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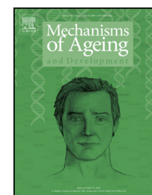

Original article

Toll-like Receptor function of murine macrophages, probed by cytokine induction, is biphasic and is not impaired globally with age

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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.

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1. Introduction

Aging is associated with a deterioration of normal immune function (Makinodan and Kay, 1980; Miller, 1991; Price and Makinodan, 1972; Thoman and Weigle, 1989). This aging-associated condition, termed "immunosenescence", has been hypothesized to be involved 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 and Goonewardene, 1990; Naylor et al., 2005; Toapanta and 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, 2001; Ershler, 1993; Ferrucci et al., 2005, 1999; Harris et al., 1999; Jeon et al., 2012; Spaulding et al., 1997; Thoman and 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 and Jiang, 2005; Panda et al., 2009; Shaw et al., 2010). Susceptibility to bacterial pathogens, in general, also increases with age (Gardner and Remington, 1977), due to reduced T cell reactivity and diminished innate immune functions of myeloid cells (Løvik and 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) family (Creagh and O'Neill, 2006; Medzhitov, 2001). One or more of multiple TLR species are

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.

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triggered by distinct bacterial, fungal, protist, and viral constituents (Gazzinelli and 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 and 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 and 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 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 antitumor 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, 2005; Bruley-Rosset and 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 and 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 TNFα 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 TNFα 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 h, 5 μg/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; Fig. 2). We focused our analyses on macrophages, using an antibody specific for F4/80, a definitive macrophage marker (Austyn and 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/ger1vi_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

2.3.1. Antibodies

Monoclonal antibodies (mAb) were obtained from BioLegend Inc. (San Diego, CA). Cytokine-specific antibodies used in this study were phycoerythrin (PE) – conjugated TNFα-specific rat mAb (IgG_1/κ, clone MP6-XT22), allophycocyanin (APC) – conjugated IL-6-specific rat mAb (IgG_1/κ, clone MP5-20F3), and IL-10-specific

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