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Effect of Senescence on Macrophage Polarization and Angiogenesis

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ABSTRACT

There is mounting evidence that as the immune system ages, a progressive deterioration in normal function occurs. Termed *immunosenescence*, aging impacts both the innate and adaptive immune responses. This review discusses the age-related alterations in the innate immune system, with a specific focus on macrophages. The downstream effect of altered macrophage function on aberrant angiogenesis in the pathophysiology of age-related eye disease is also discussed.

INTRODUCTION

IMMUNOLOGICAL DEFENSES IN VERTEBRATES consist of two immunological subsystems: the innate immune response and the adaptive immune system. These two immune responses function in a complicated interactive, cross-regulative, and synergistic fashion to protect the host against foreign microbial attack, inhibit tumor development, promote wound healing, and maintain homeostasis in several tissues. The innate immune system encompasses a collection of host defenses that range from the non-specific barrier function of epithelia to the highly selective recognition of pathogens through the use of germline-encoded receptors. A common feature of innate immune system components is a rapid and blunt response to infection or tissue destruction.[1] In contrast, the adaptive immune system uses somatically rearranged antigen receptor genes to create receptors for virtually any antigen. Although slower to respond, the adaptive immune system is more flexible and specific, and is able to combat infections that have evolved to evade the innate immune response. The induction of the adaptive immune response is dependent upon the innate immune response, including cytokine and chemokine secretion by innate immune cells and antigen presentation by macrophages and dendritic cells (DC). The adaptive immune response can differentiate into a Th1, Th2, or Th17 immune response, which is defined by their cytokine secretion profile. Th1 immune responses are responsible for cell-mediated immunity (cytotoxic T lymphocytes), while Th2 immune responses are responsible for humoral immunity (antibody production).[2] Th17 is a newly discovered arm of adaptive immune responses involved primarily in autoimmune diseases.[3] Upon activation of the adaptive immune response, the innate immune response can also be altered. For example, the secretion of IFN-γ by Th1 cells can lead to the further activation of innate immune cells, whereas the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) by regulatory T cells can downregulate innate im-

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mune responses. This cross-talk between the innate and adaptive immune responses demonstrates the highly choreographed and regulated interplay of immune responses occurring in the body. Both immune responses, however, exhibit a markedly decreased capacity to function as the host ages. This leads to increased susceptibility to infectious diseases, neoplasias, and autoimmune diseases, and reduced responses to preventative vaccination. These complex changes to the immune system over time are collectively called *immunosenescence*.[4]

MACROPHAGES

Mononuclear phagocytes are of critical importance for host immune defenses. Monocytes emigrate from blood vessels in response to antigenic stimuli and differentiate in the tissues into either macrophages or dendritic cells.[5] Macrophages are best known for initiating innate immune responses against microbes by recognition of pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRPs).[6] It has been recently identified that macrophage populations are distinctly heterogeneous. Macrophage heterogeneity arises as macrophages differentiate from monocyte precursors and is determined by the genetic background, as well as by specific tissue-related and immune-related stimuli.[5,7] More specifically, microbial antigens, tumor products, and cytokines produced by Th1, Th2, or Th17 effector cells influence the heterogeneity and the state of activation of macrophage populations.[8,9]

Some researchers have broadly subdivided heterogeneic macrophages into two subpopulations: M1 and M2 macrophages.[10,11] According to this classification, M1 macrophages, or "classically activated macrophages," are thought to be induced in a Th1-like cytokine environment (IFN-γ, TNF-α, GM-CSF) or upon recognition of PAMPs (LPS, lipoproteins, dsRNA) and endogenous "danger" signals.[12] M1 macrophages typically produce high levels of IL-12 and IL-23, low levels of IL-10, and are strong promoters of Th1 immune responses.[12] M1 macrophages also exert anti-proliferative and cytotoxic activities toward tumor and virally infected cells, resulting partly from their ability to secrete reactive oxygen species and proinflammatory cytokines such as IL-β, TNF-α, and IL-6.[11–13] M1 macrophages are efficient mediators of resistance against intracellular parasites and tumors.[10]

Macrophages classified as M2, or "alternatively activated macrophages," develop in the presence of Th2 cytokines such as IL-4 and IL-13, deactivating cytokines such as IL-10 and TGF-β, immune complexes, hormones, and apoptotic cells.[11,14] M2 macrophages promote Th2 responses, have reduced levels of inflammatory cytokines, are IL-12^{low} and IL-23^{low}, and secrete copious amounts of anti-inflammatory cytokines such as IL-10 and TGF-β.[12] Accordingly, M2 macrophages exert selective immunosuppressive functions and inhibit T cell proliferation.[15,16] The presence of M2 macrophages in healthy individuals in the placenta, lungs, and immune privileged sites, as well as in chronic inflammatory diseases like rheumatoid arthritis, suggests that M2 macrophages protect organs and surrounding tissues against detrimental immune responses. M2 macrophages orchestrate encapsulation and containment of parasites, promote tissue repair, and enhance tumor progression.[10]

Although macrophages have been classified as having an M1 and M2 phenotype, macrophages are highly plastic and the tissue microenvironment can continuously modify their function.[17] It is also thought that condensing the heterogeneity of macrophage populations into two subdivisions may be an oversimplification. Gordon et al. have stated that this dual classification "obscures important differences in macrophage responses."[14] Also, gene array analyses have shown that treatment of macrophages with different cytokines results in unique functional profiles with remarkably small overlap.[18] We therefore refrain from classifying macrophages as M1 or M2 for the remainder of this review and focus more on macrophage function as a means of classifying them.

Macrophages and angiogenesis

One of the earliest physiological responses to tissue injury or infection is an increase in vas-

cular permeability and blood flow to the affected area. This is initiated by regional vasodilation and enhanced angiogenesis to facilitate wound healing. This increase in blood flow and angiogenesis is largely achieved by factors produced by cells of the innate immune system, primarily macrophages. Inflammatory products such as LPS bind to toll-like receptors (TLR) on macrophages, resulting in the synthesis and release of vascular endothelial growth factor (VEGF). This results in vasodilation, recruitment of $CD31^+$ endothelial progenitor cells, and angiogenesis.[19,20] Also, the release of TNF-α and IL-1β by macrophages promotes blood vessel permeability and angiogenesis.[21] In addition to inducing angiogenesis, macrophages have been shown to play a central role in the remodeling of small conduit blood vessels into larger muscular arterioles.[22] Tissue-macrophage release of matrix metalloproteinases also appears to be required for the extracellular matrix remodeling essential for effective angiogenesis to occur.[23] CCR2-deficient mice, which lack the necessary chemokine receptor needed for macrophage migration, have impaired blood vessel formation following experimental arterial occlusion.[24]

However, the formation of blood vessels is not always beneficial as angiogenesis in some cases can actually exacerbate a pre-existing disease phenotype. Within tumors, increased angiogenesis leads to accelerated tumor growth, invasiveness, and metastasis. The role of tumor-associated macrophages (TAM) being either pro-angiogenic or anti-angiogenic seems to depend on their polarization state, which is influenced by cytokines present in the tissue milieu.[11,25,26] In the majority of cases, the tumor microenvironment seems to polarize macrophages towards a pro-angiogenic phenotype, resulting in TAMs to be associated with a "bad" prognosis in most tumor models.[27,28] Therapies that target TAMs have been shown to be beneficial for reduction of tumor size and/or tumor rejection.[29] However, not all TAMs support the growth of tumors, as there are multiple accounts of macrophages being tumoricidal and necessary for tumor rejection.[30–32] This again underscores the heterogeneity of macrophage populations and their influence on tumor growth or progression.

Unbridled angiogenesis also plays a role in the progression of diseases of aging. The pro-angiogenic cytokine VEGF, which is released by macrophages, is likely to play a role in the pathophysiology of arthritis, as mice with a target disruption of VEGF exhibited significantly less joint swelling and inflammation than wild-type animals.[33] Also, macrophages have been implicated to promote abnormal blood vessel growth in murine tissue injury models.[34] In atherosclerosis, macrophages have been implicated in stimulating angiogenesis in atherosclerotic plaques, further promoting inflammation and exacerbating disease.[35]

Effect of aging on macrophages

The effect of aging on the innate immune system seems to have a detrimental effect on the health of elderly individuals. There is a significant decline in the ability to resist infectious diseases and generate robust protective immune responses in the elderly. Morbidity due to infectious diseases is high in this segment of the population. There is a high incidence of infection with viral influenza, respiratory syncytial virus, and pneumococcal pneumonia in elderly individuals.[36,37] Older individuals also have an increased incidence of bacterial infections in the lungs, urinary tract, and skin. The increased incidence of infections suggests possible defects in the ability of the innate immune system to function normally as age increases. Advanced age is associated with a breakdown of the epithelial barriers of the skin, lung, and gastrointestinal tract, enabling the invasion of delicate mucosal tissues by pathogenic organisms. Also, aging seems to effect the normal function of cells of the innate immune system, including macrophages.

The effect of age on macrophages appears to be multifaceted, affecting almost every aspect of their normal cellular function. Macrophages from old mice failed to lyse tumor targets and produce nitric oxide at levels similar to young mice.[38] Also, Ding et al. found that macrophages from old mice produced 50% less hydrogen peroxide and nitric oxide than young mice when stimulated with a variety of agents, and that macrophages from old mice were less responsive to IFN-γ even though surface expression of

IFN-γ receptor was similar in old and young mice.[39] This decrease in IFN-γ signaling is indicated by reduced phosphorylation of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription-1 (STAT-1) in aged rodents.[39,40] The effect of aging on macrophages is not restricted to mice, as human monocytes from aged individuals are reported to display decreased cytotoxicity against tumor cells *in vitro* after LPS stimulation, corresponding with decreased release of reactive oxygen and nitrogen intermediates.[41,42]

Macrophages may also actively contribute to dysregulated immune function by their secretion of immunosuppressive substances, particularly prostaglandins (PGE). Macrophages from old mice have higher PGE_2 production than those from young mice.[43] This can affect multiple cells of the immune system. PGE_2 can inhibit the function of DCs, the primary professional antigen presenting cell.[44] Also, PGE_2 directly inhibits T cells,[43] and T cells from the elderly may be more susceptible to such inhibition than T cells from the young.[45] PGE_2 also suppresses IL-12 secretion, decreases surface expression of MHC class II molecules on APCs, and enhances IL-10 secretion, resulting in downregulation of T cell function.[46]

Other functions of macrophages that are affected by age include antigen presentation and phagocytosis. It has been reported that antigen-pulsed macrophages from old mice stimulated lower levels of T cell proliferation than macrophages from young mice.[47,48] This may be due to decreased expression of MHC class II levels on aged macrophages,[49] which contrasts to the stable expression of MHC class II on young and old DCs.[50] Phagocytosis and clearance of infectious organisms are reduced with advanced age.[51–53] This may be due to the inability of aged macrophages to recognize invading pathogens, as aging macrophages demonstrate decreased expression of TLRs.[54] Alternatively, aging macrophages may not be able to migrate to the site of infections, as macrophage chemotactic response to complement-derived factors is impaired in the elderly.[55,56]

Although the dysfunction of macrophages may lead to increased susceptibility to microbial infection, the continuing normal pro-inflammatory function of macrophages over time may also lead to disease in the elderly. It has been suggested that the production of pro-inflammatory cytokines by macrophages and fibroblasts is responsible for many age-associated diseases, including diabetes, osteoporosis, and atherosclerosis.[57,58] Termed *inflamm-aging*, it has been hypothesized that, as a result of constant antigenic stimulation, the continual production of inflammatory mediators could potentially trigger the onset of inflammatory diseases. This is exemplified by elevated circulating levels of pro-inflammatory mediators, including IL-6, IL-1β, TNF-α, and PGE_2.[59] Therefore, when it comes to macrophage function in the elderly, a happy medium is desirable for maintaining homeostasis. Too little macrophage activity leads to the susceptibility of infection from microbial pathogens. Too much macrophage activity leads to a pro-inflammatory state and an induction of autoimmune disease. Luckily, the plasticity of macrophages allows their state of activity to be virtually reversed.

The effect of age-associated factors on macrophage function is unknown and may be numerous. However, the plasticity of macrophages allows them to be a potential target for therapy to reverse their phenotype and promote homeostasis.[17] Oxidative stress is hypothesized to alter macrophage transcription factors and nuclear receptors, altering their ability to respond to inflammatory stimuli.[60] Anti-oxidants may be a potential treatment to reverse this cause of macrophage dysfunction, as they seem to improve macrophage inflammatory function.[61,62] Also, neuroendocrine factors and stress hormones have also been hypothesized to contribute to the immunosenescence and decreased macrophage function.[63] An approach to promote normal macrophage function in aged hosts may be to take a page from cancer therapy, and target macrophages with cytokines to promote the desired macrophage function.[29] In addition to affecting macrophage function and activation, aging seems to also affect macrophage polarization.[64] The plasticity of macrophages allows them to switch their phenotype, and cytokines may be the key to achieving a reversal of polarization.

Aging macrophages and ocular angiogenesis

Although there has been much work showing how age affects macrophage function

against microbial pathogens and tumors, little has been done to show that age affects macrophage influence on ocular angiogenesis. Immune vascular interactions can play an important role in regulating angiogenesis during diseases of aging, such as neoplasias, arthritis, and blinding eye disease.[6,65,66] Our laboratory has investigated the key role of macrophages in inhibiting the growth of abnormal blood vessels in the eye in age-related macular degeneration (AMD), the leading cause of blindness in people over 50 years of age.[66,67] Blindness in AMD occurs largely from the "wet" form of the disease that is characterized by the development of abnormal blood vessels underneath the retina, termed *choroidal neovascularization* (CNV).[68] We have demonstrated that mice that lack IL-10 are significantly impaired in their ability to generate CNV after laser-induced tissue injury to the eye.[66] IL-10 inhibits macrophages from migrating to the site of tissue injury, implicating that macrophages are serving in an anti-angiogenic manner in CNV.

Age may impact the polarization of macrophages towards an M1 or M2 phenotype. Polarization plays a pivotal role in determining the effector function of macrophages.[26] Macrophages stimulated in the presence of IFN-γ, LPS, or GM-CSF produce high levels of cytokines such as IL-12, IL-23, IL-6, and TNF-α, with low levels of IL-10. This cytokine signature indicates an anti-angiogenic macrophage, which also contributes to antibacterial and inflammatory immune functions. GM-CSF cultured macrophages inhibit CNV upon injection into the eyes of mice at the time of tissue injury.[66] In the presence of IL-10, IL-4, or IL-13, macrophages become polarized to a pro-angiogenic phenotype, with a cytokine signature of high levels of IL-10 and low levels of pro-inflammatory cytokines such as IL-6 and TNF-α. Interestingly, IL-10 production is elevated in macrophages from aged rodents and humans.[69,70] This suggests that as a macrophages age, their polarization may shift from an anti-angiogenic, pro-inflammatory phenotype to a pro-angiogenic, anti-inflammatory phenotype. Also, this suggests that one of the most important mediators of this phenotypic switch is IL-10.

As stated before, our laboratory has shown that mice that lack IL-10 are significantly impaired in their ability to generate CNV after laser-induced tissue injury to the eye. This work contrasts with the pro-angiogenic macrophage function that has been described in other CNV studies. Espinosa-Heidmann et al. and Sakurai et al. have shown that clodronate-liposome depletion of macrophages led to less CNV.[34,71] However, the work of Espinosa-Heidmann and colleagues involved the depletion of macrophages from old mice (>16 months of age). If macrophages from old mice (old macrophages) are skewed towards a pro-angiogeneic phenotype, then depletion of these macrophages may lead to reduced CNV. Our hypothesis that macrophages are anti-angiogenic in the eye is supported by the work of Lobov et al., who showed that macrophages are responsible for the induction of apoptosis in the vascular endothelial cells of the temporary hyaloid vessels of the developing eye,[72] and the work of Ambati et al., in which mice lacking a key macrophage recruitment chemokine (CCL-2) developed spontaneous CNV.[73] Taken together, this evidence suggests that there may be angiogenic differences in macrophages from young and old animals.

Additionally, as mentioned above, anti-oxidants reverse the age-associated dysfunction of macrophages by improving their inflammatory function.[61,62] It has also been found that supplementation of anti-oxidants to patients suffering from AMD delayed the progression of disease, including the development of CNV.[74] This further supports the hypothesis that macrophages function in an anti-angiogenic role in the eye, and that aging results in macrophage dysfunction, specifically related to the development of ocular blood vessels.

Our laboratory has recently performed experiments to specifically examine the ability of old macrophages to prevent neovascularization.[75] In our model of laser-induced CNV, old mice demonstrated significantly more neovascularization compared to young mice. This increased CNV seems to be due to the failure of old macrophages to prevent angiogenesis, as old macrophages injected into the eyes of young mice did not prevent CNV as effectively as when macrophages from young mice (young macrophages) were injected into the eyes of mice following laser-induced injury. We examined the cytokine profile of old macrophages in the eye following laser-induced injury, and we

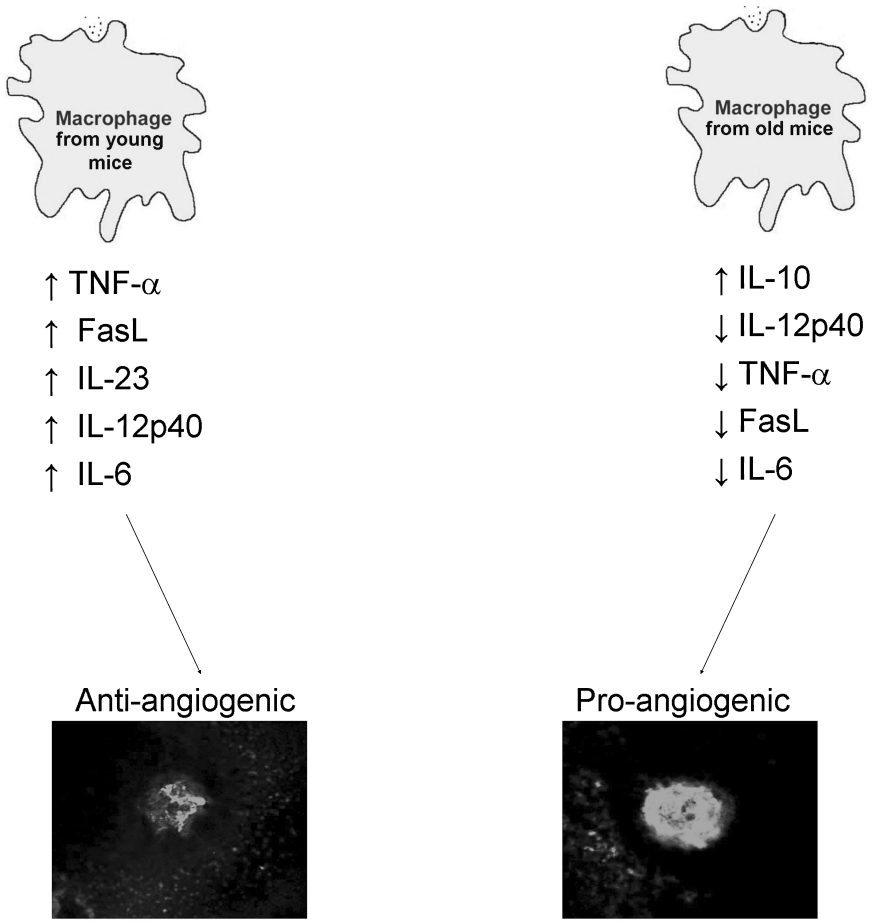

FIG. 1. The effect of senescence on macrophage cytokine expression and angiogenesis. Macrophages from young mice demonstrate increased levels of proinflammatory cytokines, including TNF-α, IL-23, IL-12, and IL-6. This results in an anti-angiogenic macrophage phenotype, leading to less choroidal neovascularization (CNV) following laser induced injury. Macrophages from old mice, however, demonstrate decreased levels of the same proinflammatory cytokines and increased levels of the immunosuppresive cytokine IL-10. These macrophages are skewed to an alternatively activated macrophage phenotype, resulting in increased CNV following laser-induced injury.

observed a significant downregulation of IL-12, TNF-α, and Fas ligand. These cytokines, however, were unaltered or upregulated in macrophages from young mice. Young macrophages upregulated the proinflammatory cytokines IL-6 and IL-23, whereas old macrophages did not.

Again, it seems that IL-10 is the key cytokine in determining the phenotypic switch of macrophages. Examining the levels of IL-10 in the eyes of young and old mice, IL-10 levels were significantly higher in old animals.[75] This suggests that the micromilieu in old mice might create an environment that promotes the polarization of infiltrating macrophages to a pro-angiogenic phenotype. The effect of IL-10 on macrophage polarization is further exemplified when we treated young macrophages with IL-10 and injected them into the eyes of laser-injured eyes; IL-10-treated macrophages failed to inhibit CNV.[75] This clearly highlights IL-10 as a pro-angiogenic cytokine and that macrophages, in the presence of IL-10, do not demonstrate anti-angiogenic properties.

We also demonstrated the effect of aging on macrophages and their ability to regulate angiogenesis *in vitro*. Thioglycollate-induced peritoneal macrophages were harvested and assessed for their ability to inhibit the growth and proliferation of vascular endothelial cells. Macrophages from young and IL-10 deficient animals significantly inhibited vascular endothelial cell proliferation, whereas macrophages from old mice had no effect.[75] This demonstrates a direct inability of senescent macro-

phages to inhibit the growth of blood vessel cells. The effect of aging on macrophage polarization and angiogenesis is summarized in Figure 1.

SUMMARY

Aging results in the dysfunction of both the innate and adaptive immune systems. Immunosenescence leads to increased susceptibility to infection and tumor formation and inflammatory diseases of aging such as osteoporosis and diabetes. Macrophages seem particularly susceptible to immunosenescence, and over time seem to polarize towards a pro-angiogeneic phenotype. This can result in the formation of unwarranted blood vessels and exacerbation of disease processes, such as AMD. Fortunately, the plasticity of macrophages renders them ideal targets for therapy, as cytokine/antibody treatment and gene therapy may result in a reversal of disease phenotype.

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