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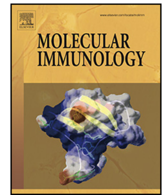

Circulating TNF and mitochondrial DNA are major determinants of neutrophil phenotype in the advanced-age, frail elderly

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ABSTRACT

Tumor necrosis factor (TNF), a potent inflammatory cytokine, and mitochondrial DNA (mtDNA), a product of inflammation-induced tissue damage, increase with age ("inflammaging") and many chronic diseases. Peripheral blood neutrophils, a critical component of innate immunity, have also been shown to be altered with age, and are exceptionally sensitive to external stimuli. Herein, we describe that the phenotype of neutrophils from the advanced-age, frail elderly (ELD) is determined by levels of circulating TNF and mtDNA.

Neutrophils from ELD donors are morphologically immature, and have higher levels of intracellular reactive oxygen species (ROS) and expression of the activation markers CD11b and HLA-DR. The frequency of CD11b^{++} neutrophils correlated with plasma TNF, and recombinant TNF elevated neutrophil CD11b *ex vivo* and *in vivo*. Furthermore, neutrophils from aged TNF-deficient mice expressed CD11b similar to young counterparts. The frequency of HLA-DR^{+} neutrophils, on the other hand, positively correlated with circulating mtDNA, which increased neutrophil HLA-DR expression in a dose-dependent manner *ex vivo*. Cell-surface TLR-9 expression, however, was unaltered on neutrophils from ELD donors.

In summary, we provide novel evidence that products of age-related inflammation modulate neutrophil phenotype *in vivo*. Given this, anti-inflammatory therapies may prove beneficial in improving neutrophil functionality in the elderly.

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1. Introduction

Dynamic alterations to the blood neutrophil compartment occur with age. For example, phagocytosis (Chiu et al., 2011), chemotaxis (Sapey et al., 2014), toll-like receptor (TLR) signaling (Qian et al., 2014) and extracellular trap formation (Hazeldine et al., 2014) are impaired, while the interferon-gamma (IFN-γ) stimulated production of reactive oxygen species (ROS) (Chaves et al., 2009) as well as the frequency of circulating neutrophils (Strindhall et al., 2007), is increased. The latter observations have also been shown to be compounded by physical frailty (Collerton et al., 2012; Fernández-Garrido et al., 2013). Another phenomenon that occurs with age and frailty is the increase in circulating products related to chronic, low-grade inflammation, such as tumor necrosis factor (TNF), interleukin (IL)-6, acute phase proteins and cell-free DNA (Bartlett et al., 2012; Jenny et al., 2012; Jylhävä et al., 2013; Leng et al., 2011). This

Abbreviations: ELD, advanced-age, frail elderly; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA-DR, human leukocyte antigen DR; IFN, γ interferon gamma; IL, interleukin; MHC, major histocompatibility complex; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; *S. pneumoniae*, *Streptococcus pneumoniae*; SEN, community dwelling seniors; Th, T helper cell; TLR, toll-like receptor; TNF, tumor necrosis factor; YNG, young adults.

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has been commonly termed "inflammaging" (Bartlett et al., 2012). It has yet to be investigated whether these circulating molecules contribute to the alterations in blood neutrophils with age and frailty. Conversely, age-related low-grade inflammation may instead be a product of neutrophils in a chronically activated state. This is supported by studies indicating that constitutive neutrophil ROS production increases with age (Chaves et al., 2009), and physical frailty is associated with elevated superoxide production in whole blood (Baptista et al., 2012).

Blood neutrophils are critical components of innate immunity, characterized by a relatively short half-life and the ability to quickly extravasate to sites of infection, providing immediate bacterial defense (Branzk and Papayannopoulos, 2013). Activated neutrophils, whether through *in vitro* stimulation or upon infiltration into tissue sites, have increased expression of the cell-surface markers CD11b and human leukocyte antigen (HLA)-DR (Alexis et al., 2000; Piskin et al., 2005; Turina et al., 2008). CD11b, a member of the integrin family of receptors, dimerizes with CD18 to form complement receptor 3 (CR3/MAC-1), and is a principal component for neutrophil adhesion and extravasation across endothelial walls (Ling et al., 2014). In addition to binding complement, CD11b has been shown to bind lipopolysaccharide (LPS) and positively regulate TLR4-induced signaling pathways (Ling et al., 2014), and its expression on neutrophils promotes phagocytosis and ROS generation (Coxon et al., 1996). Chronic inflammatory conditions (Kylänpää-Bäck et al., 2001; Muller Kobold et al., 2000) are known to result in increased expression of CD11b, which may be due to elevated levels of TNF (Volk et al., 2011) or the release of host DNA as a result of chronic tissue injury (Zhang et al., 2010). HLA-DR, a major histocompatibility complex class II (MHC-II) molecule, is not normally expressed at high levels on neutrophils, but can be induced by granulocyte macrophage colony-stimulating factor (GM-CSF) and IFN-γ *in vitro* (Lei et al., 2001) and *in vivo* (Carulli et al., 1999; Reinisch et al., 2003). Elevated blood neutrophil HLA-DR has also been shown in patients with the systemic inflammatory disease Wegener's granulomatosis (Iking-Konert et al., 2001). In humans, HLA-DR promotes the expression of IL-8 by neutrophils when cross-linked by staphylococcal enterotoxins (Lei et al., 2001), whereas in mice, MHC-II expression allows neutrophils to prime antigen-specific Th1 and Th17 responses (Abdallah et al., 2011).

Given what is already known regarding the ability of cytokines and other products of inflammation to modulate peripheral blood neutrophils, we sought to determine if neutrophil phenotype, namely markers of activation, are altered in individuals who exhibit chronic, age-related inflammation. Herein we demonstrate that in the advanced-age, frail elderly, peripheral blood neutrophils express higher levels of both CD11b and HLA-DR, and generate elevated levels of ROS constitutively. Furthermore, we demonstrate that increased CD11b expression is due to circulating TNF, while HLA-DR expression is related to circulating mitochondrial DNA.

2. Materials and methods

2.1. Participants

Healthy adults (YNG; $n = 41$, median age = 32 years, range 19–59, male:female 19:22) and community-dwelling seniors (SEN; $n = 45$, median age = 69 years, range 61–76, male:female 15:30), were recruited from Hamilton, Ontario between January and May 2012. Frail elderly participants (ELD; $n = 131$, median age = 87 years, range 67–99, male:female 34:97) were recruited from five local nursing homes between November 2011 and January 2012; they all were categorized as at least vulnerable according to the Clinical Frailty Scale (Rockwood et al., 2007). Additional healthy adults and frail elderly participants were recruited in April 2014. Participants were excluded if they were currently on immunosuppressive medication and pre-existing diseases were established by review of each participant's medical chart. These studies were approved by the McMaster Research Ethics Board and written informed consent was obtained for all participants.

2.2. Mice

C57BL/6J mice were purchased from Jackson Laboratories, while TNF knockout mice (KO) mice on a C57BL/6J background were bred in the barrier at the McMaster University Central Animal Facility (Hamilton, ON, Canada). Young mice were 3–4 months old, and aged mice were 18–22 months old. All mice were housed in pathogen-free conditions in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board as per the recommendations of the Canadian Council for Animal Care.

For TNF administration studies, murine recombinant TNF (eBioscience, CA, USA) diluted in sterile saline was injected intraperitoneally to young wildtype mice every other day for 3 weeks at a dose of 5 ng per gram of body weight in a volume of 200 μL.

2.3. Whole blood immunophenotyping

All flow cytometry was performed using a Becton Dickinson LSR II and all analyses were performed in Treestar FlowJo 7.6.4. Antibody staining for human blood was performed as described previously (Verschoor et al., 2013) using a Beckman Coulter Biomek NX^{P} Laboratory Automation Workstation. Antibodies included: CD15-PE-Cy7, CD11b-APC, CD3-PE, CD19-PE, CD56-PE (BD Biosciences, ON, Canada); HLA-DR-PerCp-Cy5.5, CD45-eFluor605 (eBioscience, CA, USA). No significant differences in CD45^{+} leukocytes were identified between the age groups, thus, all other subpopulations are presented as the percentage of CD45 expressing leukocytes. The neutrophil gating strategy can be found in Fig. 1. Antibody staining in murine whole blood was performed as above and included the antibodies CD11b-PE-Cy7, Ly6C-FITC and F4/80-APC (eBioscience, CA, USA). Blood neutrophils were distinguished as expressing low levels of Ly6C (relative to monocytes), not expressing F4/80, and were granular (side scatter high).

2.4. Peripheral blood neutrophil histology and ex vivo stimulation

Peripheral blood slides for histological analysis were prepared by red blood lysis (1× RBC lysis buffer, Biolegend, CA, USA), followed by Shandon cytospin centrifugation and staining using Hema 3 (Fisher Scientific, ON, Canada). The frequency of band and multi-lobular segmented neutrophils were determined by manual counting using ImageJ software. Counts were obtained from a minimum of 4 fields of view per donor (median, 75 cells total and 15 cells per field). Band neutrophils were defined as having a single nucleus with no distinguishable lobes.

Measurements of reactive oxygen species (ROS) was performed using 100 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) (Sigma, MO, USA). Briefly, DCFDA was added simultaneously to whole blood (100 μL) with indicated treatments. Following a 4 h incubation in non-tissue culture treated microtitre plates at 37 °C/5% CO_2, samples were fixed and red blood cells lysed using 1× Fix/Lyse buffer (eBioscience, CA, USA) and DCFDA-fluorescence was determined by flow cytometry.

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