Published in final edited form as:

Mech Ageing Dev. 2016 July ; 157: 44–59. doi:10.1016/j.mad.2016.07.008.

Toll-Like Receptor Function of Murine Macrophages, Probed by Cytokine Induction, Is Biphasic And Is Not Impaired Globally With Age

Goutham Pattabiraman[§], Karol Palasiewicz, and David S. Ucker^{||}

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612

Abstract

Aging is associated with a waning of normal immune function. This “immunosenescence” is characterized by a diverse repertoire of seemingly discreet and unbalanced immune alterations. A number of studies have suggested that aging-associated alterations in innate immune responsiveness, especially responsiveness dependent on Toll-like Receptor (TLR) engagement, are causally involved. We find, however, that the magnitude and dose-dependency of responsiveness to TLR engagement (assessed with respect to cytokine production) in distinct populations of murine macrophages are not altered generally with animal age or as a consequence of immunosenescence. Responses elicited with a wide array of TLR agonists were examined by extensive functional analyses, principally on the level of the individual cell. These studies reveal an intriguing “all-or-nothing” response behavior of macrophages, independent of animal age. Although reports to the contrary have been cited widely, aging-associated immune decline cannot be attributed to widespread alterations in the extents of TLR-dependent innate immune macrophage responses.

Keywords

Immunosenescence; Cytokines; Inflammation; Mice; Single-Cell Analysis

1. Introduction

Aging is associated with a deterioration of normal immune function (Makinodan & Kay, 1980; Miller, 1991; Price & Makinodan, 1972; Thoman & Weigle, 1989). This aging-associated condition, termed “immunosenescence”, has been hypothesized to be involved

^{||}to whom correspondence should be addressed at: David Ucker, Department of Microbiology and Immunology (MC 790), University of Illinois College of Medicine, 835 South Wolcott, Chicago, IL 60612, (312) 413 1102 (Voice), (312) 413 7385 (Fax), DUCK@UIC.EDU.

[§]current address: Department of Immunology, University of Connecticut Health Center, Farmington, CT 06030, PATTABIRAMAN@UCHC.EDU

Conflicts of Interest

The authors declare that they have no conflicts or competing commercial interests in relation to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

causally in many of the pathologies of aging (Walford, 1969). The diminution of T lymphocyte repertoire and function, B lymphocyte activity, and reduced responsiveness to vaccination with advancing age have been well-described (Gerbase-DeLima et al, 1974; Inamizu et al, 1985; Makinodan et al, 1971; Michie et al, 1992; Murasko & Goonewardene, 1990; Naylor et al, 2005; Toapanta & Ross, 2009; Zhang et al, 2002). Changes in the myeloid cell compartment have been implicated functionally in aging-associated pathologies as well (Inamizu et al, 1985). Another significant hallmark of the immunosenescent state is an increase in levels of certain inflammatory cytokines - even in the absence of evident infection (Bruunsgaard et al, 1999; Bruunsgaard et al, 2001; Ershler, 1993; Ferrucci et al, 2005; Ferrucci et al, 1999; Harris et al, 1999; Jeon et al, 2012; Spaulding et al, 1997; Thoman & Weigle, 1989; Trzonkowski et al, 2009; Walston et al, 2002).

Modified pathogen susceptibility represents a further manifestation of aging. Immune responsiveness to viral pathogens declines broadly with increasing age. Elevated susceptibility to a variety of viruses correlates with diminished interferon [especially Type I] expression as well as reduced natural killer (NK) cell numbers and cytotoxicity (Murasko & Jiang, 2005; Panda et al, 2009; Shaw et al, 2010). Susceptibility to bacterial pathogens, in general, also increases with age (Gardner & Remington, 1977), due to reduced T cell reactivity and diminished innate immune functions of myeloid cells (Løvik & North, 1985; Patel, 1981). The enhancement with advancing age of resistance to *Listeria monocytogenes* (Emmerling et al, 1979; Matsumoto et al, 1979) and *Francisella novicida* (Mares et al, 2010), in contrast, reflect paradoxical aging-associated immune alterations.

Innate immune recognition of diverse extracellular non-self determinants is accomplished primarily via receptors belonging to the Toll-like Receptor (TLR)¹ family (Creagh & O'Neill, 2006; Medzhitov, 2001). One or more of multiple TLR species are triggered by distinct bacterial, fungal, protist, and viral constituents (Gazzinelli & Denkers, 2006; Jones et al, 2001; Medzhitov, 2001; Ozinsky et al, 2000); purified molecular agonists for individual TLRs have been characterized and are of great experimental utility (see below). More generally, TLR specificities have been classified in a canonical pathogen-specific scheme, and the consequences of TLR engagement accord with those classifications, broadly (Takeda & Akira, 2004). (Physiologically, the pathogen-selective targeting of distinct TLRs appears to be more degenerate than this classification might suggest, however. For example, the discrimination of Gram-positive and Gram-negative bacteria, which often is considered to be accomplished by TLR2 and TLR4, respectively, does not follow this rule absolutely [Sun et al, 2012; Zhang et al, 1997].) TLRs are linked directly via signal transduction pathways (involving the well characterized molecules MyD88, TIRAP (Mal), TRAF6, and TBK1, among others) to the activation of specific transcription factors (such as NFκB, AP1, and IRF3 and 7) associated with particular innate immune outputs (Jones et al, 2001; Medzhitov, 2001; Moynagh, 2005; Takeda & Akira, 2004; Yamamoto et al, 2003). Those TLRs engaged by bacterial products (e.g. TLR2, 4, and 5) promote reactive oxygen bursts and inflammatory cytokine (e.g. TNFα, Interleukin- [IL-] 6) production, while those

¹**Abbreviations:** APC, Allophycocyanin; CFU, Colony Forming Unit; FBS, Fetal Bovine Serum; FITC, Fluorescein Isothiocyanate; IL, Interleukin; HKLM, Heat-killed *Listeria monocytogenes*; LPS, Lipopolysaccharide; TLR, Toll-like Receptor; mAb, monoclonal Antibody; MFI, Mean Fluorescence Intensity; PB, Pacific Blue; PE, Phycoerythrin

responding to nucleic acid and viral determinants (e.g. TLR3, 7, and 9) lead to interferon responses.

Macrophages play a pivotal role as sentinels for immune surveillance at the nexus of innate and adaptive immunity. They serve an innate immune role as sensors of stress and immunological challenge and producers of inflammatory cytokines and other factors, and as antigen-presenting stimulators of adaptive immune responses. Studies exploring the involvement of macrophages in aging-associated immune anomalies have suggested a number of intrinsic aging-associated macrophage alterations, including reductions in the production of reactive oxygen and nitrogen species (Chen et al, 1991; Ding et al, 1994; Kissin et al, 1997; Plackett et al, 2004; but see Kohut et al, 2004) and in anti-tumor activity (Wallace et al, 1995). In addition, impairment of intracellular signal transduction, especially via the JAK/Stat and mitogen-activated protein kinase (MAPK) pathways, with age has been observed (Boehmer et al, 2004; Chelvarajan et al, 2006; Panda et al, 2009; Yoon et al, 2004, also see Sun et al, 2012).

Work with human and rodent macrophages has noted diminished responsiveness to innate immune stimuli, including specific TLR agonists, with age in some (Boehmer et al, 2004; Boehmer et al, 2005; Bruley-Rosset & Vergnon, 1984; Chelvarajan et al, 2005; Ding et al, 1994; Higashimoto et al, 1993; Plowden et al, 2004; Renshaw et al, 2002; Sun et al, 2012; van Duin et al, 2007; Wallace et al, 1995), but not all (Ahluwalia et al, 2001; Beharka et al, 2001; Candore et al, 1993; Chen et al, 1996; Han et al, 1995; Segal et al, 1997; Shimada & Ito, 1996), cases (see Table 1). For example, Renshaw *et al.* (2002) reported that elicited peritoneal and splenic macrophages from older animals are substantially attenuated in their responses to TLR agonists, expressing much lower levels of TLRs, and secreting significantly lower levels of TNF α and IL-6 following TLR stimulation. Boehmer *et al.* (2004; 2005) also reported aging-associated alterations in cytokine production from bulk cultures of elicited peritoneal and splenic macrophages stimulated via TLR4 (with LPS) or TLR2 (with Zymosan), although they noted no differences in TLR2 and TLR4 expression with age on individual (F4/80⁺) macrophages. Caveats regarding several of these studies call into question their particular conclusions (see below and Table 1). More broadly, as made clear by the compilation of these published reports (Table 1), evidence of an age-dependent diminution in TLR responsiveness is quite mixed and fails largely to address issues of cell-intrinsic responsiveness. Further, the notion that TLR function declines with age is not congruent with the view of aging-associated immune dysregulation characterized simply as heightened inflammatory status. Similarly, the suggestion that isolated macrophage (and monocyte) phenotypes are skewed with age toward an anti-inflammatory (alternatively activated, “M2-like”) polarized state (Boehmer et al, 2005; Seidler et al, 2010) is not readily consistent with *in vivo* observations.

Given this contention, we found it striking that, in our examination of immunosuppressive responses to apoptotic cells (“innate apoptotic immunity”), we observed no aging-associated differences in the extents of macrophage responsiveness to TLR stimulation. We have explored this issue more comprehensively, focusing especially on TLR-dependent stimulation of cytokine expression. We employed cytokine production, assessed on the single cell level, as a robust and proximal measure of intrinsic TLR responsiveness. Here, we

report the results of our examination of intrinsic TLR responsiveness in several populations of macrophages as a function of animal age.

2. Materials and Methods

2.1 Analytical approach

In order to evaluate intrinsic macrophage function, we used sensitive and specific multiparameter cytofluorimetric analyses. We evaluated quantitatively, on the level of the single cell, macrophage responsiveness to pro-inflammatory stimulation with distinct TLR agonists. We analyzed the expression and intracellular presence of pro-inflammatory cytokines, especially TNF α and IL-6. Cytokine secretion was blocked with Brefeldin A, an inhibitor of *trans*-Golgi transport (Fujiwara et al, 1988), in order to visualize cytokine expression in cells individually; cells were immunostained intracellularly following permeabilization with saponin and fixation. Importantly, the Brefeldin A treatment used (3 hr., 5 μ g/ml) is not lethal for these cells (data not shown), and traps cytokines intracellularly, generating clearly distinct subpopulations of cells that do or do not express the relevant cytokine, and that can be quantified readily (see below; Figure 2). We focused our analyses on macrophages, using an antibody specific for F4/80, a definitive macrophage marker (Austyn & Gordon, 1981), to gate on F4/80⁺ cells. Independently, we analyzed macrophage responsiveness on the population level quantitatively in cell cultures untreated with Brefeldin A by measuring the secretion of cytokines into culture supernatants.

2.2 Mice

C57BL/6 and similarly long-lived Balb/cBy mice (of both genders) of discreet ages spanning their normal adult lifespan (young adults of 2 – 3 months of age [referred to as “young”], middle-aged adults of 15 months of age [“middle-aged”], and older adults nearing the end of mean lifespan [24 – 25 months of age; termed “old”]) were obtained from the National Institute on Aging (Bethesda, MD). The mean lifespan of these mice is about 26 months (see http://research.jax.org/faculty/harrison/gerlvi_LifeStudy1.html). All mice were housed in a single environmentally-controlled room within the UIC animal facility. All animal experiments and procedures were approved by the UIC Animal Care and Use Committee.

2.3 Reagents

Antibodies—Monoclonal antibodies (mAb) were obtained from BioLegend Inc. (San Diego, CA). Cytokine-specific antibodies used in this study were phycoerythrin (PE) - conjugated TNF α -specific rat mAb (IgG₁/ κ , clone MP6-XT22), allophycocyanin (APC) - conjugated IL-6-specific rat mAb (IgG₁/ κ , clone MP5-20F3), and IL-10-specific rat mAb (IgG_{2b}/ κ clone JES5-16E3) conjugated with phycoerythrin/cyanine (PE/Cy7) tandem dye. Surface molecule-specific antibodies were APC-conjugated CD3-specific rat mAb (IgG_{2b}/ κ , clone 17A2), PE-conjugated CD4-specific rat mAb (IgG_{2a}/ κ , clone RM4-5), fluorescein isothiocyanate (FITC) - conjugated CD44-specific rat mAb (IgG_{2b}/ κ , clone IM7), Pacific Blue (PB) - conjugated F4/80-specific rat mAb (IgG_{2a}/ κ , clone BM8), FITC-conjugated TLR2 (CD282)-specific mouse mAb (IgG₁/ κ , clone T2.5), and PE/Cy7-conjugated TLR4 (CD284)/MD2 complex-specific rat mAb (IgG_{2a}/ κ , clone MTS510). Isotype-specific

controls (fluor - matched for the relevant cytokine- or surface molecule-specific mAb) were rat IgG₁/κ isotype mAb (clone RTK2071), mouse IgG₁/κ isotype mAb (clone MOPC 21), rat IgG_{2a}/κ isotype mAb (clone RTK2758), and rat IgG_{2b}/κ isotype mAb (clone RTK4530). All antibodies were used at the manufacturer's recommended dilutions.

Other reagents, inhibitors, and drugs—Lipopolysaccharide (LPS; from *E. coli* 011:B4) and Brefeldin A (from *Penicillium brefeldianum*) were purchased from Sigma-Aldrich. TLR agonists Zymosan, Heat killed *Listeria monocytogenes* (HKLM), R848, low molecular weight Poly(I:C), purified Flagellin (from *S. typhimurium*), and synthetic bacterial lipoproteins Pam₂CSK₄ and Pam₃CSK₄, were purchased from InvivoGen (San Diego, CA).

2.4 Cell Culture

Primary murine macrophages (from the mice described above) were cultured at 37°C in a humidified 5% (v/v) CO₂ atmosphere in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with heat-inactivated fetal bovine serum (FBS; 10% v/v; HyClone Laboratories, Logan, UT; multiple lots since 2006 [AQB23239 ATD 31890, AUJ35777] have yielded comparable results), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. (This supplemented medium is referred to below as “complete RPMI medium”).

2.5 Isolation of Macrophages

Splenic Macrophages—Spleens were removed aseptically and disrupted to yield a cell suspension in complete RPMI medium. Red blood cells were lysed by resuspending the cells in Tris-buffered Ammonium Chloride solution (TAC; 140 mM NH₄Cl, 10 mM Tris-Cl, pH 7.6) for 5 min. at 37 C. Cells were washed twice in complete RPMI medium and plated (1×10^7 cells/2 ml/well) in wells of 6-well plates (Thermo Fisher [Waltham, MA], #130184). Adhesion to plastic was the criterion used for enrichment of macrophages. After 2 hr. incubation, nonadherent cells were removed; the remaining (adherent) cells were washed twice with warm complete RPMI medium. The resulting macrophage populations were >90% F4/80⁺ in all cases.

Resident Peritoneal Macrophages—Peritoneal cells were collected from unmanipulated mice by flushing the peritoneal cavity with 5 ml complete RPMI medium; recovered cells were washed twice in complete RPMI medium, plated in 100 mm dia. dishes, and allowed to adhere for 3 – 4 hours. Adherent cells were washed twice in complete RPMI medium, collected, and re-plated (0.5×10^6 cells/ml/well) in wells of 24-well plates.

Thioglycollate-Elicited Peritoneal Macrophages—Thioglycollate (2 ml of 4% thioglycollate broth; Difco; Irvine, CA) was injected intraperitoneally. Three days later, elicited cells were collected by flushing the peritoneal cavity with 5 ml complete RPMI medium. Adherent macrophages were recovered by the same washing and adhesion procedures used for resident peritoneal macrophages (above). (For cytokine secretion studies, adherent macrophages, prepared as above, were plated at 0.2×10^6 cells/ml/well.) By this approach, we were able to recover as many as 1×10^7 unactivated peritoneal macrophages per mouse. This enabled us to do a complete *ex vivo* analysis of TLR response

repertoires on cells from single animals. Results and conclusions are derived from the compiled results of these analyses of macrophages from individual mice.

2.6 Cytofluorimetric Analyses

For extra-cellular staining with primary antibodies conjugated to Pacific Blue (PB; $\lambda_{Ex} = 405$ nm; $\lambda_{Em} = 450 \pm 50$ nm), FITC ($\lambda_{Ex} = 488$ nm; $\lambda_{Em} = 530 \pm 40$ nm), PE ($\lambda_{Ex} = 561$ nm; $\lambda_{Em} = 575 \pm 25$ nm), tandem PE/cyanine dye (PE-Cy7; $\lambda_{Ex} = 561$ nm; $\lambda_{Em} > 750$ nm), or APC ($\lambda_{Ex} = 640$ nm; $\lambda_{Em} = 665 \pm 20$ nm), cells were washed twice with cold PBS containing FBS (1%) before resuspension and staining in this same buffer for 25 min. at 4°C in the dark prior to washing and cytofluorimetric analysis. For intracellular staining, cells were washed twice with cold PBS and fixed and permeabilized in a solution of 4% formaldehyde and 0.1% saponin in PBS for 20 min. at 4°C in the dark. After fixation, cells were washed twice with PBS buffer containing 0.1% saponin and 1% FBS and stained in this same buffer. In the case of intracellular cytokine detection, the normal process of secretion was blocked by treatment of cells with Brefeldin A (5 µg/ml), an inhibitor of *trans*-Golgi transport, for 3 hr. prior to fixation and permeabilization of cells. Cells were analyzed cytofluorimetrically on FACSCalibur or BD LSRFortessa instruments (BD Biosciences, San Jose CA). For each set of analyses, detectors were adjusted so that the fluorescent signals from unstained samples (autofluorescence, “background”) were less than 10 (i.e. within the first decade on a logarithmic axis that extended over a four decade range). Control staining with conjugated isotype-matched antibodies was indistinguishable from this background, in all cases. Further, we observed no changes in the levels of autofluorescence among cells from mice of different ages. Cytofluorimetric data were processed with Summit version 4.3 software (Dako, Carpinteria, CA).

2.7 Quantification of Cytokine Release

Quantification of serum cytokine levels and secreted cytokines levels were measured using Bio-Plex Multiplex System (Bio-Rad Life Sciences, Hercules, CA) as per manufacturers’ protocol. Briefly, samples were diluted as necessary (serum samples in the provided standard diluent, and the cell culture supernatants in culture media). 50 µl of samples were loaded in each well containing anti-cytokine specific beads for the multiplex assay. Following incubation and wash steps, the cytokine levels were determined by probing with Bio-Plex Detection Antibody, and followed by Streptavidin-PE. After washing wells, the plate was read using the Bio-Plex 200 System (Bio-Rad) set to read 100 beads/region and cytokine levels were analyzed and quantified using Bio-Plex Manager software (Bio-Rad).

2.8 Intravenous Mouse Infections

Infections of C57BL/6 mice were performed as previously described (Alonzo et al, 2009). Overnight cultures of a streptomycin-resistant strain of *L. monocytogenes* (strain 10403S) were diluted 1:20 into fresh medium and grown to an OD_{600 nm} of approximately 0.6 (corresponding to 6×10^8 colony forming units [CFU]/ml). Bacteria were washed twice in PBS and resuspended in PBS for intravenous (tail vein) injection. Seventy-two hr. after infection, mice were sacrificed and bacterial burdens (CFU) in livers and spleens were assessed. Organs were homogenized with a Tissue Master 125 homogenizer (Omni,

Marietta, GA) and triplicates of 10-fold serial dilutions were plated onto BHI plates containing 200 µg/ml streptomycin.

2.9 Analytical Methods and Statistical Analyses

We quantified TLR-dependent responsiveness from the compiled analyses of individual animals by two distinct analytical approaches. Importantly, these approaches allowed data from independent experiments involving distinct individual mice to be compiled and integrated, adding statistical power to this study.

Fold Induction—We evaluated the mean fluorescence intensity (MFI, obtained cytofluorimetrically following intracellular immunostaining with a response-specific conjugated antibody (e.g. an antibody specific for TNFα and conjugated with PE) for the entire stimulated (F4/80⁺) macrophage population relative to unstimulated levels in macrophages from the same animal. $\text{Fold Induction}_{\text{MFI}} = \{(\text{MFI}_{\text{Sample}} - \text{MFI}_{\text{Untreated}}) / \text{MFI}_{\text{Untreated}}\}$. It is significant that the unstimulated “background” levels of cytokine-specific staining among cells from mice of different ages did not change.

Response-positive macrophages—We evaluated the fraction (expressed as percentage) of all F4/80⁺ cells that were positive by intracellular cytokine staining for the response-specific cytokine (e.g. intracellular staining of TNFα above the unstimulated staining background; exemplified in Figure 2). Again, it is significant that the fraction of unstimulated cells that stained as cytokine-positive did not change among cell populations from mice of different ages.

The data values presented are the means of replicate determinations, and error bars represent the Standard Error of the Mean [SEM]. Statistical analysis comparing the values of two experimental sample sets involved 2-tailed Student’s t-test. Statistical analysis of multiple data sets, involving single variables, utilized 1-way Analysis of Variance (ANOVA); where two different variables (e.g. age and dose response) were involved, 2-way ANOVA was employed. The significance of differences was corrected for multiple comparisons by the Bonferroni method (with a significance level [α] of 0.05). Where indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$), results allowed the rejection of a null hypotheses [that results from different age cohorts were not different].

3. Results

3.1 The magnitude and dose-dependency of cytokine production in response to TLR stimulation in peritoneal macrophages is not altered globally with age

We compared the intrinsic responsiveness of macrophages from “young”, “middle-aged”, and “old” mice *ex vivo* to a variety of TLR stimuli, assessing cytokine production on the level of the individual cell. We evaluated several populations of macrophages, focusing our initial analyses on the large population of macrophages of the peritoneum. Figure 1A displays the induction of TNFα when elicited peritoneal macrophages from C57BL/6 mice of distinct ages are treated with *E. coli* LPS, a classical TLR4-specific pro-inflammatory agonist. As detailed in Material and Methods, we quantified TLR4-dependent

responsiveness both as the induction of intracellular TNF α accumulation over background (“TNF α Expression [Fold Induction]”) and as the fraction (percentage) of F4/80⁺ cells from a single mouse that stained positively for TNF α (“TNF α ⁺ macrophages [%]”). We found no evidence of any aging-associated alteration in the extent of this response (Figure 1A). Analogous analyses of elicited peritoneal macrophages from Balb/cBy mice gave entirely comparable results (exemplified in Figure 2A), suggesting that the absence of an aging-associated alteration is independent of mouse strain.

The compiled data from the independent analyses of macrophages from multiple individual mice of each age cohort document quantitatively that no significant differences distinguish the magnitude or dose-dependency of TLR4 responsiveness as a function of age. It also is evident that the results of the two analytical approaches yield the same conclusions. In addition, no gender-specific differences in macrophage responsiveness were observed (data not shown).

Similarly, we analyzed the responsiveness of peritoneal macrophages from “young”, “middle-aged”, and “old” C57BL/6 mice triggered via TLR2 with heat-killed *Listeria monocytogenes* (HKLM; Figure 1B). Again, the compiled data demonstrate that no significant differences distinguish the magnitude or dose-dependency of macrophage responsiveness as a function of animal age.

The primary cytofluorimetric data from which these conclusions are made are exemplified in Figure 2B, which shows C57BL/6 macrophage responses to HKLM stimulation. It is striking that these primary cytofluorimetric data reveal a distinct “all-or-nothing” pattern for TLR2 responsiveness on the level of the single macrophage. TNF α expression is, essentially, bimodal within a population, with cells manifesting either of two alternative states for TNF α expression; substantial macrophage subpopulations displaying intermediate levels of TNF α expression are absent. As demonstrated in Figure 2B, the presence within a single culture of both TNF α -negative and TNF α -positive cells reflects treatment with a suboptimal concentration of TLR2 agonist, leading to stimulation of only some of the potential responder cells. This pattern of responsiveness is not altered with age.

We further compared the responsiveness of macrophages from “young”, “middle-aged”, and “old” C57BL/6 mice to other TLR stimuli, with respect to TNF α expression. Again, we found no significant differences in the magnitude or dose-dependency of responsiveness of macrophages as a function of animal age to Zymosan (another TLR2 agonist; Figure 1C), the TLR7/8 agonist R848 (Figure 1D), the synthetic di-acylated lipopeptide Pam₂CSK₄ (an agonist for TLR2/6; Figure 1E) and the tri-acylated lipopeptide Pam₃CSK₄ (an agonist for TLR1/2; Figure 1F), and Flagellin, an agonist for TLR5 (data not shown). Gender-specific differences in macrophage responsiveness also were not observed (data not shown). While this array includes agonists that trigger endosomally-localized TLRs (e.g. TLR7/8) as well as extracellularly-exposed TLRs (e.g. TLR2 and TLR5), we did not survey the dose-dependent responses to agonists for every TLR, due to limitations of macrophage recovery from individual mice.

We found the “all-or-nothing” pattern of TNF α expression even with TLR agonists that elicited less robust TNF α responses. This can be seen by comparing Figures 1B and C, for example. Both HKLM and Zymosan are TLR2 agonists and, at their optimum doses, both triggered almost all C57BL/6 peritoneal F4/80⁺ macrophages to respond and express TNF α . However, HKLM triggered more than a 130-fold induction of TNF α expression (relative to unstimulated cells), whereas Zymosan stimulated less than a 90-fold induction. This was manifest cytofluorimetrically with respect to the TNF⁺ peak, and could be quantified in terms of the mean fluorescence intensity (MFI) of that TNF α ⁺ subpopulation (Supplemental Table 1; note that, for any agonist, the MFIs of TNF α ⁺ subpopulations vary little across doses that give significant TNF α responses). Whereas the TNF α ⁺ peak following HKLM treatment had an MFI of ~2000 (Supplemental Table 1, exemplified in Figure 2B), the TNF α ⁺ peak following Zymosan treatment had an MFI of ~1300 (Supplemental Table 1, compare Figures 1B and 1C). With secretion blocked by Brefeldin A, the induced intracellular accumulation of TNF α protein is a function of ongoing rates of [stimulated] transcription and translation. We interpret differences in TNF α ⁺ peak MFIs, then, to reflect different rates of TNF α expression in responding cells. In this comparison, the HKLM-stimulated rate of expression of TNF α was uniformly higher than it was in response to Zymosan. On the level of the single cell, and at a particular agonist dose, cells either are or are not responsive to treatment, but the rate of expression of TNF α (and other cytokines) is a function of the TLR-agonist pair, and is not dependent on the agonist dose.

As an independent test for intrinsic aging-associated alterations in macrophage responsiveness, we examined the effects of stimulation with a variety of TLR agonists on the expression of a distinct cytokine, IL-6. Just as the induction of TLR-dependent TNF α expression in macrophages was unaltered as a function of animal age, TLR-dependent IL-6 induction also was unaffected by age (Figure 3). The magnitude of IL-6 responsiveness is decidedly less robust than that of TNF α at early times, however (also see Cvetanovic & Ucker, 2004), prompting us to assess IL-6 secretion (below).

The single-cell analyses document the absence of statistically significant differences in the extents of TLR responsiveness (appraised with respect to cytokine expression) between macrophages from mice spanning the adult lifespan. Together, they support the conclusion that intrinsic TLR function - and triggered downstream steps through varied TLR-stimulated gene expression (transcription and translation) - is not impaired generally in murine macrophages as a function of animal age.

We took a distinct and complementary approach to test this conclusion additionally. We examined the secretion by macrophages from animals of discrete ages of a number of cytokines following stimulation with distinct TLR agonists. In these experiments, fixed numbers of adherent F4/80⁺ macrophages were plated per well, and cytokines secreted into culture supernatants in the absence of Brefeldin A treatment were assessed by multiplex immunoassays. The data in Figure 4 document that the secretion of pro-inflammatory cytokines (including TNF α [A], IL-1 β [B], IL-6 [C], and IFN- γ [D]) and anti-inflammatory cytokines (exemplified by IL-10 [E]) by C57BL/6 macrophages stimulated with LPS, HKLM, Zymosan, or R868 was largely unaffected by animal age (see next). (The levels of these cytokines secreted by macrophages stimulated with Flagellin or the TLR3

agonist Poly(I:C) were undetectable.) While these data reflect population behaviors, and are not unambiguously probative of intrinsic and proximal macrophage TLR responsiveness, they lead to the same conclusion derived from the intracellular staining analyses described above: as a function of animal age, TLR function in murine macrophages is not altered generally.

We did observe statistically significant aging-associated changes in two of the twenty responses we assessed (Figure 4). In one of those cases (TNF α secretion in response to LPS stimulation), aging-associated diminutions in the secreted cytokine levels were evident; in the other (IL-6 secretion in response to HKLM stimulation), the observed changes were aging-associated increases in cytokine secretion (Figure 4). Notably, this contrasts with the absence of differences seen with respect to macrophage-intrinsic TLR-dependent induction in those cases (Figure 1A and Figure 3). It may be that those idiosyncratic alterations in secretion patterns are consequences of TLR-distal or non-intrinsic factors. Certainly, they are not indicative of a coherent aging-associated alteration in the repertoire of responses elicited via any one TLR, and they do not corroborate a ubiquitous impact of aging on TLR responsiveness generally.

3.2 TLR expression in peritoneal macrophages is not diminished with age

In the absence of any apparent general diminution in TLR function with age, we examined aging-associated cell-surface expression of TLR2 and TLR4 on C57BL/6 peritoneal F4/80⁺ macrophages. (Highly specific antibodies directed to those murine molecules are available commercially.) Previous reports regarding aging-associated macrophage TLR expression have been contentious. Renshaw *et al.* (2002) reported that, with age, the expression of TLRs by elicited peritoneal macrophages is greatly reduced, although their comparison of macrophages from mice of disparate colonies compromises that conclusion. In contrast, Boehmer *et al.* (2004) reported no difference in TLR4 expression by peritoneal F4/80⁺ macrophages. The data in Figure 5 demonstrate that TLR2 and TLR4 expression on the cell surface of resident and elicited F4/80⁺ macrophages from “young” and “old” mice (Figure 5A and B) is not reduced with age and immunosenescence. In the case of resident macrophages, expression even was somewhat elevated (Figure 5A). Representative cytofluorimetric analyses of TLR2 and TLR4 expression on resident F4/80⁺ macrophages are presented in Figure 5C and D, respectively.

3.3 The magnitude and dose-dependency of response to TLR stimulation in other macrophage populations also is not altered globally with age

In order to test our findings in other populations of macrophages, we examined intrinsic TLR-specific responsiveness in resident peritoneal and splenic C57BL/6 F4/80⁺ macrophages. We analyzed these populations of macrophages recovered from individual mice. Since the yield of these cells from individual mice is limited, we restricted our analyses to the evaluation of TNF α expression (by intracellular immunostaining) stimulated by LPS or Zymosan. (While large numbers of macrophages can be generated in cell culture from bone marrow precursors, the *in vitro* differentiation of these “bone marrow-derived macrophages” confounds issues of aging. We find that bone marrow-derived macrophages do not provide a reasonable representation of the effects of aging on the animals from which

they were derived [unpublished data; see also Mahbub et al, 2012].) Just as we had found with elicited peritoneal macrophages (Figures 1 and 3), we observed no statistically significant differences in TLR responsiveness (again, assessed as TLR-dependent stimulation of TNF α expression) of resident peritoneal (Figure 6A and B) and splenic macrophages (Figure 6C and D) from “young” and “old” mice.

3.4 Manifestations of immunosenescence are evident in these aged mice

The absence of general aging-associated alterations in the extents of macrophage responsiveness to TLR stimulation was striking, and prompted us to ask whether these C57BL/6 mice, which were of chronologically distinct ages, displayed evidence of aging-associated altered immunity, including well-characterized alterations affecting T lymphocyte populations and circulating inflammatory cytokine levels. Indeed, we found that manifestations of aging-associated immune decline and immunosenescence were evident in these mice.

Alterations affecting T lymphocyte production and function are among the most dramatic and characteristic aging-associated immune effects. The steady shift with age of peripheral T lymphocytes toward a population with a “previously activated” (or “memory”) phenotype (marked especially by high levels of cell surface expression of the glycoprotein CD44 (CD44^{hi}), resulting from the impaired generation of naïve T cells and the consequent absence of new T cell emigrants to the periphery, is well-described (Michie et al, 1992; Zhang et al, 2002). Increases in the frequency of CD44^{hi} T lymphocytes were pronounced in older mice from this study (Figure 7). The fractions of CD4⁺ and CD8⁺ T lymphocytes in the periphery did not change appreciably with age (data not shown), and increases in CD44^{hi} T lymphocytes with increasing age were evident in both the CD4⁺ (Figure 7A, **left panel**) and CD8⁺ (Figure 7A, **right panel**) populations. The appearance in “old” mice of a well-defined CD44^{hi} population is particularly prominent among CD8⁺ T cells (Figure 7C).

We also found changes in the relative abundance of F4/80⁺ macrophages in the peritoneum. The fraction of adherent F4/80⁺ peritoneal macrophages among recovered peritoneal cells declined with age, even as the total number of recovered peritoneal cells increased. This apparent reduction in F4/80⁺ macrophages is due to an enormous expansion in the number of leukocytes, including neutrophils (Sletvold, 1987), in the peritoneal fluid of older mice. The change in the fractional representation of F4/80⁺ macrophages between “young” and “old” C57BL/6 mice is dramatic, both with respect to resident macrophages recovered from unmanipulated peritoneal lavage and to elicited peritoneal macrophages following thioglycollate stimulation (Figure 8A and B). While the absolute number of recovered F4/80⁺ macrophages does not change (and with no evidence of altered lifetimes or cell death susceptibility as a function of aging [Pattabiraman *et al.* manuscript in preparation]), the implication for experimentation involving macrophage cultures and the assessment of stimulated cytokine production from those *in vitro* cultures is especially significant (see Boehmer et al, 2004; Chelvarajan et al, 2005; Ding et al, 1994; Renshaw et al, 2002; Sun et al, 2012). A reduced frequency of macrophages in recovered peritoneal fluid from older animals may lead to the underrepresentation of macrophages in the resulting cultures, and be responsible for apparent aging-associated diminutions in cytokine production.

Circulating levels of pro-inflammatory cytokines were elevated significantly with age in C57BL/6 mice from this study (Table 2), consistent with aging-associated patterns described previously (Jeon et al, 2012; Ko et al, 2012; Spaulding et al, 1997). Anti-inflammatory cytokines also were elevated in older mice (Table 2), reinforcing the notion that aging-associated changes, rather than being simply pro-inflammatory, reflect an aging-associated immune “imbalance” (Saurwein-Teissl et al, 2000). In particular, we observed approximately two-fold elevations in serum concentrations of TNF α , IL-6, and IL-10 (Table 2).

We observed other aging-associated changes in the mice used in this study, including altered susceptibility to the bacterial pathogen *Listeria monocytogenes*. While susceptibility to many bacterial pathogens increases with advancing age (Gardner & Remington, 1977), susceptibility to infection by *Francisella novicida* (Mares et al, 2010) and *Listeria monocytogenes* (Emmerling et al, 1979; Matsumoto et al, 1979) diminish with age. We confirmed this behavior in our C57BL/6 mice (Figure 9; following observations of Løvik and North [1985] regarding gender-specific differences in susceptibility to infection, we tested female mice exclusively). An infectious dose of 2×10^5 *L. monocytogenes* cells was lethal to “young” mice within three days, but not to “old” mice (although the infected “old” mice evinced signs of morbidity by that time, including lethargy). “Young” mice survived with a five-fold lower infectious dose, although the resulting bacterial burdens were elevated about 50-fold relative to “old” mice receiving the higher infectious dose (Figure 9).

4. Discussion

While previous studies (Boehmer et al, 2004; Boehmer et al, 2005; Chelvarajan et al, 2005; Panda et al, 2009; Plowden et al, 2004; Renshaw et al, 2002; Sun et al, 2012; van Duin et al, 2007; Yoon et al, 2004) have suggested pronounced alterations of macrophage function with age, especially involving extensive changes in TLR expression and function, our studies of murine macrophages as a function of animal age have documented robust and aging-independent responses to TLR stimulation. We have examined macrophage responsiveness by evaluating TLR-stimulated cytokine production, deliberately utilizing a cytofluorimetric approach so as to be able to assess intrinsic macrophage activities on a single-cell level. Aging is not associated with a wholesale diminution in the magnitude or dose-dependency of macrophage responsiveness to TLR engagement. The absence of an aging-specific effect appears to be strain-independent, as well. (We have repeated these observations with several cohorts of aged mice over the course of several years, beginning in 2006.) These analyses of intrinsic TLR function in single cells allow the conclusion that the extents of TLR function in murine macrophages – including all steps through transcription and translation, at least – are not altered generally as a function of age. The results do not comport with a causal role for aging-associated TLR alterations in the hyper-inflammatory state associated with immunosenescence. Of course, TLR engagement triggers a plethora of responses. It remains possible that other outcomes (TLR-dependent phagocytosis, the production of reactive oxygen and nitrogen species, antigen presentation and co-stimulation) might be altered specifically in an aging-dependent manner.

It is important to note that the mice we have examined do exhibit evidence of aging-associated immunosenescence, including significantly elevated levels of circulating pro-inflammatory cytokines. Thus, we can conclude that widespread changes in macrophage TLR activities are neither causally responsible for aging-associated immune decline, nor are they a consequence of that pathology. The bases of those altered serum cytokine levels, and which cell types and what signaling mechanism(s) might be responsible, are intriguing and important issues, and worthwhile topics for further exploration. In this context, it is notable that TLR responsiveness is an attribute of myeloid and other cell types distinct from macrophages (especially dendritic cells). It remains to be tested whether aging-associated cell-intrinsic alterations in TLR function pertain in those cell types. It also will be imperative to evaluate these observations in equivalent human cell types.

Another consideration is that aging-associated changes in serum cytokine levels may be consequences of alterations in the termination of responses leading to cytokine gene expression, as distinct from the TLR-specific initiation of such responses. Our recent studies in murine macrophages raise this possibility (Pattabiraman *et al.*, manuscript in preparation).

Our analysis of macrophage responses on the single cell level also reveals striking details of TLR-dependent responsiveness. Macrophages appear to respond in a remarkable “all-or-nothing” manner. This is more than an artifact of the intracellular staining methodology employed, which relies on the blockade of secretion. The level of induced intracellular cytokine accumulation observed reflects an intrinsic rate of cytokine expression associated with the TLR-agonist pair. At a particular agonist dose, cells either are or are not responsive to stimulation, and the corresponding levels of intracellular cytokine accumulation observed reflect fully induced cytokine expression or its absence. Intermediate levels of cytokine expression and accumulation are not observed. This pattern is unaltered with age. Consistent with this functional “all-or-nothing” behavior, visualization of NF κ B nuclear localization following cellular stimulation with a TLR ligand also suggests that activation occurs in a binary manner (Lee et al, 2009; Tay et al, 2010).

By examining F4/80⁺ macrophages on the single-cell level, we found that there are no substantial aging-associated alterations either in TLR responsiveness or in the cell surface expression of different TLRs. (The extents to which macrophages interact with and respond to apoptotic cells also are not altered substantially with age [Pattabiraman *et al.*, manuscript in preparation], suggestive of an absence of phenotypic polarization.) We did observe dramatic aging-associated diminutions in the relative abundance of mature F4/80⁺ macrophages within recovered cell populations of the peritoneum of individual mice.

Even with unaltered responsiveness, the under-representation of macrophages from older animals in bulk cell cultures may provide an explanation for previous findings of aging-associated response declines. Given the variable, and even minor, representation of macrophages within cellular preparations, those studies (Boehmer et al, 2004; Chelvarajan et al, 2005; Renshaw et al, 2002; Sun et al, 2012) may have been compromised by the assessment of TLR expression and cytokine responses in bulk populations of variable macrophage content. This underscores the value of single-cell analyses.

It is worth reiterating as well the value of using mice from a single source, and housed together, for these comparative studies. In our work, macrophages from inbred mice of a single colony were compared with age as the single variable. Other work (Renshaw et al, 2002), using mice from disparate colonies, is further compromised by the introduction of variables independent of age.

In addition to the under-representation of F4/80⁺ macrophages in cultures from older mice, analyses of cytokine production to assess macrophage functions in these bulk cultures is limited by the inability to deconvolute the potential effects of intrinsic and extrinsic components. Finally, it is notable that cytokine production in cell culture does not reflect the observed aging-associated elevation of circulating pro-inflammatory cytokine levels *in vivo*.

5. Conclusion

Whereas an aging-associated enhancement of responsiveness of macrophages to inflammatory stimuli might be expected in light of the age-related elevation of circulating levels of inflammatory cytokines, this simple prediction has not been met generally. While previous reports of response differences to TLR stimulation correlated with aging suggest a potential and appealing explanation for aging-associated immune dysregulation, carefully controlled cellular and whole animal studies do not support the view that significant and pervasive aging-associated alterations in the levels of TLR responsiveness exist. Our studies demonstrate that extensive changes in TLR responsiveness of murine macrophages are not associated with age and cannot account for the altered inflammatory status and serum cytokine levels associated with immunosenescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Nancy Freitag for advice and guidance concerning *Listeria monocytogenes* infections.

This work was supported in part by NIH grant AG029633 to DSU.

References

- Ahluwalia N, Mastro AM, Ball R, Miles MP, Rajendra R, Handte G. Cytokine production by stimulated mononuclear cells did not change with aging in apparently healthy, well-nourished women. *Mech Ageing Dev.* 2001; 122:1269–1279. [PubMed: 11438118]
- Alonzo F 3rd, Port GC, Cao M, Freitag NE. The posttranslocation chaperone PrsA2 contributes to multiple facets of *Listeria monocytogenes* pathogenesis. *Infect Immun.* 2009; 77:2612–2623. [PubMed: 19451247]
- Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol.* 1981; 11:805–815. [PubMed: 7308288]
- Beharka AA, Meydani M, Wu D, Leka LS, Meydani A, Meydani SN. Interleukin-6 production does not increase with age. *J Gerontol A Biol Sci Med Sci.* 2001; 56:B81–88. [PubMed: 11213271]
- Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol.* 2004; 75:342–349. [PubMed: 14634059]

- Boehmer ED, Meehan MJ, Cutro BT, Kovacs EJ. Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev.* 2005; 126:1305–1313. [PubMed: 16154177]
- Bruley-Rosset M, Vergnon I. Interleukin-1 synthesis and activity in aged mice. *Mech Ageing Dev.* 1984; 24:247–264. [PubMed: 6232433]
- Brunnsgaard H, Andersen-Ranberg K, Jeune B, Pedersen AN, Skinhøj P, Pedersen BK. A high plasma concentration of TNF- α is associated with dementia in centenarians. *J Gerontol A Biol Sci Med Sci.* 1999; 54:M357–M364. [PubMed: 10462168]
- Brunnsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. *Curr Opin Hematol.* 2001; 8:131–136. [PubMed: 11303144]
- Candore G, Di Lorenzo G, Melluso M, Cigna D, Colucci AT, Modica MA, Caruso C. γ -Interferon, interleukin-4 and interleukin-6 in vitro production in old subjects. *Autoimmunity.* 1993; 16:275–280. [PubMed: 8025206]
- Chelvarajan RL, Collins SM, Van Willigen JM, Bondada S. The unresponsiveness of aged mice to polysaccharide antigens is a result of a defect in macrophage function. *J Leukoc Biol.* 2005; 77:503–512. [PubMed: 15629885]
- Chelvarajan RL, Liu Y, Popa D, Getchell ML, Getchell TV, Stromberg AJ, Bondada S. Molecular basis of age-associated cytokine dysregulation in LPS-stimulated macrophages. *J Leukoc Biol.* 2006; 79:1314–1327. [PubMed: 16603589]
- Chen L-C, Pace JL, Russell SW, Morrison DC. Altered regulation of inducible nitric oxide synthase expression in macrophages from senescent mice. *Infect Immun.* 1996; 64:4288–4298. [PubMed: 8926101]
- Chen YF, Solem L, Johnson AG. Activation of macrophages from aging mice by detoxified lipid A. *J Leukoc Biol.* 1991; 49:416–422. [PubMed: 1848273]
- Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that cooperate in innate immunity. *Trends Immunol.* 2006; 27:352–357. [PubMed: 16807108]
- Cvetanovic M, Ucker DS. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol.* 2004; 172:880–889. [PubMed: 14707059]
- Ding A, Hwang S, Schwab R. Effect of aging on murine macrophages: diminished response to IFN- γ for enhanced oxidative metabolism. *J Immunol.* 1994; 153:2146–2152. [PubMed: 7519641]
- Emmerling P, Hof H, Finger H. Age-related defense against infection with intracellular pathogens. *Gerontology.* 1979; 25:327–336. [PubMed: 116909]
- Ershler WB. Interleukin-6: a cytokine for gerontologists. *J Am Geriatr Soc.* 1993; 41:176–181. [PubMed: 8426042]
- Ferrucci L, Corsi A, Lauretani F, Bandinelli S, Bartali B, Taub DD, Guralnik JM, Longo DL. The origins of age-related proinflammatory state. *Blood.* 2005; 105:2294–2299. [PubMed: 15572589]
- Ferrucci L, Harris TB, Guralnik JM, Tracy RP, Corti MC, Cohen HJ, Penninx B, Pahor M, Wallace R, Havlik RJ. Serum IL-6 level and the development of disability in older persons. *J Am Geriatr Soc.* 1999; 47:639–646. [PubMed: 10366160]
- Fujiwara T, Oda K, Yokota S, Takatsuki A, Ikehara Y. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J Biol Chem.* 1988; 263:18545–18552. [PubMed: 3192548]
- Gardner ID, Remington JS. Age-related decline in the resistance of mice to infection with intracellular pathogens. *Infect Immun.* 1977; 16:593–598. [PubMed: 405325]
- Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol.* 2006; 6:895–906. [PubMed: 17110955]
- Gerbase-DeLima M, Wilkinson J, Smith GS, Walford RL. Age-related decline in thymic-independent immune function in a long-lived mouse strain. *J Gerontol.* 1974; 29:261–268. [PubMed: 4595417]
- Han D, Hosokawa T, Aoike A, Kawai K. Age-related enhancement of tumor necrosis factor (TNF) production in mice. *Mech Ageing Dev.* 1995; 84:39–54. [PubMed: 8719776]
- Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WH Jr, Heimovitz H, Cohen HJ, Wallace R. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med.* 1999; 106:506–512. [PubMed: 10335721]

- Higashimoto Y, Fukuchi Y, Shimada Y, Ishida K, Ohata M, Furuse T, Shu C, Teramoto S, Matsuse T, Sudo E, Orimo H. The effects of aging on the function of alveolar macrophages in mice. *Mech Ageing Dev.* 1993; 69:207–217. [PubMed: 8412370]
- Inamizu T, Chang MP, Makinodan T. Influence of age on the production and regulation of interleukin-1 in mice. *Immunology.* 1985; 55:447–455. [PubMed: 3874817]
- Jeon H, Mun GI, Boo YC. Analysis of serum cytokine/chemokine profiles affected by aging and exercise in mice. *Cytokine.* 2012; 60:487–492. [PubMed: 22884519]
- Jones BW, Means TK, Heldwein KA, Keen MA, Hill PJ, Belisle JT, Fenton MJ. Different Toll-like receptor agonists induce distinct macrophage responses. *J Leukoc Biol.* 2001; 69:1036–1044. [PubMed: 11404392]
- Kissin E, Tomasi M, McCartney-Francis N, Gibbs CL, Smith PD. Age-related decline in murine macrophage production of nitric oxide. *Journal of Infectious Diseases.* 1997; 175:1004–1007. [PubMed: 9086170]
- Ko F, Yu Q, Xue Q-L, Yao W, Brayton C, Yang H, Fedarko N, Walston J. Inflammation and mortality in a frail mouse model. *Age.* 2012; 34:705–715. [PubMed: 21633802]
- Kohut ML, Senchina DS, Madden KS, Martin AE, Felten DL, Moynihan JA. Age effects on macrophage function vary by tissue site, nature of stimulant, and exercise behavior. *Exp Gerontol.* 2004; 39:1347–1360. [PubMed: 15489058]
- Lee TK, Denny EM, Sanghvi JC, Gaston JE, Maynard ND, Hughey JJ, Covert MW. A noisy paracrine signal determines the cellular NF- κ B response to lipopolysaccharide. *Sci Signal.* 2009; 2:ra65. [PubMed: 19843957]
- Løvik M, North RJ. Effect of aging on antimicrobial immunity: old mice display a normal capacity for generating protective T cells and immunologic memory in response to infection with *Listeria monocytogenes*. *J Immunol.* 1985; 135:3479–3486. [PubMed: 3876387]
- Mahbub S, Deburghgraeve CR, Kovacs EJ. Advanced age impairs macrophage polarization. *J Interferon Cytokine Res.* 2012; 32:18–26. [PubMed: 22175541]
- Makinodan T, Kay MM. Age influence on the immune system. *Adv Immunol.* 1980; 29:287–330. [PubMed: 6998260]
- Makinodan T, Perkins EH, Chen MG. Immunologic activity of the aged. *Adv Gerontol Res.* 1971; 3:171–198. [PubMed: 4398644]
- Mares CA, Sharma J, Ojeda SS, Li Q, Campos JA, Morris EG, Coalson JJ, Teale JM. Attenuated response of aged mice to respiratory *Francisella novicida* is characterized by reduced cell death and absence of subsequent hypercytokinemia. *PLoS One.* 2010; 5:e14088. [PubMed: 21124895]
- Matsumoto T, Miake S, Mitsuyama M, Takeya K, Nomoto K. Augmented resistance to *Listeria monocytogenes* in mice at an early stage of aging. *J Clin Lab Immunol.* 1979; 2:55–58. [PubMed: 95807]
- Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol.* 2001; 1:135–145. [PubMed: 11905821]
- Michie CA, McLean A, Alcock C, Beverley PCL. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature.* 1992; 360:264–265. [PubMed: 1436108]
- Miller RA. Aging and immune function. *Int Rev Cytol.* 1991; 124:187–215. [PubMed: 2001916]
- Moynagh PN. TLR signalling and activation of IRFs: revisiting old friends from the NF- κ B pathway. *Trends Immunol.* 2005; 26:469–476. [PubMed: 16006187]
- Murasko DM, Goonewardene IM. T-cell function in aging: mechanisms of decline. *Annu Rev Gerontol Geriatr.* 1990; 10:71–96.
- Murasko DM, Jiang J. Response of aged mice to primary virus infections. *Immunol Rev.* 2005; 205:285–296. [PubMed: 15882361]
- Naylor K, Li G, Vallejo AN, Lee W-W, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ. The influence of age on T cell generation and TCR diversity. *J Immunol.* 2005; 174:7446–7452. [PubMed: 15905594]
- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA.* 2000; 97:13766–13771. [PubMed: 11095740]

- Panda A, Arjona A, Sapey E, Bai F, Fikrig E, Montgomery RR, Lord JM, Shaw AC. Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol.* 2009; 30:325–333. [PubMed: 19541535]
- Patel PJ. Aging and antimicrobial immunity. Impaired production of mediator T cells as a basis for the decreased resistance of senescent mice to listeriosis. *J Exp Med.* 1981; 154:821–831. [PubMed: 6792317]
- Plackett TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. *J Leukoc Biol.* 2004; 76:291–299. [PubMed: 15039467]
- Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: impact on macrophage function. *Aging Cell.* 2004; 3:161–167. [PubMed: 15268749]
- Price GB, Makinodan T. Immunologic deficiencies in senescence I. Characterization of intrinsic deficiencies. *J Immunol.* 1972; 108:403–412. [PubMed: 5065599]
- Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Impaired Toll-like receptor expression and function in aging. *J Immunol.* 2002; 169:4697–4701. [PubMed: 12391175]
- Saurwein-Teissl M, Blasko I, Zisterer K, Neuman B, Lang B, Grubeck-Loebenstien B. An imbalance between pro- and anti-inflammatory cytokines, a characteristic feature of old age. *Cytokine.* 2000; 12:1160–1161. [PubMed: 10880269]
- Segal R, Dayan M, Globerson A, Habut B, Shearer GM, Mozes E. Effect of aging on cytokine production in normal and experimental systemic lupus erythematosus afflicted mice. *Mech Ageing Dev.* 1997; 96:47–58. [PubMed: 9223110]
- Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol.* 2010; 11:30. [PubMed: 20565954]
- Shaw AC, Joshi S, Greenwood H, Panda A, Lord JM. Aging of the innate immune system. *Curr Opin Immunol.* 2010; 22:507–513. [PubMed: 20667703]
- Shimada Y, Ito H. Heterogeneous aging of macrophage-lineage cells in the capacity for TNF production and self renewal in C57BL/6 mice. *Mech Ageing Dev.* 1996; 87:183–195. [PubMed: 8794446]
- Sletvold O. Circadian rhythms of peripheral blood leukocytes in aging mice. *Mech Ageing Dev.* 1987; 39:251–261. [PubMed: 3626645]
- Spaulding CC, Walford RL, Effros RB. Calorie restriction inhibits the age-related dysregulation of the cytokines TNF- α and IL-6 in C3B10RF1 mice. *Mech Ageing Dev.* 1997; 93:87–94. [PubMed: 9089573]
- Sun Y, Li H, Yang M-F, Shu W, Sun M-J, Xu Y. Effects of aging on endotoxin tolerance induced by lipopolysaccharides derived from *Porphyromonas gingivalis* and *Escherichia coli*. *PLoS One.* 2012; 7:e39224. [PubMed: 22723968]
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol.* 2004; 16:3–9. [PubMed: 14751757]
- Tay S, Hughey JJ, Lee TK, Lipniacki T, Quake SR, Covert MW. Single-cell NF- κ B dynamics reveal digital activation and analogue information processing. *Nature.* 2010; 466:267–271. [PubMed: 20581820]
- Thoman ML, Weigle WO. The cellular and subcellular bases of immunosenescence. *Adv Immunol.* 1989; 46:221–261. [PubMed: 2528897]
- Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following influenza infection. *Respir Res.* 2009; 10:112. [PubMed: 19922665]
- Trzonkowski P, Mylińska J, Pawelec G, Myliński A. From bench to bedside and back: the SENIEUR Protocol and the efficacy of influenza vaccination in the elderly. *Biogerontology.* 2009; 10:83–94. [PubMed: 18563620]
- van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, Allore HG, Medzhitov R, Shaw AC. Age-associated defect in human TLR-1/2 function. *J Immunol.* 2007; 178:970–975. [PubMed: 17202359]
- Walford, RL. The immunologic theory of aging. København; Munksgaard: 1969.
- Wallace PK, Eisenstein TK, Meissler JJ Jr, Morahan PS. Decreases in macrophage mediated antitumor activity with aging. *Mech Ageing Dev.* 1995; 77:169–184. [PubMed: 7739265]

- Walston J, McBurnie MA, Newman A, Tracy RP, Kop WJ, Hirsch CH, Gottdiener J, Fried LP. Frailty and activation of the inflammation and coagulation systems with and without clinical comorbidities: results from the Cardiovascular Health Study. *Arch Intern Med.* 2002; 162:2333–2341. [PubMed: 12418947]
- Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* 2003; 301:640–643. [PubMed: 12855817]
- Yoon P, Keylock KT, Hartman ME, Freund GG, Woods JA. Macrophage hypo-responsiveness to interferon- γ in aged mice is associated with impaired signaling through Jak-STAT. *Mech Ageing Dev.* 2004; 125:137–143. [PubMed: 15037019]
- Zhang M, Caragine T, Wang H, Cohen PS, Botchkina G, Soda K, Bianchi M, Ulrich P, Cerami A, Sherry B, Tracey KJ. Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: a counterregulatory mechanism that restrains the immune response. *J Exp Med.* 1997; 185:1759–1768. [PubMed: 9151701]
- Zhang X, Fujii H, Kishimoto H, LeRoy E, Surh CD, Sprent J. Aging leads to disturbed homeostasis of memory phenotype CD8⁺ cells. *J Exp Med.* 2002; 195:283–293. [PubMed: 11828003]

Highlights

- Immunosenescence reflects the dysregulation of diverse immune activities.
- Macrophages have been implicated, but the role of TLR alterations is contentious.
- We find no global alterations of intrinsic macrophage TLR responsiveness with age.
- Macrophages exhibit an intriguing, aging-independent, binary response behavior.
- Widespread TLR alterations do not account for immunosenescent inflammatory status.

Figure 1. TLR responsiveness in macrophages is unaltered with age

Compiled data from elicited peritoneal macrophages from C57BL/6 mice of 2 – 3 months of age (“young”; □●), of 15 months of age (“middle-aged”; ○●) and of 24 – 25 months of age (“old”; △▲), treated with the TLR agonists LPS (A), HKLM (B), Zymosan (C), R848 (D), Pam₂CSK₄ (E), or Pam₃CSK₄ (F), at the indicated doses for 5 hr. After incubation, cells were fixed, permeabilized, and immunostained as described in Materials and Methods. Examples of the primary cytofluorimetric data are presented in Figure 2B. The responsiveness of gated viable F4/80⁺ macrophages is indicated as the fraction of cells that are positive for TNFα expression (□○△), and by relative TNFα expression (“fold induction” based on MFI relative to unstimulated cells, as described; ■●▲). Macrophages from individual mice ($n = 8$ [4 ♀, 4 ♂] for “young”; $n = 7$ each [5 ♀, 2 ♂] for “middle-aged” and “old”) were handled separately. There were no statistically significant differences ($p > 0.05$) between the age groups for TLR responsiveness for any of the different agonists, calculated by 2-way ANOVA.

Figure 2. Further analysis of TLR responsiveness in C57BL/6 and Balb/cBy macrophages as a function of animal age

A. Compiled data from elicited peritoneal macrophages from Balb/cBy mice of 2 – 3 months of age (“young”; □■), of 15 months of age (“middle-aged”; ○●) and of 24 – 25 months of age (“old”; △▲), treated with the TLR4 agonist LPS, at the indicated doses for 5 hr. After incubation, cells were fixed, permeabilized, and immunostained as described in Materials and Methods. The responsiveness of gated viable F4/80⁺ macrophages is indicated as the fraction of cells that are positive for TNFα expression (□○△), and by relative TNFα expression (“fold induction” based on MFI relative to unstimulated cells, as described;

■●▲). Macrophages from individual mice ($n = 3$ each [2 ♀, 1 ♂] for “young”, “middle-aged”, and “old”) were handled separately. There were no statistically significant differences ($p > 0.05$) between the age groups for TLR responsiveness for any of the different agonists, calculated by 2-way ANOVA.

B. Representative examples of primary data from the parallel cytofluorimetric analysis of TNF α expression in gated viable F4/80⁺ macrophages from individual ♀ C57BL/6 mice of 2 – 3 months of age (“Young”) and of 24 – 25 months of age (“Old”). Fixed and permeabilized cells were immunostained with a PE-conjugated antibody specific for TNF α . Results are presented for unstimulated macrophages (**a**), and macrophages stimulated with HKLM (**b**, 5×10^7 bacteria/ml; **c**, 1×10^7 bacteria/ml; and **d**, 2×10^6 bacteria/ml).

Figure 3. Analysis of stimulated IL-6 expression also indicates that TLR responsiveness in macrophages is unaltered with age

Compiled data from elicited peritoneal macrophages from C57BL/6 mice of 2 – 3 months of age (“young”; filled bars), of 15 months of age (“middle-aged”; hatched bars), and of 24 – 25 months of age (“old”; cross-hatched bars) incubated without or with the indicated TLR agonists for 5 hr. After incubation, cells were fixed, permeabilized, and immunostained as described in Materials and Methods. The responsiveness of gated viable F4/80⁺ macrophages is indicated by relative IL-6 expression (“fold induction” based on MFI relative to unstimulated cells, as described). Macrophages from individual mice ($n = 7$ each for “young” [4 ♀, 3 ♂] and “middle-aged” [5 ♀, 2 ♂], $n = 8$ [6 ♀, 2 ♂] for “old”) were handled separately. There were no statistically significant differences ($p > 0.05$) between the age groups with respect to intracellular IL-6 accumulation for any of the different agonists, calculated by 1-way ANOVA.

Figure 4. TLR responsiveness assessed by cytokine secretion following *in vitro* stimulation of macrophages

Elicited peritoneal macrophages from C57BL/6 mice of 2 – 3 months of age (“young”; filled bars), 15 mo. of age (“middle-aged”; hatched bars), and 24 – 25 months of age (“old”; cross-hatched bars) were stimulated *in vitro* (0.2×10^6 F4/80⁺ macrophages/ml) for 20 hr. with LPS (5 ng/ml), HKLM (5×10^7 cells/ml), Zymosan (100 μ g/ml), or R848 (50 ng/ml).

Cytokines secreted into culture supernatants were quantified by multiplex immunoassays. Macrophages from individual mice (“young”: $n = 8$ [4 ♀, 4 ♂]; “middle-aged”: $n = 5$ [3 ♀, 2 ♂]; “old”: $n = 6$ [3 ♀, 3 ♂]) were handled separately. The significance of differences in secreted cytokine levels by similarly stimulated macrophages from animals of different ages was tested by 1-way ANOVA; cases in which age-dependent differences were statistically significant (*: $p < 0.05$; **: $p < 0.01$) and adhered to a curve without inflection are indicated.

Figure 5. Characterization of TLR2 and TLR4 expressed on peritoneal macrophages as a function of animal age

Resident (A) and elicited (B) peritoneal macrophages were recovered from individual C57BL/6 mice of 2 – 3 months of age (“young”) and of 24 – 25 months of age (“old”). Cells were stained immediately (without culturing) for F4/80, TLR-2, and TLR-4. Macrophages from individual mice (resident: $n = 6$ [3 ♀, 3 ♂] per age cohort; elicited: $n = 4$ [2 ♀, 2 ♂] per age cohort) were handled separately. While modest differences in the levels of surface expression of TLR2 (vertically hatched bars) and TLR4 (horizontally hatched bars) between resident F4/80⁺ peritoneal macrophages from “young” (filled bars) and “old” (cross-hatched bars) mice (A) are statistically significant, no comparable statistically significant differences were observed among elicited F4/80⁺ peritoneal macrophages (B; NS: $p > 0.05$; Student’s *t*-test). Representative primary cytofluorimetric data analyzing TLR2 (C) and TLR4 (D) expression in resident F4/80⁺ peritoneal macrophages from individual “young” (a) and “old” (b) ♀ mice are presented. “Background” staining with an irrelevant isotype-matched antibody (c) is shown.

Figure 6. TLR responsiveness in splenic and resident peritoneal macrophages also is unaltered with age

Compiled data from resident peritoneal (**A, B**) and splenic (**C, D**) macrophages from individual C57BL/6 mice of 2 – 3 months of age (“young”; ■) and of 24 – 25 months of age (“old”, ▲), treated with the TLR agonists LPS (**A, C**) or Zymosan (**B, D**) at the indicated doses for 5 hr. The analysis parallels that for elicited peritoneal macrophages in Figure 1. After incubation, cells were fixed, permeabilized, and immunostained as described in Materials and Methods. The responsiveness of gated viable F4/80⁺ macrophages is indicated here exclusively by relative TNFα expression (“fold induction” based on MFI relative to unstimulated cells). Macrophages from individual mice ($n = 3$ each [2 ♀, 1 ♂] for “young”

and “old”) were handled separately. There were no statistically significant differences ($p > 0.05$) between the age groups for TLR responsiveness for any of the different agonists, calculated by 2-way ANOVA.

Figure 7. The frequency of previously-activated T lymphocytes is increased with animal age
 CD44 expression on CD4⁺ (A, left) and CD8⁺ (A, right) T lymphocytes from the spleens of C57BL/6 mice of 2 – 3 months of age (“young”; filled bars), of 15 mo. of age (“middle-aged”; hatched bars), and of 24 – 25 months of age (“old”; cross-hatched bars) was evaluated cytofluorimetrically. The fractions of CD3⁺ T lymphocytes expressing high cell surface levels of CD44 (CD44^{hi} cells) were tabulated. Splenocytes from individual mice ($n = 4$ [2 ♀, 2 ♂] per age cohort) were handled separately. CD8⁺ T cells were identified cytofluorimetrically as CD3⁺CD4⁻. Differences in the frequencies of CD44^{hi} cells among CD4⁺ and CD8⁺ T cell subpopulations between “young”, “middle-aged”, and “old” mice are statistically significant (*: $p < 0.05$; ***: $p < 0.001$), as calculated by Student’s t-test. Representative examples of cytofluorimetric analyses of individual samples evaluating

CD44 surface expression on CD4⁺ and CD8⁺ T lymphocytes are presented in Panels **B** and **C**, respectively (CD44-specific immunostaining of T lymphocytes from individual “young” [**a**] and an “old” [**b**] ♀ mice; isotype control staining: [**c**]).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8. The frequency of F4/80⁺ macrophages among peritoneal cells is diminished with animal age

Resident (A) and elicited (B) peritoneal macrophages were recovered from individual C57BL/6 mice of 2 – 3 months of age (“young”) and of 24 – 25 months of age (“old”), as in Figure 5. Cells were stained immediately (without culturing) for F4/80. Macrophages from individual mice (resident: $n = 6$ [3 ♀, 3 ♂] per age cohort; elicited: $n = 4$ [2 ♀, 2 ♂] per age cohort) were handled separately. Differences in the frequencies of F4/80⁺ macrophages among resident and elicited peritoneal cells between “young” and “old” mice are statistically significant (*: $p = 0.05$; ***: $p = 0.001$), as calculated by Student’s t-test. Representative primary cytofluorimetric data from the analysis of resident (C) and elicited (D) peritoneal cells from individual “young” (a) and “old” (b) ♀ mice are presented. “Background” staining with an irrelevant isotype-matched antibody (c) is shown.

Figure 9. Susceptibility of mice to *Listeria monocytogenes* infection is diminished with age C57BL/6 ♀ mice of 2 – 3 months of age (“young”) and of 24 – 25 months of age (“old”) were infected intravenously with *L. monocytogenes* at the indicated inocula. Three days post-infection, live animals were euthanized and bacterial burdens (CFU) in spleen (left panel) and liver (right panel) were determined (Alonzo et al, 2009). Mean bacterial burdens in organs from “young” (■) and “old” (▲) mice are indicated; infected mice that had died are noted (x).

Table 1

Compilation of previous analyses of murine macrophage TLR responses *ex vivo*.

Age	Strain	Mouse Vendor ^d	TLR agonist	Observed aging-associated effects ^b	Reference
Peritoneal (elicited) Macrophages					
6–10 wk.	BALB/c (female only)	Charles River	Zymosan	no meaningful comparison possible ^d (disparate mouse colonies)	Ding <i>et al.</i> (1994)
18–22 mo.	BALB/c (female only)	NIA			
2 mo.	CBA/CA (male only)	NIA			
22 mo.	CBA/CA (male only)	NIA			
2–3 mo.	C57BL/6 (female only)	Jax	Multiple	enhancement of NO release, no change in TNF α secretion	Chen <i>et al.</i> (1996)
18–24 mo.	C57BL/6 (female only)	NIA			
2 mo.	BALB/c (female only)	NIA	LPS (<i>E. coli</i>)	no meaningful comparison possible ^e (disparate mouse colonies)	Renshaw <i>et al.</i> (2002)
18–20 mo.	BALB/c (female only)	NIA			
3–4 mo.	BALB/c	Harlan	LPS (<i>E. coli</i>)	diminution of IL-6 and TNF α secretion	Boehmer <i>et al.</i> (2004)
22–24 mo.	BALB/c	NIA			
2 mo.	ICR	Vital River	LPS (<i>E. coli</i>), LPS (<i>P. gingivalis</i>)	no meaningful comparison possible ^f (disparate mouse colonies)	Chelvarajan <i>et al.</i> (2005)
12 mo.	ICR	Vital River			
Peritoneal (resident) Macrophages					
3–5 mo.	C57BL/6	CNRS	LPS (<i>E. coli</i>)	diminution of IL-1 secretion	Bruley-Rosset and Vergnon (1984)
18–22 mo.	C57BL/6	CNRS			
2–3 mo.	C3H/HeN (male only)	Charles River	LPS (<i>S. minnesota</i>)	diminution of O ₂ ⁻ , H ₂ O ₂ release	Chen <i>et al.</i> (1991)
15–18 mo.	C3H/HeN (male only)	Charles River			
2–3 mo.	BALB/c	Charles River	LPS (<i>S. minnesota</i>)	diminution of O ₂ ⁻ , H ₂ O ₂ release	Han <i>et al.</i> (1995)
20–24 mo.	BALB/c	Charles River			
2 mo.	C3H/HeSlc	SLC	OK-432 (TLR4 agonist)	enhancement of TNF α secretion	Wallace <i>et al.</i> (1995)
13–17 mo.	C3H/HeSlc	SLC			
24–29 mo.	C3H/HeSlc	SLC			
6–10 wk.	C57BL/6	NIA	LPS (<i>E. coli</i>) + IFN- γ	diminution of IL-1 α , TNF α secretion & NO release	Shimada and Ito (1996)
>24 mo.	C57BL/6	NIA			
3–6 mo.	C57BL/6 (female only)	Shizuoka	LPS (<i>E. coli</i>)	enhancement of TNF α secretion	Kissin <i>et al.</i> (1997)
18–31 mo.	C57BL/6 (female only)	Shizuoka			
1 mo.	BALB/c (female only)	Charles River	Peptidoglycan	diminution of NO release	

Age	Strain	Mouse Vendor ^a	TLR agonist	Observed aging-associated effects ^b	Reference
8 mo.	BALB/c (female only)	Charles River	LPS (<i>E. coli</i>)	diminution of NO release	Segal <i>et al.</i> (1997)
2 mo.	C57BL/6 (female only)	Charles River			
13 mo.	C57BL/6 (female only)	Charles River			
1.5 mo.	CB6F ₁ (female only)	Charles River	LPS (<i>E. coli</i>)	diminution of NO release	Beharka <i>et al.</i> (2001)
15 mo.	CB6F ₁ (female only)	Charles River			
2 mo.	BALB/c (female only)	Olac			
24 mo.	BALB/c (female only)	Olac	LPS (<i>E. coli</i>)	enhancement of IL-1 secretion	Kohut <i>et al.</i> (2004)
2 mo.	C3H.SW (female only)	Jax			
24 mo.	C3H.SW (female only)	Jax			
6 mo.	C57BL/6 (male only)	Charles River	LPS (<i>E. coli</i>)	no change in IL-6 secretion	Beharka <i>et al.</i> (2001)
24 mo.	C57BL/6 (male only)	Charles River			
2 mo.	BALB/c (male only)	Jax			
12 mo.	BALB/c (male only)	Jax	LPS (<i>E. coli</i>) ± IFN-γ	enhancement of IL-12 secretion; diminution of TNFα secretion & NO release; no change in IL-1 secretion	Kohut <i>et al.</i> (2004)
21 mo.	BALB/c (male only)	Jax			
Splenic (resident) Macrophages					
1 mo.	BALB/c (female only)	Charles River	LPS (<i>E. coli</i>), Peptidoglycan	diminution of NO release	Kissin <i>et al.</i> (1997)
4.5 mo.	BALB/c (female only)	Charles River			
7–20 mo.	BALB/c (female only)	Charles River			
2–3 mo.	C57BL/6 (female only)	Jax	Multiple	no meaningful comparison possible ^c (disparate mouse colonies)	Renshaw <i>et al.</i> (2002)
18–24 mo.	C57BL/6 (female only)	NIA			
2 mo.	BALB/c (male only)	Jax			
12 mo.	BALB/c (male only)	Jax	LPS (<i>E. coli</i>) + IFN-γ	enhancement of IL-12, TNFα secretion & NO release; no change in IL-1 secretion	Kohut <i>et al.</i> (2004)
21 mo.	BALB/c (male only)	Jax			
8–10 wk.	BALB/c (female only)	NIA			
18–20 mo.	BALB/c (female only)	NIA	LPS (<i>E. coli</i>), Zymosan	enhancement of LPS-stim. IL-10 secretion; diminution of IL-6 and TNFα secretion	Boehmer <i>et al.</i> (2005)
Alveolar (resident) Macrophages					
6–7 wk.	C57BL/6 (male only)	Ohmura	LPS (<i>E. coli</i>)	no change in TNFα secretion	Higashimoto <i>et al.</i> (1993)
26 mo.	C57BL/6 (male only)	Ohmura			
2 mo.	BALB/c (male only)	Jax			
12 mo.	BALB/c (male only)	Jax	LPS (<i>E. coli</i>) ± IFN-γ	enhancement of IL-1, IL-12, TNFα secretion & NO release	Kohut <i>et al.</i> (2004)
21 mo.	BALB/c (male only)	Jax			

^a Mouse vendors: Charles River – Charles River Laboratories (MA); NIA – National Institute on Aging; Jax – The Jackson Laboratory (ME); Harlan – Harlan Sprague Dawley (IN); Vital River – Vital River Laboratory Animals (Beijing); CNRS – CNRS (Villejuif); SLC – Shizuoka Institute for Laboratory Animals (Hamamatsu); Shizuoka (Shizuoka Institute for Laboratory Animals, Hamamatsu); Olac – Harlan Laboratories UK; Ohmura – Ohmura Institute for Laboratory Animals (Chiba)

^b Effects observed following primary TLR stimulation; determinations from supernatants of cultures of macrophage populations.

^c Heat-killed *Staphylococcus aureus*

^d Assessment of released H₂O₂.

^e Assessment of secreted IL-6 and TNFα.

^f Assessment of secreted IL-1, IL-6, IL-12, TNFα, and released NO.

Table 2

Aging-associated alterations in serum cytokine concentrations.

Cytokine (pg/ml)	“Young” (2–3 months of age, n = 12)	“Old” (24–25 months of age, n = 18)	p
IL-1β	127.03 $\pm$ 18.05	81.03 $\pm$ 7.59	0.011
IL-6	5.52 $\pm$ 0.44	13.16 $\pm$ 2.69	0.046
IL-10	33.87 $\pm$ 5.71	79.40 $\pm$ 7.80	0.000
IL-17	87.75 $\pm$ 17.64	97.91 $\pm$ 14.75	0.673
IFN-γ	175.15 $\pm$ 28.09	150.42 $\pm$ 9.19	0.314
TNFα	197.26 $\pm$ 35.71	286.19 $\pm$ 25.52	0.050

The serum concentrations of the indicated cytokines in individual “young” and “old” C57BL/6 mice were determined by multiplex immunoassays. The data presented (mean $\pm$ SEM) are compiled from the results of the analysis of 18 individual mice of each age cohort. The significance of differences between age groups for each cytokine, as calculated by Student’s t-test and corrected for multiple comparisons by the Bonferroni method (with a significance level [α] of 0.05), is indicated.