Experimental Gerontology 85 (2016) 95–107

Contents lists available at ScienceDirect

Experimental Gerontology

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

Strain-dependent response to stimulation in middle-aged rat macrophages: A quest after a useful indicator of healthy aging

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ARTICLE INFO

Article history:
Received 25 March 2016
Received in revised form 29 July 2016
Accepted 14 October 2016
Available online 15 October 2016

Section Editor: Chennai Guest Editor

Keywords:
Macrophages
Rat strains
Thioglycollate-induced peritonitis
Young and middle-aged rats

ABSTRACT

Rats of Albino Oxford (AO) strain in our animal facility exhibit a longer average healthy life span than rats of Dark Agouit (DA) strain. Since chronic activation of macrophages contributes to chronic low level inflammation common in older age, elucidation of the changes in middle-aged rats could be useful in prevention of unbalanced inflammatory response in advanced age. We have analysed the phenotype of unelicited and thioglycollate-elicited peritoneal macrophages from young and middle-aged DA and AO rats and tested functions of these cells following stimulation with lipopolysaccharide (LPS) in vitro. Unelicited cells from middle-aged DA rats produced higher amounts of proinflammatory mediators interleukin-6 (IL-6) and nitric oxide (NO), but have a diminished response to LPS stimulation then cells from young rats, in spite of increased frequency of TLR4- and CD14-expressing mature macrophages. Injection of thioglycollate robustly increased overall cytokine production in young rats' macrophages, while diminishing their response to LPS stimulation. In middle-aged DA rats injection of thioglycollate diminished IL-6 production, but increased it in response to LPS stimulation. Quite the contrary to DA rats, the macrophages from middle-aged AO rats have released diminished levels of TNF-α and NO, whereas urea production was strongly increased, when compared to the macrophages from young rats. Although the thioglycollate injection has increased the proportion of $CD86^{+}MHCII^{+}$ mature macrophages in young rats, and percentages of activated $TLR4^{+}$ macrophages in both age groups of AO rats, it has not affected the cytokine production in young rats' macrophages, and the TNF-α production in middle-aged rats' macrophages. Moreover, the injection of thioglycollate has robustly increased the production of urea in macrophages derived from both age groups of AO rats. Although middle-aged rats of both strains were healthy during experiment, differences between the inflammatory responses of peritoneal macrophages of middle-aged rats of these strains might be one of the contributing factors defining their health in their advanced age. Development of strategies for the prevention of undesirable inflammatory changes in the elderly would benefit from the prospective study of the middle-aged.

1. Introduction

Quick response to invading agents ensures their fast elimination and, if necessary, tissue remodelling. However both the excessive and the insufficient inflammatory responses may provoke the development of different pathologies. Even though the innate immune response seems to be better preserved than the adaptive immune response over the aging process, a chronic activation of macrophages contributes to chronic, low-level inflammation commonly observed in older age (Franceschi et al., 2000). Chronic inflammation not only results from, but also drives the cellular immunosenescence (Freund et al., 2010). However, an impaired peritoneal macrophage phagocytosis (Linehan et al., 2014) and a diminished response to lipopolysaccharide (LPS) stimulation and pro-inflammatory cytokine secretion (Shirato and Imaizumi, 2014, Boehmer et al., 2004) were observed as a result of the advanced age. In different settings, aging has been shown to increase the macrophage response to LPS and interferon-γ (IFNγ) (Barrett et al., 2015).

Several lines of evidence suggest that the genetic predisposition(s) for a particular activation pattern may be crucial in defining the efficacy of the response to inflammatory agents both in humans, and in some experimental models (Wells et al., 2003). In line with that, we have reported that peritoneal macrophages from young rats of Dark Agouti (DA) and Albino Oxford (AO) rat strains respond to stimulation by mast cell mediators (Stanojević et al., 2013), zymosan (Stanojević et

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http://dx.doi.org/10.1016/j.exger.2016.10.005
0531-5565/

al., 2007) and phorbol-myristate-acetate (Miletić et al., 2007) in a strain-dependent manner. The distinctive inflammatory responses in these rat strains were also associated with their different susceptibility to adjuvant arthritis (Miletić et al., 2007). The female rats of AO strain in our animal facility exhibit a longer average life span (116 weeks) and a longer average healthy life span (entailing the absence of any severe pathology, such as obesity, diabetes or tumors) than DA rats of the same sex (96 weeks) (Dimitrijević et al., 2014). The similar differences in longevity were observed between male rats of these same rat strains (our unpublished observation). Besides, splenocyte cultures from aged (26-months-old) DA rats produced higher levels of IFN-γ than the cultures from young rats of this strain (Djikić et al., 2015), suggesting a skewing toward the inflammatory phenotype of cells from the aged DA rats.

Up to the age of 75 weeks both DA and AO male rats remain healthy, display a good muscle tonus, and the standard rodent behaviour. It was previously suggested that a phase in rat's life that stretches between the maturation and the onset of senescence (i.e. the period characterized by the sharp increase in rate of morbidity caused by chronic degenerative diseases, and in mortality rates), may be considered as middle age. However, the exact role of middle-age changes as specific precursors of later diseases or dysfunctions is generally unclear (Finch, 1991). In line with that, it is presently not known if some inflammatory changes observed in aged DA rats may be present in these rats even at the younger age. Thus, we have chosen 71–75 weeks old rats of DA and AO rat strains to investigate to what extent the peritoneal macrophage inflammatory response in healthy (late) middle-aged rats may anticipate their health in advanced age and, consequently, their life span. The elucidation of the basis for the changes in middle-aged rats could pave a way for prevention of development of the unbalanced inflammatory response in advanced age.

Highly dynamic macrophages may undergo a phenotype and functional switch over distinctive polarized phenotypes (Xu et al., 2013), and macrophage activity, among other variables, depends on the activation state of the cells (Ehrchen et al., 2004). Hence, our research focuses on phenotype analysis of un-elicited and thioglycollate-elicited peritoneal macrophages (ex vivo), and also the functional characteristics of these cells stimulated with bacterial lipopolysaccharide LPS (in vitro), in young and middle-aged rats of DA and AO strain.

2. Materials and methods

2.1. Animals

Young (11–12 weeks) and middle-aged (71–75 weeks) DA and AO male rats were obtained from the breeding colony at Immunology Research Center "Branislav Janković", Belgrade (Serbia). Experimental groups consisted of 5–6 rats. Young rats (body weight in range of 224–287 g for DA and 153–220 g for AO) represented early adulthood when the immune system was fully matured. Healthiness of middle-aged rats (body weight in range of 275–352 g for DA and 190–323 g for AO) was confirmed for each animal by gross inspection during autopsy. Although middle-aged rats were heavier than young controls, neither of the two rat strains developed obesity. The animals were housed in standard cages (2–3 rats/cage) in a controlled environment (22 ± 2 °C, humidity ranging 40% to 70% and under 12:12-h light:dark cycle) with free access to conventional food pellets (Veterinarski zavod Subotica, Serbia) and tap water. Animals were euthanized by using increasing dose of CO_2·The experimental protocol and all procedures with animals and their care were approved by Experimental Animal Committee of the Immunology Research Center "Branislav Janković", and were in accordance with principles declared in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Directive 86/609/EEC).

2.2. Thioglycollate-elicited peritoneal macrophage isolation

Young and middle-aged rats of DA and AO strains were intraperitoneally injected with 10 ml of sterile thioglycollate medium and peritoneal cells were obtained seven days later by peritoneal lavage. Unelicited peritoneal cells were obtained from uninjected rats in the same manner. The purity of peritoneal macrophage population obtain by this method was 70–88%, according to the staining with FITC-conjugated and biotin-conjugated mouse anti-rat CD 68 antibody (followed by streptavidin-PCP) (Results, Section 3.2).

2.3. Chemicals and immunoconjugates

Lipopolysaccharide (LPS of *Escherichia coli* 0111:B4 strain), phenylmethyl sulfonyl fluoride (PMSF), α-isonitrosopropiophenone (ISPF) and RPMI-1640 medium (RPMI) were purchased from Sigma (St. Lewis, MO, USA). Foetal calf serum (FCS) was obtained from Gibco (Grand Island, NY, USA). Thioglycollate medium was acquired from the "Torlak" Institute (Belgrade, Serbia).

Monoclonal PE-conjugated mouse anti-rat CD163 (ED2-like) resident macrophage receptor (clone HIS 36), monoclonal FITC-conjugated mouse anti-rat major histocompatibility complex class II (MHC II) antibody (clone OX-6), biotin-conjugated mouse anti-rat CD 86 antibody (B7.2, clone 24F), FITC-conjugated goat anti-rabbit IgG, PE-conjugated donkey anti-rabbit IgG, streptavidin-PCP and appropriate IgG isotype controls were purchased from BD Biosciences Pharmingen (Mountain View, CA, USA). Monoclonal FITC-conjugated mouse anti-rat CD68 (clone ED1), biotin-conjugated mouse anti-rat CD 68 (clone ED1) and monoclonal mouse anti-rat CD169 (clone ED3) antibodies were obtained from Serotec (Oxford, UK). Polyclonal rabbit anti-rat C—C chemokine receptor type 7 (CCR7) and rabbit anti-rat Toll-like receptor (TLR) 4 antibodies were obtained from Abcam (Cambridge, MA, USA), whereas polyclonal goat anti-rat CD14 antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). FITC-conjugated rabbit anti-goat IgG was obtained by Sigma-Aldrich Chemie (Taufkirchen, Germany) and PCPCy5.5-conjugated goat anti-mouse IgG from Biolegend (San Diego, CA, USA).

2.4. Flow cytometric analysis (FCA)

Freshly isolated peritoneal exudate cells adjusted to 1×10^7/ml were washed and resuspended in PBS supplemented with 2% heat-inactivated FCS and 0.1% NaN_3. All incubations were performed for 30 min at +4 °C in the dark and were followed by thorough washings. Flow cytometric analysis of peritoneal cells was performed immediately following isolation. Macrophages were selected and gated by light scatter characteristics and debris was gated out on the basis of low forward scatter and low side angle scatter.

Cells were first stained with PE-conjugated anti-CD163 antibody and with goat anti-rat CD14 or with rabbit anti-rat TLR4 antibody (followed by FITC-conjugated rabbit anti-goat and goat anti-rabbit antibody, respectively). Cells were then fixed with 0.25% paraformaldehyde and permeabilised by 0.2% Tween 20 for intracellular labelling prior to the addition of biotin-conjugated mouse anti-rat CD 68 antibody (followed by streptavidin-PCP). For intracellular two-colour staining, cells were first immunolabelled with polyclonal rabbit anti-rat CCR7 antibody (followed by PE-conjugated donkey anti-rabbit IgG), or with monoclonal mouse anti-rat CD169 antibody (followed by PCPCy5.5-conjugated goat anti-mouse IgG) and then fixed with 0.25% paraformaldehyde and permeabilised by 0.2% Tween 20 prior to the addition of monoclonal FITC-conjugated mouse anti-rat CD68 antibody. For membrane three-colour staining, cells were stained with PE-conjugated anti CD163, biotin-conjugated mouse anti-rat CD 86 (followed by streptavidin-PCP) and monoclonal FITC-conjugated mouse anti-rat MHC II antibodies. After extensive washing in the flow cytometry buffer,