Mechanisms of Ageing and Development 130 (2009) 538–546

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

# Age-related alterations in innate immune receptor expression and ability of macrophages to respond to pathogen challenge in vitro

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ARTICLE INFO

*Article history:*
Received 13 April 2009
Received in revised form 5 June 2009
Accepted 18 June 2009
Available online 25 June 2009

*Keywords:*
Innate immunity
Macrophages
Pattern-recognition receptors
Ageing

ABSTRACT

The impact of ageing in innate immunity is poorly understood. Studies in the mouse model have described altered innate immune functions in aged macrophages, although these were not generally linked to altered expression of receptors or regulatory molecules. Moreover, the influence of ageing in the expression of these molecules has not been systematically examined. We investigated age-dependent expression differences in selected Toll-like and other pattern-recognition receptors, receptors involved in inflammatory amplification, and in transmembrane and intracellular regulators of inflammatory signaling. Young and aged macrophages were examined under resting conditions or upon activation with *Porphyromonas gingivalis*, a major pathogen in periodontal disease, the prevalence and severity of which increase in old age. We detected a limited number of age-dependent alterations, involving both reduction and increase of immune activity. Interestingly, surface expression of receptors that amplify inflammation (C5a anaphylatoxin receptor and triggering receptor expressed on myeloid cells [TREM]-1) was elevated in aged macrophages. No significant age-dependent differences were observed regarding the phagocytosis and intracellular killing of *P. gingivalis*, consistent with lack of significant changes in phagocytic receptor expression and induction of antimicrobial molecules. Therefore, at least at the cellular level, certain aspects of innate immune function may not necessarily decline with age.



## 1. Introduction

Infection-driven chronic inflammatory diseases generally appear rather late in life, although it is not clear whether, or what kind of, age-related alterations in innate immune recognition are responsible. Despite compelling evidence that adaptive immunity declines with age (Miller, 1996), our current state of knowledge on age-related alterations in innate immunity is less clear. A plausible hypothesis linking innate immune alterations to immunosenescence is that innate recognition of microbial pathogens declines with age, consequently resulting in defective immunosurveillance. However, this notion is not completely supported by the literature, which has not yet reached a consensus conclusion (Gomez et al., 2008; Plowden et al., 2004; Solana et al., 2006).

In addition to predisposing to increased susceptibility to a number of infectious or autoimmune diseases (Gomez et al., 2008; Miller, 1996), advanced age is also associated with poor periodontal health and increased prevalence and severity of periodontitis (Minaya-Sanchez et al., 2007; Shizukuishi et al., 1998; Streckfus et al., 1999). This oral inflammatory disease afflicts about 30% of the adult population (Oliver et al., 1998). However, an estimated 10–15% develops severe periodontitis (Papapanou, 1996), which has a systemic impact on the patients who thereby run increased risk for atherosclerotic heart disease, diabetes, pulmonary disease, adverse pregnancy outcomes, and other systemic conditions (Pihlstrom et al., 2005). In terms of etiology, periodontitis is associated with a finite group of oral pathogens that includes *Porphyromonas gingivalis* (Hajishengallis, 2009; Socransky et al., 1998) and periodontal tissue damage, including bone and tooth loss, may result from inadequate or excessive host responses to bacterial challenge (Baker, 2000; Gaffen and Hajishengallis, 2008; Waldrop et al., 1987). The mouse model has been productively used for in vitro and in vivo investigation of host–bacterial interactions and elucidation of the host response in periodontitis (Graves et al., 2008). Macrophages play important roles in the innate host response in periodontitis, as well as in other chronic infections (Linton and Fazio, 2003; Teng, 2006).

As a professional phagocyte that mediates first-line defense, the macrophage is equipped with Toll-like and other pattern-

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doi:10.1016/j.mad.2009.06.006

recognition receptors which recognize and respond to conserved microbial structures (Beutler et al., 2003). Since Toll-like receptors (TLRs) generally respond to different types of microbial structures, this property endows the macrophage (and other innate immune cells) with a degree of specificity. For instance, TLR2 responds to bacterial lipoproteins, TLR3 to double-stranded viral RNA, TLR4 to lipopolysaccharide (LPS), and TLR5 to flagellin (Beutler et al., 2003). Although most TLRs (except for TLR3) signal through the key adaptor molecule MyD88, TLR3 and TLR4 can activate MyD88-independent signaling which is mediated by the TRIF adaptor (TIR-domain-containing adapter inducing IFN-β). Moreover, TLR2 and TLR4 also utilize a MyD88-like adaptor (Mal) (O'Neill, 2006). These differences and the compartmentalization of TLRs (TLR-1, -2, -4, -5, -6 expressed on the cell surface; TLR-3, -7, -8, -9 located in endocytic vesicles) may result in qualitatively different innate responses depending on the nature of the microbial challenge.

Studies in humans suggest that a number of monocyte/macrophage functions become compromised with advancing age; these include chemotaxis, phagocytic and scavenger receptor activity, production of reactive oxygen species, the inflammatory wound healing response, and induction of certain cytokine responses (reviewed by Gomez et al., 2008; Lloberas and Celada, 2002). However, discrepant results have often been reported attributable to differences in experimental conditions and the health status of the donor subjects (Gomez et al., 2008; Lloberas and Celada, 2002). Due to several limitations associated with the use of macrophages from "healthy elderly subjects", most studies on macrophage ageing have been performed using cells from aged mice and rats (Gomez et al., 2008; Lloberas and Celada, 2002). Despite progress in understanding which macrophage activities may be affected as a function of age, the underlying mechanisms remain poorly characterized.

In that regard, age-related alterations in macrophage functions have not generally been linked to changes in expression of receptors responsible for mediating those activities. For example, although phagocytosis appears to decline in aged mouse macrophages (reviewed by Plowden et al., 2004), the impact of ageing on phagocytic receptors is largely unknown. In fact, the impact of ageing on innate immune receptor expression in macrophages has not been systematically examined, although several studies have examined a limited number of receptors. CD14, an important co-receptor for TLR2 and TLR4 (Beutler et al., 2003), was shown to be expressed at lower levels in macrophages from aged mice compared to their young counterparts (Vega et al., 2004). Another study found that aged mouse macrophages display reduced expression of TLR1-9 at the mRNA level, although at the protein level the results were confirmed only for TLR4 (Renshaw et al., 2002). Accordingly, LPS-induced cytokine responses were found to decline with age (Renshaw et al., 2002). This observation was confirmed by an independent study, although the age-dependent reduction in cytokine responses was not attributed to decreased TLR4 expression (remained intact) but rather to decreased expression of mitogen-activated protein kinases (Boehmer et al., 2004).

In this paper, we compared macrophages from young (8–10-week-old) and old (≥18 months of age) mice for expression of selected innate immune receptors at the mRNA and protein levels, including examination of the inducibility of these receptors in response to *P. gingivalis* challenge. Since age-dependent changes in macrophage responses may also involve alterations in regulatory mechanisms, we additionally investigated expression and inducibility of negative regulators of innate immune signaling. The data from these assays were then considered for correlation with findings from functional assays, including antimicrobial and cytokine responses to *P. gingivalis* and phagocytosis and intracellular killing of this pathogen.

## 2. Materials and methods

### 2.1. Bacteria

*P. gingivalis* ATCC 32277 was grown anaerobically at 37 °C in modified GAM medium (contains 5 μg/ml hemin and 1 μg/ml menadione upon reconstitution) (Nissui Pharmaceutical).

### 2.2. Mice, cell isolation, and culture

BALB/cByJ mice (8–10 weeks of age [young] or ≥18 months of age [old]) were obtained from the National Institute of Ageing. All animal procedures were approved by the Institutional Animal Care and Use Committee, in compliance with established Federal and State policies. Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of mice as previously described (Hajishengallis et al., 2005b). Specifically, mice were intraperitoneally injected with 1 ml of sterile 3% thioglycollate, and cells were harvested 5 days later by flushing the peritoneal cavity with 10 ml of ice-cold PBS three times. Isolated cells were then subjected to density gradient centrifugation (Histopaque 1.083) to remove dead cells and red blood cell contamination. The purity of macrophage preparations (>90%) was confirmed by flow cytometry using phycoerythrin-labeled anti-F4/80 (clone BM8; eBioscience). The macrophages were rested overnight (at 37 °C and 5% $CO_2$ atmosphere) prior to use in experiments. The culture medium used was RPMI 1640 (InVitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega). None of the experimental treatments affected cell viability compared to medium-only control treatments.

### 2.3. Quantitative real-time PCR

Gene expression in resting or activated macrophages was quantified using quantitative real-time PCR. Briefly, RNA was extracted from cell lysates using the PerfectPure RNA cell kit (5 Prime, Fisher) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) and quantitative real-time PCR with cDNA was performed using the ABI 7500 Fast System, according to the manufacturer's protocol (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for expression of genes shown in Figs. 1, 2 and 4, or a house-keeping gene (GAPDH) were purchased from Applied Biosystems.

### 2.4. Flow cytometry and antibodies

Macrophages were incubated with fluorescently labeled specific monoclonal antibodies (mAbs) or Ig isotype controls (or with fluorescently labeled streptavidin in assays using biotinylated mAbs), in a total volume of 100 μl staining buffer (ice-cold Dulbecco's PBS containing 0.1% BSA and 0.01% azide). Subsequently, the cells were washed, fixed, and analyzed by flow cytometry (FACSCalibur, Becton-Dickinson) and the CellQuest software. The antibodies used were from the following sources. Mouse-specific mAbs to TLR1 (clone TR23), TLR2 (6C2), TLR4 (UT41), CD14 (Sa2-8), CD11b (M1/70), CD18 (M18/2), CD36 (72-1), CXCR4 (2B11), and their isotype controls were obtained from e-Bioscience. MAbs to TLR5 (85B152.5) and Dectin-1 (2A11), and polyclonal antibody to SR-AI were from Abcam. MAbs to TLR6 (418601), TREM-1 (174031) and TREM-2 (237920), as well as polyclonal antibody to TREM-3 were from the R&D Systems. Anti-C5aR mAb (20/70) was from Cedarlane Laboratories and anti-CD206 mAb (MR5D3) was from Biolegend.

### 2.5. Cell activation assays

Induction of cytokine release in culture supernatants of activated mouse macrophages (plated at $2 \times 10^5$ cells/well) was measured using ELISA kits (eBioscience). Induction of nitric oxide (NO) production was assessed by measuring the amount of $NO_2^-$ (stable metabolite of NO) in stimulated culture supernatants using a Griess reaction-based assay kit (R&D Systems).

### 2.6. Phagocytosis

A flow cytometric method was performed to assess the uptake of FITC-labeled *P. gingivalis*, as we previously described (Wang et al., 2007). Briefly, primary mouse macrophages were incubated at 37 °C with FITC-labeled *P. gingivalis* at a MOI of 10:1 for 30 min. Phagocytosis was stopped by cooling the incubation tubes on ice. After cell washing to remove nonadherent bacteria, in some groups extracellular fluorescence (representing attached but not internalized bacteria) was quenched with 0.2% trypan blue. The cells were washed again, fixed with 1% paraforlmadehyde, and analyzed by flow cytometry (% positive cells for FITC-*P. gingivalis* and