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► **To cite this version:**

Corinne Loeuillet, Fabio Martinon, Cynthia Perez, Miguel Munoz, Margot Thome, et al.. Mycobacterium tuberculosis Subverts Innate Immunity to Evade Specific Effectors. *Journal of Immunology*, 2006, 177, pp.6245 - 6255. 10.4049/jimmunol.177.9.6245 . hal-04396203

HAL Id: hal-04396203

<https://cnrs.hal.science/hal-04396203>

Submitted on 17 Jan 2024

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***Mycobacterium tuberculosis* Subverts Innate Immunity to
Evade Specific Effectors**

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Running title: Macrophage Apoptosis and *M. tuberculosis*

Keywords: Human, macrophages, apoptosis, immunology

Abstract

The macrophage is the niche of the intracellular pathogen *Mycobacterium tuberculosis*. Induction of macrophage apoptosis by CD4⁺ or CD8⁺ T cells is accompanied by reduced bacterial counts, potentially defining a host defense mechanism. We have already established that *M. tuberculosis*-infected primary human macrophages have a reduced susceptibility to FasL-induced apoptosis.

To study the mechanisms by which *M. tuberculosis* prevents apoptotic signaling, we have generated a cell culture system based on PMA- and IFN γ -differentiated THP-1 cells recapitulating the properties of primary macrophages. In these cells, nucleotide-binding oligomerization domain 2 (Nod2) or TLR2 agonists and mycobacterial infection protected macrophages from apoptosis. Transduction of a receptor interacting protein-2 (RIP-2) dominant negative construct showed that Nod2 is not involved in protection in the mycobacterial infection system. In contrast, both a dominant negative construct of the MyD88 adaptor and a NF- κ B inhibitor abrogated the protection against FasL-mediated apoptosis, showing the implication of TLR2-mediated activation of NF- κ B in apoptosis protection in infected macrophages. Moreover, TLR2 agonists and mycobacterial infection resulted in NF- κ B nuclear translocation and up-regulation of the anti-apoptotic cellular FLICE (Fas associated death domain-like IL-1 β -converting enzyme)-like inhibitory protein (c-FLIP) protein.

The apoptosis resistance of infected macrophages might be considered as an immune escape mechanism, whereby *M. tuberculosis* subverts innate immunity signaling to protect its host cell against FasL⁺ cytotoxic lymphocytes.

Introduction

Infection by *Mycobacterium tuberculosis* (*M. tuberculosis*) is efficiently controlled by the immune system as the vast majority of the 2 billion infected humans contain infection unless their immune system is compromised (1). The preferred niche of *M. tuberculosis* is the macrophage, which fails to control the growth of *M. tuberculosis* in the absence of T cells 1. Several cytokines may act as macrophage arming factors in the control of *M. tuberculosis* 1. Mice deficient for IFN- γ or its receptor fail to generate reactive nitrogen intermediates and are unable to restrict mycobacterial growth (2,3). Humans with similar genetic defects also show an increased susceptibility to mycobacterial infections (4,5). However, the ability of human macrophages to generate reactive nitrogen derivatives in response to IFN- γ is controversial (6), and *in vitro*, treatment of human macrophages with IFN- γ enhances rather than restricts the growth of *M. tuberculosis* (7-9). Thus, the exact role of IFN- γ in anti-mycobacterial immunity in humans remains unclear.

Other cytokines have been implicated in host defences against *M. tuberculosis*. In particular, TNF- α (10) and lymphotoxin (11) have been demonstrated crucial for granuloma formation and for limiting pathology, and therapies aimed at blocking TNF- α do increase the risk of reactivation tuberculosis in humans (12). However, *in vitro* experiments have failed to demonstrate that TNF- α can arm macrophages to kill intracellular bacilli (13). These observations have led investigators to look for alternate mechanisms for the killing of mycobacteria.

Several observations have suggested ways for T cells to kill mycobacteria independent of macrophage activation, “dealing a direct blow to the pathogens” (14). Indeed, different T lymphocyte populations have the potential to restrict the intracellular growth of mycobacteria when co-cultured with infected macrophages, in part through soluble mediators and in part by a cell contact-mediated mechanism (15,16). How could T cells kill mycobacteria? Stenger *et*

al. have demonstrated that CD8⁺ T cell killing infected macrophages kill intracellular mycobacteria (17) in a granule-dependent mechanism involving granulysin (18). Others have however demonstrated that the induction of apoptosis in infected macrophages by stimuli such as FasL (19) and extracellular ATP (20,21) is accompanied by a reduction of viable intracellular bacilli independently of granulysin. FasL signalling thus provides CD4⁺ cells with a potential to participate directly to host defense mechanisms against *M. tuberculosis*. Indeed, CD4⁺ T cell responses, including responses with cytolytic activity against *M. tuberculosis* antigens, are found both in humans, especially in clinical settings characterized by an effective immunity such as tuberculous pleurisy (22-28) and in mice (29,30). Thus, induction of macrophage apoptosis by *M. tuberculosis*-specific CD4⁺ T cells may operate *in vivo* to control *M. tuberculosis* survival. Granuloma do contain CD4⁺ T cells and apoptotic figures involving macrophages (31,32) as well as cells expressing FasL (33).

If the induction of apoptosis in infected macrophages is a host defence mechanism, then preventing apoptosis may represent for mycobacteria a way to preserve their habitat and escape host immune responses. Indeed, we have observed that *M. tuberculosis*-infected macrophages are relatively resistant to apoptosis induced by FasL (19).

Two major pathways are described for mammalian apoptosis: the death receptor (type I) and mitochondrial (type II) pathways (34). The type I pathway is initiated by extrinsic signals, like the FasL-dependent triggering of the death receptor Fas, which leads to the formation of the death inducing signalling complex and the activation of the initiator caspase-8. The type II pathway is triggered by intrinsic signals with the formation of an apoptosome complex containing active caspase-9. Both pathways converge on the activation of executioner caspases and cell death.

We first hypothesized that intracellular *M. tuberculosis* may secrete a factor that may gain access to the phagocyte cytoplasm (35) and interfere with apoptotic pathways. We therefore

did a profile search of the *M. tuberculosis* genome for proteins containing motifs such as a death domain, death effector domain, caspase recruitment domain (CARD), using the Prosite database, and did not find any significant signal (L. Falquet and P. Meylan, unpublished). We then hypothesized that cell wall components, such as muramyl dipeptide (MDP), lipoarabinomannan (LAM) and 19kDa lipoprotein of *M. tuberculosis* i.e. agonists of nucleotide-binding oligomerization domain-2 (Nod2) or Toll-like receptor-2 (TLR2) (36-38), might act to prevent apoptosis through the induction of anti-apoptotic genes by the NF- κ B or other signalling pathways.

Here, we describe a model using the THP-1 cell line that recapitulates the properties of human primary macrophages with respect to the effect of *M. tuberculosis* infection on their susceptibility to FasL-induced apoptosis. We have found that following infection, the activation of NF- κ B through the TLR2 signalling pathway and the subsequent induction of c-FLIP expression is able to protect cells from FasL-induced apoptosis.

Materials and Methods

Cell culture and infection with *M. tuberculosis*

THP-1 cells (TIB 202, ATCC) were cultured in RPMI 1640/Glutamax-I medium (Invitrogen) supplemented with 10% FCS (Inotech) and 0.05 mg/ml gentamicin (Invitrogen), subsequently named R10. For their differentiation, cells were plated in R10 plus PMA (20 nM, Fluka) for 3 days, 37°C, 5% CO₂. Differentiated cells were then treated with human rIFN- γ (PBL Biomedical Laboratories), 500 U/ml, for 1 day. These cells were named THP-1/PMA/IFN- γ .

The virulent (H37Rv) and attenuated (H37Ra) *M. tuberculosis* strains were used and cultured as previously described 19. FITC labeling of mycobacteria was done as previously described 39. Single cell suspensions of *M. tuberculosis* were prepared as previously described 19 with a slight modification. Before infection, bacteria were opsonized in RPMI plus 50% AB⁺ fresh human serum (Inotech) by rocking at 200 rpm, 37°C, 30 minutes. Heat-killed inocula were generated by exposing bacteria to 70°C for 30 mn. THP-1/PMA/IFN γ were infected by replacing culture medium with the mycobacteria inoculum at a density of 10-20 bacteria per cell for 2h, 37°C, 5% CO₂. After 3 washes with RPMI, cells were placed in R10 without antibiotics.

Induction of apoptosis

THP-1/PMA/IFN- γ cells were incubated for 18h in R10 containing human rFasL (1 μ g/ml) or human rTrail (TNF related apoptosis inducing ligand, 200 ng/ml) in the presence of an enhancer for ligands (1 μ g/ml), by human rTNF- α (10 ng/ml), all from Apotech, or by staurosporine (1 μ M, Sigma). Samples and controls were treated with 1 μ g/ml of cycloheximide (CHX, Sigma).

Quantification of apoptosis

Annexin-V binding

Apoptosis was estimated by using the Annexin V-FITC apoptosis detection kit (BD Pharmingen) with a slight modification. Cells undergoing apoptosis were detached using Accutase (Inotech AG), washed 2 times with cold PBS and resuspended in 1X binding buffer. Five μ l of Annexin V-FITC were added to the cell suspension and incubated for 15 min at room temperature. Labeled cells were then washed with binding buffer and the pellet resuspended in 100 μ l of 1X Cellfix (Becton Dickinson) solution. Fixed cells were analysed by flow cytometry using a FACSCalibur system (Becton Dickinson). Data acquisition and analysis were performed using the CELL-Quest Pro software, measuring 20,000 events. Apoptosis was quantified by setting a fluorescence threshold above the fluorescence of CHX-treated cells and expressed as the percent of cells above the threshold.

Caspase activity

Apoptosis was also detected using a fluorogenic substrate for caspase 3 as previously described (40). Data were acquired on a Spectra Man Gemini reader (Molecular Devices) and analysis performed with the SOFT Max PRO3.1 software. Results were expressed as relative fluorescence units per mg of protein (RFU/mg).

Flow cytometry

Staining of THP-1/PMA \pm IFN- γ cells were washed, resuspended in PBS/0.5% BSA (Sigma) and incubated with primary mAb or isotypes, 30 min, 4°C. After washes, cells were incubated, if necessary, with secondary antibody, 30 min, 4°C. Cells were washed, fixed with CellFix and analyzed using a FACSCalibur system as described above. Primary mAb were: anti-CD95-FITC (1/20, APO1-1); anti-FasL (1/100, Mike1), anti-TRAIL-R1 (1/200, HS101), anti-TRAIL-R2 (1/200, HS201), anti-TRAIL (1/100, III6F), anti-TLR2 (1/100, TL2.1), all

from Apotech. Secondary antibodies were: anti-mouse or anti-rat IgG FITC (1/50, Sigma). Cells were analyzed using a FACSCalibur system as described above. Positive events were defined as a fluorescence level superior to that of isotypic control.

Treatment with mycobacterial cell wall components or with chemical compounds

THP-1/PMA/IFN- γ cells were treated with 2 μ g/ml of AraLAM, ManLAM, 19kDa lipoprotein (all from NIH, NIAID Contract AI-75320 and Colorado State University's contribution of Research Material) or LPS (Sigma) for 2h, 37°C, 5% CO₂ ; with 1 μ g/ml of Ac-muramyl-Ala-D-Glu-NH₂ (MDP-D, 6h, Bachem) or the inactive enantiomer Ac-muramyl-D-Ala-D-Glu-NH₂ (MDP-DD), or with Ammonium pyrrolidine dithiocarbamate (250 mM, 10h, PDTC, Fluka).

NF- κ B nuclear detection

Immunofluorescence

THP-1/PMA/IFN- γ cells were cultured in Labtek chambers (Nunc). The day after infection, the cells were washed, fixed with 4% paraformaldehyde (Fluka), 30 min, 4°C and permeabilized with acetone (Fluka) 5 min, -20°C. After 3 washes, cells were stained with TRITC-conjugated mAb anti-NF- κ B p65 (Santa Cruz Biotechnology), 30 min, room temperature (RT), washed once and stained with Hoechst 33528 (0.05 μ g/ml, Sigma), 10 min, RT. Slides were examined with an Axioplan 2 microscope (Zeiss) and images acquired with a Kappa camera using the Kappa image base control (Kappa).

Nuclear translocation of NF- κ B p65

NF- κ B p65 was measured in nuclear lysates using a kit (NF- κ B p65 Transcription factor assay, Chemicon). THP-1/PMA/IFN- γ cells were cultured in 48 wells plate. The day after infection, nuclear cell lysates were collected and the assay performed. Measure of absorbance

of the samples at 450 nm was carried out using a microplate reader with the reference length set to 650 nm (Biorad Laboratories).

Lentiviral constructions, production and transduction

The 2 lentiviral constructions used were pRDI292-CMV-MyD88DN (expressing the dominant analog of TIR domain-containing adaptor, MyD88) or pRDI292-CMV-RIP2DN (expressing the dominant analog of protein kinase receptor interacting protein-2, RIP2). The 2 genes of interest were placed under the control of CMV promoter.

Lentiviral vector (MyD88DN) expressing the dominant analog of MyD88

The plasmid pL502 containing the MyD88 DN gene (41) was digested by BamHI and ApaI, and the resulting fragment cloned into pSL1180 (Pharmacia Diagnostics). The CMV promoter was subcloned from the vector SIN.cPPT.CMV.eGFP-WHV (Dr D. Trono, Lausanne), using ClaI and BamHI, into pSL1180/MyD88 DN to obtain PSL1180/CMV/MyD88 DN. This vector was digested by ApaI and BamHI and blunted to obtain the CMV-MyD88DN fragment, which was cloned into the lentiviral vector pRDI292 (Dr R. Iggo, Lausanne) digested by BamHI and blunted. The vector obtained was designed as pRDI292-CMV-MyD88DN.

Lentiviral vector expressing the dominant analog of RIP2 (RIP2DN)

RIP2DN (42) was cloned ApaI/ClaI into PSL1180-CMV-MyD88DN digested by the same enzymes to remove the MyD88DN insert, to yield the vector pSL1180-CMV-RIP2DN. The CMV-RIP2DN fragment was then cloned into the lentiviral vector pRDI292, as described above, to obtain the pRDI292-CMV-RIP2DN construct.

Lentiviruses production

The lentiviruses were produced by cotransfecting the Gag-Pol construct (pCMV._R8.92), the Rev expression plasmid (pRSVRev), the VSV G protein envelope construct (pMD.G), and the

packaged construct of interest (expressing MyD88DN, RIP2DN or GFP as a control of transduction efficiency) (see www.tronolab.unige.ch for vector details).

Cells transduction

Transduction of THP-1 cell was performed by spinoculation of 0.5×10^6 cells with 400 μ l of lentivirus-containing supernatant in the presence of 4 μ g/ml polybrene (Sigma), 3h, 1500g, 22°C. After 48h, cells were selected overnight in R10 with 2 μ g/ml puromycin (Fluka), and maintained with 1 μ g/ml puromycin.

Western blot

THP-1/PMA/IFN- γ cells were washed with cold PBS, lysed in RIPA 1X containing protease inhibitor cocktail (Complete TM, Roche Diagnostics). The samples were mixed with sample buffer, separated on 12% SDS-PAGE, transferred to PVDF membrane (Nen Life Science Products), and immunoblotted with the appropriate dilution of c-FLIP (1/800, Dave2, Apotech), Tubulin (1/10000, B512, Sigma), FLAG M2 (10 μ g/ml, Sigma), VSV (1/1000, P5D4, Sigma). Blots were developed using an enhanced chemiluminescence detection system (Pierce).

Statistical analysis

Values are expressed as the mean + SEM. Statistically significant differences between series were assessed by t-Test using Graphpad Prism software (San Diego).

Results

Development of THP-1 cells as a model system for the study of apoptosis susceptibility

We have reported that infection of primary macrophages by *M. tuberculosis* induced a relative resistance of these cells to apoptotic stimuli (19). To facilitate the study of the mechanisms of this resistance, we aimed at developing a system using a continuous cell line to recapitulate the behavior of primary macrophages regarding infection and apoptosis susceptibility. THP-1 cells treated by PMA became phagocytic but did not express Fas (Fig. 1A). We examined whether IFN- γ treatment might affect the differentiation and apoptosis susceptibility of THP-1 cells. IFN- γ treatment of PMA-differentiated THP-1 cells induced surface expression of Fas receptor from 0 to 50 ± 12 % of the cell population (Fig. 1B). FasL expression was also up-regulated (41 ± 21 % vs. 25 ± 8 %). The IFN- γ treatment led to the diminution of Trail receptors 1 and 2 (9 and 12 % vs 19 and 18 %, respectively) and Trail expression (15% vs. 11 %), while the TLR2 receptor was equally expressed in THP-1/PMA cells, irrespective of IFN- γ treatment (25 to 30 %).

THP-1/PMA/IFN- γ cells were susceptible to FasL-induced apoptosis as demonstrated by Annexin V staining (Fig. 1C, 2A) with about 45 ± 7 % of cells undergoing apoptosis. These cells were also susceptible to Trail-induced apoptosis (up to 22 ± 5 %) but not to TNF- α -induced apoptosis. As the highest level of apoptosis induction was achieved using FasL, this ligand was used in further experiments.

Effect of *M. tuberculosis* infection on the susceptibility of THP-1 cells to FasL

To test whether infection induces apoptosis resistance in THP-1/PMA/IFN- γ cells as in primary macrophages, the cells were infected at a bacterium/cell ratio of 10-20 using *M. tuberculosis* H37Ra or H37Rv. The remaining non-phagocytosed mycobacteria were removed

by three washes. This led to the infection of a majority of the cells, with about 2-6 bacteria per cell (see Fig.2 in 19). *M. tuberculosis* infection did not by itself induce apoptosis in our system as witnessed by Annexin V staining (not shown). Two hours after infection, the cells were incubated with FasL or staurosporin to induce apoptosis. Similar to primary human macrophages (19), infection of differentiated THP-1 cells with H37Rv or H37Ra strains protected them from FasL-induced apoptosis, reducing apoptosis by close to 50% (Fig. 1D, 2B). This reduced apoptosis was not due to a diminution of Fas expression at the cell surface (not shown). *M. tuberculosis* infection did not protect cells from staurosporin-induced apoptosis (not shown). Thus, *M. tuberculosis* was able to protect cells from apoptosis mediated by death receptor binding, but not via the mitochondrial pathway.

A similarly diminished susceptibility to FasL-induced apoptosis was observed in THP-1/PMA/IFN- γ cells infected with either viable or heat-killed H37Ra bacteria (not shown) suggesting that binding and phagocytosis of mycobacteria are sufficient to induce apoptosis resistance.

Apoptosis protection was also demonstrated by the diminution of caspase 3 activity in *M. tuberculosis*-infected cells (Fig. 2C). *M. tuberculosis* infection per se reduced caspase activity suggesting a basal level of caspase activation in untreated cells that was inhibited by infection. Treatment of uninfected cells using FasL increased caspase activity by about 2-fold. This increase of caspase activity upon FasL treatment was almost completely prevented in infected cells.

Apoptosis and bacterial viability in THP-1 cells

FasL-induced apoptosis was associated with a diminution of bacterial viability (Fig. 1D) by about 38% in H37Ra-infected cells and 35% in H37Rv-infected cells. These observations are comparable to results obtained with primary macrophages (19).

Muramyl Dipeptide provides cell protection against FasL-induced apoptosis

As the apoptosis protection was also conferred by heat killed bacteria, we tested the effect of bacterial cell wall components on FasL-induced apoptosis susceptibility.

MDPs are the smallest peptidoglycan degradation products. They bind to intracellular Nod2, leading to the activation of RIP2 and subsequently of NF- κ B and anti-apoptotic genes (43).

To test whether MDP-induced NF- κ B activation was implicated in *M. tuberculosis*-mediated apoptosis resistance of macrophages, THP-1/PMA/IFN- γ cells were treated with MDP-D or with MDP-DD, an inactive enantiomer as a negative control. Apoptosis was then induced by FasL and assessed by AnnexinV staining or by caspase assay. MDP-D treatment resulted in apoptosis protection against FasL-induced apoptosis (40% reduction) whereas MDP-DD did not (Fig. 3A). Caspase activity was also reduced in MDP-D treated cells (Fig. 3B). Thus, the activation of the Nod2 pathway is a potential pathway for mycobacteria to induce apoptosis resistance.

Role of the Nod2 pathway in apoptosis resistance of infected cells

To test whether blocking the Nod2 pathway could abrogate apoptosis protection of cells infected by *M. tuberculosis*, we used a transdominant negative approach. The receptor interacting protein-2 (RIP2) is an NF- κ B activating protein interacting with Nod2 through a homophilic CARD-CARD interaction (44). A dominant negative RIP2 construct (RIP2DN), corresponding to RIP2 truncated of its CARD domain (42) was transduced into THP-1 cells using a lentiviral vector, and after several days of puromycin selection, the presence of the RIP2DN protein was detected by western blot (Fig. 3 inset).

The functionality of RIP2DN was first tested using MDP-D as an agonist of the NOD pathway. MDP-D protected THP-1/PMA/IFN- γ cells against apoptosis, and the expression of RIP2DN abrogated this protection (Fig. 3C).

In contrast to the effect of RIP2DN on MDP-D-induced resistance, the expression of RIP2DN did not abrogate the apoptosis protection in infected cells (Fig. 3D). Together, these findings suggest that the NOD pathway does not play a significant role in the apoptosis resistance of *M. tuberculosis*-infected THP-1/PMA/IFN γ cells.

***M. tuberculosis* cell wall TLR2 agonists induce apoptosis resistance**

M. tuberculosis cell wall components such as the 19kDa lipoprotein and AraLAM are known to signal through TLR2, with a potential for activating pro-inflammatory and anti-apoptotic pathways (36). We therefore tested the capability of cell wall components of *M. tuberculosis* to protect THP-1/PMA/IFN- γ cells from apoptosis (Fig. 4A). FasL-induced apoptosis of these cells was significantly reduced when cells were pretreated with the TLR2 agonists (19kDa lipoprotein and AraLAM) but not with ManLAM. In contrast, the TLR4 agonist (LPS) increased cell susceptibility to apoptosis by 110% when compared to untreated cells (Fig. 4A). Similar results were observed when apoptosis was assessed measuring caspase activity (Fig. 4B).

Thus, TLR2 agonists purified from the cell wall of *M. tuberculosis* recapitulated the ability of *M. tuberculosis* to induce a relative resistance to FasL-induced apoptosis in phagocytes.

Role of the TLR2 signaling pathway in apoptosis resistance of infected cells

To test whether TLR2 signaling was involved in apoptosis resistance in infected cells, THP-1 cells were transduced with a trans-dominant negative mutant of the adaptor molecule myeloid differentiation factor 88 (MyD88) truncated of its death domain (MyD88DN (41)), using a lentiviral vector. After puromycin selection, expression of MyD88DN was detected by western blotting (Fig. 4C, inset). No change in basal apoptosis was detected in MyD88DN expressing cells (not shown).

To verify the functionality of the MyD88DN protein, control and MyD88DN-expressing THP-1/PMA/IFN- γ cells were treated with the TLR2 agonist 19kDa lipoprotein (Fig. 4C). MyD88DN partly abrogated the apoptosis protection conferred by the 19 kDa lipoprotein, and completely abrogated the apoptosis protection conferred by *M. tuberculosis* infection (Fig. 4D). This result confirms that the TLR2 signaling pathway is involved in inducing apoptosis protection, accounting for most of the apoptosis resistance induced by *M. tuberculosis* infection in THP-1 cells.

NF- κ B activation protects cells from apoptosis

The activation of the TLR2 signaling pathway culminates in the nuclear translocation of the transcription factor NF- κ B. To assess whether NF- κ B activation was involved in apoptosis protection in our model, we first blocked NF- κ B activation by PDTC, a pyrrolidine derivative of dithiocarbamate that suppresses the release of I κ B from the latent cytoplasmic form of NF- κ B (45). Treatment of THP-1/PMA/IFN- γ cells with PDTC increased the basal rate of apoptosis by about 35% (Fig. 5A), suggesting that NF- κ B protects these cells from apoptosis in the absence of FasL-mediated signaling. In infected cells, not only did PDTC abrogate the protection induced against apoptosis, but it increased the level of apoptosis by 200% compared to infected untreated cells, thus to a degree that is beyond the apoptosis level of control cells. This confirmed the role of NF- κ B activation in apoptosis protection, in particular in infected cells.

To corroborate these data, nuclear translocation of NF- κ B was quantified using the TFA assay on nuclear cell fractions. THP-1/PMA/IFN- γ cells were infected by *M. tuberculosis*. After 24h of infection, cells were processed and NF- κ B assayed in nuclear extracts. Upon infection, there was a 3-fold increase in NF- κ B detectable in nuclear fractions (Fig. 5B).

We also looked for cellular localization of NF- κ B by immunofluorescence. THP-1/PMA/IFN- γ cells were infected using FITC-labeled H37Ra *M. tuberculosis* and, after 24h, fixed and

stained for NF- κ B p65 transcription factor and for DNA by Hoechst 33528 (Fig. 6). In uninfected cells, NF- κ B localized to the cell cytoplasm (Fig. 6A). When the cells were infected with H37Ra, NF- κ B translocated to the nucleus (Fig. 6B), co-localizing with the Hoechst DNA staining. This translocation was especially obvious in cells containing the largest number of bacteria.

Thus, these three independent approaches suggest that TLR2-mediated NF- κ B nuclear translocation is crucial in mediating *M. tuberculosis*-induced protection of phagocytes from apoptosis .

Effect of TLR signaling on the expression of proteins involved in apoptosis regulation

In an attempt to understand how TLR2 signaling may protect from apoptosis, we tested whether TLR2 agonists or infection would affect cellular levels of proteins known to regulate apoptosis. Among these, the levels of Fas, TNF receptor-associated factor-1 (TRAF1), caspase 3, caspase 8 and caspase 9 were not affected by TLR2 agonist treatment or by *M. tuberculosis* infection (not shown). We also looked for c-FLIP, a major inhibitor of death receptor-induced apoptosis (46), which is known to be regulated by NF- κ B (47) and for TRAF6, an adaptor molecule in the TLR2 signaling cascade (37), which, if regulated by NF- κ B signaling, would have a potential for feed-back amplification of this pathway.

TLR2 agonists increased the levels of c-FLIP (Fig. 7A) and, to a weaker extent, of TRAF6 (not shown), while the TLR4 agonist LPS had no effect neither on c-FLIP nor on TRAF6. In comparison, both H37Rv and H37Ra *M. tuberculosis* increased the level of c-FLIP 18 hours after infection (Fig. 7B) but had no effect on TRAF6 levels (not shown).

These data suggest that an increase of c-FLIP expression upon TLR2 activation may account, at least in part, for the relative resistance of infected cells to apoptosis.

Together, these findings suggest that *M. tuberculosis*-induced NF- κ B activation by the TLR2 but not the Nod2 pathway protects infected THP-1 cells from FasL-induced apoptosis.

Discussion

Depending on the experimental system, *M. tuberculosis* phagocytosis has been shown to promote or protect phagocytes from spontaneous apoptosis (48-51). In contrast, the effect of *M. tuberculosis* phagocytosis on apoptosis induced by various apoptotic stimuli is to reduce phagocyte susceptibility to undergo apoptosis (52). In addition, virulent *M. tuberculosis* strains, but not the avirulent ones, have been shown to evade apoptosis not only in human alveolar macrophages (53), but also in differentiated THP-1 cells (54). In some of these systems, this effect has been ascribed to the induction of a anti-apoptotic Bcl-2 family member (52,55) or s-TNF-R2 (52).

We also described a reduced susceptibility of *M. tuberculosis*-infected primary human macrophages to FasL induced apoptosis (19). As FasL-induced apoptosis was associated with a reduction in viability of intracellular bacteria, this reduced susceptibility may appear as a potential immune escape mechanism. To facilitate the study of the cellular mechanisms that underlie this altered apoptosis susceptibility, we aimed at developing a phagocyte infection system based on a continuous cell line. Upon PMA differentiation, THP-1 cells recapitulate the properties of human monocyte-derived macrophages regarding receptor mediated uptake, survival, replication and drug sensitivity of *M. tuberculosis* (56) as well as for ranking the intracellular growth characteristics of various strains (56). Recently, activated THP-1 cells have been used in a range of studies of the intracellular life style of *M. tuberculosis*, including studies that address the host cell susceptibility to apoptosis (54).

Here, we demonstrate that upon PMA-induced differentiation and IFN- γ stimulation, THP-1 cells acquire characteristics reminiscent of human primary macrophages (19) with respect to (i) susceptibility to FasL, (ii) effect of apoptosis on intracellular *M. tuberculosis* viability and (iii) effect of infection on susceptibility to apoptotic stimuli, suggesting that these cells are an appropriate model to study the determinants of this potential immune escape mechanism.

As apoptosis protection also occurred when THP-1 cells were exposed to heat killed *M. tuberculosis*, we tested the effect of various cell wall components on THP-1 apoptosis susceptibility. MDP is the smallest peptidoglycan degradation product of the bacterial cell wall. These peptidoglycan subunits are recognized by proteins of the NOD family, in particular by the Nod2 protein. Recognition of MDP by Nod2 is followed by NF- κ B activation (43) and NF- κ B-dependent activation of genes including anti-apoptotic genes. We first looked for the effect of MDP-D on FasL-induced apoptosis of THP-1 cells. Indeed, treating differentiated THP-1 cells with MDP-D induced apoptosis protection. The Nod2 protein is an activator of NF- κ B via its interaction with RIP2/RICK/CARDIAK through its CARD domain (57). A dominant negative form of RIP2 (RIP2DN) (42) abrogated protection against apoptosis induced by MDP-D, but not by *M. tuberculosis* infection. These results indicate that while MDP-D can protect cells from FasL-induced apoptosis, this signalling pathway is not involved in protection of infected cells in our system. It is unclear whether this observation reflects truly the fact that NOD signalling is not involved in apoptosis protection, or whether in our infection system, the time of apoptosis induction after infection is not long enough for *M. tuberculosis* to release peptidoglycan fragments and thus for MDP-D or similar Nod2 ligands to reach the infected cell cytoplasm, as has been described for other microorganisms (38). We can therefore not exclude that after prolonged time of infection, as in granulomas *in vivo*, Nod2 ligands may arise from bacterial metabolism or degradation after killing and thus could also protect infected cells from FasL-induced apoptosis.

As the role of pattern recognition receptors (in particular toll-like receptors) in immune defence mechanisms against microbial pathogens is increasingly recognized (37), we then turned towards the possible activation of TLRs, and subsequently of NF- κ B, by *M. tuberculosis*. These TLR receptors, especially TLR2, are expressed at the site of *M. tuberculosis* infection, i.e. macrophages in granulomas (58) and they are known to mediate

responses triggered by cell wall-associated mycobacterial lipoarabinomannans and lipoproteins (37). The lipoarabinomannan (LAM) exists in various forms with mannosylated LAM found in slow growing mycobacteria such as *M. tuberculosis* and non-mannosylated or phosphoinositide LAM found in avirulent mycobacteria such as *M. smegmatis* (37). These latter are known to signal in a TLR-dependent manner. Another important TLR2 agonist is the major 19kDa lipoprotein of *M. tuberculosis* (37).

These compounds have been described as potentially participating to the immune escape of *M. tuberculosis* by interrupting the cross talk between CD4⁺ T lymphocytes and macrophages. Indeed, they inhibit IFN- γ -mediated cell surface expression of MHC class II and Fc γ R1, potentially interfering with antigen presentation (59,60) and interfere with the IFN- γ -mediated activation of mouse macrophages, preventing their cidal effect against various targets (61). Furthermore, ManLAM has also been shown to antagonize *M. tuberculosis*-induced macrophage apoptosis, protecting the *M. tuberculosis* host cell niche (62).

Here, we present several lines of evidence that the relative refractoriness to FasL-induced apoptosis of *M. tuberculosis*-infected THP-1 cells is due to TLR2-mediated NF- κ B signalling and the resulting over-expression of c-FLIP.

First, the apoptosis resistance induced by infection could be reproduced by mycobacterial products known as TLR2 agonists (37). In our model, AraLAM and the 19kDa lipoprotein were able to protect cells from FasL-induced apoptosis with the most potent effect observed with the 19kDa lipoprotein. Others have observed that a non acylated recombinant 19kDa lipoprotein induces human macrophage apoptosis in a TLR2- and dose-dependent manner up to 200 ng/ml (63). In contrast, using purified mycobacterial 19kDa lipoprotein, we did not observe spontaneous apoptosis of our THP-1 cells even when treated with a dose as high as 2 μ g/ml, while Ciamarella *et al.* observed increasing apoptosis at 19kDa lipoprotein levels ranging from 0.5 to 10 μ g/ml (64). These discordant results may reflect differences in the

purity of 19kDa lipoprotein preparations or differences in the macrophages used (for example different relative expression of MyD88, TRIF or other signalling components) that affect the balance between NF- κ B activating and apoptosis activating TLR pathways (65,66).

Second, to confirm the involvement of TLR2 in FasL-induced apoptosis protection of *M. tuberculosis*-infected cells, we disrupted the TLR2 signalling pathway using a dominant negative form of the MyD88 adapter molecule which disconnects the receptor and the first protein of the TLR signalling pathway, i.e. interleukine-1 receptor-associated kinase, Ser/Thr kinase-1 (IRAK-1) (37). MyD88 has been described as essential in the control of *M. tuberculosis* infection and the MyD88^{-/-} mice rapidly succumb to infection (67). In mice, disrupting the TLR pathway probably prevents the induction of the production of the anti-mycobacterial agent NO which has been shown to be induced by the 19kDa lipoprotein (36). However, this mechanism is unlikely to play an important role in humans, as human macrophages produce only minimal amounts if any of nitric oxide (6,36). Our results support the idea that the MyD88DN construct abrogated the *M. tuberculosis*-induced apoptosis protection by specific inhibition of the TLR2-induced NF- κ B pathway.

Indeed, in the present model, NF- κ B was shown to translocate into the nucleus following *M. tuberculosis* infection by immunofluorescence and subcellular fractionation. To verify the involvement of NF- κ B in apoptosis protection induced by *M. tuberculosis*, THP-1 cells were also treated by a chemical compound PDTC known to antagonize the release of NF- κ B from I- κ B by inhibiting I- κ B degradation (68). Indeed, PDTC abrogated *M. tuberculosis*-mediated apoptosis protection in FasL-treated cells. Thus, NF- κ B activation is necessary to protect *M. tuberculosis*-infected cells from FasL-induced apoptosis.

As a pleiotropic factor affecting potentially the expression of many genes, NF- κ B may favor cell survival by up-regulating gene products with anti-apoptotic properties, or down-regulating pro-apoptotic factors. We therefore tested the effect of TLR2 agonists and infection

on cellular levels of a number of proteins participating in apoptotic pathways. While a number of proteins involved in apoptosis signalling were unaffected, c-FLIP protein expression was induced in cells treated with 19kDa lipoprotein and in *M. tuberculosis*-infected cells. The up-regulation of c-FLIP expression following NF- κ B activation and its involvement in preventing apoptosis has also been observed after stimulation by CD40L, LPS or TNF and may be especially effective against death ligand-induced apoptosis (47). c-FLIP protein is known to inhibit death receptor-induced apoptosis by interacting with the receptor-associated initiator caspase 8 (46,69). As the caspase 8 levels were quite stable in our cells even after infection (not shown), the increase of c-FLIP expression is likely to account for the increased FasL-resistance of the infected cells.

In summary, we present here evidences for a mechanism to explain the resistance to death receptor-mediated apoptosis observed in *M. tuberculosis*-infected human macrophages, based on c-FLIP induction secondary to TLR2-dependent NF- κ B activation (Fig. 8). Increased expression of the TRAF6 adapter may further amplify this response. In so doing, *M. tuberculosis* may protect macrophages from FasL-induced apoptosis, and thus allow their escape from CD4⁺ T cell killing. The Nod2-dependent NF- κ B activation did not play a role in our culture model but could perhaps operate *in vivo*.

Subversion of signalling pathways by microorganisms to control host cell apoptosis is not unprecedented. Thus, Herpes Simplex Virus type I (70), or *Rickettsia rickettsii* (71) have been shown to protect against apoptosis by mobilizing NF- κ B.

In conclusion, while there are no *in vitro* evidences that the old paradigm of enhanced mycobacterial activity of macrophages treated with IFN- γ holds true in humans, several alternate mechanisms for the human immune system have been identified: granulysin and perforin release by CD8⁺ cells 18, induction by TLR2 ligands of an anti-microbial activity independent of nitric oxide and TNF- α 58, induction by IFN- γ of LRG-47, a member of a

newly emerging 47 kD guanosine triphosphatase family (72) and most recently autophagy (73). The respective role of these different mechanisms in the immune defenses against *M. tuberculosis in vivo* remains to be delineated. While there are no evidence at the present time that death-receptor induced macrophage apoptosis is a host defense mechanism operating *in vivo*, the resistance to apoptosis induced by infection in macrophages might be considered as an immune escape mechanism, whereby *M. tuberculosis* subverts innate immunity signaling to escape acquired immunity effectors. As such, our observations might be an indirect testimony that death-receptor induced macrophage apoptosis is indeed a host defense mechanism *in vivo*.

Acknowledgments

We are grateful to Dr D. Trono and to Dr R. Iggo for providing the lentiviral vectors. We thank also Pr A. Telenti for helpful discussions. The authors have no conflicting financial interests.

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Footnotes

1. This study was funded by grant # 3339-064166 of the Swiss National Science Foundation (to Pascal Meylan).
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3. Abbreviations used : AraLAM, non-capped lipoarabinomannan; CARD, caspase recruitment domain; ManLAM, mannose-capped lipoarabinomannan; MDP, muramyl dipeptide; *M. tuberculosis*, *Mycobacterium tuberculosis*; Nod, nucleotide-binding oligomerization domain; PDTC, Ammonium pyrrolidine dithiocarbamate; RIP-2, receptor interacting protein-2; TLR, toll-like receptor; TRAF, TNF receptor-associated factor; TRAIL, TNF related apoptosis inducing ligand.

Figure Legends

Fig. 1. Fas expression following IFN- γ treatment and analysis of FasL-induced apoptosis in *M. tuberculosis*-infected THP-1 cells.

(A) and (B) Expression of Fas receptor was measured by flow cytometry in THP-1/PMA cells (filled black curve) before and after IFN- γ treatment. Overlay of the corresponding isotype staining control is shown (dotted line). (C) and (D) Apoptosis was detected by Annexin V staining in non infected or in *M. tuberculosis*-infected cells treated with FasL. Overlay of the Annexin V staining in cells without apoptosis induction is shown (dotted line). Percentages of positive cells (M1) are indicated.

Fig. 2. THP-1 cells as a model to study *M. tuberculosis*-induced resistance to apoptosis.

(A) THP-1 cells were treated by the indicated pro-apoptotic agents and apoptosis quantified by FACS using Annexin V-FITC staining. Shown is the mean + sem of at least 4 independent experiments. (B) The relative level of FasL-induced apoptosis was measured by Annexin V staining of THP-1 cells after *M. tuberculosis* infection (7 and 4 independent experiments were performed for H37Ra and H37Rv infection, respectively). Apoptosis was normalized to 100% in uninfected cells. (C) Infected and non-infected cells were submitted to FasL-induced apoptosis and caspase activity determined (shown is the mean + sem of 3 independent experiments). (D) Infected THP-1 cells were treated with FasL and bacterial viability determined at day 1 after infection (mean + sem of 3 independent experiments performed in duplicate).

Fig. 3. The Nod2 pathway is not involved in apoptosis protection following *M. tuberculosis* infection.

(A) THP-1 cells were treated with MDP for 6h and apoptosis induced by FasL (mean + sem of 5 independent experiments). Results are expressed as Annexin V-positive cells, normalized to 100% for untreated cells. (B) Determination of caspase activity in THP-1 cells treated with MDP (mean + sem of 3 independent experiments). (C) Effect of MDP-D and MDP-DD on FasL-induced apoptosis in THP-1 cells transduced with a lentiviral vector encoding a dominant negative form of RIP2 (mean + sem of 2 independent experiments performed in triplicate). Expression of RIP2DN was checked by western blot (inset). (D) Effect of *M. tuberculosis* infection on FasL-induced apoptosis in THP-1 cells transduced with a mock or RIP2DN construct (mean + sem of 2 independent experiments performed in triplicate).

Fig. 4. TLR2 signalling is involved in apoptosis protection following *M. tuberculosis* infection.

(A) FasL-induced apoptosis in THP-1 cells was measured after treatment with TLR2 or TLR4 agonists. Results are expressed as Annexin V-positive cells, normalized to 100% for untreated cells (mean + sem of at least 4 independent experiments). (B) Determination of caspase activity in cells treated with TLR2 or TLR4 agonists (mean + sem of 3 independent experiments performed in triplicate). (C) Effect of 19kDa lipoprotein on FasL-induced apoptosis in THP-1 cells transduced with a mock or a dominant negative construct of MyD88 (mean + sem of 2 independent experiments performed in triplicate). Expression of MyD88DN was checked by western blot (inset). (D) Effect of *M. tuberculosis* infection on FasL-induced apoptosis in THP-1 cells transduced with a mock or MyD88DN construct (mean + sem of 3 independent experiments performed in duplicate).

Fig. 5. *M. tuberculosis* infection mediates NF- κ B-dependent protection from FasL-induced apoptosis.

(A) THP-1 cells were treated with PDTC agent, infected or not with *M. tuberculosis* and apoptosis induced by FasL. Results are expressed as Annexin V-positive cells, normalized to 100% for untreated cells (mean + sem of 5 experiments). (B) Detection of NF- κ B in nuclear lysates following *M. tuberculosis* infection (mean + sem of 2 experiments performed in duplicate).

Fig. 6. Nuclear localization of NF- κ B is induced by *M. tuberculosis* infection.

(A) NF- κ B is present in the cytoplasm of non-infected cells. (B) THP-1 cells infected with FITC-labelled-*M. tuberculosis* and stained with anti-NF- κ B antibody and Hoechst. Infected cells show NF- κ B nuclear localization whose intensity is proportional to the number of ingested bacteria.

Fig. 7. Enhancement of c-FLIP expression following cell treatment with *M. tuberculosis* ligands or *M. tuberculosis* infection.

(A) Cells were treated with TLR2 or TLR4 ligands for 2 hours and c-FLIP expression determined by western blot. (B) THP-1 cells were infected with virulent or attenuated *M. tuberculosis* strains, apoptosis induced by FasL for 18h and c-FLIP expression monitored by western blot. Non-informative slots have been cut out.

Fig. 8. Model of *M. tuberculosis*-induced apoptosis evasion.

Right: In classical apoptosis, ligation of FasL to its receptor leads to caspase activation and apoptosis. Left: Infection of macrophages by *M. tuberculosis* leads through TLR2 to the nuclear translocation of NF- κ B. This leads to the expression of anti-apoptotic target genes like c-FLIP which inhibits death receptor-induced apoptosis.

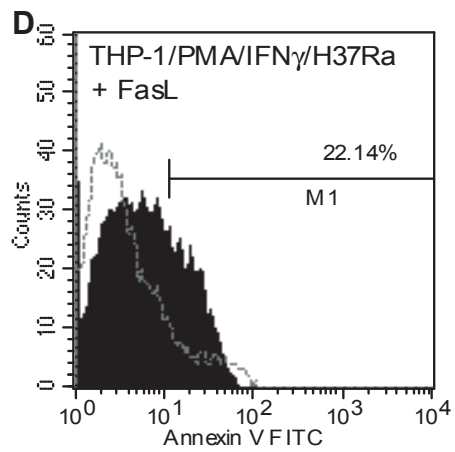
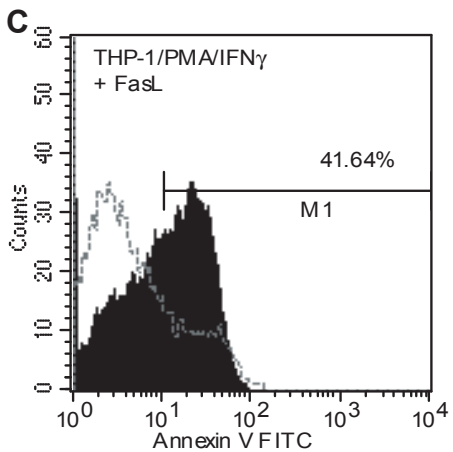
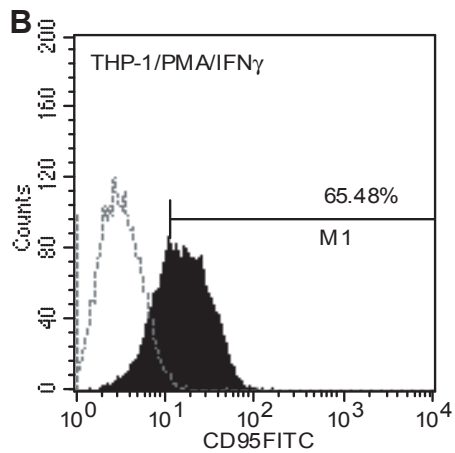
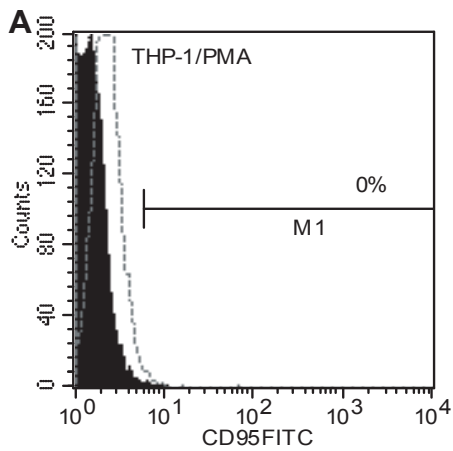


Figure 1

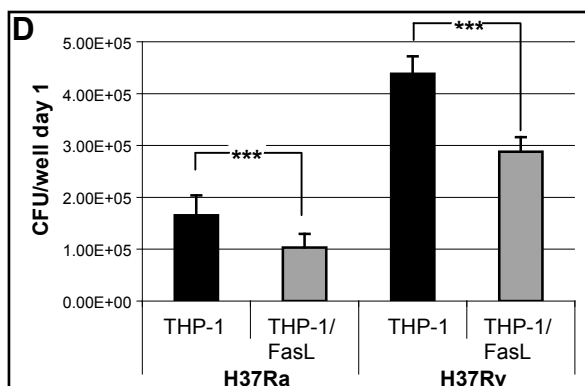
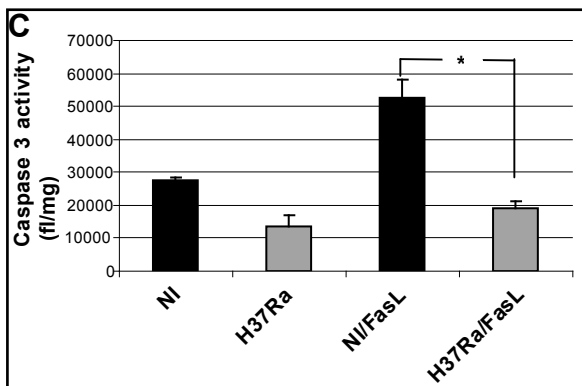
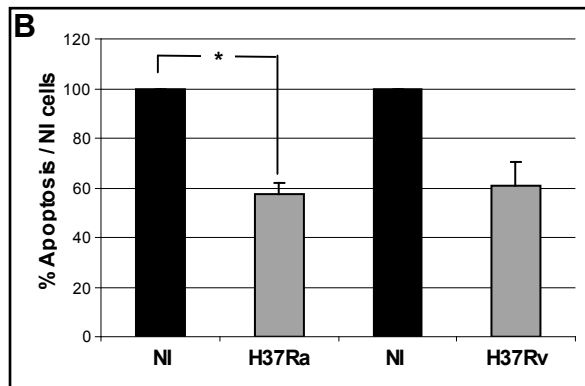
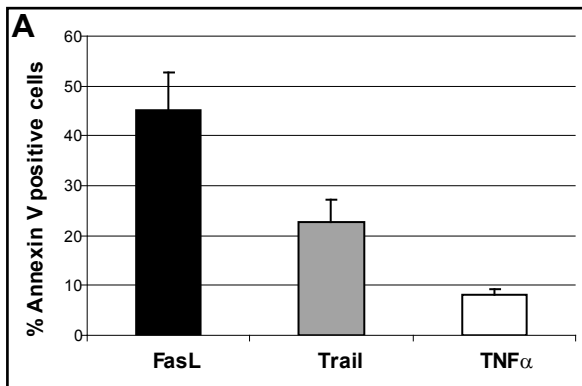


Figure 2

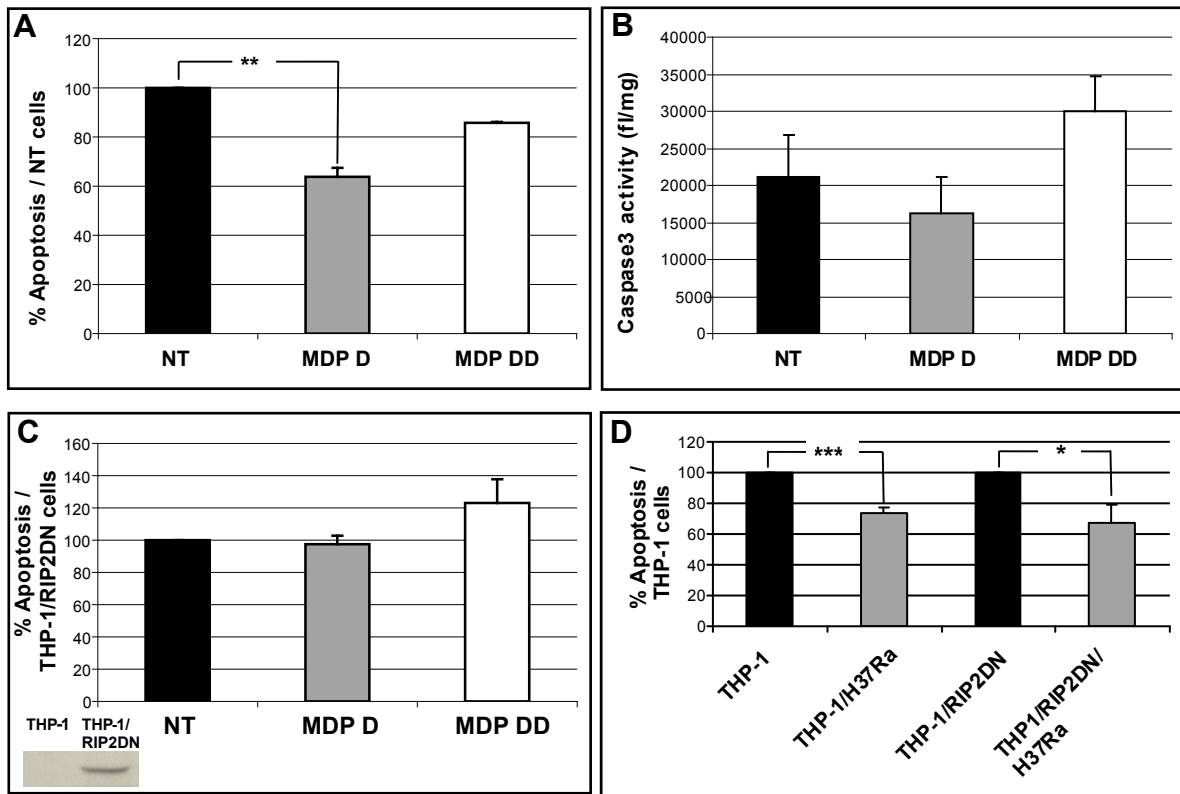


Figure 3

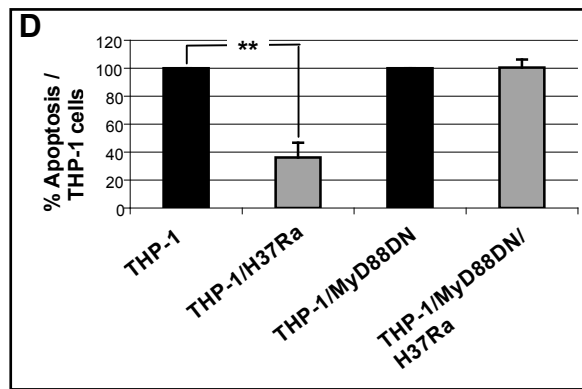
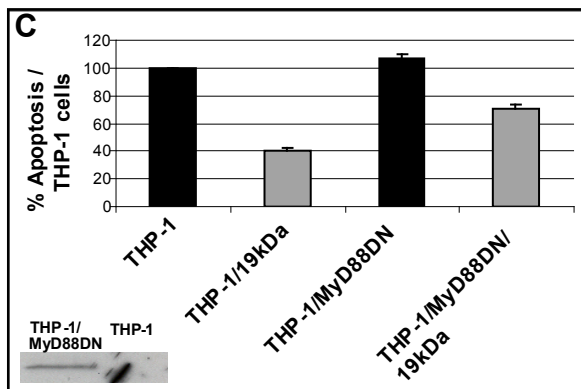
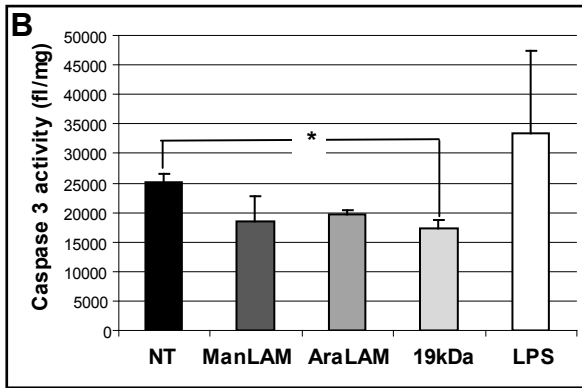
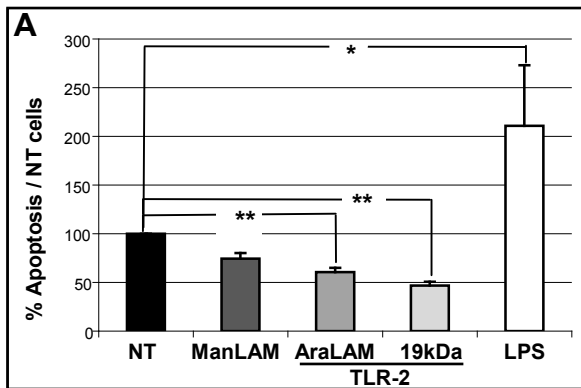


Figure 4

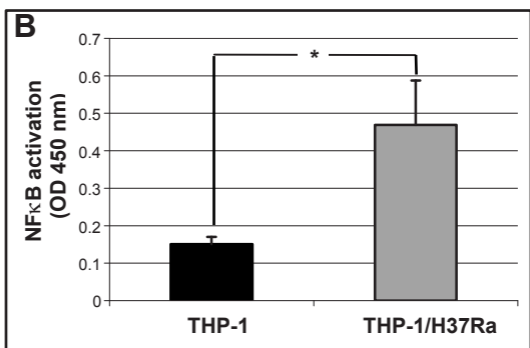
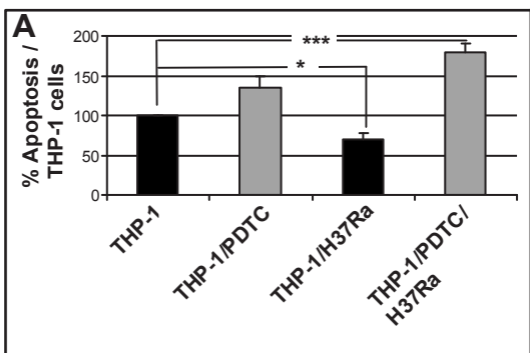


Figure 5

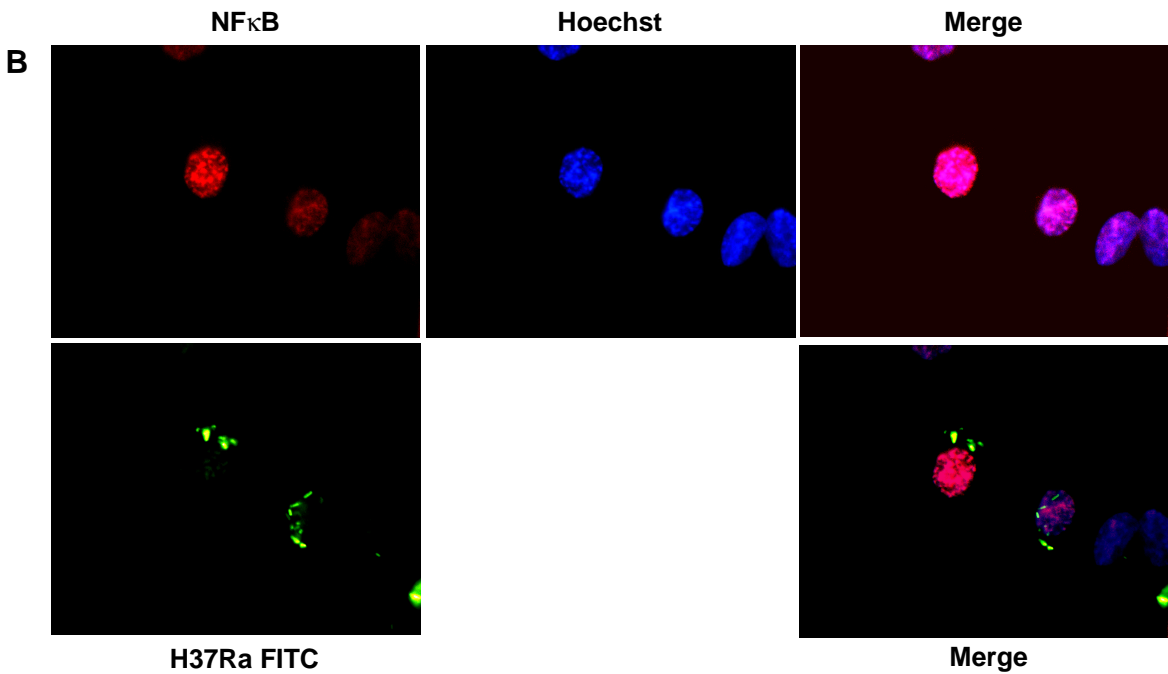
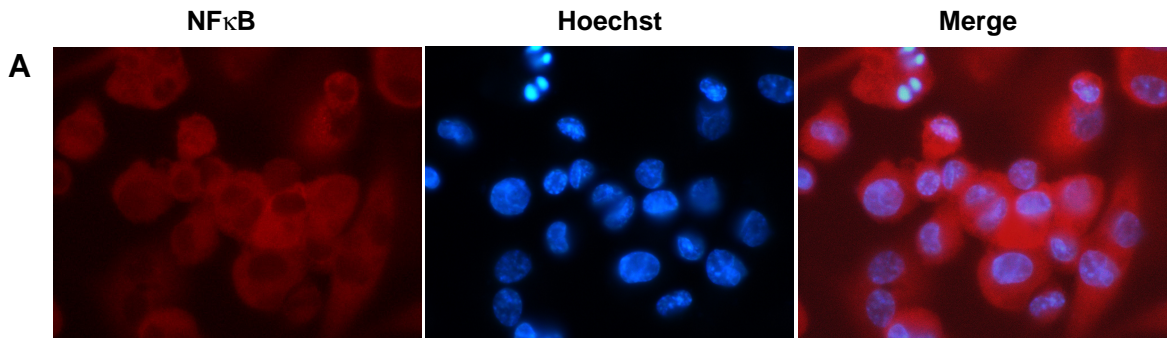


Figure 6

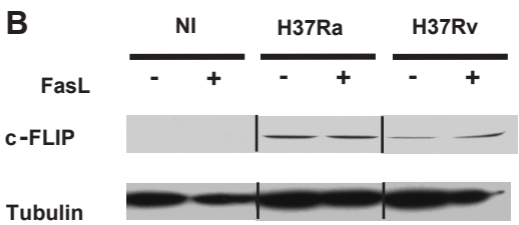
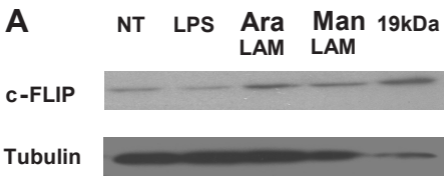


Figure 7

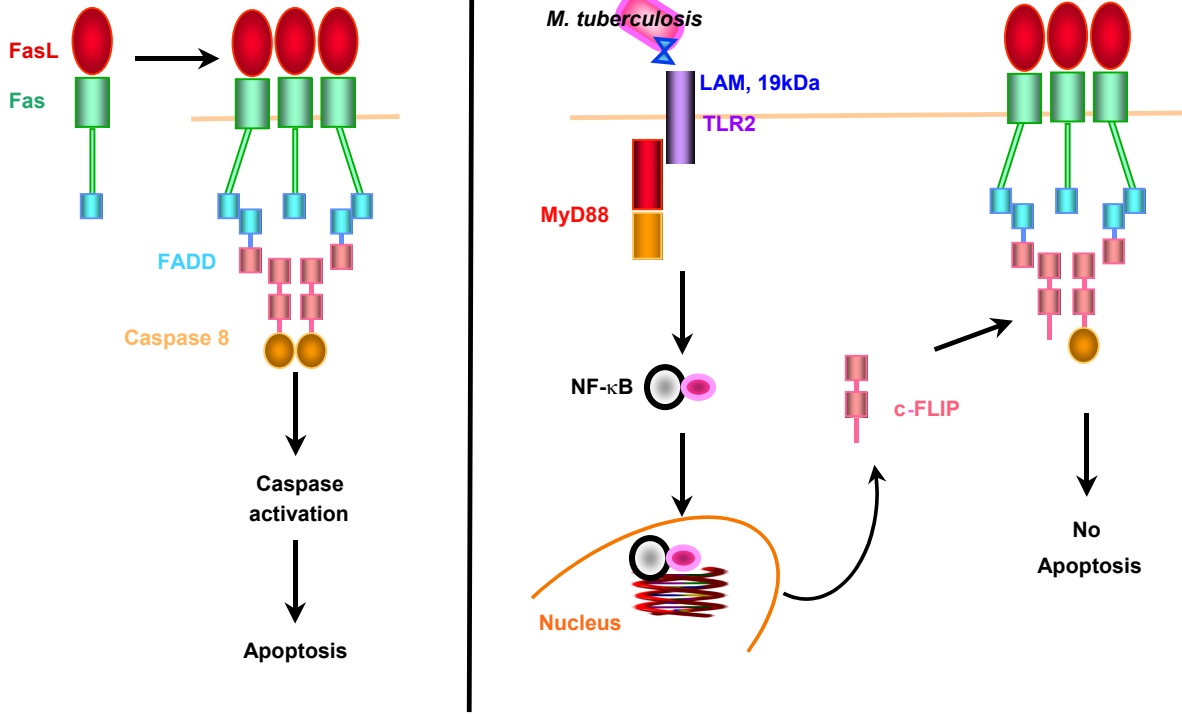


Figure 8