

Uptake of inhalable microparticles affects defense responses of macrophages infected with *Mycobacterium tuberculosis* H37Ra.

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Synopsis

Objectives: To investigate whether inhalable microparticles containing two anti-tuberculosis agents, isoniazid and rifampicin, evoke host-defense strategies in macrophages in addition to targeting the incorporated drugs.

Methods: Microparticles were prepared by spray-drying a true solution of 4:3:1 poly(lactic acid) of apparent viscosity 1.11 cps (PLA) : rifampicin : isoniazid. These were administered as "nose-only" inhalations to mice or exposed to cultured J 774 mouse macrophages. Targeting to lung macrophages was investigated by transmission electron microscopy. Reactive oxygen species (ROS) were estimated by a cytochrome c assay and flow cytometry. Reactive nitrogen intermediates (RNI) were assayed using Greiss' reagent. Cytokines in culture supernatants were estimated by ELISA.

Results: Treatment with inhalable microparticles targeted lung macrophages *in vivo* and induced intense Golgi activity in the vicinity of microparticle-containing phagosomes. Microparticles induced a respiratory burst involving NADPH oxidase and enhanced NO

production by infected macrophages. Microparticle-induced NADPH oxidase activation required optimal calcium ions. Microparticles efficiently induced tumor necrosis factor- α (TNF- α) secretion by macrophages recovered from infected mice.

Conclusions: Microparticle phagocytosis induces responses in infected murine macrophages that are indicative of activation of innate bactericidal mechanisms, and are inimical to bacterial survival. It is likely that such responses augment straightforward drug action on the bacterium and contribute to the unexpectedly high efficacy of microparticles in experimental tuberculosis

Key words: Biodegradable Microspheres, Targeting, Tuberculosis, Innate Immune Response, Free Radicals, Cytokines

Running Title: Macrophage Responses to Microparticles in TB

Introduction

The emerging view of a macrophage that harbours a tuberculosis (TB) bacterium is increasingly inclined to consider the phagocyte as 'alternatively-activated' rather than 'deactivated' or immunosuppressed.¹ There is evidence to show that the state of alternative activation changes to classical, mycobactericidal activation as a consequence of therapy with anti-TB drugs,² presumably on account of drug action on microbial metabolism; or treatment with a host-immunotropic agent such as TNF- α .³

Drug delivery systems are potentially capable of affecting the activation status of the macrophages they target. Inhalable or respirable delivery systems that contain combinations of anti-tuberculosis drugs have been proposed by several researchers.⁴⁻¹¹ The primary objective of these delivery systems is to achieve high intracellular drug concentrations in macrophages, and thereby, enhanced efficacy with a concurrent amelioration of systemic toxicity. However, the extraordinarily high efficacy of inhaled drug delivery systems in the treatment of tuberculosis, as observed by several groups,^{4, 7, 10} has not been appraised in terms of the contribution of the delivery system towards the activation status of the macrophage. Inhaled particles such as those used in this study are taken up by macrophages through phagocytosis, a process that is known to activate several innate bactericidal mechanisms of the alveolar macrophage.¹² Prior and colleagues have demonstrated that even microparticles made of components considered 'biocompatible' evoke a measurable oxidative burst from cultured murine or primary human macrophages.¹³

Observations such as those mentioned above open up interesting possibilities for investigation of the role of microparticles, not only as inert, biocompatible carriers for antimicrobial agents, but also as potential stimulators of the macrophage's innate microbicidal responses. This study therefore explored the differential abilities of anti-TB drugs— by themselves and in the form

of microparticles, to modulate various events associated with host response to infection and chemotherapy.

Materials and Methods

Materials

Isoniazid and rifampicin of the Indian Pharmacopoeia were donated by Lupin Laboratories Ltd., India. *Poly(D,L-Lactic Acid, PLA)* of intrinsic viscosity 1.11 cps (Birmingham Polymers, Inc., Birmingham, AL, USA) was generously shared with us by Dr. A.K. Panda, National Institute of Immunology, New Delhi. All other chemicals and solvents were of HPLC or Analytical Grade. Dichlorofluorescein, fluorescein isothiocyanate (FITC), ethylene glycol tetraacetic acid (EGTA), 1,2 *bis*-(2-aminophenoxy) ethane- N N N' N' tetraacetoxy methyl ester (BAPTA-AM), pigeon cytochrome c, superoxide dismutase (SOD), cell culture media and supplements were obtained from Sigma, Bangalore, India. Opt-EIA ELISA kits for cytokine assay were purchased from BD Biosciences (India).

Microparticles

Microparticles were prepared by spray drying a homogeneous solution prepared by mixing compatible volumes of 4 parts of rifampicin and 12 parts of PLA dissolved in dichloromethane with 1 part of isoniazid dissolved in methanol. This solution was spray-dried at a feed rate of 5 ml/min; air flow rate of 700–800 NL/h, aspirator level 10; inlet temperature 55 ± 1 °C and outlet temperature 32 ± 1 °C, using a Buchi 190 spray-dryer. Drugs were incorporated at efficiencies > 90% of starting material. The spray drying process is industrially scalable and allowed much higher drug incorporation as compared to emulsion methods employed earlier. The drug content of the microparticles, estimated by HPTLC (CAMAG, Switzerland) was 12.5% w/w isoniazid and 37.5% rifampicin w/w plus unquantified amounts of an adduct of isoniazid and rifampicin. The drug content of these microparticles was thus much greater than that reported previously.⁸ Size distribution

established by microscopy, laser scattering (Malvern Mastersizer 2000, UK) and cascade impaction (In-Tox Products, Albuquerque, USA) indicated a median (suspension) particle size of 5.19 μm , a mass median aerodynamic diameter (MMAD) of 3.57 μm , and a respirable, fine particle fraction (FPF $<4.6\mu\text{m}$) of 79.05+ 8.41%. These properties identified the preparation as suitable for administration as a dry powder inhalation (Muttill et al. unpublished work).

Mouse Macrophages and Bacteria

All animal experiments were conducted after approval and under supervision of the Institutional Animal Ethics Committee of the parent institute. Primary lung macrophages were obtained by bronchioalveolar lavage (BAL) as described earlier.⁸ Briefly, the trachea was cannulated and the lungs and airways lavaged with chilled saline. Recovered cells were counted and allowed to adhere overnight in 96-well plates at a density of 10^5 /well. DMEM containing 10% Foetal Calf Serum (FCS) and antibiotic-antimycotic mix as per manufacturer's instructions (complete medium) was used as culture medium. DMEM without FCS was used for several procedures of washing and dispersion and is referred to as incomplete medium. Plates were maintained in 5% CO_2 at 37°C. The mouse macrophage line J774 clone A.1 was similarly maintained. *Mycobacterium tuberculosis* strain H37Ra was grown in Sauton's medium with 0.05% Tween 80 at 37 °C until mid-log phase. Optical density at 600 nm was used as an approximation of cfu.

Infection and Treatment In Vitro

Primary alveolar macrophages or J 774 cells were infected *in vitro* after overnight incubation, with 25 multiplicities of infection (MOI) of *M. tuberculosis* for 30 min. Wells were then washed with 3 \times 300 μL portions of incomplete medium to remove extracellular bacteria. This protocol results in infection of $>50\%$ of the population as determined by flow cytometry using FITC-tagged *M. tuberculosis* (data not shown). Microparticles sterilized by γ -radiation

were dry-mixed with ~20% w/w of Bovine Serum Albumin (BSA) as suspending agent and sufficient DMEM was added to obtain a theoretical concentration of 3 µg/mL of isoniazid and 10 µg/mL of rifampicin. ⁸ These concentrations correspond to the average C_{max} of these drugs as observed in human patients under treatment with an extremely parsimonious yet efficacious therapeutic regimen.¹⁴ Equivalent amounts of the same drugs dissolved in medium or blank microparticles (by weight), were administered to infected macrophages in parallel sets for 1 hour. Incomplete medium was then used to wash out extracellular drugs as well as microparticles that had not been internalized.

Infection and Treatment In Vivo

Experiments were conducted after ethical clearance by the Institutional Animal Ethics Committee of the institute. Swiss mice weighing 20-25 g, housed in a Class 3 biosafety cabinet (Hera-safe, Kendro, USA) were infected intravenously through the tail vein with 100 µL of a bacterial suspension containing 10⁷ cfu of *M. tuberculosis* in saline. Six mice per group received treatments for three days, starting 24 hours post-infection as follows. Uninfected, untreated animals and those that were infected but not treated were used as controls. Animals receiving oral treatment alone were administered 5 mg/kg of isoniazid and 15 mg/kg rifampicin by gavage, once a day, starting 24 hours after infection, for 3 days. Another group received a combination of oral and inhalation dosing, such that the total dose was the same. The last group was administered inhalations alone. The inhalation dose was determined as described below.

A 'Nose-only' inhalation apparatus was used to administer microparticles to mice.^{7, 8} The apparatus comprised a 15-mL plastic centrifuge tube bearing two orifices: one near the rim to admit the animal's snout and the other at the apex to admit an aeration tube. The diameters of these orifices were sufficient to accommodate the snout of the animal snugly and the aeration tube loosely, leaving annular space to prevent the build-up of positive pressure in the

assembly. A turbulent airstream was admitted into the aeration tube to fluidize the microparticle bed. Animals restrained with the nares inserted in the delivery orifice thus breathed in an atmosphere of microparticulate dust.

Determination of Inhaled Dose of Microparticles

The inhalation apparatus was calibrated for dose delivery to plugs of cotton wool and to uninfected mice (Muttill *et al.* unpublished work). Briefly, weighed amounts of microparticles ranging from 10 to 50 mg in 10-mg increments (n=4 at each dose-level) were taken in the cap of the tube, the bulb actuated 30 times, once every second and fluidized microparticles were allowed to deposit on a plug of cotton wool held securely against the delivery orifice. The amounts of microparticles thus collected were determined gravimetrically and by HPLC. A similar exercise was conducted to establish the amounts deposited on the plug in 30, 60 and 90 seconds of actuation, with *ca* 20 mg of microparticles. Having observed that an average of 2.10 mg (± 0.26 SE) by weight (2.46 ± 0.26 mg by HPLC) were collected on cotton wool plugs in 30 seconds, four mice weighing 23-25 g each were subjected to inhalation under these conditions. Immediately after inhalation, the mice were exsanguinated by cardiac puncture and blood serum separated. Bronchioalveolar lavage cells, total (remaining) lung tissue, spleen, and liver were also harvested from these mice. The drug content in these tissues was estimated by a validated HPLC method to establish a partial material balance. It was thus established that of the 2 mg available for inhalation at the delivery orifice during 30 seconds, the mice inhaled 89 ± 18 μ g. This figure was rounded off to 100 μ g for convenience. Inhalation dosing as described, therefore, resulted in administration of 0.5 mg/kg isoniazid and 1.5 mg/kg rifampicin to the test animals.

Transmission Electron Microscopy (TEM)

Lung lobes of uninfected and infected mice administered drug solutions orally or microparticles by inhalation for three days were excised and stored in cacodylate buffer for a

maximum of 10 min before processing. Sections of 50-60 nm thickness were obtained from tissue embedded in epon, using an ultramicrotome (Phillips). More than 100 fields were examined per sample using a transmission electron microscope (Phillips, Tecnai MegaView).

Determination of Nitrite Accumulation.

Culture supernatants were collected 3, 9 and 24 hours after treatment of primary cells as described above. Supernatants and standards of sodium nitrite (1-200 μM) were incubated for 30 min with freshly-prepared Greiss' reagent at room temperature and the absorbance at 540 nm was measured using a microplate reader (VersaMax, Molecular Devices).

Cytochrome C assay for superoxide (O_2^-).

Superoxide dismutase (SOD)-inhibitable superoxide anion production was estimated over a two-hour period starting immediately after treatment. Primary lung macrophages or J 774 cells were plated in phenol red-free medium at a density of 10^5 /well in 96-well plates. Infection was carried out as described above, followed by a 30-min incubation during which designated wells received the intracellular calcium chelator BAPTA-AM (160 μM) or EGTA (10 mM). Pigeon cytochrome C (50 μM), with and without 100 U/mL SOD was then added to cells and incubated for a further 30 min before treatment. Cytochrome C reduction indicative of superoxide levels was measured by subtracting the absorbance at 550 nm of control wells treated with SOD from that of test wells that had received no SOD.¹⁵ Values were normalized against a cell-free blank. Superoxide formation was expressed as nanomoles/ 10^5 cells.

Cytokine Assay

Cells isolated by BAL were counted and plated as described above. Culture supernatants were collected after 24 h and assayed for $\text{TNF}\alpha$, γ -interferon (IFN- γ), interleukin (IL)-12 and IL-10 using ELISA kits as per manufacturer's instructions. Absolute amounts of cytokines were calculated from standard curves run simultaneously.

Statistics

Results have been expressed as arithmetic means, \pm SE. Normalization to a per cent value was undertaken in one case (Fig. 5), where deviation from central tendency has been expressed in terms of coefficient of variation (CV%). Statistical significance was determined by one-way ANOVA followed by the Bonferroni post-test to compare means at a level of significance of 0.1.

Results

Targeting to Lung Macrophages

Administration of inhalations to mice resulted in targeting of the microparticles to lung macrophages, whether or not the mice were infected. Electron microscopy revealed that macrophages grew to sizes approaching as much as 50 μ m in diameter, having taken up 10 or more particles. None of the fields observed showed the presence of intact bacteria. Electron-opaque debris was observed to co-localize with some microparticles within phagosomes. Such debris was not found in microparticle-containing phagosomes in sections obtained from the lungs of uninfected mice, but infected mice that were left untreated after infection did show phagosomes containing debris. Extensive Golgi vesicle accumulation was observed in the vicinity of phagosomes bearing microparticles. Vesicles were observed to interact with and release contents into phagosomes that contained microparticles as detailed in Figure 1(B).

RNI and ROS Accumulation in Infected Macrophages

NO was not detectable in primary macrophages recovered from uninfected mice (data not shown). As shown in Figure 2, however, administration of drug-containing microparticles to uninfected primary macrophages in vitro led to appreciable NO production. Infection left untreated led to a rising trend of NO production. Treating infected cells with microparticles elicited significantly larger amounts of RNI, specially at 9 hours post infection, when compared to untreated infection. Equivalent amounts of the two drugs in solution did not

induce significantly greater NO production by either infected or uninfected cells. Blank, drug-free microparticles did not evoke a significantly different response from uninfected or infected cells when Greiss' reagent was used to estimate NO (data not shown).

In contrast to NO, infection with *M. tuberculosis* did not induce significant increase in O_2^- , either immediately after adding the bacterial inoculum, or during the hour-long incubation without further treatment. This result was apparent in a SOD-inhibitable cytochrome c reduction assay detailed in Figure 3A. Treatment with drugs in solution or blank microparticles did not evoke ROS either. Drug-containing microparticles, however, induced significant O_2^- secretion by infected macrophages in the same period. There was an early peak of O_2^- within about 2 min of adding microparticles to the well, followed by a more prominent and sustained accumulation of the ROS over the succeeding hour. The extracellular Ca^{++} chelator EGTA strongly inhibited microparticle-induced O_2^- , whereas the intracellular Ca^{++} chelator BAPTA-AM showed lesser inhibition of microparticle-induced O_2^- (Figure 3B). The combination of intracellular and extracellular Ca^{++} chelators showed a cumulative effect.

Intracellular ROS was also assessed by flow cytometry using carboxy 2',7'-dichloro dihydro fluorescein diacetate (data not shown). This ester diffuses into live cells and is cleaved by ROS to generate a fluorescent base. It was observed that a sub-population of primary macrophages produced background levels of ROS, picked up as events of high fluorescence in flow cytometry. Infection resulted in halving the cell numbers showing high fluorescence. Treatment with drugs in solution did not enhance intracellular O_2^- but microparticles did so significantly.

Macrophage Cytokine Secretion

Macrophages recovered from uninfected mice secreted all four cytokines tested over 24 hours in culture, at levels depicted in Figure 4. These 'normal' cytokine levels were first compared with results from all other groups. Significant differences were observed in response to

infection, except in the case of IL-12. ANOVA was then carried out to test the significance of treatment modes, wherein all treated groups were compared with the group receiving infection but no treatment. Infection with the attenuated *M. tuberculosis* strain resulted in upregulation of TNF- α and IFN- γ , as well as IL-10 as expected.¹⁶ Oral treatment with isoniazid and rifampicin in solution, however, downregulated the secretion of these cytokines. Combining inhalations with half the dose of orally-administered drugs did not have significant effects on any of the cytokines in comparison to untreated infection, but maintained IL-12 secretion at the highest level among all treatment groups. Inhalations alone induced significant TNF- α secretion. IFN- γ appeared to be downregulated on treatment, with the group receiving inhalation alone being significantly different from the infected and untreated group. An apparent trend correlating the mode of treatment with the extent of downregulation of IFN- γ was discernible, but lacked statistical significance.

The kinetics of cytokine induction were determined using J774 cells infected with 25 MOI of *M. tuberculosis* and treated with drugs, microparticles, or blank microparticles (Figure 5). Of the four cytokines assayed, TNF- α and IL-12 were observed to be significantly induced on treatment with drug-containing microparticles. Drugs in solution and blank microparticles did not have the same effect.

Discussion

Early events in host-pathogen interaction may be expected to have a strong bearing on the fate of the invading pathogen. The experiments reported here examine a few of these events within a time-span of hours and days, in the context of drug delivery methodology. Free radicals and cytokines produced by cells in culture were evaluated within hours of infection, and cytokine profiles of alveolar macrophages recovered from infected and treated mice were evaluated after a maturation period of 5 days. The data collected are preliminary, and requires cautious interpretation since it may not necessarily extrapolate to the treatment of human TB with

microparticles. There are, nevertheless, interesting pointers to the possible role of the delivery system in our results.

Although one can understand intuitively that innate immune mechanisms might synergise with antimicrobial chemotherapy, documentation of such observations is rare. With special reference to TB, the importance of 'stimulat(ing) the phagocyte' for a favourable therapeutic outcome has been advocated for a century.¹⁷

Targeting microparticles to lung macrophages (Figure 1) induced phagocytosis and vesicle formation in the vicinity of microparticle-containing phagosomes. Golgi activity induction as a consequence of microparticle inhalation is, we believe an important observation. It is established that macrophages infected with *M. tuberculosis* retain their ability to phagocytose and process additional material, provided it is not overly hydrophobic.¹⁸ Whether or not such phagocytosis and processing in the endocytic pathway affects the activation status of the macrophage remains to be seen. Luzardo-Alvarez *et al.* have recently reported that blank biodegradable microspheres merely induce ROS in murine macrophages,¹⁹ although the same group had earlier reported macrophage activation by gentamycin-containing microparticles.¹³ Rapid maturation of the phagosome and its fusion with lysosomes is efficiently inhibited by both virulent and attenuated mycobacteria, and phagolysosome fusion is a good indicator of an effective host response.²⁰ Schlesinger's group has shown by TEM that mannose-capped lipoarabinomannan (LAM) derived from virulent *M. tuberculosis* (but not attenuated species or strains such as H37Ra used here) and inserted into the phagosome membrane is crucial to the inhibition of phagolysosome fusion.²¹ These authors observed that microparticles bearing the LAM in question formed fusion-resistant phagosomes, while removal of the LAM led to the formation of fusogenic phagosomes in human macrophages.²¹ It remains to be established whether microparticle-bearing phagosomes are capable of fusing, as is statistically possible in view of the large surface they present, with other phagosomes, some of which might contain

TB bacilli. Such phagosomes might effectively dilute the surface density of LAM and promote phagolysosome fusion.

The results shown in Fig. 2 suggest that uptake of microparticles is accompanied by significantly higher production of RNI than infection itself within 3 h after treatment. Nitrite concentrations peaked at 9 hours in all groups except untreated infection, but fell when measured at 24 hours. It is probable that downstream reactions of NO_2^- ensued in the interim, as may be expected from the concurrent elevation of ROS (Fig. 3). It is also interesting to note that infection alone did not result in similar kinetics, and that drug treatment actually had the effect of lowering RNI levels over the first 3 hours.

Phagocytosis is accompanied by activation of key macrophage enzymes such as the inducible nitric oxide synthase (iNOS) and phagocyte oxidase (phox) or NADPH oxidase complex. Such activation leads to accumulation of RNI and ROS in the forming phagosome.²² Of these two kinds of oxidative radicals, intracellular RNI are directly mycobactericidal, whereas ROS are proposed to be neutralized by a pathogen-derived catalase-peroxidase rather than SODs. This catalase also possesses peroxynitritase activity.²³ In contrast to classically-activated macrophages, those infected with *M. tuberculosis* are expected to downregulate the production of RNI and ROS.^{1,22}

Enhancement of oxidative radical production clearly indicates classical activation of macrophages as a consequence of treatment with microparticles. Such a conclusion derives greater emphasis from the observation that infection left untreated did not induce ROS. The oxidative radical response was higher when infected cells were given microparticles, rather than when uninfected cells were thus treated. It remains to be elucidated whether the very same macrophage that is infected also ingests microparticles and then proceeds to enter a program of classical activation, an experiment that is planned to be conducted using 3-colour flow cytometry. In any case, it is encouraging to observe that the microparticles were

apparently regarded as biocompatible by uninfected cells, but stimulatory when *M. tuberculosis* infection was present in the cell population.

Primary macrophages infected *ex vivo* or J 774 cells infected *in vitro* were used to assess free radical generation. This method of infection was preferred over the alternative of isolating alveolar macrophages from infected mice in order to ensure a higher degree of uniformity in infection. However, it was considered equally pertinent to examine macrophages recovered from infected animals. These were recovered from mice infected and treated as described, and the secretion of a small panel of cytokines by uninfected and infected populations was examined (Figure 5). The levels of cytokines observed in the case of the uninfected and untreated control group were taken to represent a 'normal' or baseline picture, and upregulations/downregulations were inferred with reference to these 'normal' levels.

While there is consensus on the protective roles of IL-12, IFN- γ and TNF- α in the immunology of TB,²⁴ the relative contributions of each of these remain to be ascertained in the early stages of infection when the key cytokine producers are recently-infected macrophages. In studies reported here, treatment with drug-containing microparticles most significantly affected the secretion of TNF- α . Kornfeld and colleagues have extensively demonstrated the role of TNF- α as a mediator of caspase-induced apoptosis of macrophages infected with *M. tuberculosis*H37Ra,²⁵ especially at levels of infection such as those used in the studies reported in Figure 5.²⁶ It is possible that microparticles evoke the same strategy that the host macrophage employs against *M. tuberculosis* If this were so, microparticles containing anti-TB drugs could represent a mechanism of anti-TB therapy akin to that of treatment with exogenous TNF- α .³ Investigations on the induction of apoptosis in infected human macrophages are currently in progress to address this issue.

Downregulation of macrophage-derived TNF- α and IFN- γ in response to per-oral treatment was unexpected. It was assumed that the drugs would neutralize the pathogen's survival

strategy, and the cells would respond by making 'protective' cytokines. The observations do not bear out this supposition. However, it is likely that bacterial killing had been completed in the duration of treatment and the macrophages had recovered, or were close to recovering baseline levels of cytokine secretion. No viable bacteria could be recovered from the lungs of the infected mice upon plating homogenates on 7H10 Agar-OADC (data not shown), so the issue of bacterial survival remained unresolved. To address the time-frame of cytokine secretion in response to different modes of treatment, it was decided to explore the kinetics of cytokine induction *in vitro* as shown in Figure 5.

Cytokine secretion after *in vitro* infection of the cell line resembled observations in primary macrophages recovered from infected animals, in that TNF- α and IL-12 were the only cytokines that showed significant alterations. As in living animals, drug-containing microparticles evoked the most significant response. IL-10 and IFN- γ did not change significantly from baseline values regardless of treatment. These results were interpreted to indicate that TNF- α represents the most significant facet of the host response evoked by microparticles in the early stages of infection.

Host-defense responses have traditionally been of little concern when microbicidal drugs are administered. The underlying assumption is that chemotherapy contributes the major part of antimicrobial action, and that host responses are secondary. In the case of inhalable microparticles reported by several groups,⁴ including the present authors,^{7, 8} however, there is remarkable enhancement of efficacy. Results reported here indicate that treatment of macrophages with drug-containing microparticles, but not the drugs themselves in solution, leads to several cellular and biochemical events that are in conformity with the profile of a classically 'activated' macrophage.

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Transparency Declaration

RS and PM are also co-authors of a patent application cited as reference 5 in this article.

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Legends to Figures

Figure 1 (A): Electron micrograph of resident macrophage in lung section of mouse infected with *M. tuberculosis* H37Ra and treated with inhalations of microparticles for 3 days. Arrow indicates electron-opaque debris. (B): Detail of the same cell, showing Golgi activity. Arrows indicate golgi vesicles interacting with phagosome membrane and releasing contents into the phagosome.

Figure 2: Nitric oxide production by primary lung macrophages at 3 (filled bars), 9 (hatched bars) and 24 (hollow bars) hours after treatment with. 'U+MP' refers to uninfected cells treated with MP; 'Inf' to cells receiving 25 MOI of *M. tuberculosis* but no further treatment, 'Inf+D' to infected cells exposed to drugs in solution and 'Inf+MP' to infected cells treated with an equivalent dose of microparticles. The hash sign (#) indicates significant ($P=0.1$, in one-way ANOVA, Bonferroni post-test) differences at 3 h after treatment compared with the group receiving infection alone (Inf). The asterisk marks differences at 9 h.

Figure 3: (A) Superoxide production by J774 cells in response to infection and treatment with drug-containing MP (filled circles), blank MP (open circles) and drugs in solution (filled squares). Infected cells left untreated and examined at the same time points produced negligible superoxide (open squares), but an early, low-level response was discernible when cells were examined immediately after adding the bacterial inoculum to the culture (open diamonds). (B) Calcium dependence of the superoxide response (circles) is illustrated by

inhibition with EGTA (upright triangles) or BAPTA-AM (inverted triangles) used individually or in combination (diamonds). Error bars indicate SE (n=4).

Figure 4: Comparison of cytokine secretion by cells recovered from uninfected, untreated mice (XX); mice infected with *M. tuberculosis* H37Ra and left untreated (IX); or infected and treated for three days with oral doses (IO), a combination of oral and inhalation dosing (IOI) or inhalations alone (II). Bars show group means and error bars the SEM. Significant differences at the level of 0.1 in a one-way ANOVA are indicated by the hash sign (#) when cells from uninfected mice were compared with all other groups. The asterisk (*) indicates significant differences when cells from infected but untreated animals were compared with treatment groups.

Figure 5: Induction of cytokine secretion in J774 cells receiving neither infection nor treatment (filled bars), infection alone (rising hatching), or infection in vitro with *M. tuberculosis* followed by treatment with drugs in solution (cross-hatching), drug-containing MP (hollow bars) or blank, drug-free MP (falling hatching). Uninfected cells administered drug-containing MP (vertical lines) served as an additional control. Error bars show CV%.

Figure 1

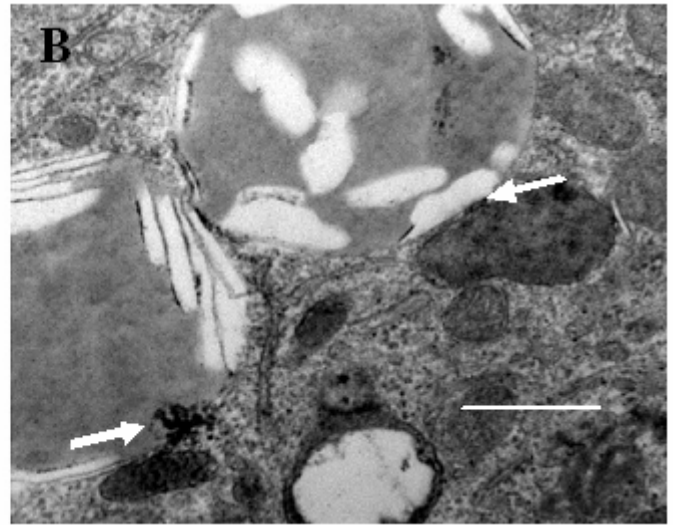
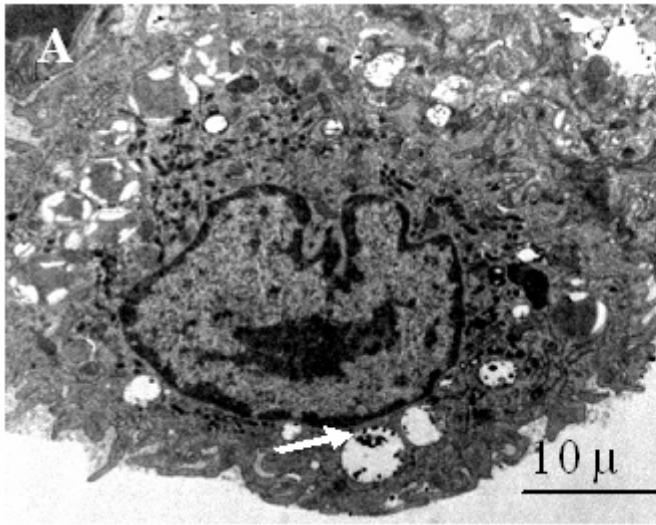


Figure 2

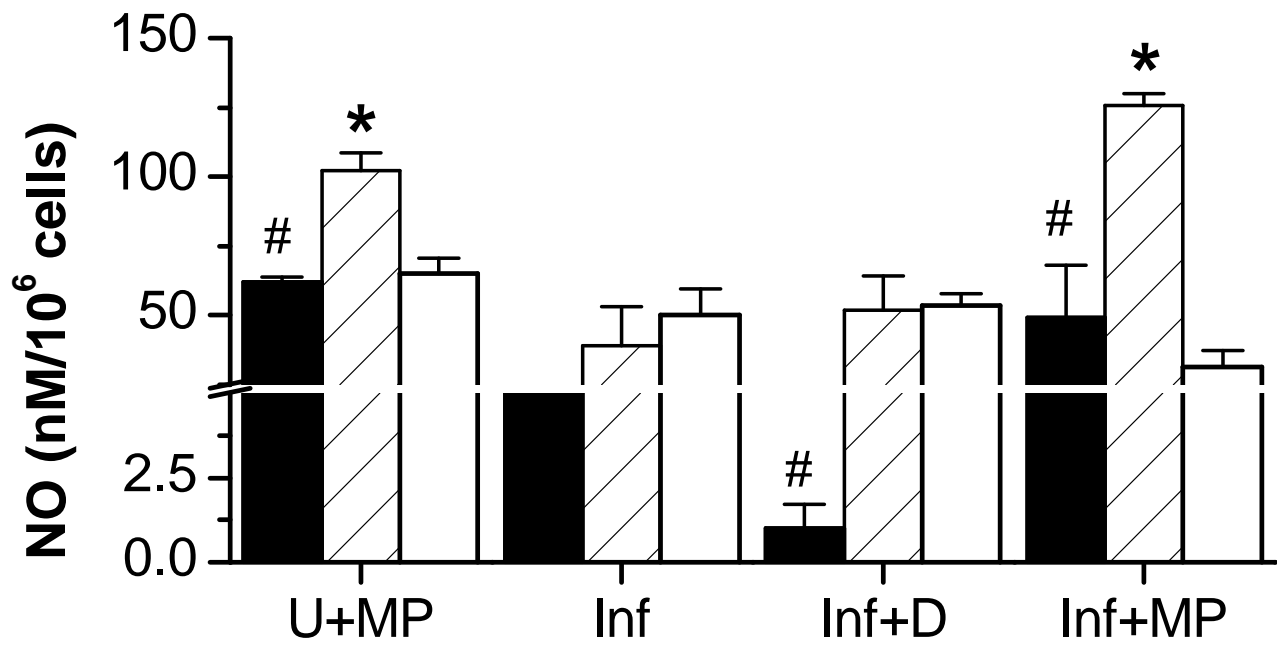


Figure 3

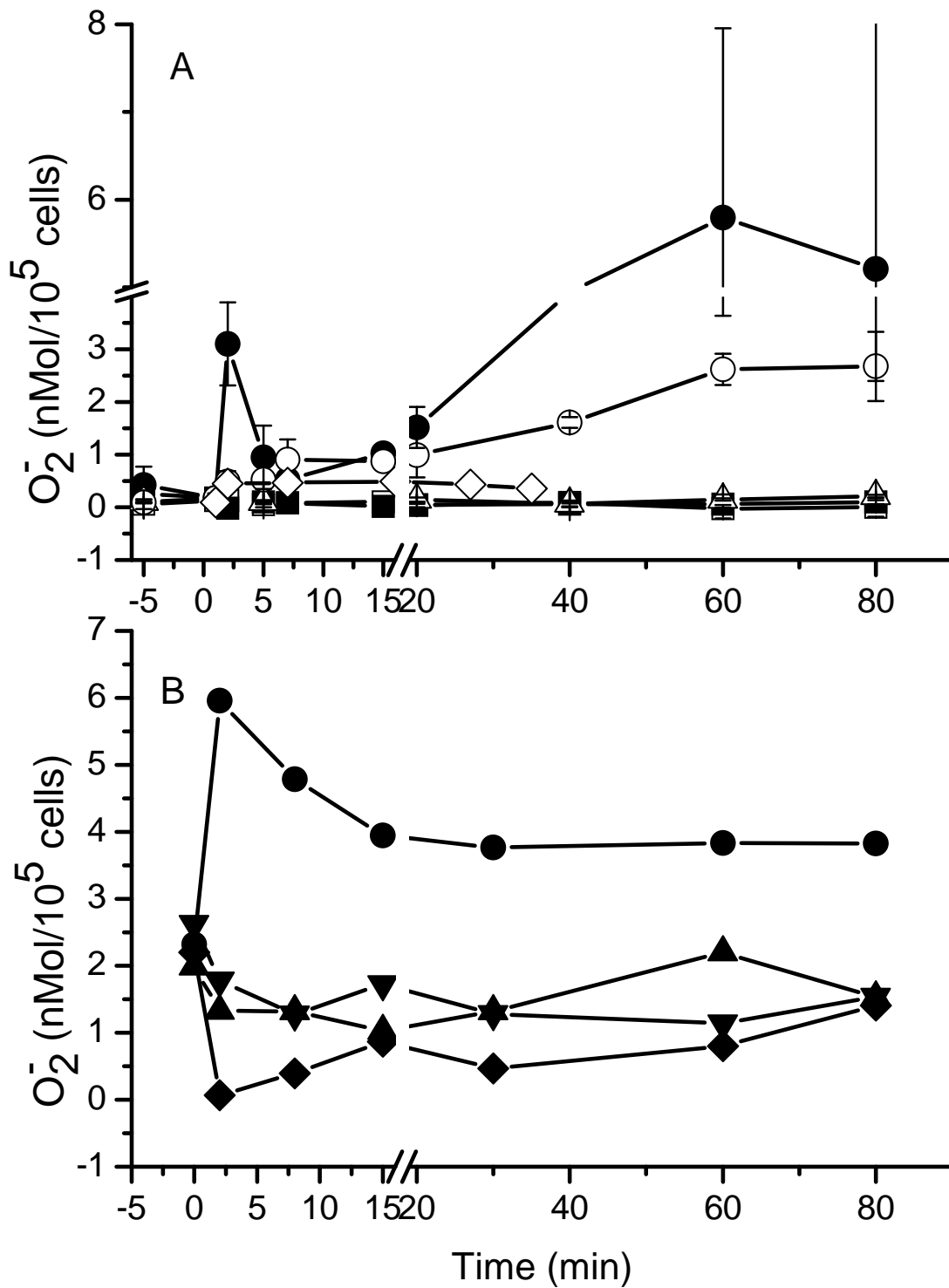


Figure 4

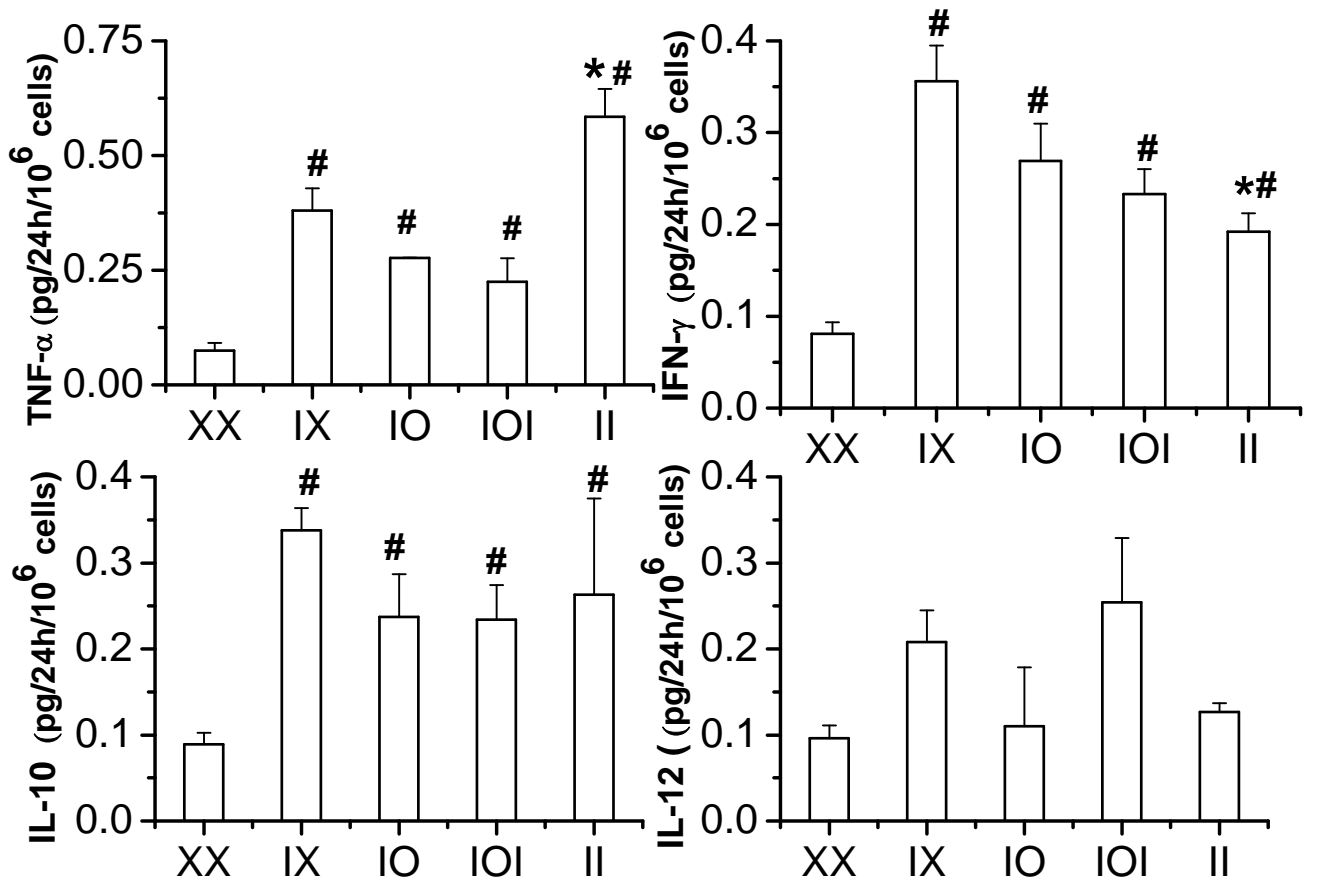


Figure 5

