Published in final edited form as:

Mech Ageing Dev. 2010 June ; 131(6): 405–414. doi:10.1016/j.mad.2010.05.006.

TLR-2 independent recognition of *M. tuberculosis* by CD11c⁺ pulmonary cells from old mice

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Abstract

The elderly are particularly susceptible to infectious diseases such as influenza, bacterial pneumonia, and tuberculosis. Current vaccines are only partially protective in old age, which makes the elderly a critical target group for the development of new vaccine strategies. The recognition of pathogens via toll like receptors (TLR) and the subsequent generation of pro-inflammatory cytokines has generated interest in incorporating TLR agonists into new vaccines to enhance immunogenicity. However, TLR function is reportedly decreased in old age, leading to questions regarding the benefit of including TLR agonists into vaccines for the elderly. It is critical that we understand the function and role of TLRs in aged hosts prior to approving new TLR based adjuvants for vaccines that will be delivered to the elderly. In this study we determine the contribution of TLRs on pulmonary macrophages from old mice to recognize and respond to infection with the virulent pathogen *Mycobacterium tuberculosis* (*M.tb*). Although pulmonary (CD11c⁺) cells from old mice were fully capable of producing cytokines in response to *M.tb* infection, we demonstrate that in contrast to young mice, *M.tb* induced cytokine production occurred independently of TLR-2. Our data indicate that the inclusion of TLR-2 agonists into new vaccines may not be fully effective in the elderly population. Investigation into such age-related differences in TLR function is of critical importance for the design of effective vaccines that will protect the elderly against infectious diseases.

Keywords

Macrophage; tuberculosis; TLR-2

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Introduction

The elderly are highly susceptible to many infectious diseases, and current vaccines are only moderately protective in this specific age group (Cabre 2009; Grubeck-Loebenstein, Della Bella et al. 2009; Sambhara and McElhaney 2009). There is a critical need to develop improved vaccines that specifically target an aged immune system. Recent evidence suggests that the inclusion of toll like receptor (TLR) agonists can serve as appropriate vaccine adjuvants that drive the production of pro-inflammatory cytokines and lead to enhanced protection (Baldrige, McGowan et al. 2004; Lahiri, Das et al. 2008). Promising studies have been performed using young animals however very little consideration has been made for whether these new vaccines will effectively target an aged immune system. Indeed, TLR expression and function in aged hosts has been examined in splenic and peritoneal macrophages from old mice, with a decrease in TLR mRNA levels and ligand induced cytokine production relative to similar cells from young mice (Renshaw, Rockwell et al. 2002). In addition, TLR-2/1 induced TNF production was decreased in monocytes from elderly individuals (van Duin, Mohanty et al. 2007), thus indicating that aged mice and humans can display diminished TLR function.

In this study we determine the capacity of pulmonary macrophages (CD11c⁺ cells) from aged mice to respond to infection with the virulent pathogen *M. tuberculosis* (*M.tb*). *M.tb* is known to interact with macrophages predominantly via TLR-2 in young mice (Underhill, Ozinsky et al. 1999) and in man, with additional contributions by TLR-9 and TLR-4 (Reiling, Holscher et al. 2002; Bafica, Scanga et al. 2005). As such, *M.tb* is an ideal experimental tool to dissect macrophage-TLR interactions in old age. Furthermore, tuberculosis (TB) is especially problematic in the elderly population, with individuals over the age of 65 having the highest TB case rate compared to other age groups (WHO 2008) and the most susceptible to TB associated death (Teale, Goldman et al. 1993), making this study particularly relevant to public health. Understanding the function of TLRs in the lungs of aged hosts will provide insight into the increased susceptibility of the elderly to *M.tb* infection, and could be critical for the effective design of vaccines that specifically target the aged population.

Our studies demonstrate that pulmonary CD11c⁺ cells from old mice are fully capable of producing IL-12p40 and TNF following *in vitro* and *in vivo* *M.tb* infection however, unlike CD11c⁺ macrophages from young mice, *M.tb* induced cytokine production occurred independently of TLR-2. Blocking studies showed that TLR-4 and TLR-9 could only partially compensate for the lack of TLR-2 responsiveness, indicating that additional receptors participate in *M.tb* recognition in old age. These data provide evidence that the incorporation of TLR-2 agonists into vaccines may not be an effective strategy for protecting the elderly and that other, currently uncharacterized receptors, may be alternate candidates when designing vaccines specifically for aged individuals.

Materials and Methods

Mice

Specific-pathogen-free, female, C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) at 2 months of age (young), or at 18 months of age (old) through a contract with the National Institute On Aging. Female wild type and TLR-2 deficient C57BL/6 retired breeders were purchased from The Jackson Laboratory (Bar Harbor, ME) at approximately 9 months of age, and aged in house to 14–18 months of age. Mice were housed in a standard vivarium in microisolator cages and were acclimated to the facility for at least one week prior to manipulation. Mice were examined at necropsy and

mice with gross lesions were excluded from the study. All procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

***In vivo* bacterial infection**

Mice were infected aerogenically with a low dose of *M. tuberculosis* Erdman (ATCC 35801, American Type Culture Collection, Manassas, VA) using the Glas-Col Inhalation Exposure System (Terre Haute, IN). Briefly, the nebulizer was filled with a suspension of bacteria calculated to deliver between 50 and 100 viable bacteria per lung during a 30 min exposure.

Lung cell isolation and CD11c⁺ cell purification

Young and old mice were euthanized by CO₂ asphyxiation and single cell suspensions were obtained from the lung using collagenase/DNAse as previously described (Vesosky, Flaherty et al. 2006). Single cell suspensions were placed in a 100mm tissue culture grade petri dish for 1 hour at 37°C, 5% CO₂. Non-adherent cells were removed and the adherent cells were washed two times with PBS. Trypsin/EDTA (Sigma Aldrich, St. Louis, MO) was added to the adherent cell layer and incubated at 37°C for 15 min. An equal volume of supplemented Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) was added to the cultures and the adherent cells were collected by vigorous pipetting, and re-suspended in supplemented DMEM. CD11c⁺ cells were isolated from the adherent cell population using the BDTM IMag (BD Biosciences, San Jose, CA) system according to the manufacturer's instructions. Briefly, cells were incubated with 2.5µg biotinylated anti-CD11c (BD Biosciences) for 15 min at 4°C, washed with supplemented DMEM and re-suspended with 50µl streptavidin beads for 30 min at 4°C. CD11c⁺ cells were purified by placing the 5ml polypropylene round bottom tube (BD Biosciences) containing the cells on the BDTM IMag for 3 cycles (1 × 8 min, 2 × 4 min). In between each cycle the supernatant was removed from the tube and the cells were re-suspended in 1ml of supplemented DMEM. CD11c⁻ cells were collected from the supernatant after the first cycle on the magnet.

***In vitro* cell culture**

0.5–1 × 10⁵ purified CD11c⁺ cells from individual mice, or 1–2 × 10⁵ cells from pooled suspensions of CD11c⁺ or CD11c⁻ cells were plated in 96-well tissue culture plates and cultured with supplemented DMEM with or without *M. tb* H37Rv (American Type Culture Collection, Manassas, VA) at a multiplicity of infection (MOI) of 1:1 for 48 or 72 hours at 37°C, 5% CO₂. Supernatants were subsequently frozen at -80°C until further analysis by ELISA. TLR-9 and TLR-4 were inhibited in these assays by culturing 1 × 10⁵ CD11c⁺ from the lungs of young and old mice with 100–200µg/ml anti-TLR-4 (clone MTS510, InvivoGen, San Diego, CA) or Rat IgG2a (BD Biosciences), or 1µM ODN 2088 (InvivoGen) for 30 min at 37°C, 5% CO₂. 1 × 10⁵ CFU *M. tb* H37Rv was subsequently added to the cultures and incubated for 48 hours at 37°C, 5% CO₂. Inhibition of cytokine production was verified with cells treated with their respective agonist/inhibitors.

Cytokine ELISA

IL-12p40 in cell supernatants was quantified by ELISA as previously described (Turner, D'Souza et al. 2001). Briefly, Falcon MicrotestTM 96-well ELISA plates were incubated at 4°C overnight in humidified chamber with purified IL-12p40/70 antibody (2.5µg/ml, BD Biosciences) in 0.1M sodium bicarbonate. The plates were blocked with supplemented DMEM, and the samples and recombinant IL-12 standard (2500–39 pg/ml) (Peprotech, Rocky Hill, NJ) were added to the wells in duplicate or triplicate. The plates were washed with phosphate buffered saline solution + 0.05% Tween 20 (PBST) and incubated with a biotinylated IL-12p40/70 antibody (2µg/ml, BD Biosciences), followed by detection with

312.5µg/ml streptavidin-horseradish peroxidase (Invitrogen, Carlsbad, CA) and TMB substrate solution (Invitrogen). 0.18M H₂SO₄ was added to stop the reaction and the plates were read on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA).

The level of TNF in cell supernatants was determined using a mouse TNF-α ELISA Ready-SET-Go! kit from ebioscience (San Diego, CA) according to manufacturer's instructions. Recombinant TNF standards were plated at concentrations of 4000–62.5 pg/ml.

Flow cytometry

All antibodies were obtained from BD biosciences unless otherwise stated. Flow cytometric analysis of extracellular markers was performed by fixing whole lung or CD11c⁺ cell cultures in deficient RPMI (dRPMI) (Irvine Scientific, Santa Ana, CA) supplemented with 0.1% sodium azide (Sigma Aldrich). Cells were incubated with 0.3µg of Fc block™ for 5 min at 4°C, and then stained with 0.3µg of anti-CD11c APC or PE (BD biosciences) or anti-CD11c APC (Serotec, Raleigh, NC), anti-CD11b PerCp-Cy5.5, anti-TLR-2 PE (ebioscience, San Diego, CA), Rat IgG2b PE (ebioscience), anti-TLR-4 PE (ebioscience), or mouse IgG1κ PE for 20 min at room temperature. Cells were subsequently washed with supplemented dRPMI and samples were read on a Becton-Dickinson LSRII and analyzed using FACSDiva software (BD Biosciences). Intracellular labeling of TLR-9 was performed with the BD Cytofix/Cytoperm™ Fixation/Permeabilization kit according to the manufacturer's instructions. Briefly, cells were fixed in supplemented dRPMI and stained with 0.3µg of anti-CD11c APC for 20 min at 4°C, washed, and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 15 min at 4°C. Cells were washed with 1X Permashield (BD Biosciences) and stained with 0.3µg anti-TLR-9 PE (LifeSpan Biosciences, Seattle, WA) or Mouse IgG1κ for 20 min at 4°C. A final two washes were performed and the cells were read with the LSRII and analyzed with FACSDiva software. Gates were set using isotype control antibodies.

Real-time PCR

Purified CD11c⁺ and CD11c⁻ cells from the lungs of young and old naïve or 12 day *M.tb* infected mice were homogenized in 1ml TRIzol® Reagent (Invitrogen) and frozen at -80°C. RNA was isolated using the Qiagen (Valencia, CA) RNeasy kit according to the manufacturer's instructions, and reverse transcribed using an Omniscript RT kit from Qiagen. Real-time PCR was performed using TaqMan® gene expression assays for IL-12p40 and TNF (Applied Biosystems, Foster City, CA) and analyzed with a BioRad IQ5 (Hercules, CA). The delta delta cycle threshold (CT) method was used for quantification of the relative units (RU) of mRNA, using 18S as an endogenous normalizer and young mice as a calibrator. $RU = 2^{-\Delta\Delta CT}$. $\Delta CT = CT \text{ gene} - CT \text{ 18S}$. $\Delta\Delta CT = \Delta CT \text{ sample} - \Delta CT \text{ calibrator (young, naïve mouse)}$.

Statistical analysis

Statistical significance was determined using PRISM 4 software (GraphPad Software; San Diego, CA). The unpaired two-tailed Student *t* test was used for comparisons.

Results

M. tb induced cytokine production by pulmonary CD11c⁺ cells remains intact in old age

We determined the capacity of pulmonary macrophages from old and young mice to respond to *M.tb* infection by secreting IL-12p40 and TNF, which are both produced upon TLR ligation and critical for the control of *M.tb*. Our initial studies compared the myeloid cell populations within the lungs of old and young naïve mice which revealed that CD11b expression was similar, but the proportion of CD11c⁺ cells was consistently increased in the

lungs of young mice (average of 5% more compared to old; data not shown). Fig 1A–B illustrate that the majority of purified CD11c⁺ cells from the lungs of both young and old mice express a CD11c⁺/CD11b^{neg-lo} phenotype, a phenotype that has previously been described for alveolar macrophages in mice (Gonzalez-Juarrero, Shim et al. 2003). In addition, we observed a minor population of cells expressing lower levels of CD11c and high levels of CD11b (Fig 1A–B), which have previously been described as monocytes (Gonzalez-Juarrero, Shim et al. 2003). As illustrated in Fig 1B, the proportion of alveolar macrophages and monocytes within the purified CD11c⁺ population differed by age. Young mice had a significantly higher proportion of alveolar macrophages in the lung in our analysis (Fig 1B and Table 1) but not a significant difference in absolute numbers (Table 1). Young mice had a reduced proportion (Fig 1B and Table 1) and reduced absolute number (Table 1) of monocytes in the lung when compared to old.

These data indicate that pulmonary cell populations may be altered in old age, and therefore we sought to determine if CD11c⁺ or CD11b⁺ cells from old mice could produce IL-12p40 and TNF in the lungs following infection with *M.tb*. CD11c⁺ and CD11c⁻ cells were purified from the lungs of young and old mice prior to, and 12 days after, a low-dose aerosol infection with *M.tb* and IL-12p40 and TNF gene expression was determined by real time (RT)-PCR. This time point was chosen based on preliminary data from our laboratory as well as previous reports illustrating that early IL-12p40 production during *M.tb* infection occurred within 2 weeks of infection (Vesosky, Flaherty et al. 2006; Rothfuchs, Egen et al. 2009). Analysis of *ex vivo* cytokine gene expression revealed that, when normalized to uninfected controls (data not shown), IL-12p40 (Fig 1C) and TNF (Fig 1D) gene expression in the lungs of infected mice were largely limited to the CD11c⁺ population of cells in both young and old mice, indicating that CD11c⁺ cells are the primary cytokine producing cells following *M.tb* infection in old age. Furthermore, these data showed that there were no significant differences in cytokine gene expression between young and old pulmonary CD11c⁺ cells at 12 days post *M.tb* infection (Fig 1C–D).

To confirm that gene expression correlated with IL-12p40 and TNF protein production we determined the ability of CD11c⁺ cells from the lungs of young and old mice to produce cytokines following an *in vitro* *M.tb* infection. Our data confirm that pulmonary CD11c⁺ cells from young and old mice produced the majority of IL-12p40 and TNF and that, *in vitro*, equivalent levels of IL-12p40 and TNF were also produced by cells from young and old mice (Fig 1E–F). These data are the first to demonstrate that pulmonary CD11c⁺ cells from old and young mice are the dominant source of IL-12p40 and TNF, and that old mice maintain the ability to produce essential cytokines following infection with the virulent pathogen, *M.tb*. Furthermore, CD11c⁺ cells from old mice are fully capable of secreting equivalent levels of TNF and IL-12p40 as cells from young mice.

TLR expression on pulmonary CD11c⁺ cells is similar between young and old mice

TLR-2, TLR-4, and TLR-9 can induce cytokine production in response to *M.tb* recognition in young hosts (Brightbill, Libraty et al. 1999; Means, Wang et al. 1999; Underhill, Ozinsky et al. 1999; Bafica, Scanga et al. 2005). The expression and function of these receptors on pulmonary macrophages from old mice is unknown, however studies involving macrophages from extra-pulmonary sites report decreases in both TLR function and expression in old age (Renshaw, Rockwell et al. 2002). Analysis of TLR-2, TLR-4, and TLR-9 expression in/on CD11c⁺ cells from the lungs of young and old mice revealed that TLR-2 was the most highly expressed of these receptors (Fig 2A–E) and was present on the surface of the majority of pulmonary CD11c⁺ cells from both young and old mice (Fig 2B). The MFI of TLR-2, indicative of the amount of receptor expression, was significantly increased on the surface of CD11c⁺ cells from the lungs of old mice (Fig 2C). TLR-9 staining within CD11c⁺ cells did not provide a clear positive and negative cell population,

and instead we observed a slight shift of the entire peak on a histogram compared to the isotype control (Fig 2A). These data indicate that the majority of CD11c⁺ cells from the lungs of young and old mice likely express TLR-9, however at a level lower than the detection limit of our assay. We therefore expressed the percentage of TLR-9⁺ cells above the threshold of detection as TLR-9^{high} and as Figure 2A, D, and E illustrate, the proportion and MFI of TLR-9 on CD11c⁺ cells from the lungs of young and old mice were similar. In contrast to other receptors, TLR-4 expression on pulmonary CD11c⁺ cells was below the level of detection of this assay (Fig 2A) and therefore the MFI and percent positive analysis of TLR-4 are not depicted in Fig 2. We did observe TLR-4 expression on peritoneal macrophages (data not shown), providing evidence that the lack of TLR-4 staining on pulmonary CD11c⁺ cells was the result of low receptor expression and not due to defective antibody labeling. Overall, we have shown that, contrary to published data for extra-pulmonary macrophages (Renshaw, Rockwell et al. 2002), TLR-2 and TLR-9 were expressed at equivalent or enhanced levels on CD11c⁺ cells from the lungs of young and old mice, and thus we hypothesize that these receptors play a role in *M.tb* induced cytokine production.

M.tb induced cytokine production by pulmonary CD11c⁺ cells from old mice occurs independent of TLR-2

TLR-2 is critical for the production of IL-12p40 and TNF by bone-marrow derived macrophages in response to *in vitro* *M.tb* infection (Underhill, Ozinsky et al. 1999; Reiling, Holscher et al. 2002). To determine whether TLR-2 plays a similar role in old age, we purified CD11c⁺ cells from the lungs of young and old wild type, and young and old TLR-2 deficient mice and infected them with *M.tb in vitro*. In accordance with data from extra-pulmonary macrophages (Reiling, Holscher et al. 2002), *M.tb* induced IL-12p40 (Fig 3A & B) and TNF (Fig 3C & D) production by CD11c⁺ cells from the lungs of young TLR-2 deficient mice was reduced, with approximately 75% reduction in cytokine production in the absence of TLR-2. In contrast, CD11c⁺ cells from the lungs of old TLR-2 deficient mice were fully capable of secreting IL-12p40 (Fig 3A) and TNF (Fig 3C) in response to *M.tb* infection, with only a 20% reduction in the production of both cytokines (Fig 3B & D). Therefore, in contrast to young mice, *M.tb* induced cytokine production by CD11c⁺ cells from the lungs of old mice occurred in a TLR-2 independent manner. These data confirm that *M.tb* induced cytokine production from pulmonary CD11c⁺ cells from young mice is highly dependent on TLR-2, and are the first to demonstrate that macrophages from old mice are capable of responding to *M.tb* in the absence of TLR-2. Furthermore, these data indicate that despite similar levels of cytokine production, pulmonary CD11c⁺ cells from young and old mice utilize different mechanisms to produce IL-12p40 and TNF in response to *M.tb* infection.

TLR-9 and TLR-4 play a minimal role in M.tb induced cytokine production by pulmonary CD11c⁺ cells from old mice

TLR-4 and TLR-9 can also contribute to cytokine production in response to *M.tb* infection in young mice (Means, Wang et al. 1999; Bafica, Scanga et al. 2005), and we therefore investigated the role of these additional TLRs in *M.tb* induced cytokine production in old mice. Due to the lack of available aged TLR-9 and TLR-4 deficient mice, CD11c⁺ cells were purified from the lungs of young and old wild type mice and incubated with *M.tb* in the presence of a competitive TLR-9 antagonist, ODN 2088, or a neutralizing TLR-4 antibody. In cultures of CD11c⁺ cells from young mice, *in vitro* inhibition of TLR-9 led to a significant reduction of IL-12p40 levels (Fig 4A & 4C), and decreased TNF production by nearly 75% (Fig 4D & F), indicating that in young mice TLR-9 plays a substantial role in *M.tb* induced IL-12p40 and TNF production. The addition of a TLR-9 competitive antagonist to *M.tb* infected CD11c⁺ cultures from old mice led to a decreased production of

IL-12p40 and TNF of approximately 30% and 10% (Fig 4A,C D & F) respectively. These data illustrated that TLR-9 plays a significant role in stimulating IL-12p40 from pulmonary CD11c⁺ cells from young mice, and a moderate role in TNF production upon *M. tb* infection in both young and old mice.

Although we were unable to detect TLR-4 expression on pulmonary CD11c⁺ cells by flow cytometry, CD11c⁺ cells were responsive to LPS (data not shown), providing evidence that TLR-4 is present on the surface of CD11c⁺ cells, but at levels lower than the detection limit of our assay. Inhibition of TLR-4 in cultures of *M.tb* infected CD11c⁺ from young mice had very little impact on IL-12p40 production (Fig 4 B, C, E, & F). In contrast, inhibition of TLR-4 resulted in an approximate 25% reduction of IL-12p40 production by CD11c⁺ cells from old mice (Fig 4 B–C), suggesting that, like TLR-9, TLR-4 can partially contribute to IL-12p40 production upon *M.tb* infection of pulmonary CD11c⁺ cells from old mice. Interestingly, in cultures from both young and old mice, TNF production was increased in the presence of a TLR-4 neutralizing antibody (Fig 4E), providing evidence that TLR-4 may play a role in dampening TNF production in response to *M.tb* infection. This inhibitory role for TLR-4 during mycobacteria infection has been reported previously (Heldwein, Liang et al. 2003). Overall, these data show that TLR-4 can partially contribute to *M.tb* induced IL-12p40 production by pulmonary CD11c⁺ cells from old mice; however neither TLR-4 nor TLR-9 could account for the total IL-12p40 production that we observe in our assay. Furthermore, TLR-9 and TLR-4 were not responsible for *M.tb* induced TNF production from pulmonary CD11c⁺ cells from old mice.

Combined inhibition of TLRs does not abrogate *M.tb* induced cytokine production by pulmonary CD11c⁺ cells from old mice

Our data indicate that, individually, TLR-4 and TLR-9 are only moderately involved in *M.tb* induced cytokine production by pulmonary CD11c⁺ cells from old mice. TLRs can have synergistic relationships (Bafica, Scanga et al. 2005; Lee, Scanga et al. 2007), and we thus sought to determine whether the function of multiple TLRs was required for *M.tb* induced cytokine production by pulmonary CD11c⁺ cells from old mice. To assess whether TLR-4 or TLR-9 played a role in the TLR-2 independent cytokine production, we inhibited TLR-4 or TLR-9 in cultures of TLR-2 deficient CD11c⁺ cells from old mice, and examined cytokine production following *in vitro* *M.tb* infection. Inhibition of TLR-9 in CD11c⁺ cells from the lungs of old TLR-2 deficient mice led to approximately 25% decrease in cytokine production (Fig 5A–D), and thus did not reduce cytokine production beyond that observed by TLR-9 inhibition in cells from old wild type mice (Fig 4). These data confirm that TLR-9 can contribute to cytokine induction in pulmonary macrophages from old mice, yet this receptor alone cannot fully account for the TLR-2 independent mechanism of *M.tb* recognition by CD11c⁺ cells from the lungs of old mice. As illustrated in Fig 3, CD11c⁺ cultures from young mice on a TLR-2 deficient background produced very little cytokine and therefore we could not accurately determine the effects of additional TLR inhibition in these cultures (data not shown).

Neutralizing of TLR-4 function on TLR-2 deficient CD11c⁺ cells led to an approximate 50% decrease in IL-12p40 production in response to *M.tb* infected CD11c⁺ cultures relative to control cultures (Fig 5A–B) however, inhibition was highly variable and this reduction was not statistically significant (Fig 5A). Similar to Fig 4E, we observed an increase in TNF production when TLR-4 was inhibited on a TLR-2 deficient background (Fig 5C). These data indicate that TLR-4 and TLR-9 partially contribute to *M.tb* induced cytokine production by pulmonary CD11c⁺ cells from old mice in the absence of TLR-2; however neither TLR was fully responsible for the TLR-2 independent induction of cytokines following *M.tb* infection of pulmonary CD11c⁺ cells from old mice.

When TLR-4 and TLR-9 were inhibited simultaneously in cultures of pulmonary CD11c⁺ cells from old wild type mice, IL-12p40 production was not reduced beyond levels observed when either receptor was inhibited alone (Fig 5E & F). Therefore, TLR-4 and TLR-9 equally contributed to the production of cytokine by pulmonary CD11c⁺ cells following *M.tb* infection in old mice, but their actions were not synergistic. When the combined role of TLR-4 and TLR-9 were determined in *M.tb* infected CD11c⁺ cultures from the lungs of young mice, IL-12p40 production was reduced by nearly 70% (Data now shown, (Media = 252.0 $\pm$ 29.9 SD pg/ml, anti-TLR-4 & ODN 2088 = 46.0 $\pm$ 16.2 SD pg/ml, $p = .013$), reflecting the significant role of TLR-9 in *M.tb* recognition by cells from young mice (Bafica, Scanga et al. 2005). Overall, our data demonstrate that *M.tb* induced cytokine production by pulmonary CD11c⁺ cells from young mice was highly dependent on TLR-2 and TLR-9. In contrast, cytokine production by pulmonary CD11c⁺ cells from old mice in response to *M.tb* infection could be partially mediated by TLR-4, and TLR-9 but neither TLR could fully account for the TLR-2 independent cytokine production that we observed.

Discussion

In this study we determined the capacity of pulmonary macrophages from old and young mice to produce IL-12p40 and TNF in response to *M.tb* infection. Our findings demonstrate that pulmonary CD11c⁺ cells (a marker associated with alveolar macrophages (Gonzalez-Juarrero, Shim et al. 2003)) were the primary cells that could secrete IL-12p40 and TNF both *in vitro* and *in vivo* in response to *M.tb*. This response was equivalent between young and old mice, indicating that early cytokine secretion by pulmonary CD11c⁺ cells was not altered in old age upon challenge with the virulent pulmonary pathogen, *M.tb*. Furthermore, we demonstrated that pulmonary CD11c⁺ cells from old mice were fully capable of secreting IL-12p40 and TNF in response to *M.tb* in the absence of TLR-2, identifying a critical change in pattern recognition receptor function in old age that has not been previously appreciated. These findings may have considerable impact for the incorporation of TLR agonists into vaccines for the elderly.

Our data show that pulmonary CD11c⁺ cells from old mice express TLRs at equivalent levels as similar cells from young mice which is in contrast to previous findings that splenic and peritoneal macrophages from old mice display decreased TLR mRNA (Renshaw, Rockwell et al. 2002). In this same study reduced ligand induced IL-6 and TNF production (Renshaw, Rockwell et al. 2002) by mouse macrophages was also reported, and it has also been shown that TLR-2/1 induced TNF production was decreased in monocytes from elderly individuals (van Duin, Mohanty et al. 2007). These data contrast our findings that CD11c⁺ cells from old mice were fully capable of producing TNF and IL-12p40 in response to *M.tb*. With such a complex stimulus as *M.tb*, which possesses multiple TLR ligands, it is perhaps unsurprising that our findings differ from published studies using purified TLR ligands. In contrast to previous studies, we defined and purified pulmonary macrophages based upon specific receptor expression and the capacity to secrete cytokine in response to *M.tb*. Pulmonary CD11c⁺ macrophages are a critical cellular niche for *M.tb* and are at the front line of innate immune responses upon recognition of this pathogen (Tian, Woodworth et al. 2005) leading us to focus our studies on this specific population. Experiments using total lung cells also showed equivalent IL-12p40 secretion between old and young lung cells (data not shown) indicating that the study of a purified cell population did not impact our general findings. Stimulation of pulmonary macrophages with LPS has also showed that cells from old mice (Kohut, Senchina et al. 2004) and old rats (Tasat, Mancuso et al. 2003) had similar cytokine production compared to young. Furthermore, Rhoades and Orme have shown that IL-12p40 and TNF gene expression was similar in bone marrow derived macrophages from young and old mice in response to culture with *M.tb in vitro* (Rhoades and Orme 1998). Therefore, in several models using pulmonary cells, and/or *M.tb* infection,

cytokine production was equivalent between young and old mice indicating that macrophage function can be highly variable and likely dependent on the original tissue location or maturation stage of the cell (Stout and Suttles 2005). Our data highlight the importance of considering the specific pathogen and cellular niche when studying macrophage function and clearly show that the capacity of pulmonary CD11c⁺ cells from old mice to secrete protective cytokines in response to *M.tb* is intact.

Our data extends upon current knowledge of pathogen-host cell interactions to show that receptor mediated recognition by pulmonary CD11c⁺ cells from old mice was divergent from CD11c⁺ cells from young mice and most significant, occurred independent of TLR-2. The essential role of TLR-2 in *M.tb* induced cytokine production in young mice has been previously described (Underhill, Ozinsky et al. 1999; Reiling, Holscher et al. 2002), and also confirmed for pulmonary macrophages in our study. In old mice however, CD11c⁺ cells were fully capable of secreting IL-12p40 and TNF in response to *M.tb*, in the absence of TLR-2. We predict that this change occurs due to the decreased function of TLR-2 observed in aged hosts (Renshaw, Rockwell et al. 2002; van Duin, Mohanty et al. 2007) and thus, CD11c⁺ cells from the lungs of old mice likely compensated for decreased TLR-2 function first by increasing surface expression of this receptor, and second by utilizing alternate receptors to compensate for diminished TLR-2 activity. The TLR-2 independent mechanism of *M.tb* recognition by pulmonary CD11c⁺ cells from aged mice is currently unknown however we hypothesize that other pattern recognition receptors involved in *M.tb* recognition, such as additional TLRs, Dectin-1, and/or NOD-2, may contribute to this process (Torrelles, Azad et al. 2008; Tsolaki 2009). In support of alternative receptor recognition, we observed a moderate decrease in IL-12p40 production following TLR-4 and/or TLR-9 inhibition by CD11c⁺ cells from old mice. These data indicated that TLR-4 and TLR-9 likely participate and contribute to the production of IL-12p40 upon stimulation with *M.tb* however, neither was absolutely critical for IL-12p40 production in old mice. Therefore, our data indicate that TLRs (particularly TLR-2 and TLR-9) play a critical role in the induction of cytokine production in response to *M.tb* infection in young mice, and yet those same receptors play only a limited role in aged mice indicating that as we age. The consequence of alternate receptor mediated recognition of *M.tb* by cells from old mice, while not fully understood, may lead to downstream events such as altered uptake or trafficking of bacteria that could have dramatic consequences for how *M.tb* is processed and presented to immune cells, and ultimately impact the control (or lack) of infection in the elderly.

In summary, we have shown that pulmonary CD11c⁺ cells from old mice are fully capable of producing cytokines in response to *M.tb* yet the pathway that leads to cytokine production is independent of TLR-2, a dominant pattern recognition receptor for *M.tb* in young mice (Underhill, Ozinsky et al. 1999). Such altered recognition may have implications for the fate of *M.tb* within macrophages (survival versus killing), and could explain why the elderly are more susceptible to TB (Rajagopalan 2001; Jo, Yang et al. 2007). Our findings also have specific relevance to vaccine design. The capacity of TLRs to initiate a robust immune response has led to their incorporation into new vaccines. TLR-9 agonists have shown promise as adjuvants for influenza and cancer vaccines (Cooper, Davis et al. 2004; Zheng, Cohen et al. 2008). Furthermore, a promising new fusion protein vaccine that targets TLR-2 has been shown to protect mice against *M.tb* infection (Wang, Henao-Tamayo et al. 2007). Our studies indicate that vaccines that rely on TLR-2 agonists as adjuvants may not be as effective in an elderly population. The identification of alternate pattern recognition receptors that can stimulate macrophages to secrete pro-inflammatory cytokines in old age may lead to the development of more effective for vaccines that are targeted to the aged population. Currently the elderly population of the world is expanding to unprecedented numbers, with the most rapid expansion expected in developing countries where TB is

endemic (Kinsella and Velkoff 2001). Therefore, to successfully reduce the global burden of TB, and other respiratory infections, we must consider immune function in the elderly when designing vaccines aimed at preventing such infections.

Acknowledgments

The project described was supported by grant number R01AG-021097 from the National Institute on Aging, and 1T32HL07946-06 from the National Institute of Health. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institute on Aging or the National Institute of Health.

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Figure 1.

M.tb induced cytokine production in young and old pulmonary CD11c⁺ cells. A–B.) CD11c⁺ cells were purified from the lungs of naive young (open) and old (closed) mice and stained with antibodies against CD11b and CD11c. A) Representative dot plot of pulmonary CD11c⁺ cell populations. B.) Proportion of CD11c⁺/CD11b^{neg-lo} and CD11c^{mid}/CD11b⁺ cells. C–D.) Young (open) and old (closed) mice were aerogenically infected with *M.tb* Erdman using the Glas-Col Inhalation Exposure System, and CD11c⁺ cells were purified from infected animals at 12 days post infection using magnetic cell separation. RNA was isolated from cells; C.) IL-12p40 and D.) TNF gene expression were analyzed using RT-PCR. E–F.) CD11c⁺ and CD11c cells were purified from the lungs of young (open) and old

(closed) mice and infected *in vitro* with *M. tb* H37Rv (MOI 1:1) for 72 hours. E.) IL-12p40 and F.) TNF production was determined by ELISA. Data represent the mean $\pm$ SD of 4–5 mice and are representative of 2 independent experiments. Statistical significance was determined by Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ND, not detected.

Figure 2.

TLR expression in young and old pulmonary CD11c⁺ cells. Single cell suspensions from the lungs of young (open) and old (closed) mice were fixed and stained extracellularly with antibodies against CD11c, TLR-2, or TLR-4, or permeabilized and stained intracellularly with antibodies against TLR-9. A.) representative histograms of TLR expression on pulmonary CD11c⁺ cells from young and old mice. Percentages of CD11c⁺ cells expressing (B.) TLR-2 or (D.) high levels of TLR-9 were analyzed, as was the MFI of (C.) TLR-2 and (E.) TLR-9 on CD11c⁺ cells. Young and old cells stained with isotype control antibodies were used to set positive gates (P3). MFI values were normalized by subtracting isotype control values from those obtained using specific antibodies. TLR-4 expression did not

exceed that of the isotype control and was therefore excluded from the analysis. Data represent the mean $\pm$ SD of 5 mice and are representative of 2 independent experiments. Statistical significance was determined by Student's *t*-test, * $p < 0.05$

Figure 3.

M.tb induced cytokine production by CD11c⁺ cells from the lungs of young and old TLR-2 deficient mice. CD11c⁺ cells were purified from the lungs of young and old wild type (open) and TLR-2 deficient (closed) mice, and pooled according to age or TLR status. Cells were infected *in vitro* with *M.tb* H37Rv for 48 hours and supernatants analyzed for A.) IL-12p40 and C.) TNF production by ELISA. Cytokine concentrations were normalized by subtracting values of cells cultured in media alone from those obtained following *in vitro* infection. ELISA values were used to calculate the percent reduction of B.) IL-12p40 or D.) TNF production. A value of zero was assigned to the experiments where no reduction was observed. Data represent the mean $\pm$ SD of pools of 5 mice from 2 independent experiments. Statistical significance was determined by Student's *t*-test, * $p < 0.05$. TLR-2 expression was not observed in old or young TLR-2 deficient mice (data not shown).

Figure 4.

TLR-4 and TLR-9 inhibition in pulmonary CD11c⁺ cells from young or old wild type mice. CD11c⁺ cells were purified from the lungs of young and old mice, pooled respective to age, and cultured with A&D.) media alone (open) or a competitive TLR-9 antagonist (closed), ODN 2088 (1 μ M), or B&E) 100–200 μ g/ml Rat IgG (open) or anti-TLR-4 (closed) for 30 min. prior to infection with *M.tb* H37Rv (MOI 1:1) for 48 hours. A–C.) IL-12p40 or D–E.) TNF levels in culture supernatants were determined by ELISA. Cytokine concentrations were normalized by subtracting values of cells cultured in media alone from those obtained following *in vitro* infection. ELISA values were used to calculate the percent reduction of C.) IL-12p40 or F.) TNF production. A value of zero was assigned to the experiments where no reduction was observed. Data represent mean $\pm$ SD of pools of 5 mice from 3 (TNF) or 4 (IL-12p40) independent experiments. Statistical significance was determined by Student's *t*-test, * $p < 0.05$, ** $p < 0.01$.

Figure 5.

Inhibition of multiple TLRs in pulmonary CD11c⁺ cultures from old mice following *M.tb* infection. CD11c⁺ cells were purified from the lungs of old A–D.) TLR-2 deficient, or E–F.) wild type mice and cultured with media alone, a competitive TLR-9 antagonist, ODN 2088 (1μM), and/or 100–200μg/ml Rat IgG or anti-TLR-4 for 30 min. prior to infection with *M. tb* H37Rv (MOI 1:1) for 48 hours. A&E.) IL-12p40 or C.) TNF levels in culture supernatants were determined by ELISA. Cytokine concentrations were normalized by subtracting values of cells cultured in media alone from those obtained following *in vitro* infection (A. 65 pg/ml; C. 234 pg/ml; E. 93 pg/ml). ELISA values were used to calculate the percent reduction of B&F.) IL-12p40 or D.) TNF production. A value of zero was assigned

to the experiments where no reduction was observed. Data represent mean $\pm$ SD of pools of 5 mice from 2 independent experiments. Statistical significance was determined by Student's *t*-test, * $p < 0.05$.

Table 1
Cell populations within purified CD11c⁺ cells

CD11c^{hi}CD11b^{lo} and CD11c^{mid}CD11b^{hi} cells in the lungs. CD11c⁺ cells were purified from the lungs of naive young and old mice and stained with antibodies against CD11b and CD11c. Proportions of alveolar macrophages (CD11c^{hi}CD11b^{lo}) and monocytes (CD11c^{mid}CD11b^{hi}) are shown for old and young mice. Data represent the mean $\pm$ SD of 4–5 mice and are representative of 2 independent experiments.

	Young mice (Mean and SEM)	Old mice (Mean and SEM)	Significance value (Student's <i>t</i> test)
Alveolar macrophages (%)	78.42 (2.97)	62.44 (1.42)	**
Alveolar macrophages (number)	11.64 (1.6)	17.84 (2.51)	NS
Monocytes (%)	10.36 (2.14)	20.76 (1.72)	**
Monocytes (number)	1.52 (0.39)	6.24 (1.53)	*

Statistical significance was determined by Student *t* test, * $p < 0.05$, ** $p < 0.001$.