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Mycobacterium tuberculosis may escape helper T cell recognition by infecting human fibroblasts

Sabrina Mariotti^a, Valeria Sargentini^a, Manuela Pardini^a, Federico Giannoni^a, Marco De Spirito^b, Maria Cristina Gagliardi^a, Emanuela Greco^c, Raffaella Teloni^a, Maurizio Fraziano^c, Roberto Nisini^{a,*}

^a Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità, Roma, Italy

^b Istituto di Fisica, Università Cattolica del Sacro Cuore, Roma, Italy

^c Dipartimento di Biologia, Università di Roma "Tor Vergata", Roma, Italy

ARTICLE INFO

Article history:

Received 13 June 2012

Accepted 19 February 2013

Available online xxx

ABSTRACT

The host immune response can limit *Mycobacterium tuberculosis* (Mtb) spreading in primary tuberculosis (TB) without eradicating all bacilli, which can persist causing latent TB infection and are responsible for reactivation TB. Persistent Mtb is confined to granulomas within phagocytes, but it is also found in other non-immune cells. We focused on fibroblasts since these cells participate to the granuloma formation and were shown to be infected in latent TB infections.

We show that *in vitro* both Mtb and Bacille Calmette-Guérin actively replicate in human fibroblasts. Mycobacterial infection of fibroblasts causes a significant inhibition of interferon (IFN)- γ induced membrane expression of major histocompatibility complex class II molecules in these cells. The functional consequence of *in vitro* infection is a significant reduction of the fibroblast capacity to present peptides and soluble proteins to autologous specific CD4⁺ T cell clones. Moreover, fibroblasts are capable of presenting antigen derived from the processing of heat-killed Mtb, but not from viable Mtb. Data indicate that IFN- γ treated fibroblasts are capable of presenting antigens derived from the processing of whole bacteria in addition to the capacity to present peptides and isolated proteins. Interestingly, Mtb infected fibroblasts lose this capacity, suggesting that Mtb may evade T helper immune surveillance by infecting fibroblasts.

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1. Introduction

The intracellular pathogen *Mycobacterium tuberculosis* (Mtb) causes tuberculosis (TB) and is able to infect antigen presenting cells (APCs), including macrophages and dendritic cells (DCs) [1]. In these cells, Mtb survives in modified phagosomes and uses multiple mechanisms to evade both innate and adaptive host immunity, including inhibition of phagosome maturation, resistance to innate microbicidal mechanisms and cytokine-mediated host defenses, as well as inhibition of antigen presentation [2,3]. Since

TB is mainly transmitted via airborne droplets from people with active respiratory disease to susceptible individuals, Mtb most commonly affects the lungs. Thus, it is generally accepted that the first cell types confronting Mtb during primary infection are alveolar macrophages and type II pneumocytes [4,5]. However, this microorganism can enter a variety of other cell types *in vitro*, as shown by several authors following the early observations by Shepard, [6] who demonstrated that Mtb can enter monolayers of HeLa, monkey kidney and human amnion cells [6,7]. More recently, Mtb has been shown to infect lung epithelial and endothelial cells [8–11] as well as adipocytes [12] and fibroblasts [13,14], in addition to professional APCs, such as monocytes and DCs, even if the capacity of Mtb to replicate within these different cell types may vary significantly [15,16]. The capacity of Mtb to infect cells different from macrophages has also been proven by *ex vivo* experiments showing Mtb or its DNA in different cell types in both TB patients and subjects with latent TB infection (LTBI), corroborating the significance of *in vitro* experiments [12,17]. In particular, Mtb was shown to persist intracellularly in lung tissue without histological evidence of tuberculous lesions and Mtb DNA was shown

Abbreviations: APCs, antigen presenting cells; BCG, Bacillus Calmette-Guérin; CIITA, class II transcriptional transactivator; CFU, colony forming unit; CM, complete medium; DCs, dendritic cells; FCS, fetal calf serum; IFN, interferon; LTBI, latent TB infection; MHC, major histocompatibility complex; MOI, multiplicity of infection; Mtb, *Mycobacterium tuberculosis*; RNFib, primary autologous fibroblasts; PPD, purified protein derivative; TB, tuberculosis.

* Corresponding author. Address: Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy. Fax: + 39 06 49387112.

E-mail address: roberto.nisini@iss.it (R. Nisini).

to be situated not only in macrophages but also in other non-professional APCs [17]. The capacity of Mtb to infect and replicate within different cell types may be dependent on both the capacity of cells to internalize and host Mtb and/or to the strength of the immune response, which may force Mtb to colonize alternative cell types possibly in relation to the TB stage. This is relevant since internalization in non-professional APCs may represent an important pathogenic feature of mycobacterial infection. In these cells major histocompatibility complex (MHC) class I expression is constitutive but class II is not expressed in normal conditions, although it may be induced by interferon (IFN)- γ release [18]. Non-professional APCs are not capable of priming antigen specific T cells, but they can present antigen to memory CD4⁺ T cells in inflammatory microenvironments where they are induced to express MHC class II molecules by locally released IFN- γ . The capacity of mycobacteria to infect, survive and interfere with antigen processing and presentation in these cells is far from being elucidated [19]. Moreover the role of non-professional APCs in TB is intriguing in the light of their possible contribution in maintaining the infection latent and possibly representing the mycobacterial reservoir in LTBI [17]. In this paper we investigated whether the known capacity of Mtb to interfere with MHC class II expression and the consequent impairment of antigen presentation could be observed in non professional APCs such as fibroblasts. In fact, fibroblasts are recruited [20] and proliferate into TB lesions where they are involved in tissue remodeling and granuloma formation, thus representing possible target for Mtb infection.

2. Materials and methods

2.1. Ethics statement

This study has been approved by the Istituto Superiore di Sanità review board and written informed consent has been obtained by the healthy volunteers who participated to the study.

2.2. Bacterial cultures

Mtb H37Rv (ATCC 27294) and Bacillus Calmette-Guérin (BCG), (ATCC 27291) were grown with gentle agitation (80 r.p.m.) in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 (Sigma Chemical Company, St. Louis, MO) and 10% Middlebrook ADC enrichment (Becton Dickinson, Mountain View, CA) as previously described [21]. Logarithmically growing cultures were washed two times in RPMI 1640. Mycobacteria were resuspended in RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS) and then stored at -80°C . Vials were thawed, and bacterial viability was determined by counting the number of colony forming unit (CFU) on Middlebrook 7H10 agar plates. For some experiments, Mtb was heat killed at 80°C for one hour (HK Mtb).

All Mtb and BCG preparations were analyzed for LPS contamination by the Limulus lysate assay (BioWhittaker) and contained less than 10 pg/ml of LPS.

2.3. Fibroblasts

MRC-5 cells (human fetal lung fibroblasts; European Collection of Cell Cultures (ECACC no. 84101801) were cultured in RPMI 1640, supplemented with 1 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 10% FCS (complete medium, CM) (all from Euroclone Ltd., UK) in tissue culture flasks (Becton Dickinson, France) maintained at 37°C in 5% CO_2 . From the same donor (RN) of antigen specific T cell clones (see below), who gave his informed consent to participate to the study, a skin biopsy was ob-

tained and primary autologous fibroblasts (RNFib) were derived as previously described [22]. Briefly, 3-mm skin punch biopsies were cut into small fragments (approx. 1-mm) and cultured in RPMI 1640 medium containing 20% FCS. After the skin fibroblasts had grown out of the explants and reached confluence, they were trypsinized and transferred to tissue culture flasks. These cells, RNFib, were maintained in culture in CM and split every 1–2 weeks as they reached confluence. All experiments were performed with skin fibroblasts taken after 10 to 20 culture passages.

2.4. Fibroblasts infection

MRC-5 and primary RNFib fibroblasts were harvested from tissue culture flasks by trypsin-EDTA (Euroclone, Italy). After washings, fibroblasts were counted and plated at 1×10^5 cell/well into 24-well plates. Cells were allowed to adhere and then cells were infected with Mtb or BCG at multiplicity of infection (MOI) of 3:1 and 10:1 respectively for 18 hours. Then wells were washed several times using warm RPMI and eventually fresh CM was added to the wells (T0). To measure mycobacterial growth, after 24 h, 72 h and 6 days of culture supernatants were collected and the cells lysed using distilled water containing 0.1% saponin for 5 min at room temperature. Then, CFU for each well were separately calculated in supernatants and cell lysates, with the exception of the well representative of T0, where only intracellular CFU were calculated. For CFU count, cell lysates and supernatants were briefly sonicated and 10-fold dilutions were performed using 0.05% tween 80 in distilled water and serial dilutions were plated on Middlebrook 7H10. After 21 days of incubation at 37°C in 5% CO_2 atmosphere, CFU in each plate were enumerated. Values are expressed as a mean of three independent experiments \pm standard deviation.

2.5. RNA extraction and qRT-PCR

Human fibroblasts were lysed in TRIzol Reagent (Invitrogen Carlsbad, CA, USA), total RNA purified with SV Total RNA Isolation System (Promega, Madison, WI, USA), DNase I treated (Promega) and reverse transcribed using ImProm-II RT (Promega), according to manufacturer's instructions. qPCR was performed in an iCycler iQ (Bio-Rad, Hercules, Calif.) with iQ SYBR Green Supermix (Bio-Rad) and 500 nM each primer for 40 cycles as follows: 40 s at 95°C , 40 s at 57°C , and 50 s at 72°C for 40 cycles with a final melting curve. *Beta-actin* mRNA was used as housekeeping gene for ΔCt determination. Forward and reverse primers used for *Beta-actin*, *HLA-A*, *B* and *C*, *CIITA* and *HLA-DR α* genes were: hBAct-S, 5'-TCCTTCTGGGCATGGAGTC-3', hBAct-AS, 5'-CAGGAGGAGCAATGATCTTGATC-3', HLA-ABC-S, 5'-CTACGACGGCAAGGATTAC-3', HLA-ABC-AS, 5'-TTCCCGTTCTCCAGGTATC-3', CIITA-S, 5'-GGCTGGGATTCCTACACAATG-3', CIITA-AS, 5'-CAACTCCATGGTGGCACAC-3' [23], HLA-DR α 3-S, 5'-CCCTGGGCCTGACTGTGG-3' and HLA-DR α 3-AS, 5'-CCATCACCTCCATGTGCCTTAC-3'. The fold change compared to control untreated fibroblasts was calculated using the $2^{-\Delta\Delta\text{Ct}}$ formula. All reactions were performed in triplicate.

2.6. Microscopy

Mtb and BCG infection of human fibroblasts was assessed using fibroblasts grown on cover-slip and stained with the Kinyoun method [21]. Intracellular localization was assessed by staining mycobacteria with auramine (Becton Dickinson, MD) [24] and analysis by confocal microscopy. Briefly, MRC-5 cell monolayer was exposed for 3 h to Mtb at the MOI of 1:1. Fibroblasts were washed with PBS to remove non-endocytosed bacilli, fixed with 4% paraformaldehyde for 10 min at 4°C and finally washed with PBS. The localization of Mtb was determined by incubating in-

fecting monolayer with auramine for 20 min at room temperature, followed by 3 min incubation in 0.5% acid alcohol and repeated washings with PBS. Finally, cover slips were mounted using Vectashield mounting medium H-1000 (Vector laboratories, CA) and their edges were sealed with nail polish. The samples were analyzed by confocal laser scanning microscopy using a Leica TCS-SP2 operating system. Auramine was excited by using the 458 nm line of an argon laser for excitation, and fluorescence emission was collected from 500 to 530 nm. Before each experiment, detector gain and laser intensity were adjusted to minimize background signals and held constant during acquisition.

2.7. Flow cytometry

Fibroblasts were infected as above described with BCG (MOI 10:1) and Mtb (MOI 3:1) for 18 h. Fibroblast monolayer was then accurately washed in order to remove all the non-endocytosed/bounded bacteria and stimulated for other 72 h with scalar doses of recombinant human IFN- γ (12.5–400 ng/ml, Pharmingen; San Diego, CA, USA). To detect cell surface expression of HLA-A, B, C and HLA-DR, IFN- γ -stimulated and non infected, BCG or Mtb-infected cells were removed from wells with trypsin, washed with PBS 1% FCS and stained with FITC conjugated anti-class I and PE-conjugated anti-class II monoclonal antibodies or with appropriate isotype controls (PharMingen) for 25 min on ice, and fixed overnight with 4% paraformaldehyde. In two experiments, intracellular HLA-DR was detected by treating infected and non-infected fibroblasts cultured with 200 ng/ml of IFN- γ with the BD Cytofix/Cytoperm™ kit for intracellular staining (BD-Pharmingen) before the addition of anti-class II monoclonal antibodies and isotype control antibody. Stained cells were finally washed and analyzed by flow cytometry using a FACScanto flow cytometer (Becton Dickinson Biosciences, San Jose, CA). From each tube, 2×10^4 viable cells were acquired. Data files were analyzed with the BDFACS Diva software.

2.8. T cell clones

The T cell clone RNppd64 specific for purified protein derivative (PPD) and the clone RNF2 specific for the *Candida albicans* antigen MP65 and for the peptide T1b, an MP65 derived peptide (Tana Laboratories, Houston, Texas) were isolated as previously described [25]. The clones were restimulated with PHA (HA-16 from Murex Diagnostics) in the presence of recombinant human IL-2 (R&D) and irradiated peripheral blood mononuclear cells as feeder for at least 20–25 days, then cells were washed twice to remove residual IL-2, counted and resuspended in CM.

2.9. Antigen presentation assays

To test the capacity of fibroblasts to function as non-professional APCs, the autologous primary human fibroblasts line RNFib was seeded in 96-well flat-bottomed culture plates at 1×10^4 cell/well and allowed to adhere. Cells were then infected with Mtb or BCG (MOI of 3:1 or 10:1, respectively) for 18 h before the addition of IFN- γ (200 ng/ml). After 72 h, fibroblasts were washed with warm RPMI to remove IFN- γ and warm CM was added together with MP65 (a kind gift from Dr. R. La Valle) at 10 μ g/ml or T1b at 1 μ g/ml and the MP65-specific T cell clone isolated from the same donor of fibroblasts at 3×10^4 cell/well. After 48 h activation was measured by IFN- γ release in supernatants using an ELISA kit (R&D Systems) according to the manufacturer's instructions. In a series of experiments, IFN- γ -treated RNFib were infected overnight with Mtb at different MOI (starting from 30:1), treated with HK Mtb or incubated with Mtb that has been cultured o/n with moxifloxacin (4 μ g/ml), kanamycin (100 μ g/ml) or isoniazid

(2 μ g/ml) or with 5 μ g/ml PPD. Then, cells were washed and co-cultured with an autologous PPD-specific T cell clone. For some experiments IFN- γ treated RNFib were infected o/n with BCG or treated with HK BCG (MOI 10:1) before the addition of autologous responsive T cell clone.

2.10. Statistical analysis

All the statistical analysis were performed using the fourth version of GraphPad Prism Software. The data were analyzed using the non-parametric Mann-Whitney *U* test. All tests for statistical significance were two-tailed and *p* values <0.05 were considered significant.

3. Results

3.1. Mycobacteria infect human fibroblasts

The human fibroblasts cell line MRC-5 and RNFib were infected with Mtb or BCG at MOI of 3:1 or 10:1 respectively. After 2 days of incubation cells were fixed and stained by Kinyoun method and microscopically observed to evaluate the cell-mycobacteria interactions. Fig. 1 reports Kinyoun staining of MRC-5 fibroblasts cultures after BCG (Fig. 1A) or Mtb (Fig. 1B) infection. Mycobacteria are associated to fibroblasts and only rare mycobacterial cells are found in the intercellular spaces. In particular, mycobacteria appear within the fibroblasts, since they often draw the contour of single cells and are observed within fibroblast vacuoli. To confirm the intracellular localization of Mtb in infected fibroblasts, we examined auramine stained mycobacteria after infection by confocal microscopy. The Z-scan of the cell cytoplasm reveals the intracellular localization of mycobacteria soon after a 3 h infection time (Fig. 1C). Moreover, Mtb or BCG growth in infected human fibroblasts was measured by counting the CFU after 1, 3 and 6 days of culture. Mtb or BCG were allowed to infect adherent fibroblasts overnight and then non internalized mycobacteria were removed by vigorous washings. Growth was measured at the time intervals indicated in Fig. 2A (Mtb) and Fig. 2B (BCG) by counting CFU both in supernatants and in lysed fibroblasts as specified in the method section. Starting from day 1, Mtb and BCG begin to replicate within fibroblasts, while only a limited number of CFU are observed in fibroblast free supernatants. Similar results were obtained using RNFib at the same time intervals (data not shown).

3.2. Mycobacteria reduce the IFN- γ mediated up-regulation of MHC class II membrane expression in human fibroblasts

MHC class II expression on fibroblasts increases almost linearly in the IFN- γ range of 12.5–400 ng/ml (Fig. 3A). Umetsu et al. [22] showed the capacity of fibroblasts to present tetanus toxoid to CD4⁺ T cell clones by inducing MHC expression on fibroblast with IFN- γ at 100 ng/ml. To test the influence of Mtb as well as BCG in the expression and IFN- γ mediated induction of MHC class II molecules, uninfected or mycobacteria infected fibroblasts were treated or not with a high dose of recombinant IFN- γ (200 ng/ml) and then cultured for three days before analysis. As shown in Fig. 3B, fibroblasts express MHC class I, but not class II molecules in the absence of stimuli. Infection with both BCG and Mtb causes a slight increase of MHC class I but not class II expression in fibroblasts. Upon IFN- γ stimulation of fibroblasts, class I molecule expression is highly up-regulated and expression of class II molecules is induced. However, IFN- γ stimulation of Mtb or BCG infected fibroblasts caused a marked reduction of MHC class II molecule expression and a reduced, but not statistically significant, MHC class I up-regulation (Fig. 3B and Table 1). Interestingly, the

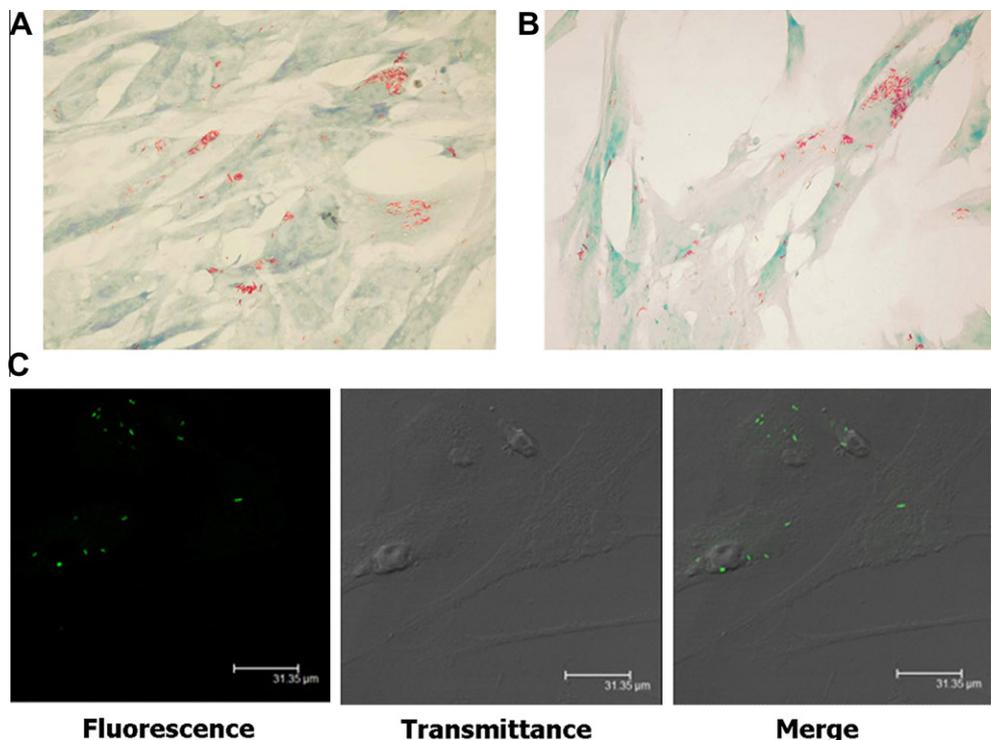


Fig. 1. Mycobacteria infect human fibroblasts. The human fibroblasts cell line MRC-5 was infected with BCG or Mtb at MOI of 10:1 or 3:1 respectively. The Kinyoun staining of fibroblasts cultures after BCG or Mtb infection is reported in (A) and (B) respectively. (C) Confocal microscopy images of human fibroblasts infected with Mtb (MOI 1:1) for three hours and then stained with auramine. To spatially localize mycobacteria and to show the integrity of fibroblast plasma membrane, the auramine fluorescence, collected from the intracellular microscope focal plane, has been merged with the transmission image by using the Leica TCS-SP2 operating system.

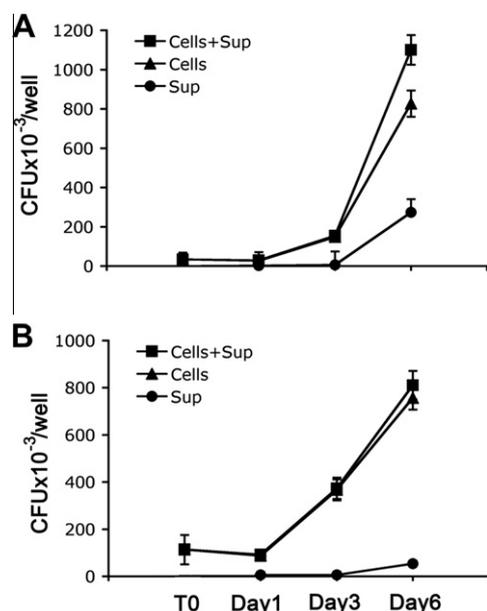


Fig. 2. Mycobacteria grow into human fibroblasts. Mtb (A) or BCG (B) growth in MRC-5 fibroblasts was followed by counting the CFU after 1, 3 and 6 days of culture in comparison to the initial infection dose (T₀). The CFU reported refer to numbers of mycobacteria counted in the culture supernatants (Sup), in the fibroblast lysates (cells) and summing the counts in supernatants and cell lysates (Cells + Sup). Values are expressed as a mean of three independent experiments \pm standard deviation. Similar results were obtained using primary human fibroblasts.

MHC class II molecule expression measured after fibroblast permeabilization was not influenced by Mtb infection. In fact, the total amount of MHC class II molecules was found unchanged when

both intracellular and cell surface expression were measured (Fig. 3C) suggesting that Mtb does not inhibit MHC class II gene expression, but rather its export to fibroblast cell membrane. To confirm that Mtb infection does not inhibit MHC molecule gene expression, we monitored mRNA changes in response to IFN- γ and Mtb infection, by qRT-PCR. We found that HLA class I mRNA was upregulated by Mtb and IFN- γ , with scarce synergistic effect in combined conditions. Differently, IFN- γ modulated the expression of HLA-DR, but we did not observe significant changes in the presence of intracellular Mtb, despite increased expression of MHC class II transcriptional transactivator (CIITA) in IFN- γ -treated Mtb-infected cells, compared to IFN- γ treated non-infected cells (Fig. 4).

3.3. Mycobacteria restrain the MHC class II dependent antigen presentation by human fibroblasts

The antigen presenting function of mycobacteria infected fibroblasts was studied in an autologous model. We first show that autologous fibroblasts RNFib are capable of presenting peptides to a specific CD4⁺ T cell clone only if previously treated with IFN- γ , that induces the up-regulation of MHC class II (Fig. 5A). Interestingly, Mtb infected fibroblasts are less efficient than uninfected fibroblasts to present the peptide to the T cell clone. This reduced stimulation of the T cell clone when Mtb infected fibroblasts are used as APCs can be explained by the reduced MHC class II molecule expression observed by immunofluorescence, since peptides do not require processing. To study the possible interference of Mtb in the processing of antigens in non-professional APC, infected fibroblasts stimulated with IFN- γ were incubated with MP65, a *C. albicans* mannoprotein and co-cultured with a specific T cell clone isolated from the same donor of fibroblasts. Fig. 5B shows that fibroblasts are able to process and

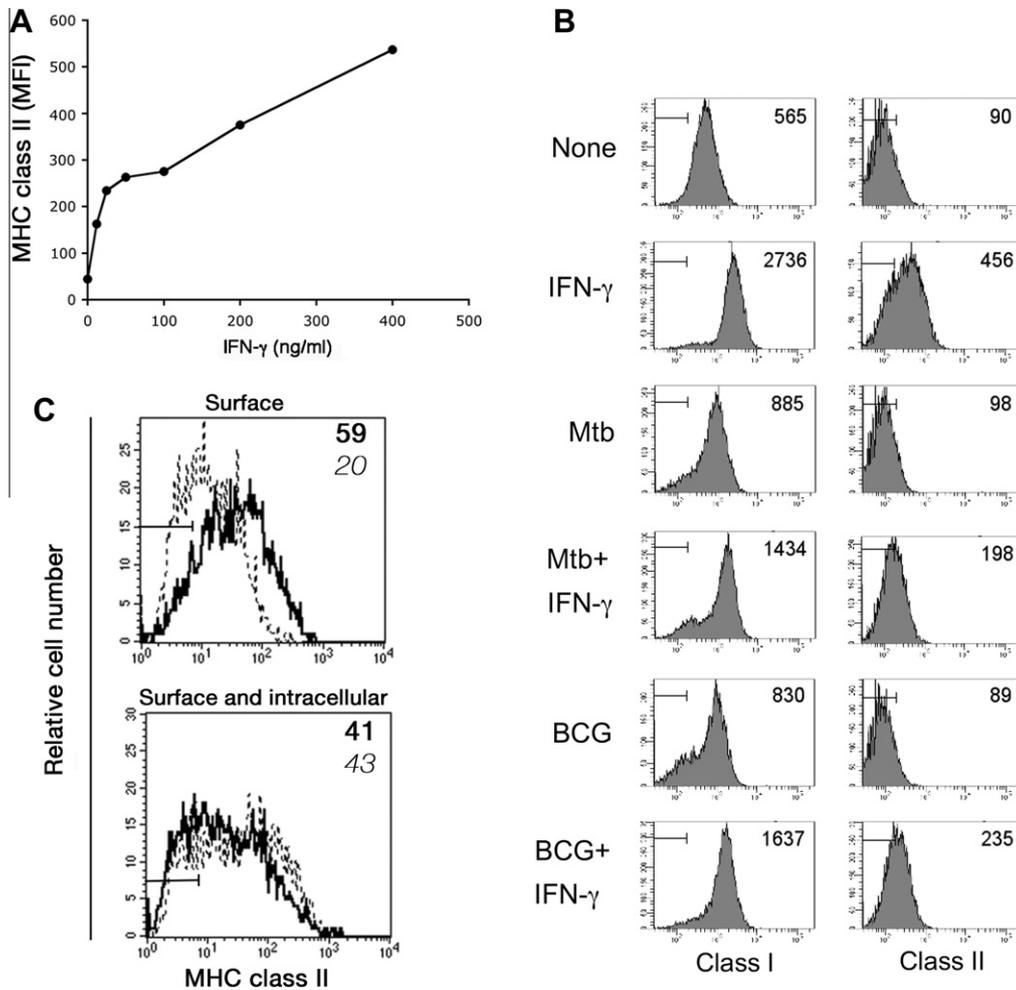


Fig. 3. Mycobacteria reduce the IFN- γ mediated up-regulation of MHC class II membrane expression in human fibroblasts. (A) Cell surface expression of MHC class II molecules (measured as median intensity of fluorescence, MFI) analyzed by flow cytometry on fibroblasts treated for three days with scalar doses of IFN- γ . (B) MHC class I and II expression was determined by flow cytometry in human fibroblasts infected or not with Mtb or BCG in the presence or absence of a further incubation with IFN- γ . None indicates non-infected cells not treated with IFN- γ . Numbers in the histogram plots indicate the median fluorescence intensity and the bar indicates the negative marker set with the appropriate isotype control. One experiment representative of three is shown. (C) Expression of MHC class II molecules in infected (dotted histograms) and non-infected (bold histograms) fibroblasts treated for three days with IFN- γ measured as MFI. Cells were permeabilized (surface and intracellular) or not permeabilized (surface) before the staining with relevant antibody. Numbers indicate the median intensity of fluorescence of non-infected (bold) or Mtb infected (italics) fibroblasts. One experiment representative of two is shown.

Table 1

Mean reduction of MHC molecule surface expression in infected fibroblasts after treatment with IFN- γ .

	BCG + IFN- γ ($n = 5^a$)	Mtb + IFN- γ ($n = 3$)
MHC class I	30.7 \pm 26 ^b $p = 0.83$	34.3 \pm 22 $p = 0.18$
MHC class II	46.4 \pm 7.9 $p < 0.01^*$	57.8 \pm 9.5 $p < 0.05^*$

^a n = number of independent experiments.

^b Percentage MFI reduction in comparison to non-infected IFN- γ treated fibroblasts \pm SD.

* p Values statistically significant.

present the soluble protein MP65 to the specific T cell clone only if treated with the MHC class II inducer IFN- γ . More interestingly, Mtb, and to a lesser extent BCG, block the presentation capacity of fibroblasts to the T cell clone, which in turns is not stimulated to secrete IFN- γ .

Moreover, to test the capacity of fibroblasts to process Mtb and to present its specific antigens, IFN- γ stimulated fibroblasts were infected with viable Mtb (MOI 3:1) or treated with Mtb killed by

heat or by o/n culture with moxifloxacin, kanamycin or isoniazid. As a read out, the IFN- γ release by an autologous PPD specific T cell clone was used. Data show that IFN- γ stimulated fibroblasts have the capacity to present antigens derived from the processing of a whole bacterium, since they are able to act as APCs for the HK Mtb (Fig. 5C). However, when infected with the viable Mtb, fibroblasts are unable to process and present Mtb derived antigens, suggesting that viable mycobacteria actively interfere with the presentation and processing of fibroblasts. Interestingly, not only HK, but also moxifloxacin, kanamycin and isoniazid treated Mtb (Fig. 5D) were also processed by fibroblasts and presented to specific T cell clones.

4. Discussion

In this work we confirm that *in vitro* Mtb infects human fibroblasts. Infection seems not to be related to the virulence of Mtb, since also the vaccine strain BCG is endowed with the same capacity to infect and grow within fibroblasts. These observations are in line with previous published data demonstrating the capacity of Mtb, as well as attenuated or non-pathogenic mycobacteria such

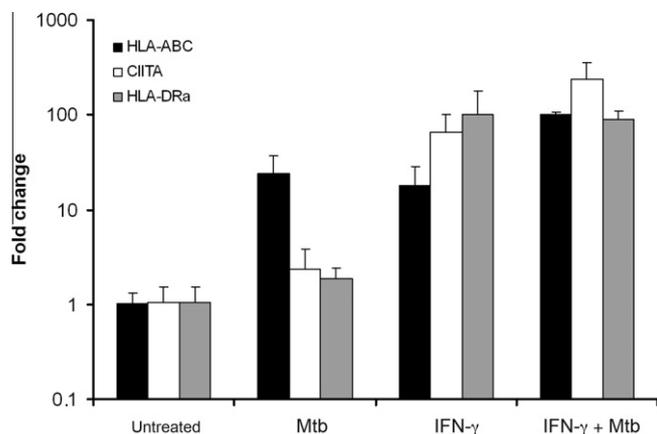


Fig. 4. MHC gene expression in Mtb-infected and IFN- γ -treated fibroblasts is not reduced. RNA from control fibroblasts, Mtb infected, IFN- γ treated and the two last conditions combined were tested by qRT-PCR with primers specific for common regions of HLA-A, -B and -C genes, for transcription factor CIITA and HLA-DR α genes. Expression was quantified as fold changes compared to control cells, using Beta-actin as housekeeping gene, calculated using the $2^{-\Delta\Delta Ct}$ formula. Data \pm SD are from three independent infection experiments.

as *M. smegmatis*, to infect non-phagocytic cells [8–11] by macropinocytosis through lamellipodia formation and cell cytoskeleton rearrangement [4,13].

Since the intracellular growth of mycobacteria in phagocytes may be limited by the intervention of the adaptive immune system, namely by the secretion of inflammatory cytokines following the recognition of specific antigens by effector T cells, Mtb has evolved an escape strategy based on the inhibition of phagosome maturation [2] and the modulation of MHC class II molecules [3,26]. Previous studies have so far analyzed the interference of mycobacteria with MHC class II expression in professional phagocytes, such as macrophages or DCs. These cells are characterized by a constitutional expression of MHC class II molecules that may be increased by the release of cytokines such as IFN- γ or by inflammatory stimuli and bacterial products [27]. Here we investigate whether mycobacteria infection also interferes with *de novo* expression of MHC class II molecules in non-phagocytic cells upon IFN- γ stimulation using fibroblasts as a prototype. In particular, we focus on fibroblasts since they are crucial players in granuloma formation [28] and they were previously found to host Mtb in LTBI [17].

We demonstrate that fibroblasts infected by mycobacteria up-regulate MHC class II molecules to a statistically significant lower extent than non-infected cells upon IFN- γ stimulation. This effect is observed *in vitro* even at very high IFN- γ doses (up to 400 ng/ml) so that the inhibition of MHC class II molecule expression induced by mycobacteria is likely to occur also *in vivo*, where IFN- γ levels may be lower than those used *in vitro* [29]. Interestingly, MHC class I molecules were up-regulated following infection with Mtb or BCG, in terms of both mRNA levels and membrane expression. IFN- γ treatment of fibroblasts caused an increase of MHC class I mRNA synthesis and membrane expression. MHC class I mRNA levels were found only weakly increased upon IFN- γ treatment of Mtb infected fibroblasts, suggesting that IFN- γ and Mtb are not additive stimuli for MHC class I mRNA synthesis. However, MHC class I membrane expression in infected and IFN- γ treated fibroblasts increased, but to a lesser and not statistically significant extent in comparison to non-infected fibroblasts. These data suggest that Mtb infection causes a reduced MHC class I membrane export of preformed molecules in IFN- γ treated fibroblasts. These findings are in agreement with previous works showing that Mtb is able to interfere with MHC class II but not class I molecules

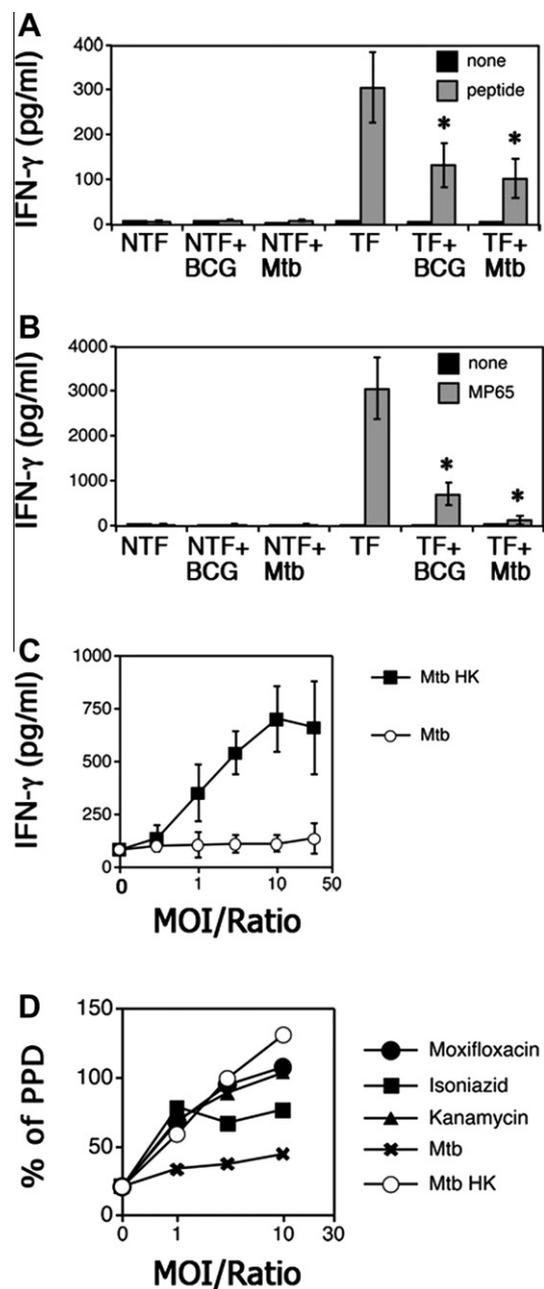


Fig. 5. Mycobacteria interfere with the MHC class II dependent antigen presentation by human fibroblasts. MHC class II dependent response of a MP65 specific T cell clone to a peptide of MP65 (A) or to the MP65 soluble mannoprotein (B) in the presence of autologous fibroblasts treated (TF) or non treated (NTF) with IFN- γ after the infection with Mtb, BCG or left uninfected. The T cell clone activation was measured as IFN- γ release and expressed as pg/ml \pm SD of three independent experiments. The asterisk indicates a significant difference ($p < 0.05$) between infected and non-infected TF. (C) IFN- γ pre-treated fibroblasts were infected with viable (Mtb) or incubated with heat-killed (HK) Mtb at different MOI or ratios, respectively, then washed and co-cultured with autologous PPD-specific T cell clone T cell activation was measured as IFN- γ release and expressed as pg/ml \pm standard deviation of three independent experiments. (D) IFN- γ -treated fibroblasts were incubated o/n with PPD or with viable or heat killed (HK) Mtb or with Mtb that has been pre-cultured o/n with moxifloxacin (4 μ g/ml), kanamycin (100 μ g/ml) or isoniazid (2 μ g/ml), then washed and co-cultured with an autologous PPD-specific T cell clone. T cell activation was measured as IFN- γ release and expressed as percentage of IFN- γ production respect to the activation obtained with PPD. One experiment representative of two is shown.

expression on macrophages [26,30], and may be linked to the constitutive expression of MHC class I molecules on both professional and non-professional APCs.

Mtb uses diverse strategies to interfere with MHC class II expression in macrophages [31], including the reduction of the MHC class II transcriptional transactivator (CIITA) activity, thus causing the reduced transcription of MHC class II mRNA [32,33]. Moreover, Mtb can interfere with the processing of the MHC class II invariant chain reducing the MHC class II export to cell membrane through the inhibition of phagosomal maturation and activity of proteolytic enzymes such as cathepsins [34,35]. Our results indicate that the observed reduced MHC class II membrane expression is not dependent on the Mtb inhibition of CIITA and MHC class II transcription. Although the aim of this study was not the identification of the mechanisms causing the observed phenomenon, our data suggest that in fibroblasts Mtb inhibits the MHC class II membrane expression by interfering with the intracellular transport of MHC class II to the external membrane, possibly inhibiting the activity of enzymes involved in the processing of the invariant chain directly or indirectly by modifying the acidity of the Mtb containing vacuoli.

The term non-professional APCs is related to cells on which expression of MHC class II molecules can be induced by IFN- γ , such as endothelial cells [36], myoblasts [37], or fibroblasts [22]. The antigen presentation by non-professional APCs can support proliferation of T cell clones or memory T cells, i.e., when co-stimulation is not required. On the contrary, they are unable to initiate a primary response by naive T lymphocytes. Accordingly, the capacity to present soluble antigen by fibroblasts has probably scarce significance in T cell priming, since professional APCs in primary lymphoid organs are the cells deputed to this function. However, the capacity of fibroblasts to present antigens during recall immune responses may have relevance in the fight against pathogens. In fact, non-professional APCs, outnumbering professional APCs in tissues, may contribute to modulate the magnitude of the efferent phase of an immune response by activating memory T lymphocytes.

To test the functional consequence of MHC class II reduced induction in non-professional APCs, we first used the capacity of infected fibroblasts to present peptides in an autologous model. We used a peptide synthesized based on the epitope sequence of MP65, an immunodominant antigen of *C. albicans* previously shown to cause the activation of our T cell clones. Since peptides do not require internalization and processing by APC, the activation of a specific T cell clone can be considered as the functional expression of MHC class II expression on fibroblasts upon IFN- γ treatment [38]. We observed that the peptide presentation is reduced but not completely abrogated in mycobacteria infected fibroblasts. This result is in agreement with the reduced but not completely inhibited MHC class II membrane expression observed in infected fibroblasts. Moreover, we tested the capacity of mycobacteria to interfere with the antigen presentation of a protein antigen by non-professional APCs. To compare the APCs function of Mtb infected fibroblasts to that of non-infected cells, we used the protein MP65, an antigen that is not shared by Mtb [25], and a MP65 specific T cell clone activation as a readout. The Mtb infection causes an almost complete inhibition of the presentation of MP65 to the specific T cell clone by fibroblasts even if the MHC class II expression is reduced but not abrogated. Moreover, using a PPD specific T cell clone, we demonstrate an almost complete inability of Mtb infected fibroblasts to present mycobacterial antigens, indicating that Mtb, by infecting fibroblasts, hides itself to the recognition of CD4⁺ T lymphocytes. The failure of fibroblasts to present Mtb antigens is not dependent on their intrinsic inability to process whole bacteria. In fact, fibroblasts induced the activation of the PPD specific clone if treated with Mtb killed by heat or treated with specific antibiotics. This result indicates that fibroblasts are cells endowed with the capacity to process whole bacteria in an environment characterized by the presence of IFN- γ , expanding our understanding of the non-professional APC function of

fibroblasts [22]. Lastly, our data suggest that the capacity of Mtb to restrain the APC function of fibroblasts is an active phenomenon requiring metabolically active and viable Mtb cells. Thus, the interference with the fibroblast antigen presentation capacity seems not to be dependent on structural components of the bacterium.

It can be hypothesized that fibrotic TB lesions with low oxygen concentrations represent a hostile microenvironment that may favor the Mtb transition to the non-replicating status of dormancy. Since our data indicate that in fibroblasts Mtb may also escape helper T cell recognition, it is possible to speculate that fibroblasts may represent a Mtb reservoir in LTBI. In this line, it is interesting to note that Mtb has been described in fibroblasts in LTBI [17], but not in active TB. Considering that *in vitro* both human and mouse [39] fibroblasts are permissive to Mtb growth, it is not clear why fibroblasts, that are associated with tuberculous loci and participate in granuloma formation have not been found to contain Mtb *in vivo*. A possible explanation is that in active diseases the growth in macrophages may be favored and infection of fibroblasts or other cells is sporadic and thus difficult to detect. The other possibility is that fibroblast may represent an immunoprivileged site for Mtb where to persist in the latent infection only. In conclusion, we have confirmed that Mtb can infect human fibroblasts [13,14] and propose a possible functional consequence of the fibroblast infection in terms of escape from the host immune response. Even if *in vitro* data cannot completely reproduce phenomena occurring *in vivo*, we report data that increase our knowledge on the possible mechanisms used by Mtb to persist in immunocompetent individuals. In fact, if infected *in vivo*, fibroblasts may represent immunoprivileged cells for Mtb homing and it can be speculated that, hidden to the helper T cell recognition, Mtb may grow in fibroblasts during active TB or to persist in a dormant status during latent TB infection.

Acknowledgments

This work was partially supported by the 7th EC Framework Programme Project “NEWTBVac” Grant #241745.

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