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Proangiogenic characteristics of activated macrophages from patients with age-related macular degeneration

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ABSTRACT

Macrophages were previously implicated in the pathogenesis of neovascular age-related macular degeneration (nvAMD). It is unclear if a specific macrophage phenotype is associated with nvAMD, and if macrophages from nvAMD patients are more pathogenic as compared with controls. To address these issues, we evaluated macrophages derived from peripheral blood monocytes of nvAMD patients and age-matched controls. Macrophages were assessed in terms of their expression profile and of their angiogenic potential in the choroid sprouting assay and the rat model of laser-induced choroidal neovascularization. Results showed a proangiogenic and inflammatory gene and protein expression profiles in classic (M[IFNγ and LPS]) and alternative (M[IL-4 and IL-13]) polarized macrophages. Furthermore, activated macrophages, particularly of the M(IFNγ and LPS) phenotype from nvAMD patients, were proangiogenic ex vivo and in vivo. These findings implicate activated human macrophages, particularly M(IFNγ and LPS) macrophages from nvAMD patients, in nvAMD. Further research is required to determine whether activated macrophages can serve as therapeutic targets in nvAMD.

1. Introduction

Innate immunity, including the complement system, was demonstrated to have an important role in the pathogenesis of age-related macular degeneration (AMD) (Anderson et al., 2002, 2013; Edwards and Malek, 2007; Haines et al., 2005; Klein et al., 2005a; Klein et al., 2005b; Klein et al., 2014; Maller et al., 2007; Scholl et al., 2008). Several lines of evidence suggest the involvement of mononuclear cells including monocytes and their macrophage descendants in AMD (Apte et al., 2006; Espinosa-Heidmann et al., 2003b; Grunin et al., 2014; Kataoka et al., 2011; Robertson et al., 2002; Sakurai et al., 2003; Sarks et al., 1980; Zhang et al., 2011). We have previously reported of altered, proinflammatory gene expression patterns in peripheral blood mononuclear cells (PBMCs) (Lederman et al., 2010), and in blood monocytes from patients with neovascular AMD (nvAMD) (Grunin et al., 2016), along with increased expression of the major chemokine receptors CCR1 and CCR2 on the CD14+CD16+ subset of monocytes in these patients as compared with controls (Grunin et al., 2012). CCR2 is the chemokine receptor involved in monocyte recruitment. In accordance with that high levels of CCR2's main ligand (CCL2/MCP1) were detected in the aqueous humor of AMD patients (Jonas et al., 2010; Kramer et al., 2012) and macrophages have been found in the vicinity of drusen, areas of retinal pigment epithelium (RPE) atrophy, Bruch's membrane rupture, and choroidal neovascularization (CNV) in AMD eyes (Cao et al., 2011; Cherepanoff et al., 2009; Eandi et al., 2016; Killingsworth et al., 1990; Penfold et al., 1985, 2001; Sennlaub et al., 2013).

Having left the peripheral circulation, monocytes differentiate into macrophages that can undergo activation into a variety of phenotypes that are largely divided into classic (M1) and alternative (M2) phenotypes. Traditionally, in nonocular tissue, M1 macrophages were reported to have a predominantly proinflammatory effect, whereas M2 cells were suggested to exert a proangiogenic effect, among other functions (Colombo and Mantovani, 2005; Mantovani et al., 2005; Martinez et al., 2006; Sica et al., 2014, 2015; Stout et al., 2005). Both M1 and M2 phenotypes were identified in aged human retina, and it was suggested that aging is associated with an M2 shift, but that both M1 and M2 macrophages are present in the vicinity of CNV (Cao et al., 2011; Nakamura et al., 2015).

Based on previous literature from other organs, it may be postulated that M1 macrophages infiltrating the retina might be involved in the activation of the complement system, induction of

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inflammation, and exertion of a toxic effect leading to RPE and photoreceptor cell death. On the other hand, M2 macrophages might express proangiogenic cytokines, thereby accelerating CNV growth. However, most of the available information was derived from studies on rodent macrophages and limited data on the function of human macrophage phenotypes in the context of AMD are available to support this hypothesis. Thus, the actual function of macrophages in nvAMD in general, and of the M1 and M2 phenotypes in particular, remains to be illustrated.

Furthermore, it is unclear if the differences in gene and protein expression which exist in nvAMD monocytes versus controls (Grunin et al., 2016) underlie or are associated with an altered function of macrophages from nvAMD patients as compared with macrophages from unaffected individuals. Characterization of the functional consequences of macrophages' presence in AMD is important to obtain a comprehensive understanding of the pathogenesis of the disease and to evaluate these cells as potential novel therapeutic targets.

To that end, we have characterized polarized macrophages from nvAMD patients and age-matched controls. Cells were evaluated in terms of expression profile, and their angiogenic characteristics were assessed in a choroid sprouting assay (CSA) and in the in vivo model of LI-CNV. Our findings suggest that AMD patients' macrophages may have a distinct expression profile for relevant genes in the context of nvAMD. Furthermore, we show that M(IFNγ and LPS) macrophages, only from nvAMD patients, but not from controls are proangiogenic in term of their expression pattern and function in relevant models in the context of nvAMD. We also demonstrate that all subsets of macrophages may be associated with a more pronounced proangiogenic effect in experimental nvAMD.

2. Materials and methods

2.1. Patients and controls

Patients with nvAMD (n = 34, 21 females, 13 males, mean age ± standard error of the mean [SEM]: 75.9 ± 1.5 years, range: 59–93) and age-matched unaffected controls (n = 25, 10 females, 15 males, mean age ± SEM: 72.3 ± 1.7 years; range: 59–89) were recruited from the retina clinic of the Department of Ophthalmology at the Hadassah-Hebrew University Medical Center. Criteria for inclusion of nvAMD patients included: age over 55 years, diagnosis of AMD according to the AREDS criteria (Age-Related Eye Disease Study Research Group, 1999) and diagnosis of CNV according to fluorescein angiogram and optical coherence tomography. Eyes with neovascular lesions that comprised less than 50% active CNV, subretinal hemorrhage greater than 25% of the lesion size, or the presence of other retinal diseases were excluded from the study. Specifically, eyes with any other potential cause for CNV, such as myopia, trauma, or uveitis were excluded. Also excluded were patients with a major systemic illness, such as cancer, autoimmune disease, congestive heart failure, or uncontrolled diabetes. Controls were older than 55 years of age and healthy, without retinal diseases (as per history and ophthalmoscopy), recent eye surgery, or systemic illness as detailed above. All patients and controls signed an informed consent form, and the study was approved by the institutional ethics committee (see Ethics Declaration).

2.2. Macrophage preparation

Blood samples (30 mL) were collected from nvAMD patients and controls in EDTA tubes (BD Bioscience). Monocytes were isolated from whole blood, differentiated into macrophages (M0), and activated into M(IFNγ and LPS) and M(IL-4 and IL-13) phenotypes (M1- and M2-like macrophages, respectively), as previously described (Bouhlel et al., 2007; Gelinas et al., 2011; Mantovani et al., 2002; Martinez et al., 2009; Pelegrin and Surprenant, 2009). Briefly, PBMCs were separated using a Histopaque-Ficoll density centrifuge according to manufacturer's recommendations (Sigma-Aldrich, Munich, Germany). PBMCs (3×10^7 cells/cm^2) were suspended in RPMI 1640 medium (Biological Industries Israel Beit Haemek Ltd) and seeded into 6-well plates coated with the amino acid poly-D-lysine, which facilitates the adherence of monocytes. An hour after incubation in a 37 °C and 5% CO_2 incubator, cells were washed with PBS, and monocytes were cultured for 7 days in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acid, 1% glutamine, 1% sodium pyruvate, 100 units/mL penicillin-streptomycin, and 50 ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ, USA). M-CSF was added to the growth medium to induce maturation of the monocytes to macrophages. Polarization of macrophages was obtained by the addition of extra cytokines as follows: 20 ng/mL IFNγ (PeproTech) and 100 ng/mL LPS (Sigma-Aldrich) were added at day 6 to obtain an M(IFNγ and LPS) phenotype. To obtain an M(IL-4 and IL-13) phenotype, 50 ng/mL IL-13 (PeproTech) and 20 ng/mL IL-4 (PeproTech) were added at day 5 of culture. Macrophages that were not given any stimulatory medium were classified as unpolarized macrophages (M0), and were used as a control group. As M(IFNγ and LPS) macrophages require 24 hours for polarization whereas M(IL-4 and IL-13) needs 48 hours (Allavena et al., 1998), macrophages were polarized on different days so that intravitreal injections would be performed on the same day. The supernatant of activated macrophages was collected and centrifuged in 1500 rpm for 5 minutes. The upper layer was collected and kept in −20 °C for protein analysis and CSA. Macrophages were collected with 0.025% trypsin (Tri Reagent; Sigma-Aldrich) which was deactivated with RPMI+FCS, following 3 washes with PBS to remove traces of supernatant and remain with pure cells. A total of 13 nvAMD patients (6 females, 7 males, mean age ± SEM: 73.3 ± 2.8 years, range: 59–93) and 12 controls (6 females, 6 males, mean age ± SEM: 74 ± 2.6 years; range: 60–89) were used for the in vivo experiments. Macrophages from 18 nvAMD patients (13 females, 5 males, mean age ± SEM: 78.27 ± 1.7 years, range: 64–87) and 14 controls (5 females, 9 males, mean age ± SEM: 70.7 ± 2.07 years; range: 59–87) were used for gene expression assays (qPCR). Supernatants of cell cultures of activated macrophages from 20 nvAMD patients (13 females, 7 males, mean age ± SEM: 74.9 ± 1.9 years, range: 59–87) and 17 controls (7 females, 10 males, mean age ± SEM: 73.7 ± 2.2 years; range: 60–89) were used for protein expression assay (ELISA). In addition, supernatants of cell cultures from 7 nvAMD patients (3 females, 4 males, mean age ± SEM: 77.6 ± 3.5 years, range: 64–93) and 8 controls (3 females, 5 males, mean age ± SEM: 70 ± 2.13; range: 61–77) were used for the CSA.

2.3. Choroid sprouting assay

An ex vivo angiogenesis assay was performed to evaluate the supernatant of M0/M(IFNγ and LPS)/M(IL-4 and IL-13) macrophages as previously described (Shao et al., 2013). Briefly, the supernatant of the 3 subtypes (M0/M[IFNγ and LPS]/M[IL-4 and IL-13]) of human macrophages was collected and kept in −20 °C until use.

C57BL6J 4–6 week old mice that were treated in accordance to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) were used. Experiments were conducted with the approval of the institutional animal care ethics committee (See Ethics Declaration). After 5 minutes of injecting ketamine,