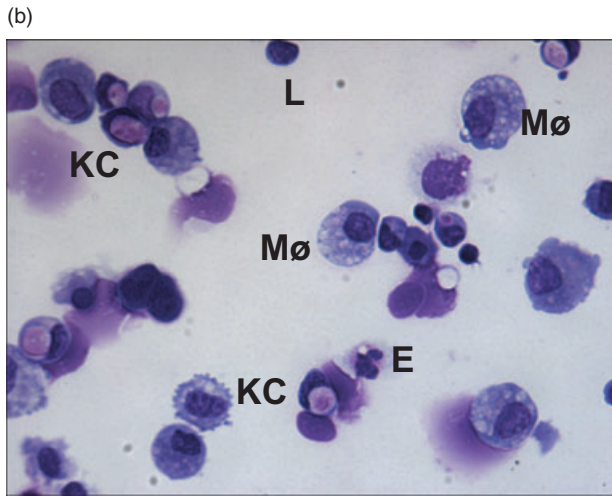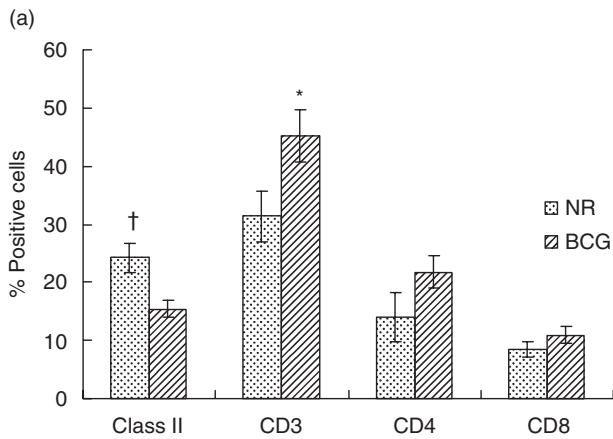
Bioassay of TNF-α

The bioassay for TNF was carried out using the protocol published previously from our laboratory [35,40]. The TNF activity in the culture supernatants was assayed by measuring their cytotoxicity on L929 cells (American Type Culture Collection).

Bioassay for IFN-γ

Concentrations of bioactive IFN in the culture supernatants were determined using a viral protection assay involving encephalomyocarditis virus (EMCV) in the guinea pig fibroblast cell line, 104C1, as described previously by us [34,43].

Statistics

The data are expressed as means ± standard errors (s.e.). The differences between naive and BCG-vaccinated groups were analysed by Student's *t*-test and values of $P < 0.05$ were considered statistically significant. The effects of treatments were assessed by analysis of variance (ANOVA) and the differences between means were analysed by the Tukey *post hoc* test. There were three to 10 guinea pigs per group and each experiment was performed at least three times.

by flow cytometry (Fig. 1a) and by Diff-Quik staining (Fig. 1b). There was a significant reduction ($P < 0.04$) in the proportions of MHC class II+ cells in the lung digests of BCG-vaccinated animals compared to the naive animals. In contrast, the proportions of T cells in the BCG-vaccinated group were increased significantly ($P < 0.007$) compared to the naive group; however, no significant changes in the proportions of T cell subsets in two groups were observed (Fig. 1a). Lung digests from both naive and BCG-vaccinated guinea pigs contained approximately 8–29% MHC class II positive cells, 28–73% CD3+ T cells, 8–48% CD4+ T cells and approximately 6–21% CD8+ T cells.

The cytospin preparations of lung digests were stained using the Diff-Quik method and the differential counts revealed significant differences in the number of cells between unvaccinated and BCG-vaccinated groups (Fig. 1b and Table 1). Macrophages were seen in abundance in the lung digests of both naive and BCG-vaccinated guinea pigs (Fig. 1b); however, there was a significant reduction in the percentage of cells ($P < 0.01$) in the latter. Eosinophils and neutrophils were seen frequently in the cytospin preparations but they were not clearly distinguishable from each other and, thus, these two types of granulocytes were enumerated together. In the BCG-vaccinated guinea pigs, the proportion of these cells increased significantly ($P < 0.0001$). Similarly, there were numerous Kurloff cells, mononuclear cells with a large cytoplasmic inclusion body that are uniquely present in guinea pig tissues, in the lung digests. BCG vaccination induced a significant increase ($P < 0.03$) in the proportion of Kurloff cells in the lung. The percentage of lymphocytes in the lung digests of unvaccinated and BCG vaccinated guinea pigs, however, remained similar. Identification of certain cell types in the digests was confounded by the fact that the integrity and anatomical location of these cells in the tissue was lost due to the nature of isolation procedures after enzymatic digestion. Figure 1c indicates that lung digest cells yielded mainly macrophages after

Results

Phenotypic analysis of lung digest cells

In order to characterize the cell populations in the lung digests, lung tissue from both naive and BCG-vaccinated guinea pigs was digested with collagenase and DNase. Figure 1 shows the phenotypic analysis of lung digest cells

Table 1. Differential counts of guinea pig lung digest cells (mean percentage $\pm$ s.e.m.).

Groups	Lymphocytes	Macrophages	Neutrophils/ eosinophils	Kurloff cells
Unvaccinated	23·4 $\pm$ 1·3	43·3 $\pm$ 2·0	13·6 $\pm$ 1·2	19·8 $\pm$ 1·4
BCG-vaccinated	21·3 $\pm$ 1·6	34·9 $\pm$ 2·4†	22·6 $\pm$ 1·7*	24·0 $\pm$ 1·6†

†$P < 0.003$–0.01 and *$P < 0.0001$ compared to the unvaccinated group as determined by Student's *t*-test; BCG: bacille Calmette–Guérin; s.e.m.: standard error of the mean.

plastic-adherence as assessed by non-specific esterase staining. Thus, BCG vaccination resulted in a decrease in the proportions of MHC class II$^+$ cells accompanied by a reduction in the percentage of macrophages. In contrast, the proportions of CD3$^+$ T cells increased in the lung and the percentage of neutrophils/eosinophils and Kurloff cells increased after BCG vaccination.

Lymphoproliferation of lung digest cells

In order to assess the function of lung digest cells, the proliferative capacity of total lung cells from naive and BCG-vaccinated guinea pigs to ConA and PPD was examined. Total lung digest cells proliferated well to ConA but poorly in response to PPD (Fig. 2). Therefore, non-adherent cells were
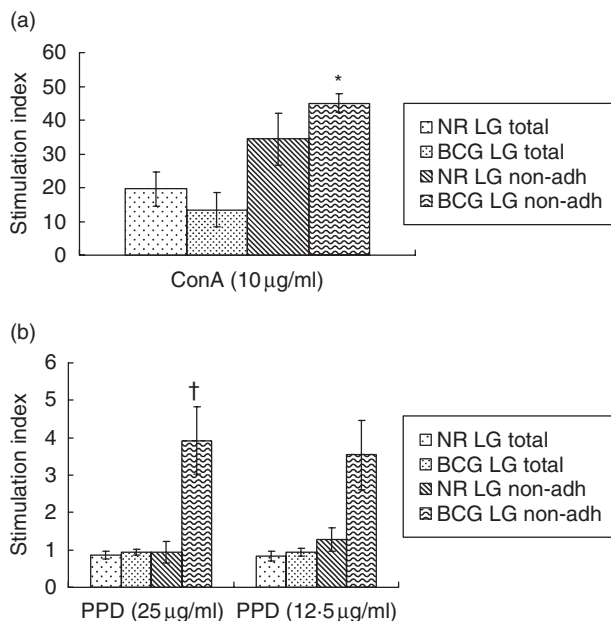

Fig. 2. Proliferation of total and non-adherent cells from guinea pig lung digests. Lung digest cells from naive and bacille Calmette–Guérin (BCG)-vaccinated guinea pig cells (2×10^6/ml) were cultured for 4 days in the presence of concanavalin A (ConA) (10 μg/ml) and purified protein derivative (PPD) (12·5 and 25 μg/ml). The cells were harvested after the addition of [^{3}H]-thymidine (1 μg/well) 6 h earlier. The results are expressed as stimulation index calculated by dividing the counts per minute (cpm) of stimulated cells by the cpm of unstimulated cells. The results are the mean and standard error of the mean from three experiments. There were five animals per group. *$P < 0.005$ in comparisons between groups by Student's *t*-test.

obtained by incubating the lung digest cells in 100-mm-diameter Petri dishes for 2 h at 37°C. As is clear from Fig. 2, the ConA-induced proliferative capacity of the non-adherent population from lung digests from both naive and BCG-vaccinated guinea pigs was significantly enhanced after removing the adherent population. As expected, proliferation to PPD of non-adherent cells from naive guinea pigs remained low, while that of the BCG-vaccinated group was increased significantly ($P < 0.01$) after removing the adherent cells. These results indicated that the adherent population in the lung digests suppressed the proliferative capacity of lung T cells to ConA and PPD.

Lymphoproliferation in the co-cultures

Because the proliferation of non-adherent cells from lung digests in response to ConA and PPD was affected by the adherent population, we examined whether T cells obtained after nylon wool purification would act differently when co-cultured with macrophages obtained from different anatomical sites. Nylon wool-purified T cells from the lung digests of BCG-vaccinated guinea pigs were co-cultured with macrophages obtained from BAL (alveolar macrophages, AMøs), peritoneal macrophages (PMøs) or lung parenchymal macrophages (LMøs) at a ratio of 3 : 1. This ratio of lymphocytes to macrophages was used based on our previous work with spleen T cells and peritoneal macrophages [31] and also because our preliminary experiments showed no change in the proliferation trend with other combinations (data not shown). The co-cultures were stimulated for 4 days in the presence of ConA or PPD and the proliferation was assessed by tritiated thymidine uptake. Results from Fig. 3 indicate that total unseparated cells or T cells alone from the lung digests proliferated well to ConA (Fig. 3a). Similarly, T cells co-cultured in the presence of all macrophage populations showed an enhanced response. The proliferative response to PPD was lower than the response to ConA. Total lung digest cells as well as purified T cells responded poorly to PPD when cultured alone; however, in the co-cultures the PPD-induced proliferation of lung T cells was significantly higher in the presence of PMøs (Fig. 3b). In contrast, the proliferative capacity of lung T cells to PPD in the presence of AMøs and LMøs was significantly suppressed, although the response was greater than that observed with either total digest cells or T cells alone (Fig. 3b). Therefore, AMøs and LMøs inhibited T cell proliferation to PPD.

We then determined whether addition of rgpIFN-γ would rescue the ability of LMøs to induce proliferation of T cells to PPD. Therefore, total lung digest cells, purified T cells and co-cultures of T cells and macrophages from different anatomical sites were treated with the rgpIFN-γ at the beginning of the culture period. As is clear from Fig. 3c, the addition of 200 ng/ml or 500 ng/ml rgpIFN-γ to any of these cell cultures did not influence proliferation.

Intracellular growth of *M. tuberculosis* in lung macrophages

Because LMøs suppressed the proliferation of lung T cells compared to peritoneal macrophages, we wanted to determine whether these cells retained other functions, such as restricting the intracellular growth of *M. tuberculosis* after rgpIFN-γ treatment. LMøs from BCG-vaccinated guinea pigs were cultured with different doses of rgpIFN-γ for 24 h. The medium was removed, fresh medium without antibiotics was added, and the cultures were infected with *M. tuberculosis* and incubated for 7 days. In some cultures, IFN-γ was added both before and after infection (2×). Figure 4 shows the [³H]-uracil uptake by *M. tuberculosis* in LMøs. rgpIFN-γ significantly ($P < 0.02$–0.0004) reduced the intracellular
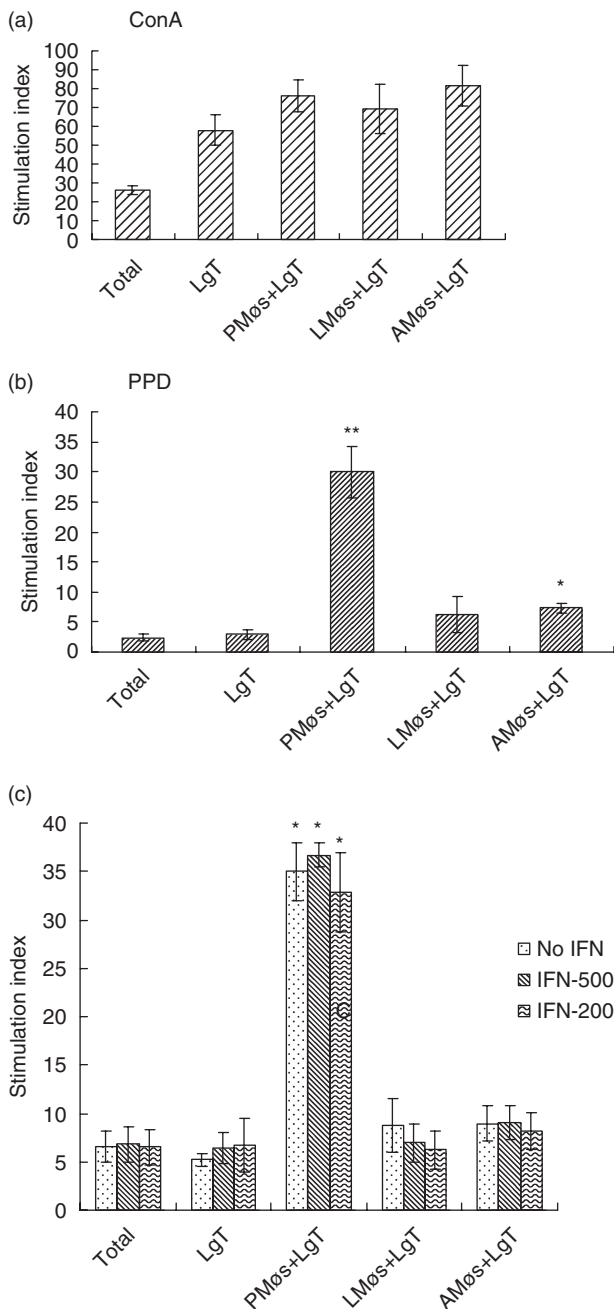

Fig. 3. Proliferation in co-cultures of guinea pig lung T cells and macrophages from different anatomical sites. Total and lung T (LgT) cell proliferation in the presence of macrophages from peritoneal (PMøs), lung (LMøs) and alveolar (AMøs) sites. Lung T cells (3×10^5/well) purified on nylon wool columns from bacille Calmette–Guérin (BCG)-vaccinated guinea pigs were co-cultured with peritoneal, lung or alveolar macrophages (1×10^5/well) in the presence of concanavalin A (ConA) (a) or purified protein derivative (PPD) (b) for 4 days and the [³H]-thymidine uptake was assessed. The cell populations were also treated with recombinant guinea pig interferon-γ (rgpIFN-γ) (200 and 500 ng/ml) at the beginning of the culture period and these results are shown in (c). The results represent mean ± standard error of the mean (s.e.m.) from five to 10 animals. Results are expressed as stimulation index. *$P < 0.01$ and **$P < 0.005$ when compared with total cells as determined by analysis of variance.
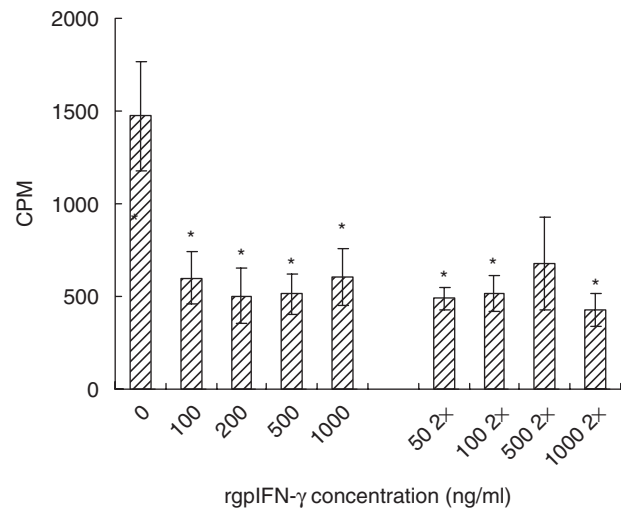
Fig. 4. Effect of recombinant guinea pig interferon-γ (rgpIFN-γ) on intracellular growth of *Mycobacterium tuberculosis* in lung macrophages. LMøs from bacille Calmette–Guérin (BCG)-vaccinated guinea pigs were treated with varying doses (50–1000 ng/ml) of rgpIFN-γ for 24 h. The RPMI-1640 medium was removed and fresh medium without antibiotics was added and the cultures were infected with *M. tuberculosis* (multiplicity of infection 1 : 1) for 3 h. The extracellular bacteria were removed and some cultures were treated with 50–1000 ng/ml also after infection. The [³H]-uracil uptake by viable *M. tuberculosis* was measured on day 7 and is expressed as counts per minute (cpm). The results represent mean ± standard error of the mean (s.e.m.) from five experiments. The differences between unstimulated and stimulated cultures were examined by a Student's *t*-test. *$P < 0.005$ when compared with the untreated cultures.

growth of bacteria in LMøs at doses ranging from 100 to 1000 ng/ml compared to the untreated LMøs. No additional effect was seen when the cytokine was added again after infection (Fig. 4). It is clear from these experiments that although LMøs were lymphocytostatic, they were capable of restricting the intracellular growth of mycobacteria after activation with rgpIFN-γ.

Cytokine mRNA expression and protein production

The expression of TNF-α and IFN-γ mRNA was examined by real-time PCR in the total, non-adherent and adherent populations of lung digest cells stimulated with ConA, LPS or PPD for 3–24 h. The results are illustrated in Fig. 5. Total lung digest cells showed a significant increase in IFN-γ mRNA expression after stimulation with ConA or PPD for 24 h, although the fold induction was much higher with ConA. TNF-α mRNA was also increased significantly in total lung digests stimulated for 24 h with ConA but not with PPD (Fig. 5a). Similarly, the non-adherent cells also showed a significant increase in IFN-γ mRNA expression after PPD stimulation at 12 h and the mRNA level was reduced at 24 h (Fig. 5b). The TNF-α mRNA expression was increased modestly in the lung non-adherent cells after stimulation with both ConA and PPD at 12 and 24 h; however, the levels did not attain statistical significance (Fig. 5c). Surprisingly, IFN-γ mRNA expression was increased significantly in the lung adherent cells after LPS stimulation at 3 h and after PPD stimulation at 3 and 12 h (Fig. 5d). In contrast, stimulation of lung adherent cells with either LPS or PPD had no significant effect on TNF-α mRNA expression (Fig. 5e). Thus, the level of IFN-γ mRNA was higher than TNF-α mRNA in all the lung cell types stimulated with various stimulants.

The bioactive IFN protein levels were measured in the culture supernatants of lung digest cells stimulated with ConA, LPS or PPD based on their ability to reduce the cytopathic effect of EMCV in guinea pig fibroblast cell line. IFN protein was not detected in the culture supernatants from total or non-adherent cells stimulated with ConA or PPD. The supernatants from adherent lung cells stimulated with LPS or PPD showed low levels of IFN protein (data not shown). Thus, IFN-γ mRNA expression in the lung digest cells stimulated with various antigens was not associated with the release of IFN protein.

Bioactive TNF-α levels were assessed by the L929 cytotoxicity assay in the culture supernatants from total, non-adherent and adherent lung cells stimulated with LPS or PPD. Both total and non-adherent cells produced significant levels of TNF-α protein in response to PPD 24 h after stimulation when compared to the unstimulated cultures (Fig. 6a). Similarly, the adherent lung cells stimulated for 3, 12, and 24 h with LPS or PPD secreted copious amounts of TNF-α protein compared to the unstimulated cultures. The maximum induction of protein was observed at 3 h after LPS or PPD stimulation and the levels receded by 12–24 h (Fig. 6b). The protein levels in the lung adherent population were much higher after PPD stimulation than with LPS.

Discussion

Despite our knowledge of the functions of immune cells isolated from peripheral lymphoid organs such as spleen or lymph nodes, or from the peritoneal cavity or BAL fluid of guinea pigs [28–33,37], little is known about the local cellular responses that occur in the lung after BCG vaccination. Our study provides the first evidence that functional lymphocytes and macrophages can be isolated from the lungs of guinea pigs. The lung digest cells were prepared by the enzymatic digestion of collagenase and DNase using modified mouse protocols in order to allow for the processing of a larger amount of tissue. Although initial characterization of cell types was carried out in naive and BCG-vaccinated guinea pigs, the functional assays for macrophages and lymphocytes were performed only in BCG-vaccinated guinea pigs because the assays required antigen-specific T cell reactivity. Flow cytometric analysis indicated that the BCG-vaccinated group had significantly lower proportions of MHC class II+ cells but higher proportions of CD3+ cells in the lung digests in comparison with the unvaccinated animals (Fig. 1a). There were no changes in the proportions of T cell subsets in the lung after BCG vaccination, although our previous findings indicated that CD4+ T cells increased in the spleens of BCG vaccinated guinea pigs [31]. The significant decrease in the proportions of MHC class II+ cells in the lung after vaccination is due clearly to the reduction in the number of macrophages after BCG vaccination, as revealed by the differential counts. It is quite probable that macrophages migrate from one location to the other after bacterial encounter.

The differential counts of lung digest cells from the naive guinea pigs revealed that macrophages were seen abundantly while lymphocytes, granulocytes and Kurloff cells were comparatively fewer (Table 1). However, after BCG vaccination, the lung digest cells showed a reduction in the percentage of macrophages but an increase in the number of granulocytes and Kurloff cells. These macrophages are apparently replaced with granulocytes and Kurloff cells. It is known that neutrophils [44,45] and eosinophils [46] are recruited to the site of *M. tuberculosis* infection, and are found in the granulomas of human, mice and guinea pigs. The unique presence of Kurloff cells in the guinea pig lung is noteworthy, as these cells are found only in tissue preparations including spleen homogenates [32] and are not seen in the resident cells collected from peritoneal or alveolar space (data not shown). It has been reported that Kurloff cells are NK-like cells mediating TNF-dependent cytotoxic activity [47] and also cytolytic activity on a guinea pig leukaemic cell line [10]. Its cytotoxic activity has been demonstrated using ^{51}Cr release assay with human and murine targets. The increased influx of Kurloff cells in the lung following BCG vaccination

(a)

Total lung cells

(b)

Lung non-adherent cells

(c)

Lung non-adherent cells

(d)

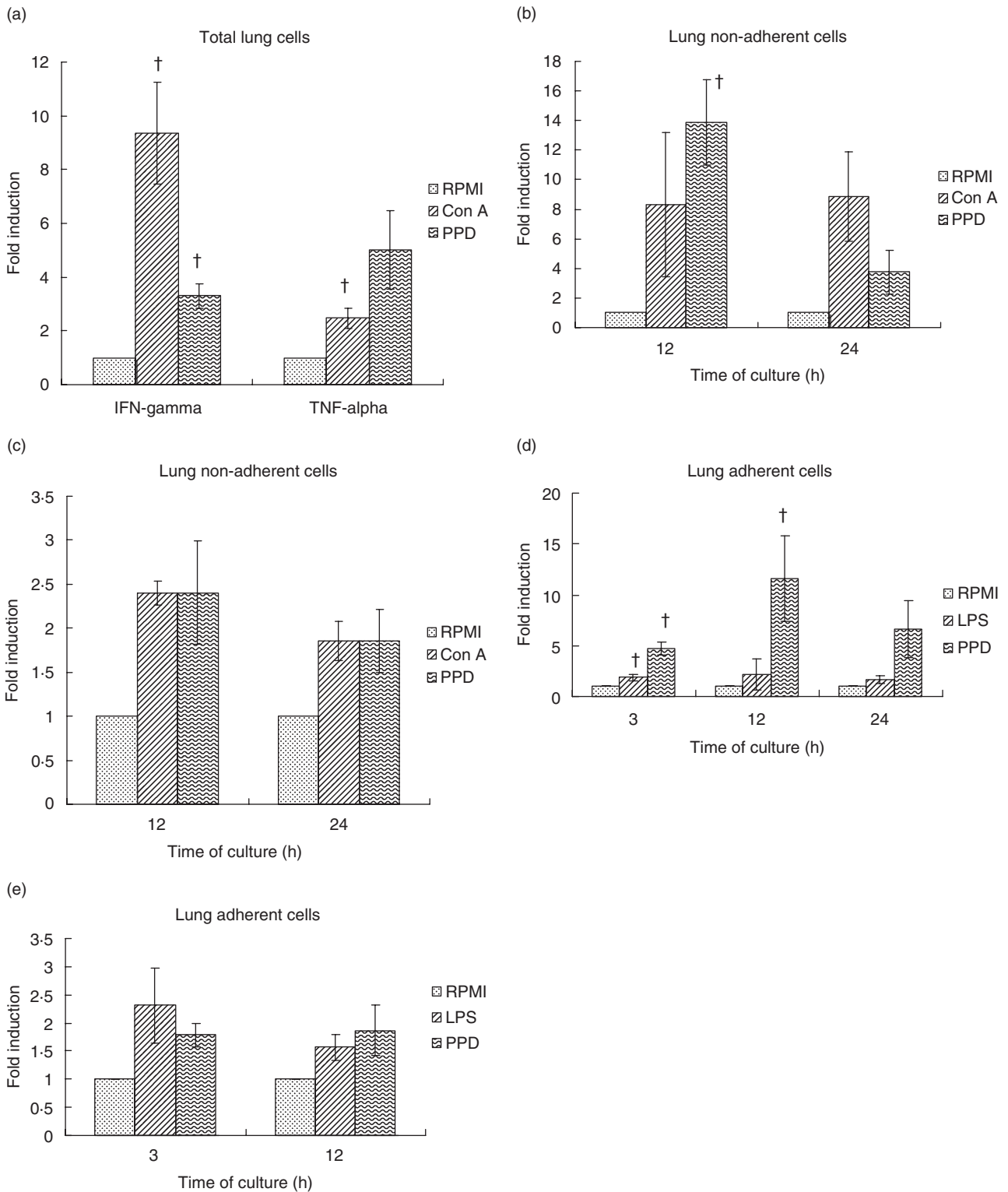**Lung adherent cells**

(e)

Lung adherent cells

Fig. 5. Cytokine mRNA expression in guinea pig lung digest cells. Total (a), non-adherent (b, c) and adherent lung digest cells (d, e) from bacille Calmette–Guérin (BCG)-vaccinated guinea pigs were stimulated with concanavalin A (ConA) (10 µg/ml), lipopolysaccharide (LPS) (1 µg/ml) or purified protein derivative (PPD) (25 µg/ml) for 3, 12 or 24 h. Interferon (IFN)-γ (b, d) and tumour necrosis factor (TNF)-α mRNA (c, e) expression was quantified using real-time polymerase chain reaction (PCR). Fold induction of mRNA was calculated from the threshold cycle values (Ct) normalized to hypoxanthine-quanine phosphoribosyl transferase (HPRT) Ct values and then to unstimulated cultures. The results are expressed as mean ± standard errors of means (s. e.m.) from four to six animals. The differences in the fold induction between unstimulated and stimulated cultures were determined by a Student's t-test or by analysis of variance. †$P < 0.05$–0.01.
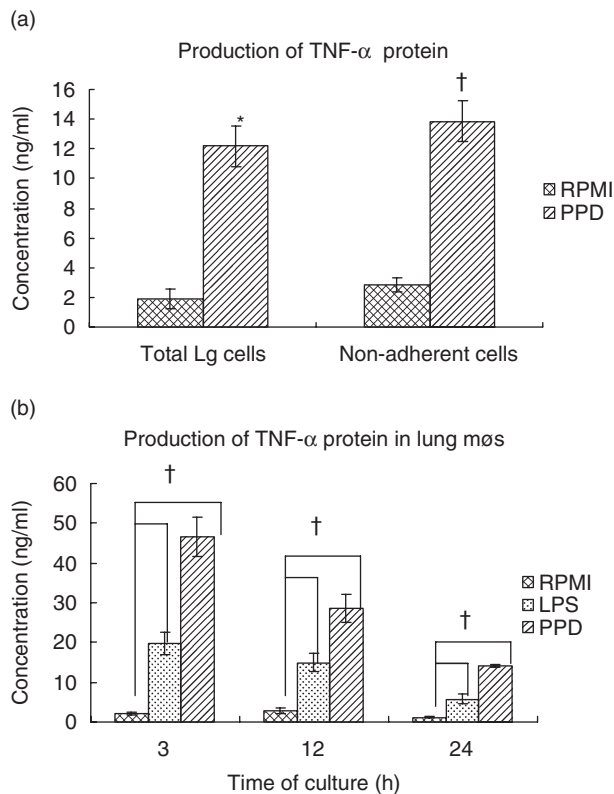
(a)

(b)

Fig. 6. Production of tumour necrosis factor (TNF)-α protein in guinea pig lung digest cells. Total and non-adherent lung digest cells (a) were stimulated with purified protein derivative (PPD) (25 μg/ml) for 24 h and the adherent population (b) was stimulated with lipopolysaccharide (LPS) (1 μg/ml) or PPD for 3, 12 or 24 h. The culture supernatants were collected and assayed for the presence of TNF-α protein production by the L929 cytotoxicity assay. The results are expressed as concentration in ng/ml and denote mean and standard errors of means from four animals. *$P < 0.006$ and †$P < 0.05$ when compared to the response in untreated cultures determined by a Student's *t*-test [results in a)] or by analysis of variance [results in (b)].

suggests that they may be components of a positive innate immune response against mycobacteria. Identification of other cells types such as epithelial cells, fibroblasts or mast cells was confounded because the integrity of normal cellular network was lost due to the nature of isolation procedures.

In co-cultures, the proliferation of T cells to ConA was similar in the presence of macrophages from various anatomical sites (Fig. 3a). However, lung T cells proliferated well to PPD only in the presence of PMøs and not when co-cultured with AMøs or LMøs (Fig. 3b). Previous research from our laboratory demonstrated that spleen T cells from guinea pig cells proliferated poorly to ConA in the presence of AMøs and the effect was not due to nitric oxide (NO), prostaglandin E_2 (PGE$_2$) or H_2O_2 production by macrophages. Those studies indicated that cell-to-cell contact was necessary for suppression to occur and the

suppression was mediated by soluble factors released in the co-cultures [27].

Similarly, AMøs from human and other mammalian species are known to suppress proliferation of T cells [21,48]. AMøs from humans and rodents selectively inhibited T cell proliferation, while other indices of T cell activation (e.g. CD3-down-modulation, IL-2R expression, IL-2 secretion) proceeded normally [23,49]. The suppression of T cell proliferation by AMøs is thought to represent a protective immunoregulatory mechanism within the lung [21], which is challenged constantly with both pathogenic and non-pathogenic organisms. Therefore, it is important to maintain the balance between appropriately effective responses against infectious organisms and unnecessary or excessive responses to non-pathogenic stimuli [23]. In our studies, the suppression in T cell proliferation by AMøs was not abrogated by the stimulation of cultures with rgpIFN-γ (Fig. 3c). In some studies using mouse and human alveolar macrophages, the addition of cytokines such as TNF-α or GM-CSF bypassed the suppression caused by pulmonary alveolar macrophages [50]. Furthermore, LMøs behaved in a similar manner to the resident AMøs in suppressing T cell proliferation. Our previous studies indicated that AMøs produce lower levels of cytokine mRNA and protein when cultured in the presence of whole mycobacteria or PPD [29]. Lung digest cells, when stimulated with ConA, LPS or PPD, showed enhanced mRNA expression for IFN-γ and TNF-α, although the levels of IFN-γ mRNA were significantly higher than TNF-α mRNA in total, non-adherent or adherent populations (Fig. 5). However, a higher expression of IFN-γ mRNA was not accompanied by the production of IFN protein. Expression of TNF-α mRNA in the total, non-adherent or adherent lung populations was associated with the release of large quantities of TNF-α protein. These results indicate a dissociation between IFN-γ mRNA expression and protein production, which has been reported previously for both TNF-α and IFN-γ mRNAs in the rectal samples of Shigella-infected patients [51]. It is clear that the frequency of cytokine mRNA-expressing cells and the corresponding protein-synthesizing cells are highly variable. Alternatively, limitations of the protein assay may also contribute to this phenomenon.

Surprisingly, adherent population from the lung digest cells that were mainly macrophages as assessed by non-specific esterase staining showed significant induction of IFN-γ mRNA. Stimulation with LPS induced IFN-γ mRNA expression in murine macrophages [52]. Similarly, mouse pulmonary macrophages isolated from BCG-vaccinated mice produced IFN-γ protein when cultured *in vitro* with LPS or PPD [53]. These studies also demonstrated that IFN-γ was released in the culture supernatants of freshly isolated lung macrophages from naive mice after stimulation with LPS and IL-12 or BCG and IL-12 [53]. Human AMøs, when stimulated with virulent or attenuated *M. tuberculosis* for 24–48 h, showed induction of IFN-γ mRNA and release of IFN-γ protein [54]. Thus, it is clear that multiple cytokine

signalling may be required for the release of IFN-γ protein by macrophages and that macrophage activation occurs in an autocrine fashion by responding to, as well as producing, IFN-γ.

We have reported recently that rgpIFN-γ up-regulated MHC class II expression, enhanced H_2O_2 production and restricted the intracellular growth of virulent *M. tuberculosis* in guinea pig PMøs [34]. Although LMøs were lymphocytostatic, they restricted the intracellular replication of virulent *M. tuberculosis* when activated with rgpIFN-γ (Fig. 4). It is quite possible that, similar to AMøs, the suppression of T cell proliferation by LMøs might also be an important immunoregulatory mechanism in preserving lung function.

Our studies indicate clearly that functionally active lymphocytes and macrophages can be purified from guinea pig lung digests after enzymatic digestion. We have demonstrated that LMøs, like AMøs, suppress T cell proliferation compared to peritoneal macrophages in co-cultures. Furthermore, treatment of LMøs with rgpIFN-γ induced a reduction in intracellular accumulation of virulent *M. tuberculosis*. These experimental approaches will enhance significantly our understanding of the mechanisms of vaccine-induced resistance, as well as the cellular and molecular events that occur at the site of *M. tuberculosis* infection in the guinea pig model.

Acknowledgements

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