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

José M. Guerra-Laso · Sandra González-García ·
Carolina González-Cortés · Cristina Díez-Tascón ·
Ramiro López-Medrano ·
Octavio M. Rivero-Lezcano

Received: 11 October 2011 / Accepted: 24 June 2012 / Published online: 12 July 2012
© American Aging Association 2012

Abstract The elderly account for a disproportionate share of all tuberculosis cases, and the population ageing may not fully explain this phenomenon. We have performed in vitro infection experiments to investigate whether there is an immunological basis for the apparent susceptibility of elders to

tuberculosis. In our infection model, *Mycobacterium tuberculosis* induces a higher production of interleukin (IL)-6 and reactive oxygen species in macrophages from elders than from younger adults. This response did not prevent, however, an increased multiplication of *M. tuberculosis* in macrophages from elders as compared with the growth observed within cells from adults. By performing a factorial experiment, we have found that IFN- γ , but not IL-1 β , IL-6 or TNF- α , stimulate the macrophages to restrict the multiplication of the bacterium in macrophages from elders. Although monocytes from elders seem to be in a higher level of activation, we present evidences that protein tyrosine phosphorylation response induced by *M. tuberculosis* is stronger in monocytes from adults than from elders. Using a protein array that detects 71 tyrosine phosphorylated kinases, we identified Pyk2 as the only kinase that displayed a difference of intensity larger than 50 % in adults than in elders. Furthermore, monocytes from elders that were incubated in the presence of tyrosine kinase inhibitors (genistein and PP2) allowed a higher level of bacterial multiplication. These observations may help to explain the susceptibility of elders to tuberculosis. An unexpected result was that both genistein and its negative control, daidzein, abundant soy isoflavones, promoted intracellular mycobacterial growth.

J. M. Guerra-Laso
Servicio de Medicina Interna, Hospital de León,
Altos de Nava s/n,
24008 León, Spain

S. González-García · C. González-Cortés ·
O. M. Rivero-Lezcano (✉)
Unidad de Investigación, Hospital de León,
Edif. S. Antonio Abad,
Altos de Nava s/n,
24008 León, Spain
e-mail: omrivero@iecsyl.com

C. Díez-Tascón
Servicio de Anatomía Patológica, Hospital de León,
Altos de Nava s/n,
24008 León, Spain

R. López-Medrano
Servicio de Microbiología, Hospital Comarcal del Bierzo,
Médicos sin Fronteras, 7,
24411 Fuentesnuevas, Spain

O. M. Rivero-Lezcano
Fundación Instituto de Estudios de Ciencias de la Salud de
Castilla y León,
Parque de Santa Clara s/n,
42002 Soria, Spain

Keywords Immunosenescence · Cytokines ·
Phagocytosis · Reactive oxygen species · Design of
experiments · Protein tyrosine phosphorylation

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 USA, 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, 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 IFN- γ in response to mycobacterial antigens in the presence of IL-2 (Orme et al. 1993). An initial protection observed in the 3 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 nonspecific production of IFN- γ 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

M. 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 $100\times g$ 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 less than or equal to five 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 -80°C .

Monocyte derived macrophages

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 appropriately 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 h from blood collection, in RPMI-1640/10 % autologous serum, at 37°C 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 . Infected were 10^5 cells with 10^3 bacteria (multiplicity of infection (MOI) = 0.01). When indicated, cells were incubated in 25 ng/mL of the following cytokines: IL-1 β ($\geq 1\times 10^9$ units/mg), IL-6 ($\geq 1\times 10^7$ units/mg), TNF- α ($\geq 2\times 10^7$ units/mg) and IFN- γ ($\geq 2\times 10^7$ units/mg, Peprotech, London, UK). In neutralization studies, 0.5 μg of either mouse anti-IL-1 β (eBioscience, CA, USA) or goat polyclonal anti-IL-6 (Abcam, Cambridge, UK) was added. In tyrosine kinase inhibition experiments, cells were preincubated for 1 h before infection with either 100 μM genistein or daidzein and either 10 μM PP2 (Cayman Chemical, MI, 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 4 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 37°C in 7H9 broth supplemented with 0.2 % glycerol and 10 % Middlebrook enrichment ADC (Becton Dickinson Microbiology Systems) for 10 days. Colony forming units (CFU) were determined under an inverted microscope at $\times 100$ 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 h in a volume of 500 μL (24-well plates).

Quantitative polymerase chain reaction

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, MD, USA). Real-time polymerase chain reaction (PCR) was performed on a Bio-Rad iCycler system (Barcelona, Spain) using SYBR-Green (Molecular Probes, Invitrogen, Barcelona, Spain). The threshold cycle (C_t) values for each of the target genes were normalized to the C_t of the reference gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). The efficiency (E) of the PCR reaction for each target gene was calculated using the slope of the standard curve obtained from the C_t of one eighth dilutions of each amplicon ($E = 10^{-1/\text{slope}}$). Calculated efficiencies were: IL- β , 1.980; IL-6, 1.950; TNF- α , 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): $(E_{\text{target}})^{\Delta C_t, \text{target (calibrator-test)}/(E_{\text{ref}})^{\Delta C_t, \text{ref (calibrator-test)}}$ (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) GGCCATCTGCTTAGTA GAGCTTTT, reverse (R) TTAAACAA CAATCCGCCCAA; IL-1 β (F) AAGCTCGC CAGTGAAATGATG, (R) GGTGGTCGGA GATTCGTAGC; IL-6 (F) TGCAATAACCAACCCT GACC, (R) CATTGCCGAAGAGCCCTC; TNF- α (F) TCTTCTCGAACCCCGAGTGA, (R) CCTCTGA TGGCACCACCAG.

Quantification of cytokines in supernatants

To remove bacteria from supernatants, samples were centrifuged for 3 min at 10,000 $\times g$ at room temperature in ultrafree MC filter units (Millipore Iberica, Madrid, Spain) of 0.45 μm and frozen at -80°C . Cytokines were quantified by ELISA using BD OptEIA Human IL-1 β 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 (Sørensen

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 *hsp60*prF (GCTCTAGATTGATTAGCACGTC GATGC) and *hsp60*prR (AATCATATGCATTGCGAA GTGATTCCT), which contain an *XbaI* and an *NdeI* site, respectively, and was subcloned upstream the fluorescent protein gene in the pNBV1 plasmid. Purified plasmid, named pMDsRed, was electroporated (1.8 kV, 25 μF , 1,000 Ω) into the BCG strain by standard protocols (Jacobs Jr. et al. 1991). Transformants were grown on supplemented 7H11 agar/hygromycin 50 $\mu\text{g}/\text{mL}$. Individualized bacteria were frozen at -80°C in single use aliquots.

Monocytes of 10^5 were incubated with 5×10^5 BCG bacteria (MOI = 5) in RPMI-1640/10 % autologous serum in a vertical rotator at 37°C . After 10 min, the cells were placed in ice and analysed in a FACScan instrument (Becton Dickinson).

Western blot analysis

Monocytes of 7×10^5 were incubated with 1.4×10^7 bacteria in RPMI-1640/10 % autologous serum in a vertical rotator at 37°C . After 30 min, the cells were centrifuged at $800 \times g$ for 5 min at 4°C , 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, CA, USA) and incubated in ice. After 5 min, lysates were centrifuged at $14,000 \times g$ for 5 min at 4°C , and supernatants were boiled in Laemli loading buffer and frozen at -40°C . Proteins were fractionated by sodium dodecyl sulphate–7.5 % polyacrylamide gel electrophoresis and transferred by electroblotting to polyvinylidene difluoride membranes (Millipore Iberica, Madrid, Spain). Membranes were incubated in blocking buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl and 10 % bovine serum

albumin (Applichem, Darmstadt, Germany) for 1 hour and with anti-phosphotyrosine PY20 antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology), 1:3,000 in blocking buffer, for 1 h. 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

Monocytes of 3×10^6 were infected with 6×10^7 bacteria in RPMI-1640/10 % autologous serum in a vertical rotator at 37 °C. After 30 min, the cells were centrifuged at $800 \times g$ for 3 min at 4 °C, washed with cold RPMI-1640 and centrifuged again in the same conditions. After supernatant removal, the cell lysates were obtained in the buffer included in the Ray Bio[®] Human receptor tyrosine kinases phosphorylation antibody array I (RayBiotech, GA, USA) and frozen at -80 °C. Arrays, which include 71 human tyrosine kinases, are incubated with the lysates (120 μ g of total protein) following the manufacturer's instructions. Tyrosine phosphorylated proteins are detected by chemiluminescence with a biotinylated anti-phosphotyrosine antibody and streptavidin coupled to horseradish peroxidase. Arrays were developed using the Chemidoc-XRS image analyser and analysis was performed with Quantity One 4.4.1. (Bio-Rad). Units were intensity $\times$ square millimetre, 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 chemiluminescence. Either 5×10^5 bacteria (MOI=5) or 150 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Calbiochem), luminol 500 μ M (Sigma-Aldrich Spain) and Hanks balanced salt solution were added to 10^5 monocytes in a total volume of 100 μ L, and incubated for 30 min at 37 °C. After 30 min, emitted light was measured for 5 sec at 21 °C 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 μ L of the cell lysis obtained by sonication for measuring antimicrobial activity (see “Cellular infection”) were diluted one tenth. The dilution (22 μ L) was mixed with 11 μ L of the luminogenic peptide substrate, and 30 μ L of the mix were incubated 30 min at 21 °C, and measured for 5 s in a TopCount-NXT microplate scintillation and luminescence counter (Packard). Results were expressed as 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 analysed 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 analysed 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-1 β , IL-6, TNF- α and IFN- γ) are low, 0 and high, 25 ng/mL, and for the factor ‘age’, low: <50, adults and high: >70 years, elders. The analysed 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 four 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. Two adults and two 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 four 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 μ M in all cases and high levels were 100 μ M genistein (daidzein as negative control) or 10 μ M PP2 (PP3 as negative control). The third factor in both experiments was IFN- γ , used as a control, with 0 and 25 ng/mL as low and high levels, respectively. Results were analysed with Design-Expert 8.0.6 (Stat-Ease, Minneapolis, MN, USA). In all statistical analysis, a P value <0.05 was considered significant.

Table 1 Allocation of treatments among fractions of the factorial design

Fraction 1	Fraction 2
No treatment	IL-1 β
IL-1 β , IL-6	IL-6
IL-1 β , TNF- α	TNF- α
IL-1 β , IFN- γ	IFN- γ
IL-6, TNF- α	IL-1 β , IL-6, TNF- α
IL-6, IFN- γ	IL-1 β , IL-6, IFN- γ
TNF- α , IFN- γ	IL-1 β , TNF- α , IFN- γ
IL-1 β , IL-6, TNF- α , IFN- γ	IL-6, TNF- α , IFN- γ

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-1 β , IL-6 and TNF- α 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 analysed 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 analysed. While the production of IL-1 β and TNF- α 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 analyse the protein production in supernatants from *M. tuberculosis* infected monocytes (Fig. 1). We also found that the production of IL-1 β and TNF- α was not significantly higher in elders than in adults. Although the amount of these cytokines was somehow higher in

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

	Adults	Elders	P
IL-1 β	373.8 (8,649.4)	135.8 (1,537.5)	0.343
IL-6	1,956.2 (18,660.7)	292.1 (15,511.7)	0.755
TNF- α	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 $HPRTIC_t$, 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. MDM, 5×10^5 , from aged (*continuous line*) and from young (*dashed lines*) donors were infected with *M. tuberculosis* (MOI=1) in 500 μ L of RPMI-1640/10 % autologous serum. Medium of 50 μ L was removed after 6, 24, 48 and 72 h. IL-1 β , IL-6 and TNF- α in culture supernatants were measured by ELISA. Data represent the median of concentration (nanograms per millilitre), and *vertical lines* are the 95 % confidence interval. Sample sizes for adults and elders were six and four 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

elders, the variability was large. A different case was IL-6 because at 24 h there were significantly higher amounts in elder cell supernatants (* $P = 0.014$), and this trend was kept at 48 h ($P = 0.067$, nearly significant). The quantities measured for the three cytokines at 72 h 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 IFN- γ (Nagabhushanam et al. 2003) or TNF- α (Bermudez et al. 1992) to inhibit anti-mycobacterial 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 IFN- γ or TNF- α beneficial influence on the anti-mycobacterial activities of monocyte-derived macrophages (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 IFN- γ or TNF- α , and included IL-1 β because it is also produced by infected MDM in high quantities. We analysed the same data as a 2^4 factorial design, using as factors the cytokines IFN- γ , IL-1 β , 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 IFN- γ 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 IFN- γ 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 analysed because they all were non-significant (data not shown).

When the results were analysed 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

Table 3 Factorial experiments of monocyte anti-mycobacterial activity

Factors	Effect estimate	<i>P</i>
2^4 design		
Adults		
IFN- γ	−0.117 (−0.240–0.002)	0.053
IL-1 β	0.026 (−0.092–0.144)	0.661
IL-6	0.033 (−0.086–0.152)	0.571
TNF- α	0.061 (−0.058–0.180)	0.303
Elders		
IFN- γ	−0.120 (−0.188–0.052)*	0.001
IL-1 β	−0.020 (−0.088–0.048)	0.553
IL-6	0.011 (−0.058–0.080)	0.738
TNF- α	0.014 (−0.054–0.082)	0.683
2^5 design		
Age	0.578 (0.440–0.720)*	<0.001
IFN- γ	−0.118 (−0.260–0.032)	0.121
IL-1 β	0.003 (−0.148–0.154)	0.970
IL-6	0.022 (−0.128–0.172)	0.769
TNF- α	0.037 (−0.114–0.188)	0.623

Monocytes, 10^5 , from either adults or elders 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 4 days of incubation was determined as CFU and was statistically analysed 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 analysed in the 2^4 designs. The same results from both groups were analysed together in the 2^5 design. * P <0.05 was considered significant

MDM from elders after 4 days of infection. The estimate of the effect of IFN- γ , 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-1 β neutralizing antibodies as factors, together with IFN- γ (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 IFN- γ 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-IL-1 β : −0.02 [−0.16–0.12], P =0.764), although the effect of IFN- γ remained significant (effect estimate: −0.19 [95 % confidence interval: −0.34 to −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-1 β . For illustration purposes, we present the log CFU of the IFN- γ (Fraction 2, Table 1) and ‘no cytokine’ (Fraction 1) treatments: elder IFN- γ , 3.62 and 3.76; elder ‘no cytokine’, 4.11 and 3.07; adult IFN- γ , 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 an *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 4 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 ($\pm$ SD) was 2.90 (0.10), and surviving bacteria after 4 days were 2.77 (0.41). Data analysed 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

Fig. 2 Phagocytic activity of monocytes from aged donors. Monocytes, 10^5 , 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 analysed 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 cutoff 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

influencing the *M. tuberculosis* intracellular growth rate. For this reason, we analysed 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- and a 118-kD protein 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

Fig. 3 Protein tyrosine phosphorylation in infected monocytes. Monocytes, 7×10^5 , were infected with *M. tuberculosis* (MOI, 20) for 30 min and 12 μ g of total protein were analysed 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 2 days after with an anti- β -actin antibody

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 analysed tyrosine phosphorylated kinases in lysates from infected monocytes. In Fig. 4a, we confirmed a higher level of tyrosine phosphorylation

Fig. 4 Identification of tyrosine phosphorylated kinases. **a** Cells, 3×10^6 , were infected with *M. tuberculosis* (MOI, 20) for 30 min and 12 µg of total protein were analysed by immunoblot with an anti-phosphotyrosine antibody. **b** Arrays incubated with lysates from either an adult or an elder (120 µg of total protein). Chemiluminescence was measured as counts with an image analyser, and data were 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, ROR1; 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

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. 4b, 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). IFN- γ , included as a control of the anti-mycobacterial 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 IFN- γ and genistein was the only one that was statistically significant ($*P = 0.016$). A puzzling result was obtained, however, with the negative controls because daidzein also promoted bacterial intracellular

Fig. 5 Effect of tyrosine kinase inhibitors on protein tyrosine phosphorylation in infected monocytes. Monocytes, 7×10^5 , from an adult were preincubated with protein kinase inhibitors (100 μ M genistein or 10 μ M PP2) for 1 h before infection with *M. tuberculosis* (MOI, 20) for 30 min and 12 μ g of total protein were analysed by immunoblot with an anti-phosphotyrosine antibody. Molecular weight markers are indicated on the left. For loading control, the same blot was probed 2 days after with an anti- β -actin antibody

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 analysed 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.

Table 4 Factorial experiments of monocyte anti-mycobacterial activity in the presence of protein tyrosine kinase inhibitors

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

Monocytes, 10^5 , from elders ($n=4$) were infected with 10^3 bacteria in the presence of IFN- γ 25 ng/mL, tyrosine kinase inhibitors (genistein, 100 μ M and PP2, 10 μ M) or negative controls of the inhibitors (daidzein, 100 μ M as the genistein negative control and PP3, 10 μ M as the PP2 negative control). The number of surviving bacteria after 4 days of incubation was determined as CFU, and statistically analysed as log CFU. Data represent the estimate of the main effects (95 % confidence interval). The results were analysed as independent 2^3 designs. $*P < 0.05$ was considered significant

Fig. 6 ROS production in activated monocytes. Cells, 10^5 , 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

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 analyse this biological phenomenon, 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 IFN- γ

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*+IFN- γ).

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-1 β (Rudd and Banerjee 1989) or fewer amounts of IL-1 β , IL-6 and TNF- α 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'

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

(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 a 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-1 β , 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 TNF- α (Bermudez et al. 1992). Furthermore, IL-6 inhibits the macrophage responses to IFN- γ 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 IFN- γ 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 analysed by the mean comparison tests Student's *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 analysed in the 2⁵ 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 phagocytic activity of mononuclear phagocytes from elders either (Fietta et al. 1993, 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 analysed 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 therapeutic use of these products may be detrimental to tuberculosis.

Finally, we have found that MDM from elders have a higher level of cellular death than 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 (one 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.

Acknowledgments We thank the nurses who helped us with the blood collection and Ignacio Rodríguez-Barbosa for the flow cytometry technical assistance. This study was supported by grants from the Consejería de Sanidad de la Junta de Castilla y León (2010 and 2011) and Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III [PI05/1288]. Dr. Rivero-Lezcano is a member of the Fundación Instituto Ciencias de la Salud de Castilla y León and participates in the SACYL research program. González Cortés was supported by the Instituto de Salud Carlos III program for national health system research support.

References

- Álvarez E, Santa María C (1996) Influence of the age and sex on respiratory burst of human monocytes. *Mech Ageing Dev* 90:157–161

- Beharka AA, Meydani M, Wu D, Leka LS, Meydani A, Meydani SN (2001) Interleukin-6 production does not increase with age. *J Gerontol A Biol Sci Med Sci* 56:B81–B88
- Bermudez LE, Wu M, Petrofsky M, Young LS (1992) Interleukin-6 antagonizes tumor necrosis factor-mediated mycobacteriostatic and mycobactericidal activities in macrophages. *Infect Immun* 60:4245–4252
- Boehmer ED, Goral J, Faunce DE, Kovacs EJ (2004) Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol* 75:342–349
- Cabre M (2009) Pneumonia in the elderly. *Curr Opin Pulm Med* 15:223–229
- Cardona PJ (2009) A dynamic reinfection hypothesis of latent tuberculosis infection. *Infection* 37:80–86
- Chatta GS, Dale DC (1996) Aging and haemopoiesis. Implications for treatment with haemopoietic growth factors. *Drugs Aging* 9:37–47
- DelaRosa O, Pawelec G, Peralbo E, Wikby A, Mariani E, Mocchegiani E, Tarazona R, Solana R (2006) Immunological biomarkers of ageing in man: changes in both innate and adaptive immunity are associated with health and longevity. *Biogerontology* 7:471–481
- Delpedro AD, Barjavel MJ, Mamdouh Z, Faure S, Bakouche O (1998) Signal transduction in LPS-activated aged and young monocytes. *J Interferon Cytokine Res* 18:429–437
- Ernst JD (1998) Macrophage receptors for *Mycobacterium tuberculosis*. *Infect Immun* 66:1277–1281
- Fazal N, Bartlett R, Lammas DA, Kumararatne DS (1992) A comparison of the different methods available for determining BCG–macrophage interactions in vitro, including a new method of colony counting in broth. *FEMS Microbiol Immunol* 5:355–362
- Fietta A, Merlini C, De Bernardi PM, Gandola L, Piccioni PD, Grassi C (1993) Non specific immunity in aged healthy subjects and in patients with chronic bronchitis. *Aging (Milano)* 5:357–361
- Fietta A, Merlini C, Dos Santos C, Rovida S, Grassi C (1994) Influence of aging on some specific and nonspecific mechanisms of the host defense system in 146 healthy subjects. *Gerontology* 40:237–245
- Fortin CF, Larbi A, Lesur O, Douziech N, Fülöp T Jr (2006) Impairment of SHP-1 down-regulation in the lipid rafts of human neutrophils under GM-CSF stimulation contributes to their age-related, altered functions. *J Leukoc Biol* 79:1061–1072
- Fortin CF, Larbi A, Dupuis G, Lesur O, Fülöp T Jr (2007) GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals. *Biogerontology* 8:173–187
- Franceschi C, Bonafè M, Valensin S, Olivieri F, De LM, Ottaviani E, Giovanna DB (2000) Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* 908:244–254
- Fülöp T Jr, Varga Z, Jacob MP, Robert L (1997) Effect of lithium on superoxide production and intracellular free calcium mobilization in elastin peptide (κ -elastin) and FMLP stimulated human PMNS. Effect of age. *Life Sci* 60:PL325–PL332
- Gomez CR, Nomellini V, Faunce DE, Kovacs EJ (2008) Innate immunity and aging. *Exp Gerontol* 43:718–728
- Gon Y, Hashimoto S, Hayashi S, Koura T, Matsumoto K, Horie T (1996) Lower serum concentrations of cytokines in elderly patients with pneumonia and the impaired production of cytokines by peripheral blood monocytes in the elderly. *Clin Exp Immunol* 106:120–126
- Howard NS, Gomez JE, Ko C, Bishai WR (1995) Color selection with a hygromycin-resistance-based *Escherichia coli*–mycobacterial shuttle vector. *Gene* 166:181–182
- Huang R, Singh M, Dillon GH (2010) Genistein directly inhibits native and recombinant NMDA receptors. *Neuropharmacology* 58:1246–1251
- Jacobs WR Jr, Kalpana GV, Cirillo JD, Pascopella L, Snapper SB, Udani RA, Jones W, Barletta RG, Bloom BR (1991) Genetic systems for mycobacteria. *Methods Enzymol* 204:537–555
- Kamen LA, Schlessinger J, Lowell CA (2011) Pyk2 is required for neutrophil degranulation and host defense responses to bacterial infection. *J Immunol* 186:1656–1665
- Karim AF, Chandra P, Chopra A, Siddiqui Z, Bhaskar A, Singh A, Kumar D (2011) Express path analysis identifies a tyrosine kinase Src-centric network regulating divergent host responses to *Mycobacterium tuberculosis* infection. *J Biol Chem* 286:40307–40319
- Kim HJ, Ahn HS, Choi BH, Hahn SJ (2011) Inhibition of Kv4.3 by genistein via a tyrosine phosphorylation-independent mechanism. *Am J Physiol Cell Physiol* 300:C567–C575
- Lee J, Kornfeld H (2010) Interferon- γ regulates the death of *M. tuberculosis*-infected macrophages. *J Cell Death* 3:1–11
- Lee J, Hartman M, Kornfeld H (2009) Macrophage apoptosis in tuberculosis. *Yonsei Med J* 50:1–11
- Liang S, Domon H, Hosur KB, Wang M, Hajishengallis G (2009) Age-related alterations in innate immune receptor expression and ability of macrophages to respond to pathogen challenge in vitro. *Mech Ageing Dev* 130:538–546
- McLachlan JA, Serkin CD, Morrey KM, Bakouche O (1995) Antitumoral properties of aged human monocytes. *J Immunol* 154:832–843
- Meyer KC (2005) Aging. *Proc Am Thorac Soc* 2:433–439
- Montgomery Douglas C (2001) Design and analysis of experiments, 5th edn. Wiley, New York
- Mori T, Leung CC (2010) Tuberculosis in the global aging population. *Infect Dis Clin North Am* 24:751–768
- Nagabhushanam V, Solache A, Ting LM, Escaron CJ, Zhang JY, Ernst JD (2003) Innate inhibition of adaptive immunity: *Mycobacterium tuberculosis*-induced IL-6 inhibits macrophage responses to IFN- γ . *J Immunol* 171:4750–4757
- O'Mahony L, Holland J, Jackson J, Feighery C, Hennessy TP, Mealy K (1998) Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. *Clin Exp Immunol* 113:213–219
- Orme IM (1987) Aging and immunity to tuberculosis: increased susceptibility of old mice reflects a decreased capacity to generate mediator T lymphocytes. *J Immunol* 138:4414–4418
- Orme IM, Griffin JP, Roberts AD, Ernst DN (1993) Evidence for a defective accumulation of protective T cells in old mice infected with *Mycobacterium tuberculosis*. *Cell Immunol* 147:222–229
- Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E (2005) Control of human host immunity to mycobacteria. *Tuberculosis (Edinb)* 85:53–64

- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45
- Rajagopalan S, Yoshikawa TT (2000) Tuberculosis in the elderly. *Z Gerontol Geriatr* 33:374–380
- Reyes-Ruvalcaba D, González-Cortés C, Rivero-Lezcano OM (2008) Human phagocytes lack the ability to kill *Mycobacterium gordonae*, a non-pathogenic mycobacteria. *Immunol Lett* 116:72–78
- Rhoades ER, Orme IM (1998) Similar responses by macrophages from young and old mice infected with *Mycobacterium tuberculosis*. *Mech Ageing Dev* 106:145–153
- Rottinghaus EK, Vesosky B, Turner J (2010) TLR-2 independent recognition of *Mycobacterium tuberculosis* by CD11c⁺ pulmonary cells from old mice. *Mech Ageing Dev* 131:405–414
- Roubenoff R, Harris TB, Abad LW, Wilson PW, Dallal GE, Dinarello CA (1998) Monocyte cytokine production in an elderly population: effect of age and inflammation. *J Gerontol A Biol Sci Med Sci* 53:M20–M26
- Rudd AG, Banerjee DK (1989) Interleukin-1 production by human monocytes in ageing and disease. *Age Ageing* 18:43–46
- Sadeghi HM, Schnelle JF, Thoma JK, Nishanian P, Fahey JL (1999) Phenotypic and functional characteristics of circulating monocytes of elderly persons. *Exp Gerontol* 34:959–970
- Sakai T, Kogiso M (2008) Soy isoflavones and immunity. *J Med Invest* 55:167–173
- Schaaf HS, Collins A, Bekker A, Davies PDO (2010) Tuberculosis at extremes of age. *Respirology* 15:747–763
- Sörensen M, Lippuner C, Kaiser T, Mißlitz A, Aebischer T, Bumann D (2003) Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Lett* 552:110–114
- Turner J, Frank AA, Orme IM (2002) Old mice express a transient early resistance to pulmonary tuberculosis that is mediated by CD8 T cells. *Infect Immun* 70:4628–4637
- van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC (2007) Age-associated defect in human TLR-1/2 function. *J Immunol* 178:970–975
- Vesosky B, Turner J (2005) The influence of age on immunity to infection with *Mycobacterium tuberculosis*. *Immunol Rev* 205:229–243
- Vesosky B, Flaherty DK, Turner J (2006) Th1 cytokines facilitate CD8-T-cell-mediated early resistance to infection with *Mycobacterium tuberculosis* in old mice. *Infect Immun* 74:3314–3324
- Warwick-Davies J, Dhillon J, O'Brien L, Andrew PW, Lowrie DB (1994) Apparent killing of *Mycobacterium tuberculosis* by cytokine-activated human monocytes can be an artefact of a cytotoxic effect on the monocytes. *Clin Exp Immunol* 96:214–217
- Yoshikawa TT (1981) Important infections in elderly persons. *West J Med* 135:441–445
- Zaffran Y, Zhang L, Ellner JJ (1998) Role of CR4 in *Mycobacterium tuberculosis*-human macrophages binding and signal transduction in the absence of serum. *Infect Immun* 66:4541–4544
- Zevallos M, Justman JE (2003) Tuberculosis in the elderly. *Clin Geriatr Med* 19:121–138