Macrophages from elders are more permissive to intracellular multiplication of *Mycobacterium tuberculosis*

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Introduction

Respiratory infections are one of the leading causes of disease in the elderly. Several factors contribute to their development, including malnutrition, age-associated diseases like diabetes, cardiac or structural lung diseases, and age-associated alterations in immunity (Meyer 2005). The most common agents that cause pneumonia are *Streptococcus pneumoniae* followed by respiratory viruses (Cabre 2009). Another important pathogen in respiratory infections is *Mycobacterium tuberculosis* (Yoshikawa 1981). In this regard, a noteworthy observation evidenced in countries with low prevalence of tuberculosis, like the United States, is that the incidence rate in the elderly is much higher than in the African Region which, according to the World Health Organization, represents high prevalence areas. The drift of tuberculosis into aged people seems to be explained by the aging of the population (Mori and Leung 2010). Additionally, the elderly account not only for a disproportionate share of all tuberculosis cases, but also of tuberculosis-related mortality (Zevallos and Justman 2003). Consequently, tuberculosis is becoming a serious health issue for the elderly population in low-prevalence countries.

Immunosenescence, understood as the changes in the immune system associated with age, is one of the reasons often claimed to influence the course of tuberculosis in the elderly (Rajagopalan and Yoshikawa 2000; Schaaf et al 2010). Most studies have focused on the analysis of the deterioration of adaptive immunity with age. In fact, it has been observed that the number of naïve T cells is lower in the elderly while, reciprocally, the number of memory and effector-memory cells is higher, as a result of exposure to pathogens through life. Thus, it has been defined the concept of "immune risk phenotype", characterized by an inverted CD4/CD8 ratio and low lymphoproliferative response (DelaRosa et al 2006). Regarding the innate immunity,

cells seem to suffer from defects that limit their functionality. Neutrophils, although in similar numbers in both young and elder people (Chatta and Dale 1996), exhibit in the latter less chemotaxis (Fortin et al 2006), impaired ability of priming agents to delay apoptosis (Fortin et al 2007) and less phagocytosis (Fülöp, Jr. et al 1997). Variations in the function of aged monocytes/macrophages are less clear. Activation of macrophages leads to a decrease in the production of proinflammatory cytokines in the mouse model (Boehmer et al 2004), but contradictory results have been reported for the human model. Some studies describe that stimulation of monocytes or mononuclear cells from peripheral blood induced a higher production of cytokines in elders (O'Mahony et al 1998; Roubenoff et al 1998; Sadeghi et al 1999), but in other works a decrease is reported (Beharka et al 2001; Delpedro et al 1998; Gon et al 1996; van Duin et al 2007). Among functional activities, phagocytosis does not seem to be altered (Fietta et al 1993; Fietta et al 1994) but it has been reported a decrease in the reactive oxygen species (ROS) production (Álvarez and Santa María 1996). There is an agreement in the idea that several Toll-like receptors are less expressed in aged macrophages (Gomez et al 2008).

Several studies deal with the influence of immunosenescence in the immune response to tuberculosis. In the mouse model an associated negative effect has been found on $CD4^+$ T cell-mediated responses (Orme 1987), including an inferior capacity of $CD4^+$ to produce IFNγ in response to mycobacterial antigens in the presence of IL-2 (Orme et al 1993). An initial protection observed in the three weeks after infection in old, but not young mice, has been attributed to the activity of $CD8^+$ T-cells (Turner et al 2002). The early production in old mice of the Th1 cytokines IL-2, IL-12 and IL-18, collaborate with $CD8^+$ T-cells in the nonspecifical production of IFNγ that lead to the transient control of *M. tuberculosis* growth (Vesosky et al 2006). Other reports indicate

that biological activities of aged as compared with young mouse macrophages against *M. tuberculosis* seem unaltered (Rhoades and Orme 1998; Vesosky and Turner 2005). Nevertheless, a recent study identified an important difference. Production of cytokines by $CD11c^+$ pulmonary cells infected with *M. tuberculosis* is TLR2 dependent in young mice but not in old mice (Rottinghaus et al 2010).

A literature search for *in vitro* infection studies with human cells from elders yielded no results. We have performed experiments with human monocytes to test the hypothesis that the increase of tuberculosis in the elderly is not only a consequence of an aged population, but also of an intrinsic susceptibility related to immunosenescence. We have found that the intracellular multiplication of the bacteria in aged monocytes is faster than in monocytes from adults, which may partially explain the impaired ability of the elderly to contain the development of tuberculosis. Although we are not certain about the biological reason for this finding, we have observed that monocytes from elders exhibit lower levels of tyrosine phosphorylation when infected, and that protein tyrosine phosphorylation inhibitors allow a further increase in the intracellular multiplication of *M. tuberculosis*.

Materials and Methods

Bacterial strains

Mycobacterium tuberculosis HL186T, isolated at the Hospital de León (Microbiology Service) and kindly provided by Julio Blanco and Manuela Caño, and *Mycobacterium. bovis* BCG (RIVM strain derived from the 1173-P2 strain) were grown on 7H11 agar supplemented with 0.2% glycerol and 10% Middlebrook enrichment OADC (Becton

Dickinson Microbiology Systems, San Agustín de Guadalix, Madrid, Spain). *M. bovis* BCG was used only for the phagocytosis assay. Bacteria from fresh culture were suspended in RPMI-1640 medium, without serum (Sigma-Aldrich Spain, Tres Cantos, Madrid, Spain). To obtain isolated mycobacteria, they were sonicated using an S-450 digital ultrasonic cell disruptor (Branson Ultrasonics, Danbury, Connecticut, USA). Pulses of 10 s were applied with a microtip at an amplitude of 10% (2 W), and sonicated bacteria were centrifuged at 100 × g for 1 min at room temperature. After recovering the supernatants, sonications were repeated as many times as necessary to obtain individualized bacteria, usually three or four rounds. At the end most bacteria were alive and very few groups remained, with ≤ 5 bacteria per group, as determined by the LIVE/DEAD Baclight bacterial kit (Molecular Probes, Invitrogen, Prat de Llobregat, Barcelona, Spain). After addition of glycerol to 20%, single use aliquots were frozen at – 80ºC.

Monocyte derived macrophages (MDM)

Peripheral blood was collected from aged (73-92 years, mean 81.1 years) and younger adults (20-48 years, mean 29.1 years) donors, following consent and approval of the protocol by the Hospital of León Clinical Research Ethics Board. Peripheral blood mononuclear cells were isolated by Ficoll-Paque Plus density gradient sedimentation (GE Healthcare, Life Sciences, Uppsala, Sweden), and $CD14^+$ cells (monocytes) were purified by magnetic cell separation (Miltenyi Biotec, Pozuelo de Alarcón, Madrid, Spain). We ascertained the purity of cells by flow cytometry with appropriate labelled antibodies (Becton Dickinson, San Agustín de Guadalix, Madrid, Spain) and > 94 % of cells in the monocyte preparation were $CD14^+$. The number of cells was calculated by

counting in a Neubauer chamber and were cultivated, within 4 hours from blood collection, in RPMI-1640 /10% autologous serum, at 37ºC in 95% air/5% CO_2.

Cellular infection

For antimicrobial activity determination monocytes were incubated in 96-well plates in a total volume of 100 µl. 10^5 cells were infected with 10^3 bacteria (Multiplicity of Infection, MOI = 0.01). When indicated, cells were incubated in 25 ng/ml of the following cytokines: IL-1β (≥ 1×10^9 units/mg), IL-6 (≥ 1×10^7 units/mg), TNFα (≥ 2×10^7 units/mg) and IFNγ (≥ 2×10^7 units/mg, Peprotech, London, UK). In neutralization studies 0.5 µg of either mouse anti-IL-1β (eBioscience, California, USA) or goat polyclonal anti-IL-6 (Abcam, Cambridge, UK) were added. In tyrosine kinase inhibition experiments cells were preincubated for 1 hour before infection with either 100 µM genistein or daidzein and either 10 µM PP2 (Cayman Chemical, Michigan, USA) or PP3 (Calbiochem, Merck Spain, Madrid, Spain). Inhibitors stocks in dimethyl sulfoxide (500 mM genistein and daidzein, 10 mM PP2 and PP3) were diluted to the appropriate concentrations with RPMI-1640 and 0.2 % dimethyl sulfoxide was added to cells that were not treated with inhibitors. Cells were lysed after four days by sonication with a microtip (Branson Ultrasonics), at an amplitude of 10% (2 W) for 3 s, to release bacteria. At this setting, ultrasounds were able to lyse cells without affecting the bacterial viability. Decimal dilutions of the sonicates were inoculated and incubated at 37ºC in 7H9 broth supplemented with 0.2% glycerol and 10% Middlebrook enrichment ADC (Becton Dickinson Microbiology Systems) for 10 days. CFU (Colony Forming Units) were determined under an inverted microscope at × 100 magnification (Fazal et al 1992).

For total RNA purification and supernatant recovery for cytokines quantification, immediately after purification, 5×10^5 monocytes were infected with 5×10^5 bacteria (MOI = 1) for 24 hours in a volume of 500 µl (24-well plates).

Quantitative polymerase chain reaction (qPCR)

Total RNA from infected cells was prepared using the Speedtools Total RNA Extraction Kit (Biotools B&M Labs, Madrid, Spain) and reverse transcribed into cDNA by qScript cDNA synthesis kit (Quanta Biosciences, Maryland, USA). Real-time PCR was performed on a Bio-Rad iCycler system (Barcelona, Spain) using SYBR-Green (Molecular Probes, Invitrogen, Barcelona, Spain). The threshold cycle (Ct) values for each of the target genes were normalized to the Ct of the reference gene *HPRT1* (hypoxanthine phsophoribosyltransferase 1). The efficiency (E) of the PCR reaction for each target gene was calculated using the slope of the standard curve obtained from the Ct of 1/8 dilutions of each amplicon ($E = 10^{-1/slope}$). Calculated efficiencies were: IL-β, 1.980; IL-6, 1.950; TNFα, 1.955 and HPRT1, 1.952. The delta delta C_t method was used for quantification of the relative gene expression as the following ratio (Pfaffl 2001): $(E_{target})^{\Delta Ct,\ target\ (calibrator-test)}/(E_{ref})^{\Delta Ct,\ ref\ (calibrator\ -\ test)}$ (target gene: cytokine genes; reference gene: *HPRT1*; calibrator: RNA from non-infected cells and test: RNA from *M. tuberculosis* infected cells). The primers used were as follows: *HPRT1* forward (F) GGCCATCTGCTTAGTAGAGCTTTT, reverse (R) TTAAACAACAATCCGCCCAAA; *IL-1β* (F) AAGCTCGCCAGTGAAATGATG, (R) GGTGGTCGGAGATTCGTAGC; *IL-6* (F) TGCAATAACCACCCCTGACC, (R) CATTTGCCGAAGAGCCCTC; *TNFα* (F) TCTTCTCGAACCCCGAGTGA, (R) CCTCTGATGGCACCACCAG.

Quantification of cytokines in supernatants

To remove bacteria from supernatants, samples were centrifuged for 3 min at 10.000 × g at room temperature in ultrafree-MC filter units (Millipore Iberica, Madrid, Spain) of 0.45 μm and frozen at –80ºC. Cytokines were quantified by ELISA using BD OptEIA Human IL-1β ELISA Set II, Human IL-6 ELISA Set and Human TNF ELISA Set (Becton Dickinson).

Phagocytosis assay

For detection of bacteria by flow cytometry we constructed an expression plasmid with the gene of the fluorescent protein DsRed .T3_S4T (Sörensen et al 2003). From plasmid pDsRed.T3_S4T, kindly provided by Ignacio Rodríguez-Barbosa, we obtained the XbaI-HindIII fragment that contains the fluorescent protein gene, and subcloned it in the mycobacterial expression vector pNBV1 (Howard et al 1995), kindly provided by Carlos Martín, which contains a hygromycin-resistance antibiotic cassette. To facilitate the expression of the protein, a 613 bp fragment of the mycobacterial *hsp60* promoter was amplified with the oligonucleotides hsp60prF (GCTCTAGATTGATTAGCACGTCGATGC) and hsp60prR (AATCATATGCATTGCGAAGTGATTCCT), which contain an XbaI and an NdeI sites, respectively, and was subcloned upstream the fluorescent protein gene in the pNBV1 plasmid. Purified plasmid, named pMDsRed, was electroporated (1.8 kV, 25 μF, 1000 Ω) into the BCG strain by standard protocols (Jacobs, Jr. et al 1991).

Transformants were grown on supplemented 7H11 agar /hygromycin 50 μg/ml. Individualized bacteria were frozen at -80ºC in single use aliquots.

10^5 monocytes were incubated with 5×10^5 BCG bacteria (MOI = 5) in RPMI-1640/ 10% autologous serum in a vertical rotator at 37ºC. After 10 min cells were placed in ice and analyzed in a FACScan instrument (Becton Dickinson).

Western blot analysis

7×10^5 monocytes were incubated with 1.4×10^7 bacteria in RPMI-1640/ 10% autologous serum in a vertical rotator at 37ºC. After 30 min cells were centrifuged at 800 × g for 5 min at 4ºC, washed with cold RPMI-1640 and centrifuged again in the same conditions. After supernatant removal cells were lysed in 10 mM Tris-HCl pH 7.5, 1% Triton X-100, 5 mM EDTA and 10% glycerol, supplemented with a protease inhibitor cocktail set I (Calbiochem) and a phosphatase inhibitor cocktail B (Santa Cruz Biotechnology, California, USA) and incubated in ice. After 5 min lysates were centrifuged at 14.000 × g for 5 min at 4ºC, and supernatants were boiled in Laemli loading buffer and frozen at -40ºC. Proteins were fractionated by sodium dodecyl sulphate- 7.5% polyacrylamide gel electrophoresis and transferred by electroblotting to polivinylidene difluoride membranes (Millipore Iberica, Madrid, Spain). Membranes were incubated in blockin buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 10% bovine serum albumin (Applichem, Darmstadt, Germany) for one hour and with anti-phosphotyrosine PY20 antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology), 1:3000 in blocking buffer, for 1 hour. After extensive washing blots were developed with the ECL Advance Western Blotting Detection Kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) using the Chemidoc-XRS image analyzer (Bio-Rad).

β-actin was used as the loading control, and was detected with anti-β-actin antibody (AC-15) (Santa Cruz Biotechnology).

Protein tyrosine phosphorylation array analysis

3×10^6 monocytes were infected with 6×10^7 bacteria in RPMI-1640/ 10% autologous serum in a vertical rotator at 37ºC. After 30 min cells were centrifuged at 800 × g for 3 min at 4ºC, washed with cold RPMI-1640 and centrifuged again in the same conditions. After supernatant removal, cell lysates were obtained in the buffer included in the Ray Bio® Human RTK (Receptor Tyrosine Kinases) Phosphorylation Antibody Array I (RayBiotech, Georgia, USA) and frozen at -80ºC. Arrays, which include 71 human tyrosine kinases, are incubated with the lysates (120 μg of total protein) following the manufacturer's instructions. Tyrosine phosphorylated proteins are detected by chemiluminiscence with a biotinylated anti- phosphotyrosine antibody and streptavidin coupled to horse radish peroxidase. Arrays were developed using the Chemidoc-XRS image analyzer and analysis was performed with Quantity One 4.4.1. (Bio-Rad). Units were intensity × mm^2, in which signal intensity was measured in counts. Variations in intensities between membranes were normalized using a positive control included in the array. Comparison results were expressed as the percentage of the difference in dots intensities.

Analysis of ROS production

Intracellular production of ROS was measured as luminol-enhanced chemiluminiscence. Either 5×10^5 bacteria (MOI = 5) or 150 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Calbiochem), luminol 500 μM (Sigma-Aldrich Spain) and Hanks balanced salt solution were added to 10^5 monocytes in a total volume of 100 μl, and incubated for 30

min at 37ºC. After 30 min emitted light was measured for 5 sec at 21ºC in a TopCount-NXT microplate scintillation and luminescence counter (Packard, Perkin Elmer España, Madrid, Spain) and ROS formation was expressed as counted photons per second (cps).

Cellular death quantification

Cellular death was measured with the CytoTox-Glo Cytotoxicity Assay (Promega Biotech Ibérica, Madrid, Spain). The main reagent of this kit is a luminogenic peptide substrate (alanyl-alanyl-phenylalanyl-aminoluciferin) that measures "dead-cell protease activity", an intracellular activity that is released from cells that have lost membrane integrity. Briefly, 5 µl of the cell lysis obtained by sonication for measuring antimicrobial activity (see Cellular infection, above) were diluted 1/10. The dilution (22 µl) was mixed with 11 µl of the luminogenic peptide substrate, and 30 µl of the mix were incubated 30' at 21ºC, and measured for 5 sec in a TopCount-NXT microplate scintillation and luminescence counter (Packard). Results were expressed as counted photons per second (cps), and subtracted from background, measured in medium RPMI-1640. The quantities correspond to the activity in live cells present at the moment of lysis. The lower the number of cps, the higher the number of dead cells.

Statistical analysis

Cellular death and phagocytosis data followed a normal distribution (Shapiro-Wilks test), but CFU and ROS data needed a log transformation to be normally distributed. Comparisons between adult and old were analyzed using the Student's *t*-test. When variances were not homogeneous (Levene test) the unequal variance *t*-test was used.

Gene expression and ELISA data were not normally distributed and were analyzed by the Mann-Whitney *U* test. Analysis was performed with PASW Statistics 18 (International Business Machines, Madrid, Spain).

Antimicrobial activity was analyzed in two-level factorial experiments using the "Design of experiments" methodology. Factorial designs are characterized by the investigation in each complete trial or replication of all the possible combinations of the levels of the factors. The two levels that we have chosen for the factors cytokines (IL-1β, IL-6, TNFα and IFNγ) are low: 0 and high: 25 ng/ml, and for the factor 'age', low: < 50, adults and high: > 70 years, elders. The analyzed response was log CFU. Factorial designs are more efficient than one-factor-at-a-time designs like ANOVA, because in a single experiment we obtain several estimates for every factor, and a smaller sample size is required. Additionally, they are necessary when interactions may be present to avoid misleading conclusions (Montgomery, 2001). Because we used 4 factors (cytokines) in each age group, in every experiment we need to perform 2^4 (16) infections, and we decided to use a one-half fractional design, in which the combinations are allocated in two fractions. Distribution of treatments is not random and in our design follows the pattern shown in Table 1. The arrangement of these treatments within the 96-well plate was randomized. 2 adults and 2 elders were studied in each fraction, which allowed reaching a statistical power of 83% to detect differences of 0.48 log CFU. In this way we obtain two replicates for every cytokine treatment and age group. In each fraction we included two additional central points (12.5 ng/ml of each of the 4 cytokines together). These points provide us with information regarding the variability within groups and the linearity of the statistical model. Studies with tyrosine kinase inhibitors were designed as 2^3 full factorial experiments. Two experiments were performed, one with tyrosine kinase inhibitors and the other with

relevant negative controls. Low levels were 0 μM in all cases and high levels were 100 μM genistein (daidzein as negative control) or 10 μM PP2 (PP3 as negative control). The third factor in both experiments was IFNγ, used as a control, with 0 and 25 ng/ml as low and high levels, respectively. Results were analyzed with Design-Expert 8.0.6 (Stat-Ease, Minneapolis, USA). In all statistical analysis, a P value < 0.05 was considered significant.

Results

M. tuberculosis infected monocytes from elderly produce more IL-6.

The capacity of aged human monocytes to produce cytokines when infected with live bacteria is very little characterized. For this reason, we measured the gene expression and protein production of IL-1β, IL-6 and TNFα by monocytes from old and younger adults infected for 24 h with *M. tuberculosis* (MOI = 1). As expected, monocytes from both adults and elders increased dramatically the gene expression of the three cytokines upon infection (data not shown). Nevertheless, no statistical difference in the increased relative expression was found between infected monocytes from adults and elders, although the median was higher for adults in the three analyzed cytokines (Table 2).

In several studies in which either monocytes (O'Mahony et al 1998; Sadeghi et al 1999) or peripheral blood mononuclear cells (Roubenoff et al 1998) from aged subjects were activated, the production of proinflammatory cytokines was analyzed. While the production of IL-1β and TNFα was not uniformly increased, in cells from elders the IL-6 production was always higher as compared with young individual cells. For this reason, and despite the absence of differential gene expression, we decided to analyze

the protein production in supernatants from *M. tuberculosis* infected monocytes (Fig. 1). We also found that the production of IL-1β and TNFα was not significantly higher in elders than in adults. Although the amount of these cytokines was somehow higher in elders, the variability was large. A different case was IL-6 because at 24 hours there were significantly higher amounts in elder cell supernatants (*P = 0.014), and this trend was kept at 48 hours (P = 0.067, nearly significant). The quantities measured for the three cytokines at 72 hours were very similar to those at 48 h. We think that the reason is that by this time *M. tuberculosis* had destroyed the cell culture, as we observed under the microscope. From these results we conclude that post-transcriptional regulation may determine differences in the production of cytokines, which may be biologically important.

Multiplication of *M. tuberculosis* is increased in monocytes from elderly

IL-6 has been found to negatively interact with either IFNγ (Nagabhushanam et al 2003) or TNFα (Bermudez et al 1992) to inhibit antimycobacterial activity in macrophages. Because *M. tuberculosis* infected monocytes from aged donors produce more IL-6, we reasoned that this excess of cytokine might be inhibiting the IFNγ or TNFα beneficial influence on the antimycobacterial activities of MDM in elders. For this reason we decided to study in a factorial experiment the influence of IL-6 on the intracellular multiplication of *M. tuberculosis* in MDM activated with IFNγ or TNFα, and included IL-1β because it is also produced by infected MDM in high quantities. We analyzed the same data as a 2^4 factorial design, using as factors the cytokines IFNγ, IL-1β, IL-6 and TNF-α and, similarly, as a 2^5 factorial design, with the addition of the factor "Age". When the 2^4 designs were applied independently to MDM from either adults or elders

we found that the addition of IFNγ had an important effect in the MDM from elders, because a smaller number of CFU was recovered. The same trend was appreciated in the case of the MDM from adults, and although the figures were comparable to the ones observed for elders (-0.117 versus -0.120 log CFU) the difference did not reach statistical significance (Table 3). These results are in accordance with the known clinical importance of IFNγ in the immune response to tuberculosis (Ottenhoff et al 2005). Regarding the remaining proinflammatory cytokines, none of them showed a major influence in the intracellular multiplication of the bacilli. The same result was obtained when interactions were analyzed, because they all were non-significant (data not shown).

When the results were analyzed as a 2^5 design, introducing "Age" as a factor, a new aspect was, however, evidenced: an estimate of more than half an order of magnitude of bacteria was recovered from MDM from elders after four days of infection. The estimate of the effect of IFNγ, although non-significant in this analysis, remained alike (-0.118 log CFU, Table 3). Non-significant were also both the interactions (data not shown) and the effect of the other cytokines. The negative results regarding the influence of IL-6 prompted us to perform a new factorial experiment with anti-IL-6 and anti-IL-1β neutralizing antibodies as factors, together with IFNγ (sample size = 2). With this experiment we expected to learn whether neutralization of the IL-6 excess production in MDM from aged donors would allow a better IFNγ control of the *M. tuberculosis* intracellular growth. This was not, however, the case, because the neutralizing antibodies treatments gave non significant results (anti-IL-6 effect estimate: 0.04 [95% confidence interval: -0.10 - 0.19], P = 0.503; anti-IL1β: -0.02 [-0.16 – 0.12], P = 0.764), although the effect of IFNγ remained significant (effect estimate: -0.19 [95% confidence interval: -0.34 – -0.05]; *P = 0.012). Altogether, these results suggest

that MDM from elders are more permissive to the intracellular multiplication of the bacilli than those from younger individuals. On the other hand, we have obtained no evidence that this permissiveness depends on the influence of either IL-6 or IL-1β. For illustration purposes we present the log CFU of the IFNγ (Fraction 2, Table 1) and 'no cytokine' (Fraction 1) treatments: elder IFNγ 3.62 and 3.76; elder 'no cytokine' 4.11 and 3.07; adult IFNγ 3.53 and 2.81 and adult 'no cytokine' 3.15 and 2.70.

Phagocytic activity of monocytes from elders remains unaltered.

The detected increased level of *M. tuberculosis* multiplication in experiments with monocytes from elders might be a consequence of an augmented phagocytic activity, rather than faster intracellular multiplication. To test this possibility we have constructed an expression plasmid with the DsRed fluorescent protein and electroporated it in a *M. bovis* BCG strain. Biosafety reasons precluded the use of the *M. tuberculosis* strain because it was going to be used in flow cytometry. When monocytes from either adults or elders were incubated with the BCG bacterium in the presence of 10% autologous serum their phagocytosis was fast. In each experiment two samples were processed simultaneously, one from an adult and the other from an elder. In only two out of five experiments more monocytes from elders than from adults had phagocytosed the bacteria (Fig. 2). The statistical comparison between the number of phagocytic monocytes from adults and elders showed no significant difference ($P = 0.453$).

Another possibility that we needed to rule out was the extracellular growth of the bacteria. When we incubated for four days 10^3 bacteria in RPMI-1640/10% human serum and quantified the number of CFU, we observed that the bacteria had survived,

but did not multiply. Inoculated bacteria average (±SD) was 2.90 (0.10), and surviving bacteria after four days were 2.77 (0.41). Data analyzed by paired t-test showed no significant difference between both groups (P = 0.439). From these evidences we conclude that the difference in the multiplication level of *M. tuberculosis* in monocytes from elders may be attributed to intracellular growth.

Protein tyrosine phosphorylation promoted by *M. tuberculosis* infection.

The only characteristic feature of monocytes from elders that we have observed is the higher production of IL-6, and we have found no evidence that it may be influencing the *M. tuberculosis* intracellular growth rate. For this reason we analyzed other biologic activities like signalling. It is known that *M. tuberculosis* infection induces protein tyrosine phosphorylation in monocytes (Zaffran et al 1998), and we decided to investigate whether there were differences in the protein phosphorylation pattern between monocytes from adults and elders. When the cells were infected for 30 min with a MOI 20 some changes were observed (Fig. 3). A low level of basal phosphorylation was detected in non-infected cells, although perhaps higher in monocytes from adults. A phosphorylated 44 kD protein present in non-infected cells was no longer apparent after infection. Two higher size proteins (46 and 103 kD) seemed to be little phosphorylated in non-infected monocytes from elders, although the 103 kD protein became phosphorylated after infection. Phosphorylation of a 49 kD and a 118 kD proteins was higher in both age groups after infection, while two proteins of 58 and 56 kD did not show much change in their level of phosphorylation. The more important change was detected for two proteins of 75 and 78 kD, which were

dramatically phosphorylated in monocytes from adults. Although suggestive, conclusive results may not be reached out of such a small sample.

To gain further insight into the nature of this protein phosphorylation we analyzed tyrosine phosphorylated kinases in lysates from infected monocytes. In Fig. 4.A we confirmed a higher level of tyrosine phosphorylation in infected monocytes from adults than from elders. We used these same lysates to probe a Human Receptor Tyrosine Kinases Phosphorylation Antibody Array, which detects protein phosphorylation levels rather than protein expression. The obtained results may be more relevant for the identification of biological pathways which are active in the tested cells. In Fig. 4.B we observe the simultaneous exposition of both membranes, in order to allow appropriate comparisons. For this purpose data were normalized with the positive control included in the kit that was closer to the general dot intensities observed for the tested kinases (POS3, dots A5-6). Many of the tyrosine kinases included in the array exhibit basal levels of phosphorylation, but the pattern is practically identical when the lysate from the adult is compared with that from the elder. Increased levels of phosphorylation are detected in the following kinases: FGFR2 (dots F9-10), Hck (G7-8), JAK2 (H7-8), Lyn (I3-4), MATK (I5-6), Pyk2 (J5-6), TNK1 (L5-6) and TXK (L9-10). The apparent stronger intensity of the dots in the adult membrane is the consequence of a darker background. When the local background that surrounds each dot is subtracted, differences disappear in most cases. Nevertheless, three of the kinases had more than 20% intensity increase for the elder, namely, FGFR2 (22%), Hck (22%) and TXK (23%), and one for the adult, MATK (23%). The only kinase that exhibited more than a 50% difference was Pyk2, which was 51% stronger in the adult.

Inhibition of protein tyrosine phosphorylation allows higher levels of *M. tuberculosis* intracellular multiplication.

Higher levels of bacterial multiplication and lower levels of tyrosine phosphorylation might be unrelated events. For this reason we investigated whether the addition of protein tyrosine kinase inhibitors (genistein and PP2) further increased the mycobacterial multiplication rate in MDM from elders. Genistein, a soy isoflavone, is a substrate competitive inhibitor of protein tyrosine kinases, and daidzein, an analog that lacks this property, is frequently used as a negative control. On the other hand, PP2 is a selective inhibitor of the Src-family of protein tyrosine kinases and PP3 is the corresponding negative control. When cells were preincubated with either 100 μM genistein or 10 μM PP2 we observed that genistein induced a slighter inhibition of protein tyrosine phosphorylation than PP2 (Fig. 5). We designed two additional 2^3 full factorial infection experiments using MDM from elders, one with the protein tyrosine kinase inhibitors, and the other with the negative controls (Table 4). IFNγ, included as a control of the antimycobacterial activity in both experiments, again induced a significant effect ($*P \leq 0.011$). Both genistein and PP2 allowed a higher multiplication level of *M. tuberculosis*, more apparent with genistein, a result that supported the influence of a lower level of phosphorylation in the ability of MDM from elders to interfere with the mycobacterial multiplication. In this experiment the interaction between IFNγ and genistein was the only statistically significant ($*P = 0.016$). A puzzling result was obtained, however, with the negative controls because daidzein also promoted bacterial intracellular multiplication. This result does not allow obtaining a definitive conclusion about the effect of genistein which, in addition, displayed a low

level of tyrosine kinase inhibition (Fig. 5). Results with PP2 and PP3 were clearer, because PP2 allowed a higher multiplication rate of the bacteria, but PP3 had no effect.

Infected monocytes from elders produce a higher amount of ROS.

To further characterize the response of monocytes to the infection with *M. tuberculosis* we analyzed the production of ROS, a classical marker of cellular activation. As the negative controls we used non infected monocytes and TPA activated monocytes, inducer of the oxidative burst, as a positive control. In both cases, the ROS basal level and the ROS produced in response to activation by TPA were higher in monocytes from elders (Fig. 6). This same pattern was observed in *M. tuberculosis* infected monocytes, and differences of ROS production between monocytes from elders or adults resulted significant in both non infected and infected cells. This result was surprising, because we would have expected a stronger response by monocytes from adults, but it showed the same trend observed for the production of IL-6, another sign of monocyte activation.

Cellular death is higher in MDM from elders.

While performing the aforementioned infections we observed under the microscope that MDM from adults looked usually healthier than those from elders. To objectively analyze this biological phenomenom we decided to study the level of cellular death by a chemiluminescent assay based on the measure of a "dead-cell protease activity" by using a luminogenic peptide substrate. This protease activity is intracellular and cell lysis releases it. The higher the number of viable cells before lysis the higher the amount of light produced. Four days after lysis no activity could be detected (Fig. 7, t =

0). We did not measure the protease activity in non-lysed cultures because cells that had died at an early point would not be detected. With this assay we confirmed that the highest amount of light emitted corresponded to the MDM from adults in both non-infected and *M. tuberculosis* infected cells. Additionally, both MDM from elders or adults seemed to be protected from cellular death by *M. tuberculosis* infection, but the difference was not statistically significant when compared with non infected cells. Although it has been reported in the murine model that IFNγ promotes survival of macrophages with a low intracellular *M. tuberculosis* load (Lee and Kornfeld 2010), we did not observe such effect in human cells, although differences between MDM from adults and elders remained significant (Fig.7, *M. tuberculosis* + IFNγ).

Discussion

One of the reported effects of immunosenescence on monocytes is the decrease in the production of proinflammatory cytokines or ROS after activation. Several authors have observed that aged monocytes activated with LPS produce the same amount of IL-1β (Rudd and Banerjee 1989) or fewer amounts of IL-1β, IL-6 and TNFα than young monocytes (Delpedro et al 1998b; Gon et al 1996a; McLachlan et al 1995; Sadeghi et al 1999). These results are counterintuitive because macrophages are one of the major sources of proinflammatory cytokines, and the elderly exhibit a heightened proinflammatory milieu with higher levels of proinflammatory cytokines and markers such as C-reactive protein, a condition termed 'inflamm-aging' (Franceschi et al 2000). Nevertheless, other authors have observed an increased production of proinflammatory cytokines in activated macrophages (O'Mahony et al 1998; Roubenoff et al 1998; Sadeghi et al 1999), and our results are consistent with theirs and with the 'inflamm-aging' condition. There is not a clear reason for this discrepancy but in our model the

activating stimulus is live bacilli instead of LPS, and *M. tuberculosis* has a very complex cell wall that display different ligands for numerous macrophage receptors (Ernst 1998) besides TLR4, the LPS receptor. The importance of specific receptors has recently been underscored by Rottinghaus et al, who have found that in macrophages from old mice *M. tuberculosis* induce cytokine production but, in contrast to young mice, the induced cytokine production occurred independently of TLR2 (Rottinghaus et al 2010). In the murine model, Liang et al. also found that *Porphyromonas gingivalis* infected monocytes from aged mice produce more IL-6 (Liang et al 2009). As in our case they observed limited relationship between mRNA expression and protein production. This disparity may be a result of post-transcriptional or post-translational modifications, or even differences in the protein secretion. For example, McLachlan et al. described that although human monocytes activated with LPS produced less IL-1β, they displayed the intracellular 31- kDa IL-1 precursor (McLachlan et al 1995).

ROS production by human monocytes follows the same pattern exhibited by IL-6 production. Although there are numerous studies regarding the oxidative burst in aged neutrophils, the analysis of monocytes has not attracted such interest (Gomez et al 2008). In any case, it has been observed that LPS activated monocytes from elders show a lower superoxide production (Álvarez and Santa María 1996; McLachlan et al 1995). In our study, monocytes from elders produced more ROS by both non-infected and TPA activated cells. These monocytes also showed a good capacity to produce ROS when infected with *M. tuberculosis*, with significant differences when compared with monocytes from adults. These results are again congruent with the basal activated state of these cells in 'inflamm-aging'.

The most important finding presented in this work concerns the impaired ability of aged MDM to restrict the intracellular growth of *M. tuberculosis*. We wondered

whether the higher production of IL-6 influenced the antimicrobial activity of aged MDM. It is possible that an excess of these cytokines have a detrimental influence on the biological functions of MDM. In this regard, it has been reported that IL-6 antagonizes the *M. avium* bacteriostatic activity of TNFα (Bermudez et al 1992). Furthermore, IL-6 inhibits the macrophage responses to IFNγ in the murine model (Nagabhushanam et al 2003). We performed a factorial experiment, rarely used in life sciences, to test this hypothesis. Regardless of variations in our infection protocol that allow a higher production of IL-6, neither the tested cytokines nor their interactions seem to affect the antimicrobial activity of MDM from either elders or adults. It is noteworthy that IFNγ does have a negative influence on the intracellular multiplication of the bacteria. Although this result would be expected, given the importance of this cytokine in mycobacteriosis (Ottenhoff et al 2005), *in vitro* experiments analyzed by the mean comparison tests Student *t*-test or ANOVA do not usually reach statistical significance (Reyes-Ruvalcaba et al 2008; Warwick-Davies et al 1994).

When the factor "Age" was analyzed in the 2^5 factorial very significant differences were found. MDM from elders allowed a higher level of *M. tuberculosis* multiplication than MDM from younger adults. The higher number of CFU quantified after lysis of elders MDM does not seem to be a consequence of extracellular multiplication or differential phagocytic activity, because phagocytosis of mycobacteria does not seem to be impaired in elders. Fietta et al have not observed alterations in the phagocityc activity of mononuclear phagocytes from elders either (Fietta et al 1993; Fietta et al 1994). These results support the hypothesis that the epidemiological increase of elders with tuberculosis is not only a consequence of the population aging, but that they show an intrinsic susceptibility to the disease. Any immunological disturbance in an individual with latent tuberculosis may promote the development of the disease. In

this situation, returning to equilibrium with the pathogen will be more difficult for aged macrophages, which are more permissive to intracellular growth. Our results suit particularly well the dynamic hypothesis of latent tuberculosis infection that proposes that the bacillus do not remain dormant for a long time in the pulmonary parenchyma, but in stationary phase with a slower metabolism. According to this hypothesis there is a constant endogenous reinfection that may last for many years, although not an entire life (Cardona 2009). If this hypothesis is correct, macrophages from elders with a smaller capacity to restrict intracellular growth will increase the chance that the individual develops the disease, which would help to explain its higher rate among the elderly.

Because all the biological activities analyzed are heightened in monocytes from elders no clear mechanism that explains their impaired ability to restrict the intracellular growth of *M. tuberculosis* may be deduced. The only exception is that protein tyrosine phosphorylation is stronger in infected monocytes from adults than from elders. Our results need to be confirmed, however, because no final conclusion may be obtained out of such a small sample (n = 2). In initial approaches to characterize the nature of the kinases involved we identified Pyk2 as a protein with a higher amount of tyrosine phosphorylation in infected monocytes from adults than from elders. This kinase is related to the Focal Adhesion Kinase (FAK) and is necessary for neutrophil degranulation in host defense against bacterial infection (Kamen et al 2011), which makes it an interesting candidate to participate in the innate response to mycobacteria. Nevertheless, although there are several Src-family kinases in the array that we studied (Blk, Fgr, FRK, Fyn, Hck, Lyn and SRMS), some others are missing, like Yes, Lck, Src and Yrk, which may have an important influence in the antimicrobial mechanism, as may be deduced from the observed effect that PP2 (a Src-kinase family specific inhibitor) has on the intracellular multiplication of *M. tuberculosis*. A significant report

has recently indicated that Src is the key regulator of cellular responses to *M. tuberculosis* infection and regulates phagolysosome acidification and *M. tuberculosis* targeting to the autophagosome (Karim et al 2011). Consequently, protein tyrosine phosphorylation might be a major mechanism of intracellular growth control. A disturbing result was finding that both genistein and daidzein, abundant soy isoflavones, promotes intracellular mycobacterial growth. Unlike PP2, this role in genistein does not seem connected to its tyrosine kinase inhibitory properties because daidzein, the negative control, shows an even larger effect. Both molecules exert other actions not related to tyrosine phosphorylation, like the inhibition of the N-methyl-D-aspartate receptor (Huang et al 2010) or the voltage-dependent K^+ (Kv) 4.3 channel (Kim et al 2011). Soy consumption is high in Asian countries like Japan, and there are several studies related to the effect of isoflavones on the immune system, with either positive or negative outcomes (Sakai and Kogiso 2008). Our results should be taken into account because therapeutical use of these products may be detrimental to tuberculosis.

Finally, we have found that MDM from elders have a higher level of cellular death that those from adults. Although cellular death, and specially apoptosis, has been extensively studied in neutrophils from elders (Gomez et al 2008), much less is known regarding macrophages. We have observed that MDM from adults survive better than those from elders. Nevertheless, we found no statistical differences between non-infected and *M. tuberculosis* infected MDM for both age groups. It has been suggested that the induction of apoptosis in heavily infected macrophages (> 20 bacteria per cell) represents a means to exit the cell (Lee et al 2009). Our conditions of infection with very low MOI (1 bacterium per 100 cells) would not induce this apoptotic mechanism. Although we measured cellular death rather than apoptosis, a lower survival of aged

MDM does not seem to bear any relationship with the faster multiplication of *M. tuberculosis*.

In conclusion, although monocytes from elders show a higher basal level of activation, evidenced by increased cytokine and ROS production after infection, they are more permissive to intracellular growth of *M. tuberculosis*, which may have an important influence on the susceptibility to the disease. Despite the increasing importance of tuberculosis among the elderly in low incidence countries there is a surprising lack of studies that target the consequences of immunosenescence on the tuberculosis infection. We hope that reports like ours encourage other research groups to get interested in this prominent issue.

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Table 1 Allocation of treatments among fractions of the factorial design.

FRACTION 1	FRACTION 2
No treatment	IL-1β
IL-1β IL-6	IL-6
IL-1β TNFα	TNFα
IL-1β IFNγ	IFNγ
IL-6 TNFα	IL-1β IL-6 TNFα
IL-6 IFNγ	IL-1β IL-6 IFNγ
TNFα IFNγ	IL-1β TNFα IFNγ
IL-1β IL-6 TNFα IFNγ	IL-6 TNFα IFNγ

Table 2 Gene expression of proinflammatory cytokines in adults and elders measured by qPCR.

	Adults	Elders	P
IL1β	373.8 (8649.4)	135.8 (1537.5)	0.343
IL6	1956.2 (18660.7)	292.1 (15511.7)	0.755
TNFα	17.9 (201.5)	10.1 (5.0)	0.432

Data represent the median (interquartile range) of the relative gene expression calculated by the delta delta C_t. Cytokine C_t was normalized to *HPRT1* C_t and gene expression in *M. tuberculosis* infected monocytes was calculated relative to non-infected monocytes. Mann-Whitney test was performed for comparisons between adults (n = 5) and elders (n = 7). Results were considered significant with $P < 0.05$.

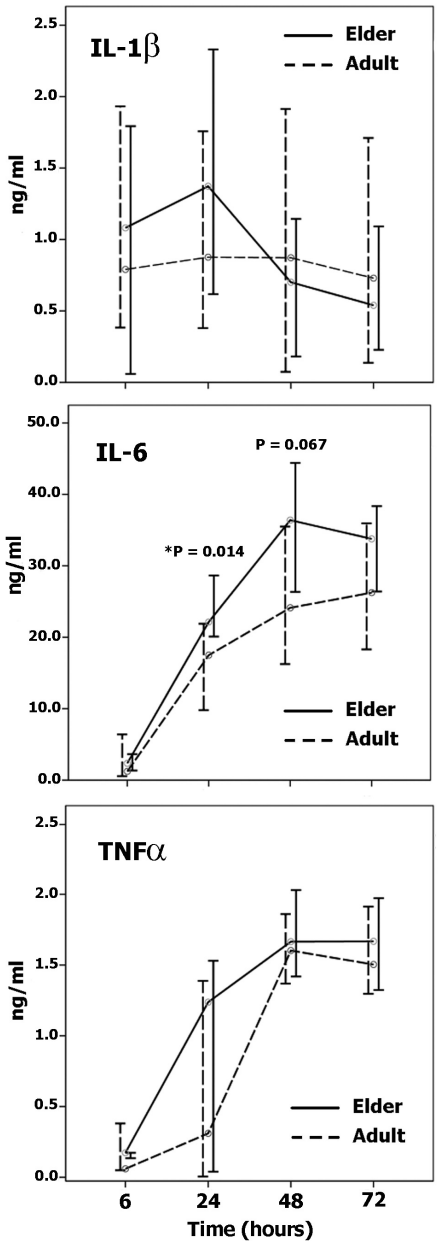

Fig 1 Cytokine production by MDM from aged donors. 5 X 10^5 MDM from aged (continuous line) and from young (dashed lines) donors were infected with *M. tuberculosis* (MOI = 1) in 500 µl of RPMI-1640/10% autologous serum. 50 µl of medium was removed after 6, 24, 48 and 72 h. IL-1β, IL-6 and TNFα in culture supernatants were measured by ELISA. Data represent the median of concentration (ng/ml), and vertical lines are the 95% confidence interval. Sample sizes for adults and elders were 6 and 4 in time points 6, 48 and 72 h, and 15 and 12 in time point 24 h, respectively. At each time point a Mann-Whitney's test was performed for comparisons between adults and elders. *P < 0.05 is considered significant.

Table 3 Factorial experiments of monocyte antimycobacterial activity.

Factors	Effect estimate	P
2^4 design		
Adults		
IFNγ	-0.117 (-0.240 – 0.002)	0.053
IL1β	0.026 (-0.092 – 0.144)	0.661
IL6	0.033 (-0.086 – 0.152)	0.571
TNFα	0.061 (-0.058 – 0.180)	0.303
Elders		
IFNγ	-0.120 (-0.188 – 0.052)*	0.001
IL1β	-0.020 (-0.088 – 0.048)	0.553
IL6	0.011 (-0.058 – 0.080)	0.738
TNFα	0.014 (-0.054 – 0.082)	0.683
2^5 design		
Age	0.578 (0.440 – 0.720)*	< 0.001
IFNγ	-0.118 (-0.260 – 0.032)	0.121
IL1β	0.003 (-0.148 – 0.154)	0.970
IL6	0.022 (-0.128 – 0.172)	0.769
TNFα	0.037 (-0.114 – 0.188)	0.623

10^5 monocytes from either adults (n = 4) or elders (n = 4) were infected with 10^3 bacteria in the presence of each of the cytokines (25 ng/ml) or their combinations. The number of surviving bacteria after four days of incubation was determined as CFU, and statistically analyzed as log CFU. Data represent the estimate of the main effects (95% confidence interval). The results from experiments performed with monocytes from either adults or elders were analyzed in the 2^4 designs. The same results from both groups were analyzed together in the 2^5 design. $P < 0.05$ was considered significant.

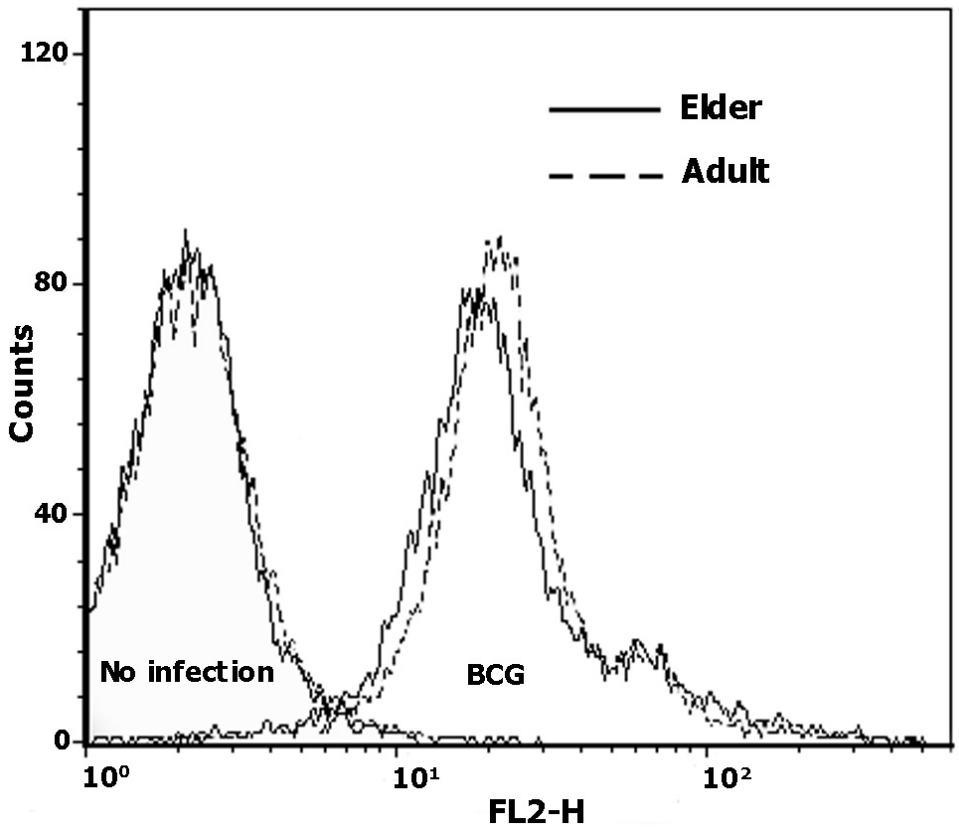

Fig.2 Phagocytic activity of monocytes from aged donors. 10^5 monocytes from either an elder (continuous line) or an adult (dashed line) were incubated with 5×10^5 BCG-DsRed bacteria for 10 min. Non-infected cells were used as controls. Fluorescence intensity (red) was analyzed by flow cytometry and 10^4 events were measured. The curves that correspond to control cells have inside the label 'No infection'. The curves that correspond to infected cells are marked with the label 'BCG'. For statistical analysis a cut-off value for defining red fluorescent positivity was set arbitrarily at 10^1 to exclude over 97% of non-infected cells. This result is representative of five independent experiments.

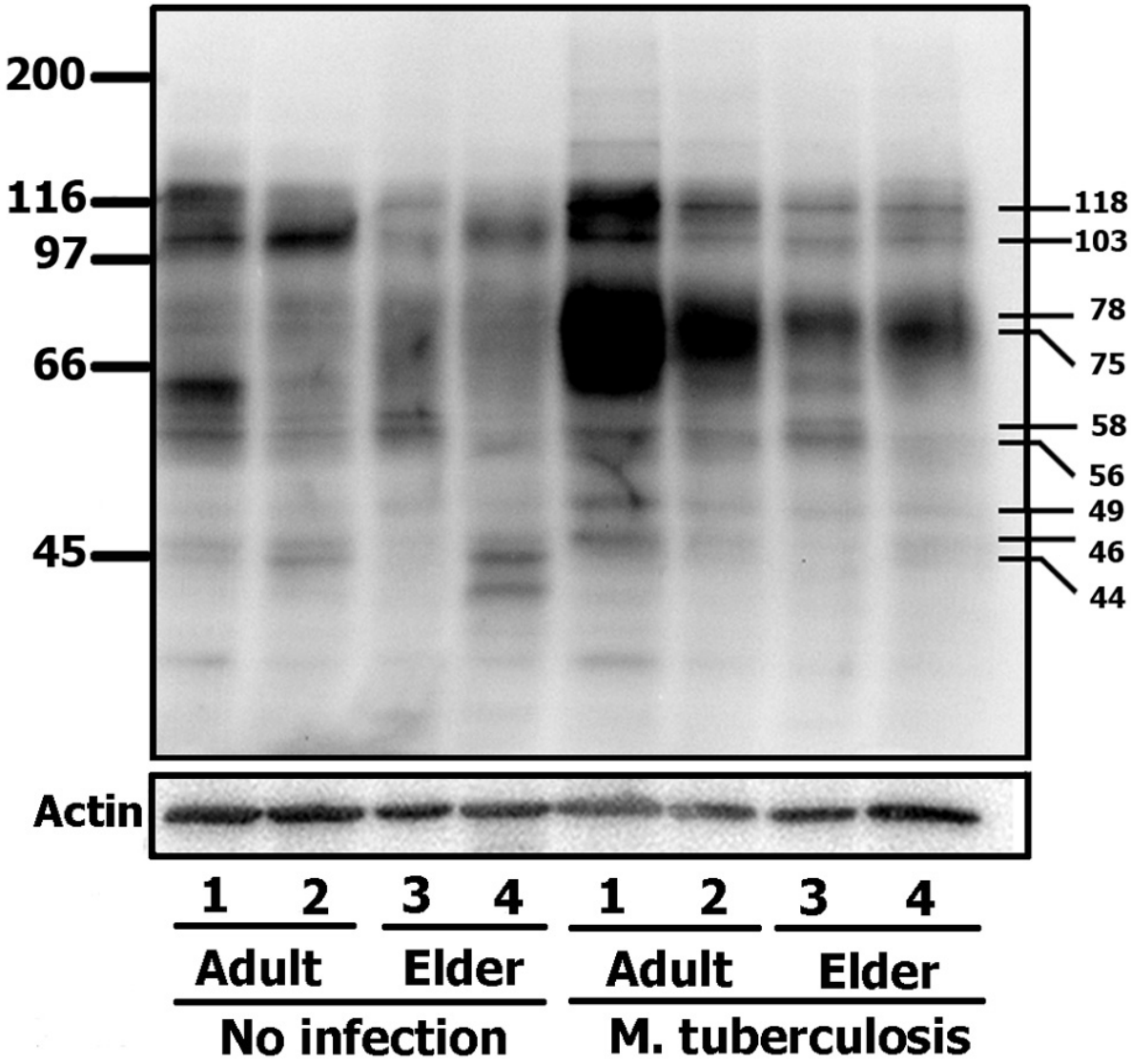

Fig. 3 Protein tyrosine phosphorylation in infected monocytes. 7×10^5 monocytes were infected with *M. tuberculosis* (MOI 20) for 30 min and 12 μg of total protein were analyzed by immunoblot with an anti-phosphotyrosine antibody. Two donors from each age group were studied and *M. tuberculosis* infected cells were compared with non-infected cells. Molecular weight markers are indicated on the left, and the sizes of specifically phosphorylated proteins are shown on the right. For loading control the same blot was probed two days after with an anti- β-actin antibody.

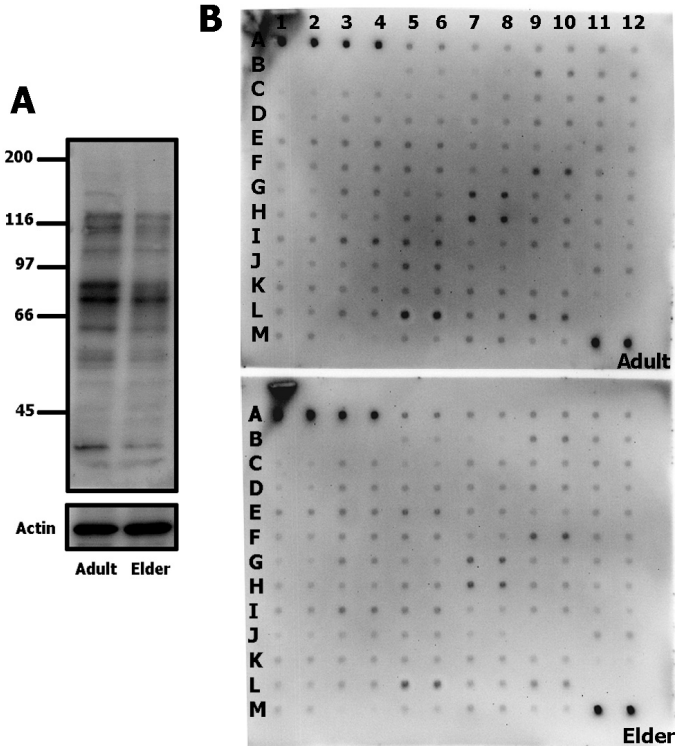

Fig. 4 Identification of tyrosine phosphorylated kinases. **A.** 3×10^6 cells were infected with *M. tuberculosis* (MOI 20) for 30 min and 12 μg of total protein were analyzed by immunoblot with an anti-phosphotyrosine antibody. **B.** Arrays incubated with lysates from either an adult or an elder (120 μg of total protein). Chemiluminiscence was measured as counts with an image analyzer and data was normalized with dots A5-6, which are positive controls included in the kit. Local background was subtracted from each dot, and differences in dot intensities were determined. Dots were included in duplicates in the kit, and correspond to the following kinases: Positive controls, A1, A3, A5 and M11. Negative controls: B1, B3 and L11. A7, Abl1; A9, Ack1; A11, ALK; B5, Axl; B7, Blk; B9, BMX; B11, Btk; C1, Csk; C3, Dtk; C5, EGFR; C7, EphA1; C9, EphA2; C11, EphA3; D1, EphA4; D3, EphA5; D5, EphA6; D7, EphA7; D9, EphA8; D11, EphB1; E1, EphB2; E3, EphB3; E5, EphB4; E7, EphB6; E9, ErbB2; E11, ErbB3; F1, ErbB4; F3, FAK; F5, FER; F7, FGFR1; F9, FGFR2; F11, FGFR2 (α); G1, Fgr; G3; FRK; G5; Fyn; G7, Hck; G9, HGFR; G11, IGF-I R; H1, Insulin R; H3, Itk; H5, JAK1; H7, JAK2; H9, JAK3; H11, LCK; I1, LTK; I3, Lyn; I5, MATK; I7, M-CSFR; I9, MUSK; I11, NGFR; J1, PDGF-α; J3, PDGF-β; J5, Pyk2; J7, RET; J9, RORI; J11, ROR2; K1, ROS; K3, RYK; K5, SCFR; K7, SRMS; K9, SYK; K11, Tec; L1, Tie-1; L3, Tie-2; L5, TNK1; L7, TRKB; L9, TXK; M1, Tyk2; M3, TYRO10; M5, VEGFR2; M7, VEGFR3; M9, ZAP70.

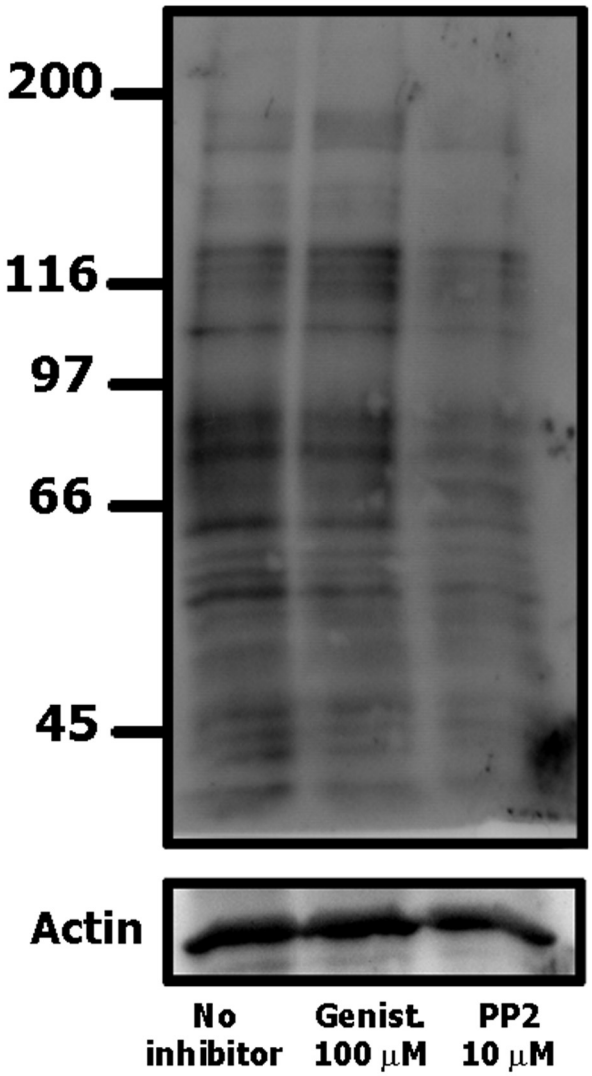

Fig. 5 Effect of tyrosine kinase inhibitors on protein tyrosine phosphorylation in infected monocytes. 7×10^5 monocytes from an adult were preincubated with protein kinase inhibitors (100 μM genistein or 10 μM PP2) for 1 hour before infection with *M. tuberculosis* (MOI 20) for 30 min and 12 μg of total protein were analyzed by immunoblot with an anti-phosphotyrosine antibody. Molecular weight markers are indicated on the left. For loading control the same blot was probed two days after with an anti- β-actin antibody.

Table 4 Factorial experiments of monocyte antimycobacterial activity in the presence of protein tyrosine kinase inhibitors.

Factors	Effect estimate	P
Kinase Inhibitors		
IFNγ	-0.089 (-0.150 – -0.026)*	0.007
Genistein	0.180 (0.120 – 0.240)*	< 0.001
PP2	0.070 (0.008 – 0.132)*	0.030
Negative Controls		
IFNγ	-0.085 (-0.148 - -0.022)*	0.011
Daidzein	0.220 (0.154 – 0.280)*	< 0.001
PP3	-0.015 (-0.078 – 0.048)	0.633

10^5 monocytes from elders (n = 4) were infected with 10^3 bacteria in the presence of IFNγ 25 ng/ml, tyrosine kinase inhibitors (genistein 100 μM and PP2 10 μM) or negative controls of the inhibitors (Daidzein 100 μM as the genistein negative control and PP3 10 μM as the PP2 negative control). The number of surviving bacteria after four days of incubation was determined as CFU, and statistically analyzed as log CFU. Data represent the estimate of the main effects (95% confidence interval). The results were analyzed as independent 2^3 designs. $P < 0.05$ was considered significant.

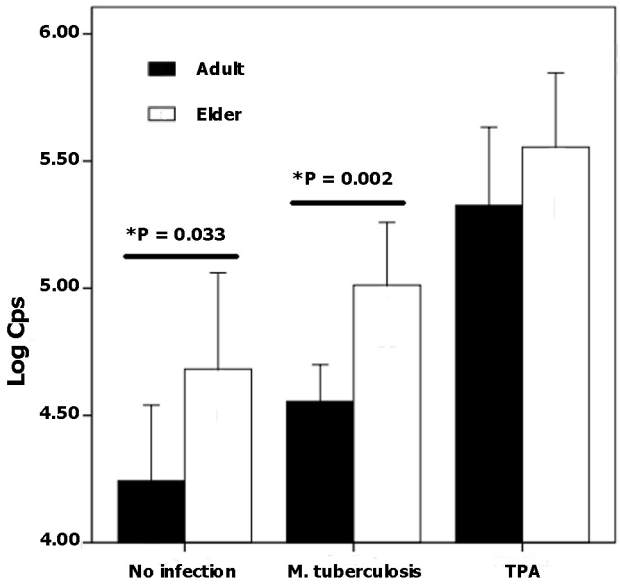

Fig. 6 ROS production in activated monocytes. 10^5 cells were activated by *M. tuberculosis* infection (MOI 5) or by incubation with the strong activator TPA for 30 min in the presence of luminol. Non activated cells ('No infection') were included as negative controls. Data are the cps + standard deviation and Student's *t*-test was performed for comparisons between adults (n = 7) and elders (n = 7). *P < 0.05 is considered significant.

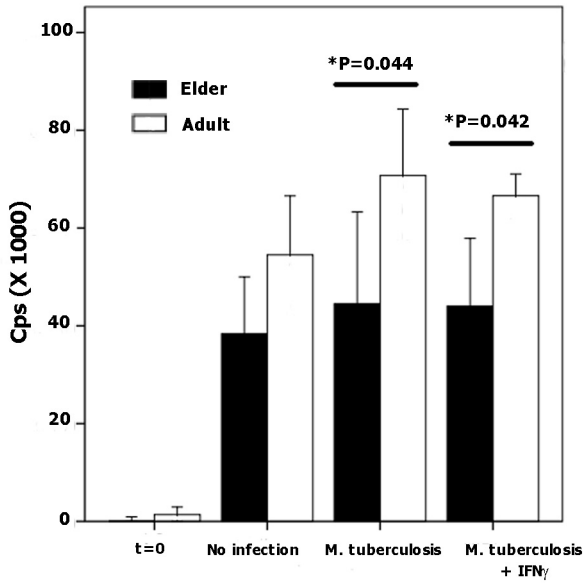

Fig 7 Cellular death in infected monocytes. After cell lysis by sonication, the lysate was diluted 1/10 and mixed with a luminogenic substrate that detect a 'dead-cell protease activity' (2 volumes diluted lysate: 1 volume substrate). After 30 min chemiluminiscence was measured in a luminometer as cps. Data are the log of the cps + standard deviation. Student's *t*-test test was performed for comparisons between adults (n = 5) and elders (n = 4). *P < 0.05 is considered significant.