

On phagocytes and macular degeneration

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On Phagocytes and Macular Degeneration

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Abstract:

Age related macular degeneration (AMD) is a complex multifactorial disease caused by the interplay of age and genetic and environmental risk factors. A common feature observed in early and both forms of late AMD is the breakdown of the physiologically immunosuppressive subretinal environment and the protracted accumulation of mononuclear phagocytes (MP). We here discuss the origin and nature of subretinal MPs, the mechanisms that lead to their accumulation, the inflammatory mediators they produce as well as the consequences of their chronic presence on photoreceptors, retinal pigment epithelium and choroid. Recent advances highlight how both genetic and environmental risk factors directly promote subretinal inflammation and tip the balance from a beneficial inflammation that helps control debris accumulation to detrimental chronic inflammation and destructive late AMD. Finally, we discuss how changes in life style or pharmacological intervention can help to break the vicious cycle of inflammation and degeneration, restore the immunosuppressive properties of the subretinal space, and reestablish homeostasis.

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Abbreviations:

ABCA1 *ATP-binding cassette transporter A1*

AD Alzheimer's disease

aHUS atypical hemolytic uremic syndrome

AMD Age related macular degeneration

APOA-1 Apolipoprotein A-1

APOE apolipoprotein E

ARM age-related maculopathy

ATP adenosine triphosphate

AZ atrophic zone

Blam basal laminar deposits

BlinD basal linear deposits

BM Bruch's membrane

CCL2 chemokine (C-C motif) ligand 2

CCR2 C-C chemokine receptor type 2

CD11b/CD18 Complement 3 Receptor, Mac-1

CEPT cholesteryl transfer protein

CFH complement factor H

CNS central nervous system

CNV choroidal neovascularization

CS cone segments

CX3CL1 chemokine (C-X3-C motif) ligand 1

CX3CR1 CX3C chemokine receptor 1

DAMP damage-associated molecular pattern

DC dendritic cells

FASL Fas ligand

GA geographic atrophy

GAG glycoaminoglycans

HDL high-density lipoprotein

HTRA1 high-temperature requirement A serine peptidase 1

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IBA1 ionized calcium-binding adapter molecule 1

IIRC innate immunity receptor cluster

IL-1β Interleukin 1 β

iMϕ inflammatory macrophages

KPSG keratan sulfate proteoglycans

LAP latency-associated peptide

LCAT lecithin:cholesterol acyltransferase

LDL low-density lipoprotein

LPS lipopolysaccharide

LTBP latent TGF-β binding protein

MAC membrane attack complex

MC microglial cells

Mo monocytes

MP mononuclear phagocytes

MPGN II Membranoproliferative glomerulonephritis, type II, dense-deposit disease

NFκB nuclear factor κB

NLR Nod-like receptors

NLRP3 NOD-like receptor family, pyrin domain containing 3

OTX2 orthodenticle homeobox 2

P2RX7 P2X purinoceptor 7

PAMP pathogen-associated molecular pattern

RAP retinal angiomatous proliferations

RCT reverse cholesterol transport

RDH5 retinol dehydrogenase 5

rMϕ tissue resident macrophages

RP retinitis pigmentosa

RPE retinal pigment epithelium

SCR short Consensus Repeat domains

SIRP1α Signal regulatory protein α

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TGF-β tumor growth factor β

TLR toll like receptor

TNF-α Tumor necrosis factor α

TRE2/3/4 targeted replacement mice expressing the human APOE-isoforms

TSP-1 Thrombospondin 1

TTR transthyretin

TZ transitional zone

VLDL very-low density lipoproteins

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1. Age related macular degeneration

The macula consists of a small cone-dominated fovea, responsible for high acuity vision, surrounded by a rod-dominated parafovea and peripheral retina. The degeneration of the macula and central retina is a common medical problem called age-related macular degeneration (AMD), and a leading cause of visual impairment in the world (Wong et al., 2014). Early AMD, also called age-related maculopathy (ARM) (Bird et al., 1995), is characterized by pigmentary abnormalities and accumulation of membranous, lipoproteinaceous debris located between the basal lamina of the retinal pigment epithelium (RPE) and the inner collagenous layer of Bruch's membrane (BM) of the central retina. They can either take the form of basal linear deposits (BlinD) or clinically visible sizeable (>125 μm), ill-defined protrusions called large (soft) drusen (Curcio and Millican, 1999; Sarks, 1976). The protrusions deform the over-lying retina leading to metamorphopsia, and are associated with a local thinning of the photoreceptor cell layer (Schuman et al., 2009) and local loss of sensitivity (Midena et al., 2007). Eyes with large-sized soft Drusen can progress and develop late AMD (~ 15% in the Beaver Dam study over 10 years; ~30% in the Blue mountain study over 6 years), regress (~ 25%, Beaver Dam study) or stay stable for years (Klein et al., 2004; Wang et al., 2003). It is not clear whether the constituents of drusen are derived from the RPE, choroidal vasculature or both. Additionally, reticular pseudodrusen are strongly associated with AMD (Klein et al., 2008b; Mimoun et al., 1990). They are ~30-150 μm-sized pale fundus lesions that are believed to be caused by subretinal drusenoid deposits located between the RPE and photoreceptors (Alten and Eter, 2014; Rudolf et al., 2008; Zweifel et al., 2009). Small, globular, well-defined, hard drusen (<63 μm) are not associated with AMD when few are present, but large areas of macular hard drusen are a risk factor for ARM (Klein et al., 2004).

ARM afflicts more than 150 million people worldwide and 10 million people suffer

from late AMD (Wong et al., 2014). The reasons that patients progress to late AMD are incompletely understood, but the interactions of certain protective and predisposing genetic variants, alongside with environmental factors, seem to play a role (Yu et al., 2012). There are two clinical forms of late AMD: exudative AMD and geographic atrophy (GA) (Sarks, 1976): In exudative AMD, subretinal neovascularization develops below (occult) or above (classic) the RPE. They originate for the most part from the choroid (choroidal neovascularization; CNV), but in about 10-15% of exudative AMD, subretinal neovascularization arises intra-retinally from the retinal vasculature (retinal angiomatous proliferations; RAP) (Ghazi, 2002; Yannuzzi et al., 2001). Interestingly, eyes with CNV also present a loss of choriocapillaries in the surrounding areas (McLeod et al., 2009). CNV is commonly associated with retinal edema, subretinal exudation as well as blood and lipid deposits. Untreated, exudative AMD leads to the formation of a neovascular fibrous membrane and the formation of a disciforme subretinal scar (Sarks, 1976). In recent years, treatments for neovascular AMD have dramatically improved with the development of anti-angiogenic therapy (anti-vascular endothelial growth factor (VEGF)). The anti-angiogenic therapy decreases the permeability and inhibits the formation of neovessels and the associated degenerative changes. It does however not halt vessel-independent degenerative processes and the decline in visual functions in 30% of patients that occurs in the long term (Rofagha et al., 2013).

In GA, an extending atrophic zone (AZ) forms, characterized by the loss of the RPE and degeneration of the photoreceptor cell layer (Sarks, 1976). The initial lesion in GA often develops parafoveally (Sarks et al., 1988) and slowly expands through the central retina and eventually the fovea. In the AZ, despite the absence of RPE, residual cones (and to a lesser extent rods) survive, but they lack their inner and outer cone segments (CS) necessary for light perception (Bird et al., 2014; Eandi et al., 2016). Similar findings are observed in disciform subretinal scars, the end-stage of exudative AMD (Curcio, 2001). In a perilesional

transitional zone (TZ), directly peripheral to the area of RPE-loss of the scar and AZ, the number of rods drops dramatically compared to regions more distant from the lesion, despite the presence of the RPE (Bird et al., 2014; Curcio, 2001; Eandi et al., 2016). These anatomical changes translate clinically to decreased perilesional retinal sensitivity (Meleth et al., 2011). The number of cones changes little in the TZ, but they lack their cone segments (CS) (Bird et al., 2014; Curcio, 2001; Eandi et al., 2016). One could assume that CS loss in the TZ of AMD patients is due to a primary RPE dysfunction, however CS loss is also observed in patients with retinitis pigmentosa (RP) with rod-gene mutations and unremarkable RPE (Mitamura et al., 2013). Clinically GA lesions provoke central scotomas, which severely affect visual acuity when the lesion involves the fovea itself. Patients with late AMD also suffer from impaired dark adaptation, which can be measured as an increase in recovery time after bleach, (Flamendorf et al., 2015; Owsley et al., 2001). During dark adaptation, the bleached retinal (all-trans retinal) of the rod outer segments is transported into the RPE where it is re-isomerized into 11-cis-retinal by the isomerohydrolase RPE65 and retinol dehydrogenase 5 (RDH5). Together, this complex process is called the visual cycle. Any slowing of the visual cycle, will increase both dark adaptation and recovery times after bleach, as observed in RDH5-deficient patients (Cideciyan et al., 2000). Interestingly, a significant increase in recovery time after bleach is already observed with reticular pseudodrusen in ARM (Flamendorf et al., 2015; Owsley et al., 2001) and is associated with an increased incidence of AMD (Owsley et al., 2016) and this quite some time before visible RPE or photoreceptor lesions occur. There is currently no therapy that has been approved for atrophic AMD.

AMD is a common, complex disease that results from interplay of aging, and genetic and environmental risk factors. The importance of genetic factors is illustrated by the fact that the AMD-risk increases between 5- and 10-fold when a parent or sibling is affected

(Chakravarthy et al., 2010; Shahid et al., 2012), making AMD one of the most heritable complex diseases. Polymorphisms in the complement factor H (CFH), the Apolipoprotein E isoforms and on chromosome 10q26 (that harbors the gene for the high-temperature requirement A serine peptidase 1 [HTRA1] and age-related maculopathy susceptibility 2 [ARMS2]) are the major genetic factors that predispose to AMD (Swaroop et al., 2007). Age, smoking, obesity, and possibly exposure to light are the main environmental factors, but other factors such as hypertension might also play a role (Adams et al., 2011; Chakravarthy et al., 2010; Schick et al., 2016). How the coaction of these mainly non-ocular risk factors fuels the pathomechanisms of AMD remains unclear but will be discussed in this article.

Current hypotheses on the pathomechanisms of AMD can be grouped into three main axis: (1) choriodal vascular insufficiency results in hypoxia, CNV and dysfunction and death of RPE and photoreceptors; (2) dysfunction of RPE (induced by oxidative stress or complement activation) impairs photoreceptor renewal and leads to progressive accumulation of deposits at the basal membrane of the RPE, visible as drusen, that ultimately cause hypoxia and CNV, or progressive loss of RPE and photoreceptor cells; (3) chronic non-resolving inflammation (mainly the accumulation of MPs and over-activation of the innate immune system) leads to neovascularization and degeneration of RPE and photoreceptor cells. None of theses hypothesis is exclusive and they likely all play into the disease development to different degrees in individual patients. This review will concentrate on the possible implication of phagocytes and their mediators in the pathogenesis of AMD.

2. Mononuclear phagocytes

Mononuclear phagocytes (MPs) comprise a family of cells that include monocyte (Mo), monocyte-derived inflammatory macrophages (iMϕ), dendritic cells (DC), and tissue resident macrophages (rMϕ) such as microglial cells (MCs) (Chow et al., 2011; Gautier et al., 2012; Ransohoff and Cardona, 2010; Wynn et al., 2013). rMϕs, which serve trophic as well as

sentinel roles, are present in virtually all organs and develop early during embryogenesis or pre-natal life from either embryonic Mϕs found in the yolk-sac or from embryonic monocyte found in the liver, respectively (Gautier and Yvan-Charvet, 2014). In the retina, microglia are involved in the clearance of apoptotic neurons and pruning of synapsis in retinal development, and constitutively required for the maintenance of synaptic structure in the adult retina and for synaptic transmission underlying normal visual function (Wang et al., 2016). The vast majority of long-lived rMφs are not replaced by Mos in the adult, but rather self-renew by proliferation. Few exceptions might remain as for a sub-population of rMφs in the gut (Gautier and Yvan-Charvet, 2014). During inflammation, short-lived circulating Mos that strongly express the chemokine receptor CCR2 are recruited to the inflammatory site where they differentiate into iMϕs (Geissmann et al., 2003). Thus, under inflammatory conditions, Mϕs from different origins cohabite but little is known about their specificity of function and action. At the end of the inflammatory response, iMϕs disappear from the site, contrary to rMφs that are dedicated to remain in tissues after resolution and return to homeostasis.

In summary, distinct types of resident and infiltrating MPs, exert distinct functions during tissue development, inflammation and homeostasis. Insight on individual roles of each type of MP has been complicated given the limitation of currently available tools and the immunohistochemical similarities of these cell populations in diseased tissues, as they constitutively express or induce similar markers (Gautier et al., 2012; Ransohoff and Cardona, 2010).

2.1. Mononuclear phagocytes in AMD

Physiologically, the posterior segment of the eye contains several types of MPs. Numerous rMϕs reside in the choroid, a network of MCs is located in the inner layers of the retina, perivascular rMϕs are found along the major retinal and choroidal vessels, while DCs are rare in the retina (Forrester et al., 2010; Kumar et al., 2014; Streilein, 2003). The adult

central photoreceptor cell layer and subretinal space (located between the RPE and the photoreceptor outer segments) are physiologically devoid of resident and infiltrating MPs in healthy subjects (Combadiere et al., 2007; Eandi et al., 2016; Gupta et al., 2003; Lad et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013). Subretinal MPs, can however be found in the extreme periphery and during fetal development of the photoreceptor cell layer (McMenamin and Loeffler, 1990).

Histological evidence of the association of subretinal leucocytes and exudative AMD has first been described in 1916 (Hegner, 1916) and in numerous publications since (Penfold et al., 2001). A number of studies have observed inflammatory cells, particularly MPs, but also lymphocytes in excised neovascular membranes from patients with AMD (Gehrs et al., 1992; Lopez et al., 1991; Oh et al., 1999; Seregard et al., 1994).

In GA, MPs have been observed within the atrophic area (Combadiere et al., 2007; Eandi et al., 2016; Gupta et al., 2003; Lad et al., 2015; Levy et al., 2015a; Penfold et al., 2001), on RPE cells of the TZ and on large drusen (Eandi et al., 2016; Gupta et al., 2003; Lad et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013), within large drusen and BlinD (Lad et al., 2015; Sennlaub et al., 2013), and their numbers in the choroid increase compared to controls (McLeod et al., 2016). The MPs were identified using the MP markers Ricinus communis agglutinin-I (Gupta et al., 2003), CX3CR1 (Combadiere et al., 2007), CD18 (Combadiere et al., 2007; Levy et al., 2015a; Sennlaub et al., 2013), IBA1 (Sennlaub et al., 2013), CCR2 (Sennlaub et al., 2013), CD163 (Lad et al., 2015), and CD14 (Eandi et al., 2016). Interestingly, the classically used marker CD68 only reveals negligible numbers of MPs in sections of patients with AMD, which has been suggested to be the reason why changes in the MP population went unnoticed in some earlier studies (Lad et al., 2015). IBA-1-staining (to visualize subretinal MPs) of central RPE flatmounts, reveal that IBA-1+MPs are seldom present in healthy age-matched central donor RPE (Eandi et al., 2016; Levy et al., 2015a) and

in donors with small drusen that are not associated with AMD (Fig. 1 A and B). However, they are invariably observed on large drusen, within the AZ of patients with GA, and on the apical side of the RPE in the TZ adjacent to GA lesions (Levy et al., 2015a) (Fig. 1 C and D).

Interestingly, a subset of MPs might be clinically visible in AMD patients. A significant number of MPs (identified using immunohistological markers) in AMD donor eyes contain melanosomes, presumably from ingested RPE debris (Lad et al., 2015; Sennlaub et al., 2013). We have recently shown, using serial in vivo flood-illumination adaptive optics imaging (FIAO), that highly mobile melanin-containing cells (MCCs), of a similar size to the observed MPs, precede and accompany the emergence and progression of atrophy in GA (Gocho et al., 2013). Examination by Optical Coherence Tomography (OCT), identifies similarly sized hyper-reflective dots, proposed to be MCCs, that are associated with photoreceptor loss in ARM (Schuman et al., 2009), predictive progression from ARM to GA (Christenbury et al., 2013; Leuschen et al., 2013) and associated with AMD-genetic risk variants (Altay et al., 2016). It is thus tempting to speculate that MCCs are melanin-containing MPs, possibly additionally to migrating RPE cells (Zanzottera et al., 2015). Surprisingly this view was recently contested (Curcio and Ach, 2016), even though evidence for MCCs being MPs is supported by immunohistochemical data using three MP markers (CD163, IBA-1 and CCR2), while the existence of migrating RPE cells in AMD is solely based on the histological observation of melanin containing cells without the use of an additional RPE cell marker.

Contrary to fast evolving autoimmune lesions, characterized by cytotoxic T lymphocytes, neutrophils and MPs (Caspi, 2002; Kerr et al., 2008), infiltrating leukocytes in slowly-evolving GA are predominantly MPs which is similar to other protracted age-related diseases including atherosclerosis, neurodegenerative diseases and cancer (Grivennikov et al., 2010; Hotamisligil, 2010), However, the choroid of patients with AMD and surgically removed neovascular membranes are infiltrated by lymphocytes, (Ezzat et al., 2008; Lopez et

al., 1991; Penfold et al., 1984; Penfold et al., 1985), including $IL17^{+}$T lymphocytes (Camelo et al., 2016). Patients with GA are also characterized by an increased number of degranulated (activated) choroidal mast-cells (Bhutto et al., 2016). While it has been suggested that the absolute number of infiltrating MPs during progression of AMD is too low to play a meaningful pathogenic role in either CNV or retinal degeneration, a growing number of animal studies have demonstrated that a comparable infiltrate in mice plays an important role in both, CNV and photoreceptor degeneration (see 4. and 5.2).

Additionally, increased intra-ocular concentrations of CCL2, a potent chemokine that attracts Mo to diseased tissue, are observed in humans with neovascular- (Fauser et al., 2015; Jonas et al., 2010; Kramer et al., 2012; Rezar-Dreindl et al., 2016) and atrophic-AMD (Newman et al., 2012; Sennlaub et al., 2013). Moreover, from a systemic perspective, patients whose Mos express the greatest amount of TNFα, have a higher prevalence of choroidal neovascularization (Cousins et al., 2004), and circulating blood Mo of patients with AMD express higher levels of CCL2, IL8, and VEGF (Lechner et al., 2017). The percentage of IL-6-expressing circulating Mos is higher in patients with neovascular AMD (Lechner et al., 2017) and these Mos more generally display an altered immune-related transcription signature (Grunin et al., 2016). Increased serum levels of IL-6 correlate with the incidence of ARM (Klein et al., 2014) and with late AMD (Klein et al., 2008a; Seddon et al., 2005).

In summary, AMD is associated with systemic pre-activation of circulating Mos, and a local infiltration of the photoreceptor cell layer by MPs (iMφs and rMφs) around large drusen, in and around GA lesions and adjacent to choroidal neovascularization (Fig. 2). Their presence on AMD-associated large drusen, in the advancing TZ of GA lesions as well as in CNVs puts them at the right time and place to possibly partake in early pathogenic changes of AMD, the growth of the GA lesion, and ultimately, in the formation CNV.

2.2. Mononuclear phagocytes and inflammation

2.2.1. Inflammation, a crucial process for survival

MPs survey the local tissue environment, and are involved in innate and adaptive immune responses that occur during inflammation induced by tissue injury (sterile inflammation), or infection (the invasion of potentially disease-causing agents). Most often tissue injury, such as injury of the skin and mucous membranes, is associated with the invasion of microbes from the commensal flora, or worse, with pathogens. This invasion will quickly become life threatening if their proliferation and spread is not controlled. The inflammatory process is typically triggered by activation of tissue mast cells and rMϕs, followed by early neutrophil recruitment, and an infiltration of blood Mos that differentiate into Mϕs. These recruited neutrophils and Mϕs first display an anti-microbial and pro-inflammatory phenotype (iMϕs), characterized by bactericidal mechanisms (release of reactive oxygen species and complement, and phagocytosis of opsonized pathogens) and secretion of inflammatory cytokines (such as IL-1β, TNF-α, IL-6, and CCL2) that alter the tissue to facilitate the immune response (vaso-dilation, increased permeability, increased sensitivity (Wynn et al., 2013)). The rapid differentiation of Mos into iMϕs requires considerable amounts of energy and building materials, as the small precursor Mo with little cytoplasm, transforms into an iMϕ that is several times its initial size and secretes a number of bactericidal mediators and cytokines. iMϕs meet this demand in energy and substrates via increased glucose consumption, and a predominantly glycolytic metabolism with minimal reliance on oxidative phosphorylation in the Krebs cycle (similar to the Warburg effect in tumors) (Kelly and O'Neill, 2015). This switch allows iMϕs to use oxygen to produce bactericidal reactive oxygen species and citrate to (i) produce antimicrobial itaconic acid, (ii) generate large amounts of fatty acids, required for the synthesis of new membrane lipids, arachidonic acid, and prostaglandins (iii) synthesize oxaloacetate, necessary to generate

reactive oxygen species and nitric oxide (El Kasmi and Stenmark, 2015). Due to this metabolic switch, iMϕs are very reliant on surrounding glucose concentrations for survival and function, which they insure by inducing insulin resistance via IL-1β and TNF-α in adjacent stromal cells, thereby decreasing their competing glucose consumption (Medzhitov, 2008; Shoelson et al., 2006). The release of inflammatory cytokines in microscopic injuries of our skin and mucosae that characterize our daily life does not cause symptoms, but they are at the origin of the cardinal features of inflammation in bigger localized- (calor, dolor, rubor, tumor (Nathan, 2002)) and systemic- (sleepiness (Roerink et al., 2017), fever, hyperglycemia (McGuinness, 2005)) infections and can participate in shock and organ failure if the infection gets out of hand.

At the site of injury, once the wound is disinfected, neutrophils undergo death within hours and are cleared together with tissue debris by Mϕs. Phagocytosis of dead neutrophils and other stimuli trigger the production of mediators, which generate Mϕs that facilitate tissue repair, scar formation, and inflaation resolution (Fadok et al., 2001; Huynh et al., 2002). Finally, iMϕs disappear from the site and the tissue is left with the tissue-specific rMϕ, as before the injury. Similar to neutrophils, iMϕs are mostly eliminated by local death rather than clearance through emigration to lymph nodes (Gautier et al., 2013). It is not clear what triggers the death of iMϕs in inflammation resolution. It might be due to the decrease of growth factors and inflammatory cytokines necessary for their survival, an intrinsic maximal life span and a death rate that surpasses the recruitment of new iMϕs, or an active induction of apoptosis by stromal cells or other leukocytes.

In summary, injury and infection potentially coincide. The innate immune system reacts very quickly, to terminate the spread of a possible infection that would otherwise cause sepsis and death but there may be a cost of further tissue damage. In Carl Nathan’s words:

"Evolution did not anticipate surgery with aseptic technique. Thus, the body reacts to trauma as if the emergency is infection, until proven otherwise." (Nathan, 2002).

2.2.2. Nonresolving inflammation

In order to re-establish tissue homeostasis after the elimination of persistent tissue stress, invading pathogens and tissue repair, the inflammatory reaction needs to rapidly and efficiently resolve. If the inflammatory response is not quickly controlled, it can become pathogenic and contribute to disease progression, as seen in many chronic inflammatory diseases. Nonresolving and low-grade chronic inflammation (also called dysregulated parainflammation, see 5.1. (Medzhitov, 2008)) signifies that tissue homeostasis was not reestablished. It is observed in contexts such as metabolic diseases (obesity, atherosclerosis), neurodegenerative diseases and cancers (Glass et al., 2010; Grivennikov et al., 2010; Hotamisligil, 2010). Nonresolving inflammation is not a primary cause or trigger of disease, but it contributes significantly to pathogenesis as pro-inflammatory mediators produced by neutrophils and iMϕs (reactive oxygen species, proteases and inflammatory cytokines, etc.) can also cause considerable collateral damages to host cells, which itself fuels inflammation. It is often not clear if chronic inflammation persists because of a continuous primary problem or the incapacity to exit a vicious circle that leads to collateral damage and fosters inflammation. Perhaps no single phenomenon contributes more to the medical burden in industrialized societies than nonresolving inflammation, as it is involved in many of the most prevalent diseases (Nathan, 2002).

3. The immuno-suppressive environment of the retina

3.1. Immune privilege and immune suppression

Certain sites of the human body (eye, brain, testis, ovaries) are considered as "immune privileged", meaning that innate and adaptive immune responses are dampened or suppressed in these tissues, possibly because they are particularly vulnerable to inflammation induced collateral damage (Streilein, 2003), and the damage to these tissues puts the fitness of the

individual at risk (survival or reproductive capacity). All tissues are potentially threatened by pathogen invasion. The immune response consists of a compromise between fast effective elimination and neutralization of pathogens, and avoidance of collateral tissue damage that interferes with vital functions. In particular, the skin and mucous membranes are most exposed to tissue injury and microbial invasion, but also have one of the greatest regenerative capacities. On the other extreme, the retina and brain are especially vulnerable to immunopathogenic damage as they have very limited regenerative capacities, but they are endowed with structures that protect them particularly well from direct infection (cranium, sclera, eye lids), and also from blood-born microbial invasion (blood-tissue barriers). Additionally, these tissues are sites of "immune privilege", which further protects them from inflammation-mediated injury.

Strictly speaking the term "immune privilege" describes the observation first reported by Medawar in 1948 (Medawar, 1948) that skin allografts introduced into an "immune privileged" site or organ such as the brain and the eye anterior chamber do not elicit an immune response and are not rejected, unless the recipient animal was previously immunized against the graft. Nowadays, the term immune privilege is often more widely used to describe organs or sites where the innate and adaptive immune reaction is diminished and inflammation reduced. Factors that determine immune privilege include the lack of DCs and a lymphatic drainage system (*e.g.* eye and brain) through which antigen-presenting cells migrate to the lymph nodes, the lack of blood vessels through which effector cells infiltrate the tissue (cornea, subretinal space), and locally produced factors that induce immune tolerance. Importantly, this privilege is also mediated by tonic inhibitory signals in the retina that set the threshold for activation high and the particularly efficient clearance of infiltrating inflammatory cells (immunosuppressive microenvironment) compared to non-immune privileged tissues (Streilein et al., 2002) (Fig. 3). In that way, potential antigen-presenting

cells and effector cells (lymphocytes, Mϕs) can be neutralized before they develop cytotoxicity.

3.2. Tonic inhibitory signals in the retina

Physiologically, neurons express a number of factors that continually repress MC activation, such as CX3CL1, CD200L, SIRP1α, CD22 (Galea et al., 2007), and translocator protein (Karlstetter et al., 2014; Wang et al., 2014b) among others. *CX3CL1 is an atypical chemokine. It is expressed as a transmembrane protein that mediates integrin-like intracellular adhesion and can be cleaved by proteases into a soluble form that has chemotactic properties (Bazan et al., 1997). CX3CL1 has only one receptor, CX3CR1. In blood, CX3CR1 is expressed on "non inflammatory" or "patrolling" Mos* (~10% of circulating Mos in humans) (Geissmann et al., 2003) and its genetic deletion in mice *reduces MP accumulation in diseases of peripheral tissues, where "patrolling" Mos partake in the MP infiltrate (Combadiere et al., 2003)*. Among MPs, rMϕs express high levels of CX3CR1, which is by far the highest in MCs (Gautier et al., 2012). CX3CR1/CX3CL1 signaling between neurons and MCs plays important roles in *synaptic pruning, transmission and plasticity and* additionally mediates a tonic inhibitory signal on MCs *(Limatola and Ransohoff, 2014). In mouse experimental models of brain pathologies, Cx3cr1-deficiency most often leads to increased neuroinflammation and degeneration (Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, epilepsy (Cardona et al., 2006; Limatola and Ransohoff, 2014 ; Wolf et al., 2013)) except in models of Alzheimer's disease, where uninhibited Apolipoprotein E secretion from Cx3cr1$^{-/-}$MPs might explain its protective effect (Lee et al., 2010) (see 6.1.1.).* In the eye, transmembrane CX3CL1 is constitutively expressed in inner retinal neurons (Zieger et al., 2014). We and others showed that genetic deletion of Cx3cr1 in mice leads to an accelerated age-dependent increase of subretinal MPs in pigmented animals in both *Cx3cr1*$^{-/-}$ knockout (Combadiere et al., 2007) and *Cx3cr1*$^{GFP/GFP}$ knockin mice (Chinnery et al.,

2011; Combadiere et al., 2007; Levy et al., 2015a; Sennlaub et al., 2013) compared to wildtype animals kept in the same conventional light conditions (~250lux). *Cx3cr1*-deletion also increases MP accumulation in young albino mice (Chinnery et al., 2011; Combadiere et al., 2007), and in pigmented mice exposed to a non-toxic light-challenge that does not induce degeneration in control mice (Levy et al., 2015a; Sennlaub et al., 2013). Raising albino *Cx3cr1*$^{-/-}$mice in darkness (Combadiere et al., 2007) or letting pigmented C57BL/6 *Cx3cr1*$^{GFP/GFP}$ mice age in very dim light conditions prevents the accumulation (Combadiere et al., 2013; Luhmann et al., 2013), which confirms the importance of light in subretinal MP accumulation (Ng and Streilein, 2001). Furthermore, the age- and light-dependent accumulation of subretinal *Cx3cr1*-deficient MPs is associated with a significant loss of rods (Calippe et al., 2017; Chinnery et al., 2011; Combadiere et al., 2007; Hu et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013), cone segments (in acute models such as light-challenge) (Eandi et al., 2016), and cone numbers (in chronic age-related inflammation) (Calippe et al., 2017). The subretinal accumulation of MPs in *Cx3cr1*-deficient mice *is not associated with drusen development or RPE atrophy*, but the MP accumulation in combination with rod and cone degeneration observed in these mice is strikingly similar to the TZ of GA patients *(Eandi et al., 2016)*. Taken together, the deletion of *Cx3cr1*, a gene that is exclusively expressed on MPs (and not on RPE or retinal neurons) in combination with aging under normal light conditions is sufficient to trigger a pathogenic non-resolving subretinal inflammation with age. The *Cx3cr1 knockout mouse therefore represents a model of "primary" subretinal inflammation, where inflammation is not the consequence of a tissue injury but solely due to "over reacting" MPs, triggered by a non-toxic initiating stimulus (aging under normal light conditions). In the inflammatory reaction that appears secondary to tissue injury, such as a laser-injury*, Cx3cr1$^{-/-}$-mice develop exaggerated MP accumulation and associated amplified CNV (Combadiere et al., 2007). A strikingly similar phenotype is observed in CD200R$^{-/-}$mice

(Horie et al., 2013) that lack the tonic inhibitory signal of neuronal CD200 (Broderick et al., 2002).

Mechanistically, we demonstrated that *Cx3cr1*-deficient MPs are characterize by an over-expression of surface P2RX7, which stimulates IL-1β maturation and secretion (Hu et al., 2015) (see 5.2.2.1.) and by increased apolipoprotein E secretion (Levy et al., 2015a) (also observed in muscle repair (Arnold et al., 2015)) that activates the expression of inflammatory cytokines (see 6.1.1.). The increased expression of inflammatory cytokines leads to the recruitment of neurotoxic blood-derived iMϕs (CCL2), the resistance to subretinal MP clearance (IL-6), and photoreceptor toxicity (IL-1β) in *Cx3cr1*-deficient mice (see 4., 5.2.2.3, and 5.2.2.1. respectively) (Eandi et al., 2016; Hu et al., 2015; Sennlaub et al., 2013). Cx3cr1 deletion also increases MP accumulation and associated degenerative changes in other retinal disease models, such as diabetes (Beli et al., 2016; Cardona et al., 2015; Kezic et al., 2013; Mendiola et al., 2016), retinitis pigmentosa (Peng et al., 2014; Zabel and Kirsch, 2013), Glaucoma (Wang et al., 2014a) and paraquat-induced retinopathy (Chen et al., 2013). Importantly, increased CCL2, APOE and IL6 expression are also observed in AMD (Anderson et al., 2001; Jonas et al., 2010; Klaver et al., 1998; Klein et al., 2008a; Levy et al., 2015a; Seddon et al., 2005; Sennlaub et al., 2013). *Cx3cr1*-deficient mice might therefore share important pathogenic similarities with human disease. Although they *do not mimic all aspects of AMD such as drusen formation and RPE atrophy, they do model subretinal MP accumulation and associated photoreceptor degeneration, which are important features of AMD (Combadiere et al., 2007; Eandi et al., 2016; Gupta et al., 2003; Levy et al., 2015a; Sennlaub et al., 2013).* Furthermore, CX3CL1 expression in the brain decreases with age (Fenn et al., 2013) and *Cx3cr1* polymorphisms have been associated with AMD in several studies (Anastasopoulos et al., 2012; Combadiere et al., 2007; Schaumberg et al., 2014; Tuo et al., 2004; Zhang et al., 2015), which suggest a possible direct role of CX3CL1/CX3CR1

signaling in AMD. In addition, in animal models, the injection of soluble CX3CL1 has been shown to restore tonic MP inhibition and curb pathogenic inflammation (Mendiola et al., 2016; Zabel and Kirsch, 2013) and might be useful to treat inflammation in patients.

In summary, constitutive repression of MP activation by neuronal inhibitory signals is essential for retinal homeostasis. The lack of only one of these signals, as observed in Cx3cr1-deficient mice, can be sufficient to trigger a vicious cycle of chronic inflammation and collateral damage under normal "healthy" aging conditions that do not cause any significant degeneration in age-matched Cx3cr1-competent mice.

3.3. The immunosuppressive retinal pigment epithelium

3.3.1. Fas/FasL signaling

Additionally to the tonic inhibitory signals of the inner retina that restrict the activation of retinal MCs, the subretinal space is an actively immunosuppressive environment. The subretinal space harbors the photoreceptor outer segments and is shielded from the surrounding tissue by tight junctions of the RPE and zonula adherens between the photoreceptors and Müller glial cells that form the outer limiting membrane (van de Pavert et al., 2004). It is devoid of blood and lymphatic vessels that facilitate leukocyte infiltration and egress in other tissues and the RPE expresses immunosuppressive mediators that induce the death of infiltrating immune cells. In fact RPE allografts survive in non-immunosuppressed hosts for prolonged periods of time even when grafted to non-immune privileged sites, such as the kidney capsule (Wenkel and Streilein, 2000), where conjunctival control cells are quickly rejected. Interestingly, this immunosuppressive capacity is dependent on RPE FasL expression, as FasL-deficient RPE (obtained from $FasL^{gld/gld}$ mice with inactive FasL) is quickly destroyed under the same circumstances (Wenkel and Streilein, 2000). However, FasL over-expression alone in an allograft, such as a Langerhans cell graft, is not sufficient to convey immune privilege (Kang et al., 1997). Therefore, FasL expression by RPE allografts

seems a necessary but not sufficient factor to escape immune rejection and other factors expressed by the RPE or MPs must be involved. In agreement with the transplant experiments described above, we reported that FasL-defective (*FasL*$^{gdl/gld}$-mice) and Fas-defective (*Fas*$^{lpr/lpr}$-mice) mice develop significant subretinal MP accumulation after a light-challenge (designed to cause little inflammation and no damage in wild-type mice) (Levy et al., 2015a). This increase in subretinal MPs is likely the result of their deficient elimination, as subretinally injected MPs survived better when FasL/Fas signaling was defective (WT MPs injected in *FasL*$^{gld/gld}$ mice and *Fas*$^{lpr/lpr}$ MPs injected in WT mice compared to WT MPs injected in WT mice) (Levy et al., 2015a). Interestingly, in the resolution phase of lung injury *in vivo*, FasL preferentially eliminates iMϕs and not rMϕs (Janssen et al., 2011) and a Fas agonist induced monocyte but not Mϕs apoptosis *in vitro* in our hands (Levy et al., 2015a). In contrast, our experiments showed that Mo, Mϕ and MCs all undergo apoptosis when adoptively transferred to the subretinal space, suggesting that a synergism of different factors acts to physiologically eliminate all types of MPs in contact with the RPE (Levy et al., 2015a).

3.3.2. TSP-1/CD47 signaling

Similarly to *FasL*$^{gld/gld}$ and *Fas*$^{lpr/lpr}$ mice, Thrombospondin 1 (TSP-1)- deficient mice display increased and prolonged subretinal inflammation with age, and after light- and laser-induced injury (Ng et al., 2009; Wang et al., 2012), and develop more severe uveitis (Chen et al., 2012a). They also develop increased inflammation in a variety of peripheral diseases (extensive acute pneumonia, leukocytosis, pancreatitis, and inflammatory infiltrates in the lacrimal glands) (Lopez-Dee et al., 2011). TSP-1 is synthesized by a wide variety of cell types, notably the RPE (Miyajima-Uchida et al., 2000), iMφs (Fordham et al., 2012) and rMφs (Gautier et al., 2013) and plays a role in multiple biological processes including phagocytosis, angiogenesis, and immune regulation (Bornstein, 2009; Housset and Sennlaub, 2015; Lopez-Dee et al., 2011). It contains a Willebrand type C domain, necessary for its trimerization,

followed by three properdin-like type 1 repeats, which include the regions that bind the CD36 receptor (anti-angiogenic signals (Jimenez et al., 2000)) and the latency-associated peptide (LAP) of the latent TGF-β binding protein (LTBP). TSP-1 thereby binds CD36 and LTBP and liberates active TGF-β (Bornstein, 2001; Lawler and Hynes, 1986). The COOH-terminal cell-binding domain (CBD) of TSP-1 contains two valine-valine-methionine (VVM) sequences that can each interact with a CD47 receptor (Bornstein, 2001; Lawler and Hynes, 1986) (Fig. 4). Independently of its role as a TSP-1 receptor, the N-terminal domain of CD47, also acts as a ligand for the signal regulatory protein alpha (SIRPα) of MPs and inhibits phagoptosis ("don't eat me" signal) (Navarro-Alvarez and Yang, 2011). The efficient activation of CD47 by TSP-1 is dependent on the presence of both VVM sites of TSP-1 (McDonald et al., 2003), but can be mimicked by the TSP-1-derived 4N1K peptide (KRFYVVMWKK) (Martinez-Torres et al., 2015), at 100 fold higher molar concentrations (McDonald et al., 2003). CD47 activation has been shown to sensitize endothelial cells and lymphocytes to FasL-induced death in a CD47-dependent manner (Manna et al., 2005; Quesada et al., 2005). We recently confirmed that $Tsp1^{-/-}$-mice develop age-, and exaggerated light- and laser-induced subretinal MP accumulation. Interestingly, this phenotype was shared by $CD47^{-/-}$, but not $CD36^{-/-}$mice (Calippe et al., 2017). $Tsp1^{-/-}$- and $Cd47^{-/-}$-MCs, adoptively transferred to the subretinal space of wildtype recipients significantly resisted the elimination observed in wildtype MCs and recombinant TSP1 very significantly accelerated the elimination of wiltype-MCs, reversed the phenotype of $Tsp1^{-/-}$-MCs but had no effect on $Cd47^{-/-}$-MCs. This suggests that the interaction of TSP1 and CD47 mediates subretinal macrophage elimination (Calippe et al., 2017). Taken together, these results demonstrate that the activation of CD47 on infiltrating subretinal MPs participates in their homeostatic elimination and during the resolution of acute inflammation. CD47 signaling likely sensitizes MPs to FasL-induced death as described for other cell types (Manna et al., 2005; Quesada et al., 2005) (Fig. 3). In diseases where subretinal MPs

accumulate on the RPE, in areas such as the TZ and drusen of AMD, subretinal RPE-mediated immune-suppression has clearly become ineffective. It is currently not clear if the deficient immune-suppression arises due to insufficient RPE signals, increased resistance of MPs to elimination, or both.

Taken together, the capacity of the RPE to induce leukocyte death is a crucial part in repressing the accumulation of potentially pathogenic MPs and maintaining homeostasis in the photoreceptor cell layer and the subretinal space. TSP-1 mediated activation of CD47 and FasL signaling through Fas are essential, but likely not the only molecular pathways involved in this mechanism.

4. The origin of infiltrating mononuclear phagocytes

The photoreceptor cell layer and the subretinal space are physiologically devoid of MPs, and any subretinal MPs found in AMD must therefore have actively infiltrated the space through the OLM, or the RPE. Infiltrating MPs could originate from retinal MCs, circulating Mos or rMφs from the choroid or ciliary body. Most studies concerning MP accumulation in AMD patients and animal models do not allow for the differentiation of distinct types and origins of MPs, as they express or can induce similar markers (Gautier et al., 2012; Ransohoff and Cardona, 2010). Mo-derived MPs are also not morphologically distinguishable from MCs, best evidenced by the blood borne GFP^+microglia-like cells that replace MCs in whole-body irradiated, GFP^+bone marrow transplanted mice (Chen et al., 2012b). The distinction between rMφ or Mo-derived iMφs might be important, as the sustained presence of iMϕs, evolved to neutralize pathogens, has been shown to be detrimental in neurodegenerative conditions such as multiple sclerosis and stroke (Conductier et al., 2010; Ransohoff, 2009).

A molecular marker that differentiates inflammatory Mos and iMϕs from MCs is CCR2, the receptor of the major chemokine CCL2 (Geissmann et al., 2010). Contrary to MCs, inflammatory Mos express high levels of CCR2 (Geissmann et al., 2010; Mizutani et al.,

2011; Sennlaub et al., 2013) and CCR2 cannot be induced in MCs (Saederup et al., 2010; Sennlaub et al., 2013). CCR2 is also not constitutively expressed or induced (light-challenge) in RPE cells, clearly demonstrated by the absence of red fluorescence protein (RFP) in the RPE of $Ccr2^{RFP/RFP}$-mice (Sennlaub et al., 2013). CCR2 is therefore a good marker to identify subretinal Mo-derived Mφs, but likely underestimates this population, as *Ccr2 transcription is quickly downregulated when Mo differentiate to* Mφ *(Sennlaub et al., 2013; Wong et al., 1997).*

CCL2 (the main ligand of CCR2) expression in the retina is physiologically low but it increases with age and is induced in situations of stress such as laser- and light-injury or retinal detachment in animal models (Chen et al., 2012a; Nakazawa et al., 2007; Sennlaub et al., 2013; Yamada et al., 2007). Initially CCL2 is likely secreted by resident MCs that sense local danger signals, but it is also strongly produced by infiltrating iMφs (Sennlaub et al., 2013), and other resident cells, such as Müller glial cells (Rutar et al., 2012) and the RPE (Chen et al., 2008; Elner et al., 1991). Importantly, increased intra-ocular levels of CCL2 are observed in human neovascular- (Fauser et al., 2015; Guymer et al., 2011; Jonas et al., 2010; Kramer et al., 2012; Newman et al., 2012; Rezar-Dreindl et al., 2016) and atrophic-AMD (Newman et al., 2012; Sennlaub et al., 2013). Using CCR2 immunohistochemistry, we showed that subretinal MPs observed in human sections of GA patients are in part blood-derived inflammatory $CCR2^+$ Mos (Sennlaub et al., 2013). Indeed, all studied diseased eyes, but no healthy control eye, contained $CCR2^+$ Mos in the atrophic lesions and $CCR2^+$ Mos were observed on the RPE of the TZ, in BlinD and in and around large drusen (Sennlaub et al., 2013). In inflammation-prone Cx3cr1-deficient mice (in which CCL2 is induced with age and during a light-challenge) we demonstrated using *genetic Ccl2 or Ccr2 deletion, monocyte depletion, and inhibitors of CCR2* that approximately 50% of the MPs infiltrating the photoreceptor cell layer are recruited from circulation. This recruitment from blood occurred,

despite the absence of a visible breech in the blood retinal barrier (Sennlaub et al., 2013), indicating that the blood retinal barrier is not an obstacle for Mo infiltration. Importantly, *the inhibition of CCR2^{+} monocyte recruitment nearly completely prevents the inflammation-associated photoreceptor degeneration in these mice. These results suggest that (i) the CCR2^{+} monocyte derived MPs are the main mediators of inflammation-associated neurotoxicity in this model and (ii) that the subretinal accumulation of MPs is in part due to CCR2^{neg} cells, possibly retinal microglial cells that display no or little toxicity. Our in vitro experiments comparing wildtype and Cx3cr1$^{-/-}$ Mo and MC toxicity on photoreceptors of retinal explants in a co-culture system confirmed increased toxicity of Mos compared to MCs and of Cx3cr1$^{-/-}$ MPs compared to wildtype MPs (Sennlaub et al., 2013).*

Similarly, infiltrating MPs are in part derived from circulating Mos in RD models (Guo et al., 2012; Kohno et al., 2015) and photooxydative stress (O'Koren et al., 2016; Suzuki et al., 2012). Mo-derived iMφs contribute significantly to photoreceptor degeneration in light-induced models (Hu et al., 2016; Rutar et al., 2012; Suzuki et al., 2012), in the *Abca4$^{-/-}$Rdh8$^{-/-}$* mouse Stargardt/AMD model (Kohno et al., 2013), the rd10 mouse (Guo et al., 2012), and in the carboxyethylpyrrole immunization-induced AMD model (Cruz-Guilloty et al., 2013). Although we observed little MC neurotoxicity in light-challenged *Cx3cr1-deficient mice, MCs have been shown to contribute* to photoreceptor degeneration in rd10 mice (Zhao et al., 2015). The rd10 mice lack the exon 13 of the phopshodiesterase 6b that is physiologically expressed in rods. MCs participate in photoreceptor degeneration in rd10 mice as they phagocytose diseased cells, but living, rods in a process called phagoptosis (Zhao et al., 2015). It will be interesting to see to what degree photoreceptor phagoptosis by MCs occurs in other degeneration models, where the instigator event does not lie within the photoreceptors.

In experimental, laser-induced CNV approximately 50-70% of MPs are Mo-derived and 30-50% are derived from resident MPs (MCs, and choroidal rMφs) (Caicedo et al., 2005).

Depletion of circulating Mo (Sakurai et al., 2003a) and inhibition of Mo-recruitment by genetic deletion of *Ccr2* and *Ccl2* (Liu et al., 2013b; Luhmann et al., 2009; Robbie et al., 2016; Tsutsumi et al., 2003), or *CD18 / ICAM-1* signaling (important for Mo diapedesis (Sakurai et al., 2003b)) very significantly inhibits CNV formation in numerous studies. These results might seem somewhat surprising as $CCR2^{+}$Mo derived iMφs are generally supposed to be anti-angiogenic (M1 polarized, see 5.) (Sica and Mantovani, 2012; Wynn et al., 2013), contrary to MCs (M2 polarized) that participate in physiological retinal angiogenesis (Checchin et al., 2006) and are pro-angiogenic in the subretinal space (Ma et al., 2009). However, the phagocytosis of damaged RPE by the Mo-derived Mφs (Liu et al., 2013b), which characterize the microenvironment of CNV, might lead to this "quick switch" to a pro-angiogenic profile of the infiltrating subretinal iMφs.

Taken together, MP populations that infiltrate the photoreceptor cell layer and subretinal space are likely constituted of resident Mφs such as MCs and possibly choroidal rMφs but also of blood Mo-derived iMφs as seen in animal models and in the human disease (Sennlaub et al., 2013). As outlined above, iMφs evolved to react as quickly as possible to terminate the spread of potential deadly infections, even at the cost of tissue damage (Nathan, 2002). The threshold to induce the secretion of potentially damaging inflammatory cytokines in iMφs is therefore much lower compared to rMφs (Nathan, 2002). Experimental data from a number of "secondary" subretinal inflammation models, where inflammation occurs secondary to RPE or photoreceptor disease or injury, demonstrate that the chronic presence of subretinal Mo-derived iMφs aggravate photoreceptor degeneration additionally to promoting CNV (Fig. 5). Importantly, this was also the case in "primary" models of subretinal inflammation, in which the inflammation is not a secondary event to photoreceptor or RPE dysfunction but due to (i) the lack a tonic inhibitory signal in the inner retina (such as Cx3cr1-deficient mice (Calippe et al., 2017; Combadiere et al., 2007; Eandi et al., 2016; Hu et al., 2015; Sennlaub et al., 2013))

or (ii) an induced autoimmune reponse (carboxyethylpyrrole immunization-induced AMD model (Cruz-Guilloty et al., 2013)). These primary models strongly suggest that the chronic infiltration of iMφs can be sufficient to induce degenerative changes in the outer retina.

Crb1^{rd8} mutation induced confusion in the field

In 2003 Ambati *et al.* reported that aged Ccl2$^{-/-}$ and Ccr2$^{-/-}$ mice develop drusen, spontaneous neovascularization and photoreceptor degeneration (Ambati et al., 2003). The authors suggested that deficiency in CCL2-/CCR2-dependent Mφ recruitment from the choroidal circulation may prevent the clearance of accumulating debris in BM (Ambati et al., 2003), which, over time, would lead to drusen formation. In an attempt to accelerate the development of AMD-like features, Tuo *et al*. generated Ccl2$^{-/-}$Cx3cr1$^{-/-}$-mice that develop "drusen," pigment alterations, and retinal degeneration by the age of 6 weeks in 100% of mice and CNV in 15% of mice (Tuo et al., 2007). This mouse strain was subsequently shared with a number of laboratories and used in numerous publications as a model of AMD, because of its phenotypical similarities. How deficiency in CCL2/CCR2- and CX3CL1/CX3CR1-signaling led to the phenotype was not investigated. In these studies, it remained unclear if CCL2-deficiency protected against the more pronounced phenotype observed in *Cx3cr1*-deficient mice (or vice versa), as data from single knockout controls were never presented.

Later reports first failed to reproduce spontaneous CNV in aged Ccl2$^{-/-}$ and Ccr2$^{-/-}$ mice and the appearance of drusen was recognized due to subretinal lipid bloated MPs (Chen et al., 2011; Luhmann et al., 2009) similar to those observed in Cx3cr1-deficient mice (Combadiere et al., 2007). In our laboratory, aged *Ccl2*$^{-/-}$ mice only developed discreet subretinal accumulation of MP, which represented only a fraction of the accumulation observed in age-matched *Cx3cr1*-deficient mice kept under matched conditions in the same animal facilities (see supplementary data of Sennlaub et al. (Sennlaub et al., 2013)). Furthermore, none of our independently bred *Ccl2*$^{-/-}$*Cx3cr1*$^{-/-}$-, *Ccl2*$^{-/-}$*Cx3cr1*$^{GFP/GFP}$-, and *Ccr2*$^{RFP/RFP}$*Cx3cr1*$^{GFP/GFP}$-

mice developed the early onset phenotype. In fact CCL2 or CCR2 deficiency, pharmacological inhibition of CCR2, or circulating Mo depletion, protected against the pathogenic accumulation of subretinal MPs observed in *Cx3cr1*$^{-/-}$ and *Cx3cr1*$^{GFP/GFP}$-mice. These observations are in line with the tonic inhibitory role of CX3CL1/CX3CR1 signaling and the neurotoxicity of CCR2+-Mo-derived Mφs observed not only in the retina, but also in the central nervous system in general (see above). It is still however not clear how deletion of CCL2 or CCR2 induces subretinal MP accumulation and what the significance this observation has for AMD research as intraocular CCL2 concentrations are increased in both late forms and CCR2^{+}Mos accumulate in GA (see above).

Ultimately, in 2012, *the early onset phenotype of Ccl2*$^{-/-}$*Cx3cr1*$^{-/-}$ *mice was* explained by a contamination with a mutation in the *Crumbs homologue-1 gene (Crb1), known as* the retinal degeneration 8 (Crb1^{rd8}) mutation (Luhmann et al., 2013; Mattapallil et al., 2012). The Crb1^{rd8} mutation produces a secreted truncated CRB1 protein (Mehalow et al., 2003). CRB1 localizes specifically to the adherens junction complex at the outer limiting membrane in the retina. Homozygous Crb1^{rd8} mice can develop prominent focal inferior retinal degeneration, photoreceptor loss, and retinal thinning *(Mehalow et al., 2003) as observed in* Crb1$^{-/-}$ mice (van de Pavert et al., 2004). At later stages the mice might present vascular lesions that resemble CNV, but are in fact derived from the retinal vasculature, and subretinal MP accumulation similar to AMD (Luhmann et al., 2014). Crb1 mutations in humans can lead to a comparable range of symptoms (Bujakowska et al., 2012) but there are currently no studies that would link variants of Crb1 to AMD pathology. Akin to human mutations the phenotype in homozygous Crb1^{rd8} mice is dependent on additional yet unknown genetic modifiers (located on chromosome 15 for the mouse) and the absence of macroscopic lesions does not exclude the presence or even homozygosity for the mutation (Luhmann et al., 2014). The *Cx3cr1*$^{-/-}$- and *Cx3cr1*$^{GFP/GFP}$-mice we used in our 2007 (Combadiere et al., 2007) study did

not seem to have been contaminated with the Crb1^{rd8} mutation as their pro-inflammatory phenotype was since consistently reproduced in mice that tested negative for the Crb1^{rd8} mutation (Calippe et al., 2017; Eandi et al., 2016; Hu et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013).

It is likely that numerous mouse studies using knockout- and transgenic-mice contaminated Crb1^{rd8} wrongly implicated genes in subretinal inflammation and the pathomechanism of AMD because of the similarity of the features caused by the Crb1^{rd8} mutation. Taken together, it is crucial that any transgenic mouse strain used in retinal and in particular AMD research must be genotyped and the Crb1^{rd8} mutation eliminated. In our hands, the targeted replacement mice that express human APOE isoforms (TRE2-, 3-, and 4 - C57BL/6N mice; a generous gift from Dr. Patrick Sullivan (Sullivan et al., 1997)) and TSP-1$^{-/-}$ C56BL/6J-mice (purchased from Jackson laboratories) were all homozygote for the Crb1^{rd8} contamination and were backcrossed to eliminate the mutation before experimentation. The absence of the typical Crb1^{rd8} phenotype or the background strain (C57BL/6N versus C56BL/6J) does not exclude its presence and interference with ocular phenotypes.

5. Function and consequences of chronic subretinal mononuclear phagocyte accumulation

5.1. A “homeostatic” role

In tissues with high regenerative potential such as the skin, a homeostatic role of MPs is clearly observed in the resolution of acute inflammation, where they enhance the proliferation of epithelial progenitor cells and promote wound healing (Wynn and Vannella, 2016). Similarly, in the central nervous system of fish that have retained the capacity to regenerate neurons after injury, MPs trigger the regeneration of neurons and photoreceptors (Kyritsis et al., 2012; White et al., 2017). However, this reparative aspect of the resolution of acute inflammation is not observed in mammals, as we lost the regenerative capacity of neurons and photoreceptors.

In chronic low-grade tissue inflammation triggered by tissue stress and malfunction MPs might help preserve tissue function for some time, albeit the persistence of the noxious conditions. This might be in particular the case in situations where tissue stress is accentuated or caused by accumulation of toxic extracellular material that MPs can remove by phagocytosis. For example, we showed that infiltration of iMφs can be partially neuroprotective in the (admittedly extreme) case of experimental subretinal injection of toxic doses of β-amyloid, as iMφs neutralize this protein (Bruban et al., 2011). Theoretically, in this context, MP infiltration is beneficial as long as the toxicity associated with the MP infiltration is outweighed by the toxicity of the material it removes. It depends therefore on the presence, amount, and nature of the accumulating toxins and the composition and activation state of the infiltrate (iMφs, rMφs and others). Medzhitov proposed to call the beneficial, "homeostatic" accumulation of rMφs, "which helps a tissue to adapt to the noxious conditions and restore tissue functionality" that is observed with milder tissue malfunction, "para-inflammation". Pathogenic chronic low grade inflammation that includes Mo-derived Mφs on the other hand would be called "dysregulated para-inflammation" (Medzhitov, 2008). "Para-inflammation" might take place in patients with early AMD, as MPs that are observed in and around large drusen might participate in the removal of debris, preventing drusen growth or participate in drusen absorption. It has become increasingly clear that large drusen are dynamic structures that grow, but are also absorbed (Querques et al., 2016; Smith et al., 2010; Toy et al., 2013; Yehoshua et al., 2011) and MPs are likely candidates to play an important part in this mechanism. Furthermore, patients with large sized soft drusen do not necessarily progress to late AMD. Over 10 years, only 15% (Beaver Dam study)-30% (Blue mountain study) progressed to late AMD in these population based epidemiological studies (Klein et al., 2004; Wang et al., 2003) and MP-mediated "para-inflammation" might have prevented patients from progression to late AMD. As mentioned above such a mechanism has been proposed,

based on the observation for spontaneously appearing drusen-like fundus lesions in $Ccl2^{-/-}$ and $Ccr2^{-/-}$-mice (Ambati et al., 2003). However, these lesions were later identified to be caused by subretinal lipid-bloated MPs (Chen et al., 2011; Luhmann et al., 2009) and not drusen. It is generally difficult to study the implication of MPs in drusen regression with current rodent animal models, as none develop lesions similar to human drusen. In humans, AMD-associated drusen are thick (10-50µm) lipoproteinaceous debris located between the basal lamina of the RPE and the inner collagenous layer of BM (in the same anatomical location as BlinD). In Mice, sub RPE debris accumulation can be observed in $APOE^{-/-}$ mice (Ong et al., 2001), TRE4-mice (Malek et al., 2005) and $Cfh^{-/-}$mice (Toomey et al., 2015) but these accumulations are thin (<3µm) and located between the RPE and their basal infoldings. They are therefore similar to basal laminar deposits (BlamD), that are associated with age but not with AMD (Curcio and Millican, 1999).

Aged C57BL/6 wildtype mice also present subretinal MP accumulation (at a significantly older age compared to Cx3cr1-/- mice) that does not seem to be associated with photoreceptor degeneration. This accumulation has been suggested to be a form of para-inflammation (Chen and Xu, 2015), but experimental evidence that the infiltrate protects these mice from photoreceptor or RPE dysfunction is lacking so far. Contrary to mice, donor eyes from healthy humans (even of advanced age) do not present significant subretinal MP accumulation that could be akin of a para-inflammatory infiltrate and subretinal MPs are only observed in diseased eyes (Eandi et al., 2016; Gupta et al., 2003; Levy et al., 2015a; Sennlaub et al., 2013).

In summary, during the resolution phase of acute inflammation MPs fulfill an important role in the regeneration and reestablishment of homeostasis in tissues with high regenerative potential. In vertebrates such as fish this mechanism is preserved in the central nervous system (CNS) including the retina that has retained the ability to regenerate, but is restricted

to tissues such as the skin in mammals. In chronic inflammation MPs can help prevent the accumulation of toxic extracellular material and help preserve tissue function. Even though experimental data is difficult to produce due to the lack of appropriate models, this might well be the case for hard drusen during "healthy" aging and for large drusen in non-progressing ARM patients, where moderate MP accumulation might help control drusen growth.

5.2. Collateral damage

In some patients the compounded combination of several AMD risk factors likely leads to particularly inflammatory MPs (see 6.), which pushes the balance towards a pathogenic chronic inflammation and late AMD. The degree to which chronic inflammation constitutes a major or minor pathogenic factor in these patients likely depends on the extent of the infiltrate and on its production of pathogenic mediators.

5.2.1. M1 and M2 polarization

Polarization states of MPs are often divided into two activation states in analogy to the adaptive immune system, where immunologists observed that two distinct subpopulations of $CD4^+$ T helper cells could be subdivided on the basis of the pattern of their cytokine production, which predicted their role in the immune response (Th1/Th2 polarization) (Liew, 2002). *In vitro*, Mϕs activated by bacterial molecular patterns (such as LPS) are defined as classically activated Mϕs (M1). They are pro-inflammatory, anti-microbial, anti-angiogenic, potentially neurotoxic and defined by the expression of mediators such as IL-1β, TNF-α, IL-6, CCL2 and iNOS. Alternative activation of Mϕs (M2) is traditionally induced by IL-4 and IL-13 which polarizes them towards a phenotype that is anti-inflammatory, promotes phagocytosis, neovascularization, wound healing and ultimately fibrosis (scarring). They are characterized by the expression of VEGF, Arginase, IL-10, and IL-1RA among others (Sica and Mantovani, 2012; Wynn et al., 2013). However these two types of polarization are only two of a multitude of activation states and the M1/M2 defining proteins are not regulated by a general transcriptional switch but individually. In many studies the identification of M1 or M2

markers is used to infer if the MPs are “good” or “bad”, without investigating the effect of the individual mediators that are used as markers. However, *in vivo* subretinal MPs have been shown to express M1 and M2 markers simultaneously (Camelo et al., 2012; Horie et al., 2013; Liu et al., 2013b) and they are often both, neurotoxic and angiogenic (see 4.). Hence, in subretinal inflammation, but also in peripheral and central inflammation in other settings, the dated differentiation of M1 and M2 polarized MPs is not helpful to characterize Mo-derived Mφ polarization (Wynn et al., 2013) or MC polarization (Ransohoff, 2016) and should no longer be used. Therapeutically, it would also seem ill advised to direct subretinal MP polarization from M1 to M2, as their pro-angiogenic/fibrotic phenotype would promote CNV and fibrosis.

5.2.2. Phagocyte-derived cytokines and collateral damage

As outlined above, Mo-derived iMφs play a pathogenic role in many models of subretinal inflammation and AMD (Cruz-Guilloty et al., 2013; Guo et al., 2012; Hu et al., 2016; Kohno et al., 2013; Rutar et al., 2012; Sennlaub et al., 2013; Suzuki et al., 2012), in addition to MCs (Zhao et al., 2015). As Mo-derived iMφs lack the receptors for tonic inhibitory signals from the retina (see above) at the beginning of their differentiation into Mφs in the retinal microenvironment, they are prone to produce high levels of inflammatory cytokines, including the classical inflammatory cytokines IL-1β, TNF-α, IL-6, CCL2 when they infiltrate the tissue. Over the last years, the understanding of the multiple modes of action of these cytokines and how they affect chorioretinal homeostasis has evolved significantly (Fig. 6):

5.2.2.1. Interleukin-1β

The *Il-1β* gene is transcribed and pro-IL-1β protein produced after pro-inflammatory stimuli, such as those ensuing from the activation of toll-like receptors (TLR) that recognize pathogen- or damage-associated molecular patterns (PAMPs, DAMPs) (Allan et al., 2005; Simi et al., 2007a). Activation by a second stimulus, can trigger the assembly of the NLRP3

inflammasome, and activate caspase-1 that cleaves the IL-1β precursor protein and produces a mature, secretable IL-1β (Schroder and Tschopp, 2010). During sterile inflammation, this second stimulus is often extracellular adenosine triphosphate (ATP), released by degenerating cells, which activates the P2RX7 receptor on MPs (Mariathasan et al., 2006). IL-1β is a potent survival factor for endothelial cells and induces similar genes than VEGF (Schweighofer et al., 2009) and we and others have shown that it exacerbates CNV (Lavalette et al., 2011; Olson et al., 2009). Additionally to its pro-angiogenic effect, it also has neurotoxic properties. In the brain, IL-1β is a potent mediator of neuronal apoptosis (Simi et al., 2007b) and the increased degeneration observed in mice lacking the tonic inhibitory CX3CR1 signaling is due to increased IL-1β secretion from *Cx3cr1*-deficient MPs (Cardona et al., 2006). We recently showed that IL-1β maturation and secretion in *Cx3cr1*-deficient MPs is due to their spontaneous and sustained release of ATP and the increased expression and activation of surface P2RX7, which constitutively activate the inflammasome (Hu et al., 2015). Interestingly, human blood Mos, contrary to differentiated Mφs, also constitutively release endogenous ATP, which activates NLRP3 and caspase-1 that can mature IL-1β. Human Mos can therefore secrete mature IL-1β after transcriptional induction of the *Il-1β* gene without an additional second exogenous stimulus (Netea et al., 2009). Correspondingly, *Cx3cr1*-deficient MPs and human Mos are able to induce IL-1β-dependent rod apoptosis in Mo/retina co-cultures (Eandi et al., 2016; Hu et al., 2015). Importantly, IL-1β inhibition (using a P2RX7 inhibitor) also significantly diminished rod death in light-induced subretinal inflammation of *Cx3cr1*-deficient mice (Hu et al., 2015), and in rd10 mice *in vivo* (Zhao et al., 2015). Using human blood-derived $CD14^{+}$MPs *in vitro* and inflammation-prone $Cx3cr1^{GFP/GFP}$ mice *in vivo*, we also recently showed that MP derived IL-1β leads to rapid cone segment degeneration (Eandi et al., 2016) and chronic exposure to IL-1β might be responsible for the observed cone loss in aged $Cx3cr1^{GFP/GFP}$- and TRE2-mice (Calippe et al., 2017).

This inflammation-induced, IL-1β-dependent cone and rod degeneration could help explain the photoreceptor loss (rods and cone segments (CS)) in the TZ of GA patients that occurs despite the presence of RPE in the TZ. One could assume that photoreceptor loss in the TZ of AMD patients is due to RPE dysfunction (prior to its disappearance), but cone segment loss is also observed in patients with retinitis pigmentosa (RP) with rod-gene mutations and unremarkable RPE. Gupta et al. proposed several years ago that the subretinal inflammation secondary to rod degeneration in RP could be the "missing link" to explain secondary CS and cone degeneration observed in RP (Gupta et al., 2003). Cone maintenance in RP has recently been shown to depend on cone glucose uptake and aerobic glycolysis (Ait-Ali et al., 2015) that is stimulated by insulin (Punzo et al., 2009) and rod-derived cone viability factor (Ait-Ali et al., 2015). Indeed, cones (contrary to rods) express the mediators of the insulin receptor-signaling cascade, including the phosphoinositide 3-kinase (PI3K) and m-TOR (Rajala et al., 2013) and the cell specific deletion of PI3K (p85α) resulted in age-related cone (Ivanovic et al., 2011b) but not rod (Ivanovic et al., 2011a) degeneration. As mentioned above, iMϕs have high glucose consumption as their metabolism mainly depends on glycolysis (Kelly and O'Neill, 2015). Due to this metabolic switch, iMϕs are very reliant on surrounding high glucose concentrations for survival and function, which they insure by inducing insulin resistance and decreasing glucose consumption of stromal cells via IL-1β, TNF-α□, and IL-6 (Shoelson et al., 2006). Cytokine-induced insulin-resistance is increasingly recognized to play an important role in in the pathogenesis of type 2 diabetes (Shoelson et al., 2006). In subretinal inflammation, associated with AMD and RP, cytokine-induced cone insulin resistance, could thereby decrease glucose uptake and induce cone starvation, which would lead to cone segment loss in the short run and cone cell loss chronically.

5.2.2.2.2. Tumor Necrosis Factor α (TNFα)

Similar to IL-1β, TNFα participates significantly in driving CNV formation (Lichtlen et al., 2010; Shi et al., 2006) and patients with monocytes that express the greatest amount of TNFα have a higher prevalence of CNV (Cousins et al., 2004). It also has profound effects on RPE homeostasis as it represses a key transcription factor of the RPE, the orthodenticle homeobox 2 (OTX2) (Mathis et al., 2017). OTX2 is a critical transcription factor for the development of the brain and sensory organs (Acampora et al., 1995; Cantos et al., 2000; Fossat et al., 2006). During retinal development, OTX2 controls the RPE and is maintained in the adult RPE where it regulates the expression of a number of essential genes, including crucial genes for the retinol visual cycle: Transthyretin (TTR), a retinol carrier, and retinol dehydrogenase 5 (RDH5) that re-isomerizes all-trans-retinal into 11-cis-retinal (Housset et al., 2013). Complete OTX2 ablation in adult mice induces progressive photoreceptor degeneration (Housset et al., 2013). Reduced OTX2 in RPE cells and therefore TTR and RDH5 expression likely leads to the impaired capacity to import and revert all-trans retinal into 11-cis-retinal, therefore slowing the visual cycle, which increases the recovery time after bleach, as observed in RDH5 deficient patients (Cideciyan et al., 2000). Using a co-culture model of human $CD14^+$blood monocytes (Mo) and RPE, we showed that activated Mos resist elimination when in contact with immunosupressive RPE and markedly inhibit their OTX2 expression (Mathis et al., 2017). We demonstrated that TNFα, secreted from activated Mos, induces a dramatic down-regulation of OTX2 and the genes it regulates including RDH5. As mentioned above, early AMD is associated with a marked increase in recovery time after bleach before significant loss of RPE or photoreceptors, which suggests that the visual cycle is slowed before degeneration appears (Flamendorf et al., 2015; Owsley et al., 2001). In our study we used LPS, but also APOE, which we showed to be strongly expressed in subretinal MPs in AMD and to activate Mos *in vitro* (see 6.1.1.) (Levy et al., 2015a). TNFα can be induced by diverse stimuli and patients with the CFH AMD-risk variant have higher systemic

levels of TNFα (Cao et al., 2013). Even though there is not yet direct evidence for OTX2 down-regulation, it is tempting to speculate that TNFα, produced by infiltrating MPs associated with large drusen (Lad et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013), slows the visual cycle (as observed in early AMD), before RPE lesions appear (Flamendorf et al., 2015; Owsley et al., 2001). Interestingly, TNFα-dependent OTX2 down-regulation might also have implications in congenital microphthalmia and auditory defects, common after intrauterine infections, notably with cytomegalovirus (CMV) (Becroft, 1981). CMV potently induces TNFα (Smith et al., 1992), which in turn might downregulate OTX2 necessary for eye and ear development (Acampora et al., 1995; Cantos et al., 2000; Fossat et al., 2006).

5.2.2.3. Interleukin 6 (IL-6)

IL-6 is a pleotropic cytokine that belongs to the gp130 family of cytokines. IL-6 binds to an 80-kilodalton (KDa) type 1 cytokine α receptor subunit (IL-6R, CD126), which together activate a universally expressed 130- KDa signal-transducing β-receptor subunit (gp130, CD130) that is ubiquitously expressed (Hunter and Jones, 2015). 'Classical' IL-6 receptor signaling is mediated via the membrane-bound IL-6R subunit and gp130. In 'trans-signaling', IL-6 forms a complex with soluble IL-6R, which increases the half-life of IL-6 and can bind and signal with the gp130 expressed ubiquitously on almost every cell (Hunter and Jones, 2015). The ability of IL-6 signaling to promote angiogenesis maybe best seen in Kaposi sarcoma, a multifocal angioproliferative disorder, that is caused by the human herpesvirus-8, which carries a viral IL-6 gene, structurally homologous to human IL-6 (Aoki et al., 1999). In CNV formation, others and we showed that MP-derived IL-6 is an important contributor to neovascularization (Izumi-Nagai et al., 2007; Levy et al., 2015a) in concert with IL-1β, TNFα, and VEGF. Intraocular concentrations of IL-6 are significantly associated with macular edema in patients with CNV (Miao et al., 2012) and the percentage of IL-6 expressing circulating Mos is higher in patients with neovascular AMD (Lechner et al., 2017). In fact,

aqueous humor levels of IL-6 showed a better correlation with macular edema than levels of VEGF (Chalam et al., 2014). Interestingly, intravitreal injection of antivascular endothelial growth factor drugs did not change the intraocular level of IL-6 (Miao et al., 2012) suggesting independent modes of action.

IL-6 has also an important influence on the immune privilege in the eye: IL-6 antagonizes TGF-β and abolishes the immune privilege in the anterior chamber (Ohta et al., 2000). In subretinal inflammation, we showed that IL-6 reduces RPE FasL expression that physiologically contributes to the elimination of infiltrating subretinal leukocytes (see 3.3.1.) (Levy et al., 2015a). *Cx3cr1*$^{-/-}$ MPs (that lack the tonic inhibitory CX3CL1 signal) and *TRE2*-MPs (that express the AMD-risk APOE2 isoform) secrete elevated amounts of IL-6, which helps explain the reduced clearance of subretinal MPs in these models and their exaggerated age-, light-, and laser-induced subretinal MP accumulation (Levy et al., 2015a; Levy et al., 2015b). On the other hand, IL-6 protects against photoreceptor death in experimental retinal detachment (Chong et al., 2008) and inner retinal neurons from ischemia reperfusion injury (Sanchez et al., 2003). Taken together, IL-6 lowers the ocular immune privilege while protecting retinal neurons in the eye. This dual mechanism might have evolved to ensure an efficient host defense against pathogen invasion, while minimizing excessive tissue damage. In sterile experimental inflammation we showed that inhibition of IL-6 significantly reduced subretinal MP infiltration and CNV (Levy et al., 2015a). These results suggest that inhibiting IL-6 in the context of AMD or RP could help restore subretinal immune-supression, and reduce the subretinal infiltrate and the associated degenerative changes.

5.2.2.4. Chemokine (C-C motif) ligand 2

As mentioned above, CCL2 (MCP-1) is the main chemokine for inflammatory Mo recruitment that robustly express its main receptor CCR2 (Geissmann et al., 2010). Its role in subretinal inflammation and AMD is discussed in detail in the section 4.

The discussed studies demonstrate how classical inflammatory mediators such as IL-1β, TNFα, IL-6 and CCL2 influence photoreceptor degeneration, RPE function, and monocyte recruitment. These four inflammatory cytokines are however only a small fraction of possible mediators produced by the subretinal infiltrate. Others include reactive oxygen species, krebs cycle intermediates, prostaglandins and more. Future studies will increase our understanding of how mediators produced by the subretinal infiltrate affect chorioretinal homeostasis.

6. AMD-risk factors and subretinal mononuclear phagocytes

As outlined above, subretinal accumulation of MPs characterizes large drusen and late AMD. In a multitude of *in vitro* and animal models, a similar kind of inflammation has been shown to participate in CNV development, RPE dysfunction and photoreceptor degeneration. However, these studies do not decipher if subretinal inflammation is one of the "primary" events that drive AMD pathogenesis or purely a "secondary" reaction that would be similarly observed in any type of retinal degeneration. "Primary" events for AMD pathogenesis must somehow be triggered by the AMD risk-factors that are strongly associated with disease. The main identified risk-factors comprise genetic variants, age, and environmental factors such as light-exposure, smoking, and obesity. How the complex interactions of genetic- and environmental-risk factors affect photoreceptor, choroid, and RPE function remains largely unknown. However, recent studies demonstrate how major genetic and environmental risk factors for AMD directly affect MP function and promote subretinal inflammation, thus emphasizing the role of inflammation in AMD.

6.1. Genetic risk factors

The risk of developing AMD increases between 5- and 10-fold when a parent or sibling is also affected (Chakravarthy et al., 2010; Shahid et al., 2012), making AMD one of the most heritable complex diseases. A haplotype of chromosome 10q26, a common variant of the complement factor H (CFH), and the Apolipoprotein E isoforms account for a large part of this genetic risk to develop AMD (Fritsche et al., 2016; Swaroop et al., 2007).

6.1.1. Apolipoprotein E

In humans, the Apolipoprotein E (APOE) gene has three common genetic variants (APOE2, APOE3, and APOE4). APOE2-allele carriers are at increased risk of developing late age-related macular degeneration, while the APOE4-allele protects against AMD when compared to the most common APOE3-allele (Mahley and Rall, 2000; McKay et al., 2011).

APOE is expressed in the liver and is the main lipoprotein of the brain and the retina (Anderson et al., 2001; Mahley and Rall, 2000). It is strongly secreted by hepatocytes, but also by the RPE (Ishida et al., 2004) and by mononuclear phagocytes (MPs), such as Mφs and MCs (Levy et al., 2015a; Peri and Nusslein-Volhard, 2008). APOE plays a major role in macrophage lipid efflux and in reverse cholesterol transport (RCT) in conjunction with *apolipoprotein A-I* (APOA-I) (Mahley and Rall, 2000; Mahley et al., 2009). The RCT pathway directs excess cholesterol by high-density lipoproteins (HDL) to the liver for elimination, to avoid toxic overload of cholesterol in peripheral cells. *Lipid-poor APOA-I particles (pre- HDL) are particularly efficient in accepting cholesterol transferred by the ATP-binding cassette transporter A1 (ABCA1) from peripheral cells (including* Mφs). *The cholesterol is then etsterified by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) and packaged to* form small HDL. The addition of APOE molecules to the small HDLs helps to further bind choletsrol esters and HDL increases in size (Mahley et al., 2006; Tall et al., 2000). The HDLs can be taken up via the scavenger receptor BI or its cholesterol esters can be transferred to very-low density lipoproteins (VLDLs) by the cholesteryl transfer protein (CEPT). The VLDLs are thereby processed to low-density lipoproteins (LDL) that are cleared from the circulation by the LDL receptor. In atherosclerosis it is well established that low levels of HDL and high levels of non-HDL cholesterol participate significantly in cholesterol accumulation and plaque formation (Moore and Tabas, 2011). Accordingly, Apoe$^{-/-}$ mice have extremely high lipid levels and massive amounts of cholesterol-rich β-VLDL and spontaneously develop atherosclerotic lesions (Meir and Leitersdorf, 2004).

Large drusen and atherosclerotic lesions contain similar extracellular proteins (eg. vitronectin, complement factors, and APOE) and important amounts of extracellular cholesterol and cholesterol esters (Curcio et al., 2001; Hageman and Mullins, 1999; Moore and Tabas, 2011; Mullins et al., 2000; Pikuleva and Curcio, 2014). These observations led to the hypothesis that drusen develop secondary to a deficit in the reverse cholesterol transport (RCT) similar to atherosclerotic plaques (Malek et al., 2005; Ong et al., 2001; Pikuleva and Curcio, 2014). Therefore, one would expect a similar deregulation of the RCT with both diseases. Curiously, the reverse is the case: High levels of HDL, protective against atherosclerosis, are associated with AMD (Delcourt et al., 2001; Klein et al., 1993; Klein et al., 1997; Klein et al., 2003; Paun et al., 2015; van Leeuwen et al., 2004). A variant of the ABCA1 gene, linked with low HDL and impaired RCT, has been shown to be protective against advanced AMD (Chen et al., 2010). APOA-I levels are elevated (not decreased) in the vitreous (Koss et al., 2014) and serum (Paun et al., 2015) of AMD patients. Maybe most importantly, the APOE*4*-isoform, associated with decreased APOE-levels (Bales et al., 2009; Levy et al., 2015b; Riddell et al., 2008; Sullivan et al., 2011) (see below) and impaired RCT (Heeren et al., 2004; Mahley et al., 2009), protects against AMD (McKay et al., 2011). The implication of APOE in AMD pathogenesis is therefore likely profoundly different from that in atherosclerosis and likely not directly linked to impaired RCT.

APOE, APOA-I, and cholesterol can also influence innate immunity. Cholesterol crystals can activate the innate immunity receptor cluster (IIRC), formed by toll-like receptors (eg. TLR2, TLR4) and obligate co-receptors (such as CD14) that activate Myd88, NFκB and induces inflammatory cytokines (CCL2, IL-6, TNFα…). APOE and APOA-I can also bind and neutralize hydrophobic TLR ligands (such as β-amyloid and LPS) and inhibit the induction of inflammatory cytokines (Azzam and Fessler, 2012). On the other hand, an excess of APOE and APOA-I can activate the TLR2-TLR4-CD14-dependent IIRC, in the absence of

extracellular ligands (Fig. 7). Under physiological conditions, the different components of the IIRC of Mφs are separated from each other, as some elements cluster to cholesterol-rich membrane domains called lipid rafts (eg. CD14) while others are located in the non-raft plasma membrane (eg. TLR2 and TLR4) (Pfeiffer et al., 2001). IIRC-ligands, such as bacterial lipopolysaccharides overcome this separation, as they bind to the extracellular domains of both co-receptors (CD14 and TLRs), bringing the receptors and their intracellular domains closely together, which activates Myd88, NFκB and induces the transcription of inflammatory cytokines (Schmitz and Orso, 2002). However, in absence of IIRC ligands, excessive APOA-I and/or APOE can extract enough cholesterol from the lipid raft to overcome the physiological separation of the receptors that form the IIRC and trigger intracellular signaling (Levy et al., 2015a; Smoak et al., 2010). Although this activation is often less strong than ligand induced activation, it can lead to very significant cytokine induction. In sterile inflammation, excessive APOA-I and APOE can thereby induce inflammatory cytokines such as IL-6 and CCL2 (Levy et al., 2015a; Smoak et al., 2010).

In AMD, APOA-I levels are elevated in vitreous (Koss et al., 2014) and serum (Paun et al., 2015) and APOE accumulates in drusen (Klaver et al., 1998) and is strongly expressed by subretinal MPs (Levy et al., 2015a). In mice, we showed that subretinal MPs of *Cx3cr1*$^{GFP/GFP}$-mice that develop subretinal inflammation and cardinal features of AMD (Combadiere et al., 2007), express similar high levels of APOE (Levy et al., 2015a), but also IL-6 (Levy et al., 2015a) and CCL2 (Sennlaub et al., 2013). Increased levels of CCL2 and IL6 are also observed in late AMD (Chalam et al., 2014; Jonas et al., 2010; Seddon et al., 2005; Sennlaub et al., 2013). *ApoE* deletion in *Cx3cr1*$^{GFP/GFP}$-mice nearly completely prevented age- and stress-induced pathogenic subretinal MP accumulation (Levy et al., 2015a). Mechanistically, we showed that APOE-induced IL-6 release from MPs represses RPE immune-suppression and prolongs subretinal MP survival and accumulation (Levy et al.,

2015a). Additionally, the APOE-dependent increase of CCL2 recruits pathogenic inflammatory $CCR2^{+}$ monocytes to the subretinal space (Sennlaub et al., 2013). Excessive APOE, and possibly APOA-I, can thereby induce chronic, pathogenic MP accumulation due to decreased MP elimination (IL-6) and increased MP recruitment (CCL2).

As mentioned above, in humans, the *APOE* gene has three common genetic variants (*APOE2*, *APOE3*, and *APOE4*) that lead to 6 possible diplotypes. *APOE4*, believed to be the ancestral form (Raichlen and Alexander, 2014), evolved into *APOE3*, and *APOE3 to APOE2* due to two polymorphisms (rs429358 and rs7412 respectively) that are imbedded in a well-defined CpG island, and lead to two cysteine-arginine interchanges at residues 112 and 158 (Yu et al., 2013). In most populations, *APOE3* is now the most common isoform (80% frequency) compared to *APOE4*- (14%frequency) and *APOE2*-isoforms (6% frequency). The *APOE*-isoforms are associated with several common, age-related diseases. Notably, the *APOE4*-allel is the most important genetic risk factor for Alzheimer's disease (AD) and a risk factor of atherosclerosis, while *APOE2* is protective for AD and atherosclerosis (except if associated with type III familial hyperlipiproteinemia) (Davignon et al., 1988; Herz and Beffert, 2000; Mahley et al., 2009). Curiously, the association with AMD is inversed: homozygote *APOE2*-allele carriers are at increased risk for developing late AMD (OR=1.83 for homozygote carriers), while the *APOE4*-allele protects from AMD (OR=0.72 per haplotype) compared to the most common *APOE3*-allele recently confirmed in a world wide study comprising 20 000 subjects (McKay et al., 2011). This association was found for both clinical forms of late AMD, but not for early/intermediate AMD (Paun et al., 2015), suggesting the APOE isoforms are not primarily implicated in the lipid accumulation in drusen genesis. Indeed, the targeted replacement mice expressing the human APOE4 (TRE4), and not APOE2 (TRE2), accumulate most lipids in Bruchs membrane compared to APOE3-expressing TRE3 mice (Malek et al., 2005), although *APOE4*-allele plays a protective role in

human AMD (McKay et al., 2011). Similar, lipid deposits are also observed in APOE$^{-/-}$ mice (Ong et al., 2001), but AMD is associated with increased APOE immunoreactivity (Anderson et al., 2001; Klaver et al., 1998; Levy et al., 2015a) in the human disease. Theses results suggest that the reasons for the association of the APOE isoforms with AMD are not due to a decreased capacity to evacuate cholesterol from Bruchs membrane.

The *APOE* isoforms are also associated with differences in APOE abundance: APOE plasma concentrations are significantly higher in homozygous *APOE2*- diplotype carriers and lower in *APOE4*-carriers compared to the *APOE3* homozygous diplotype (*E2/E2*: 13.8 mg/dl; *E3/E2*: 7.3 mg/dl; *E4/E2*: 6.7; *E3/E3*: 5.5 mg/dl; *E3/E4*: 5 mg/dl; *E4/E4*: 4.4 mg/dl) (Smit et al., 1988). The increased APOE concentrations associated with the *APOE2* isoform might be in part due to increased transcription in certain cell types (astrocytes and Mφs), caused by the loss of a CpG site present in the *APOE3*-alleles (Levy et al., 2015b; Yu et al., 2013). Most importantly, APOE2 is characterized by a severely decreased affinity of APOE2 for the LDL receptor, which impairs its cellular uptake and clearance (Mahley and Rall, 2000). HDLs contain several APOE molecules, which is likely the reason why APOE concentrations in *E3/E2* and *E4/E2* carriers, that do not carry an increased risk for AMD, are relatively normal, as HDL particles can be cleared via the APOE3 and APOE4 they contain. Homozygote *APOE2*-carriers also display higher APOE concentrations in cerebrospinal fluid, brain tissue, and the retina (Bales et al., 2009; Levy et al., 2015b; Riddell et al., 2008). Compared to the *APOE3*-allele, the *APOE4*-allele is transcribed similarly in neurons, astrocytes and Mφs (Levy et al., 2015b; Yu et al., 2013), but its protein concentration in plasma (see above), CSF, brain, and retina are decreased (Bales et al., 2009; Levy et al., 2015b; Riddell et al., 2008; Sullivan et al., 2011). The structural changes in the APOE4 protein also lead to diminished association with HDL (Dong and Weisgraber, 1996) and impaired reverse cholesterol transport (Heeren et al., 2004; Mahley et al., 2009).

Using the targeted replacement mice expressing the human APOE isoforms (TRE2, TRE3, and TRE4), we recently showed that the increased levels of APOE in MPs of homozygote *TRE2*-mice, activates the IIRC and induces IL-6, and CCL2 (Levy et al., 2015b) similar to *Cx3cr1*$^{GFP/GFP}$-mice (Levy et al., 2015a). As a consequence, *TRE2*-mice develop subretinal MP accumulation, photoreceptor degeneration and exaggerated choroidal neovascularization akin to AMD (Levy et al., 2015b). In the context of APOE-dependent subretinal inflammation in *Cx3cr1*$^{GFP/GFP}$-mice, the *APOE4*-allele led to diminished APOE tissue levels and CCL2 levels and protected *Cx3cr1*$^{GFP/GFP}$-mice against harmful subretinal MP accumulation observed in *Cx3cr1*$^{GFP/GFP}$*TRE3* mice (Levy et al., 2015b).

A possible pathogenic role of excessive cholesterol extraction and IIRC activation in AMD is also supported by increased APOA-I levels in AMD patients (Koss et al., 2014; Paun et al., 2015), the protective effect of an ABCA1 polymorphism (associated with impaired RCT) (Chen et al., 2010), and clinical studies showing that statins (that inhibit cholesterol synthesis) can accelerate the progression to late AMD (VanderBeek et al., 2013).

Our studies also shed an interesting light on the puzzling differences of the APOE-isoform association with AMD (McKay et al., 2011) and AD (Mahley and Rall, 2000), two major age-related neurodegenerative diseases: In AD, the *APOE4*-allele is associated with greater β-amyloid burden, possibly due to decreased APOE tissue concentrations and reduced efficacy in clearance of β-amyloid clearance via multiple pathways (Bales et al., 2009; Mahley et al., 2009). Accordingly, Cx3cr1$^{-/-}$mice that express increased amounts of APOE in MPs, including MCs (Levy et al., 2015a), are protected against beta-amyloid deposition in Alzheimer disease mouse models (Lee et al., 2010).

In summary, while a lack of APOE and RCT might play a pathogenic role due to reduced efficacy of cholesterol and β-amyloid clearance in atherosclerosis and AD (Bales et

al., 2009; Mahley et al., 2009), excessive APOE and RCT might be responsible for IIRC-activation and chronic inflammation in AMD. The *APOE2*-allele leads to increased APOE expression and reduced APOE uptake. The increased concentration of APOE extract excessive amounts of cholesterol from the MP lipid rafts, which leads to their destabilization and activation of the IIRC, which supports subretinal inflammation. The APOE4-isoform is less efficient at extracting cholesterol from the lipid rafts, which stabilizes the IIRC and keeps the inflammatory cytokine expression and inflammation low. These findings reflect the clinical association of the genetic predisposition. They emphasize the role of APOE in inflammation and inflammation in AMD.

6.1.2. Complement factor H

A common variant of complement factor H (CFH) accounts for a major part of the genetic risk for AMD (Fritsche et al., 2013; Fritsche et al., 2014; Magnusson et al., 2006). CFH is best known for its ability to inhibit the alternative complement cascade. The complement cascade is an innate component of the immune system that evolved to kill pathogens, such as bacteria. Complement components are activated in a cascade on the pathogen's surface and result in their opsonization and the assembly of a pore-forming complex in the pathogen's membrane, the membrane attack complex (MAC). The complement cascade can be activated by specific antibodies that are directed to the pathogen's surface epitopes, by bacterial sugars that initiate the lectin pathway, or via the alternative path that is initiated by the spontaneous hydrolysis of C3, the most abundant complement factor, that forms C3a and C3b. C3b can attach itself to bacterial cell surfaces and assemble with the complement Factor Bb (a fragment that results from the activation of Factor B by Factor D) to form the C3 converting enzyme C3bBb. C3bBb catalyzes an avalanche of C3a and C3b from C3. This amplification loop allows for deposition of several million molecules of opsonizing C3b on bacteria within a few seconds, which triggers their

phagocytosis and neutralization by neutrophils and MPs (Morgan and Harris, 2015). The recruitment of an additional C3b molecule to C3bBb leads to the formation of the C3bBbC3b complex, the C5 convertase that cleaves C5 into C5a and C5b. C5b recruits C6, C7, C8, and C9 to form a pore (MAC) that can induce lytic pathogen death. The C3a and C5a fragments are potent chemotactic agents that recruit neutrophils and Mos that are important for pathogen neutralization and phagocytosis (Flierman and Daha, 2007; Ricklin et al., 2010; Zipfel and Skerka, 2009) (Fig. 8).

The liver secretes these complement factors and their concentrations are high in the plasma. Plasma complement is important to prevent disastrous, uncontrolled pathogen growth and spread in blood and participates in tissue pathogen neutralization through inflammation-induced plasma extravasation. Locally, infiltrating Mos and Mo-derived Mφs of the infiltrate also strongly secrete the complement factors (Ezekowitz et al., 1984). Among MPs, we recently showed that *C3* transcription in Mos is ~50 fold higher compared to MCs, which might reflect its bactericidal functions (supplementary Figure 4 (Calippe et al., 2017)).

There are several mechanisms that inhibit alternative complement activation in order to prevent complement consumption in blood and to protect host cells from spontaneous complement activation and MAC formation: CD55 (decay accelerating factor or DAF), CD35 (complement recteptor 1 or CR1), complement factor I (CFI), and CFH. CFH acts as a cofactor of CFI that degrades already formed C3bBb, inhibits new formation of C3bBb (the crucial initiating step in the alternative cascade), and cleaves C3b into iC3b, which has no hemolytic or amplification potential. Additionally, CFH sterically blocks C3b deposition onto eukaryotic host cells by binding to glycoaminoglycans (GAG) that are present on virtually all host cell surfaces (Atkinson and Goodship, 2007; Zipfel and Skerka, 2009).

The CFH gene is located on human chromosome 1q32. It produces two transcripts, a full-length mRNA encoding a 1231 amino acid protein composed of 20 globular Short

Consensus Repeat domains (SCR, also called Complement control protein modules or Sushi domains) and an alternatively spliced mRNA coding for a 431 amino acid protein, the CFH-like protein 1, that contains only SCR 1 to 7 (Zipfel et al., 2002). SCR domains are formed by 60 amino acids and have many activities: SCRs 1-4, SCRs 12-14, and SCRs 19-20 bind C3b (Alsenz et al., 1984; Kuhn et al., 1995; Sharma and Pangburn, 1996). Binding of C3b to SCRs 1-4 is important for the CFI cofactor activity (Alsenz et al., 1984; Kuhn and Zipfel, 1996). The SCR7 and SCR19-20 of CFH mediate cell adhesion to GAGs (Blackmore et al., 1998; Clark et al., 2006; Sharma and Pangburn, 1996) and to the integrin CD11b/CD18 (Complement 3 Receptor, Mac-1 (Kopp et al., 2012; Losse et al., 2010)). CD11b/CD18 is strongly expressed by neutrophils and MPs and CFH binding to this integrin receptor mediates cell adhesion and migration as well as the phagocytosis of CFH- and iC3b-opsonized cell debris. The phagocytosis of apoptotic cell debris is not associated with the induction of inflammatory cytokines, contrary to the phagocytosis of pathogens and is an important part of the resolution phase of acute inflammation (DiScipio et al., 1998; Kang et al., 2012; Losse et al., 2010; Martin and Blom, 2016).

Like other complement factors, CFH is secreted by the liver and is an abundant soluble plasma factor (Zipfel and Skerka, 2009). Blood CFH is necessary to avoid spontaneous intra-vasal complement activation and resulting plasma C3 and CFB consumption that is observed in CFH-deficiency in humans and in $Cfh^{-/-}$ mice (Pickering et al., 2002; Zipfel and Skerka, 2009). CFH is also expressed by certain stromal cells such as RPE (Anderson et al., 2010) and strongly secreted by MPs (Calippe et al., 2017; Gautier et al., 2012; Luo et al., 2011; Schlaf et al., 2001). Among MPs *Cfh* transcription is higher in Mφ towards the end of the inflammatory reaction (5 days into experimental peritonitis) and in long-living rMφs such as MCs compared to Mos (Calippe et al., 2017).

CFH deficiency and mutations in SCR 19-20 are associated with atypical hemolytic

uremic syndrome (aHUS), and Membranoproliferative glomerulonephritis, type II (MPGN II; also known as dense-deposit disease) (Holers, 2008; Zipfel and Skerka, 2009). aHUS is a rare episodic disease, characterized by hemolytic anemia, thrombocytopenia, thrombotic microangiopathy and renal failure, while MPGNII is a type of glomerulonephritis caused by deposits in the glomeruli. Low plasma C3 levels, due to intra-vasal complement activation and consumption, characterize both diseases, and C3 fragments partake in the deposits in the glomeruli of MPGNII patients (Zipfel and Skerka, 2009). In addition, ~10% of aHUS patients (but not MPGNII patients) produce autoantibodies (mainly monoclonal) that bind to the C-terminal domain of CFH and block its binding to cell surfaces (Dragon-Durey et al., 2005; Jozsi et al., 2007).

AMD is associated with the single nucleotide polymorphism (SNP) rs1061170 of the CFH gene (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005) that leads to the substitution of histidine 402 for tyrosine (Y402H) in the SCR7 domain. The association with AMD is found for both advanced forms, but the association is stronger for geographic atrophy (Fritsche et al., 2013; Seddon et al., 2007). CFH402H is also associated with early disease stages (Fritsche et al., 2014), suggesting that CFH402H drives a pathomechanism implicated at the onset of disease. Additionally, this variant is strongly associated with other conditions such as smoking-associated lung cancer (Zhang et al., 2012) and increased mortality after cerebral hemorrhage (Appelboom et al., 2011), but interestingly not with aHUS or MPGNII (Zipfel and Skerka, 2009). Complement activation can be detected in donor eyes with AMD (Mullins et al., 2000), and plasma complement components and activation-fragments are elevated in patients with neovascular and atrophic AMD (Machalinska et al., 2009; Reynolds et al., 2009; Scholl et al., 2008; Sivaprasad et al., 2007) contrary to aHUS and MPGNII patients and $Cfh^{-/-}$ mice. However, the increase in plasma activation-fragments in AMD patients is likely independent of the CFH variant, as it is

observed in CFHY402 and CFH402H carriers alike (Reynolds et al., 2009; Sivaprasad et al., 2007) and might be due to the AMD-risk variants of CFI (van de Ven et al., 2013), CFB (Montes et al., 2009), and C3 (Zhan et al., 2013), or to a less specific activation of the innate immune system, suggested by concomitantly increased cytokines (see 2.1.). $Cfh^{+/-}$-heterozygous mice have been suggested to model the implication of CFH402H in AMD pathogenesis (Toomey et al., 2015), although there is no clinical evidence of diminished CFH expression in AMD patients and $Cfh^{+/-}$-heterozygous mice are characterized by diminished circulating FB and C3 levels compared to $Cfh^{+/+}$-wildtype mice.

We recently reported a rather unexpected role of CFH in the control of subretinal MP accumulation in both, early and late AMD. We showed that Cfh-deficiency completely prevented, age-related, subretinal MP accumulation and photoreceptor degeneration in AMD-prone Cx3cr1-deficient mice and mice expressing the AMD-associated APOE2 isoform (Calippe et al., 2017). Interestingly, a similar age- and CFH-dependent increase in MPs was also described in the choroid of $Cfh^{+/-}$ compared to $Cfh^{-/-}$ mice (Toomey et al., 2015). Using acute light-induced inflammation and subretinal adoptive transfer experiments, we showed that CFH does not influence initial MP recruitment but inhibits MP elimination during inflammation resolution. We further demonstrated that MP-derived CFH, rather than RPE- and liver-derived CFH, inhibits MP clearance (Calippe et al., 2017).

As described above, CFH can act as a cofactor of complement factor I (CFI) to cleave C3b into iC3b, which helps to opsonize apoptotic bodies with iC3b (Martin and Blom, 2016). However, we did not detect C3 or activated C3 fragments in subretinal MPs of $Cx3cr1^{-/-}Cfh^{-/-}$ mice and Mos and MCs do not express detectable levels of *Cfi* mRNA. Together, these observations suggest that implication of C3, C3b, or iC3b in CFH-mediated inhibition of MP elimination is unlikely. However, CFH also binds directly to CD11b/CD18 that is strongly expressed by MPs (DiScipio et al., 1998; Kang et al., 2012; Losse et al., 2010). We

demonstrated that CD47 (also called the *integrin associated protein*) that mediates the physiological role of TSP-1 in subretinal MP elimination (see 3.2.2.), interacts with the integrin CD11b/CD18 on the surface of MPs. We showed that CFH binding to CD11b blocks the activation of CD47 by TSP-1, thereby inhibiting MP elimination. TSP-1 and more specifically CD47 activation efficiently accelerated MP elimination similar to Cfh-deficiency (Calippe et al., 2017) (Fig. 9).

Importantly, we found that the AMD-associated CFH402H variant has an increased capacity to inhibit the elimination of certain MP populations, such as MCs, strengthening the causal link to AMD etiology. Although CFH402H affinity is decreased to certain GAG species, it is higher in particular to GAG sulfates (Clark et al., 2006). GAG sulfate profiles differ greatly between MPs and different microenvironments. For example, keratan sulfate proteoglycans (KPSG) are strongly present on ramified brain MCs but not on blood Mos (Wilms et al., 1999). The differential expression of GAG sulfates, such as KPSG, on MCs might explain why the CFH402H variant differentially influences MC but not Mos elimination (Calippe et al., 2017). Subretinal MPs originate from infiltrating Mo and MCs (Sennlaub et al., 2013) invariably robustly express KPSG, suggesting that KPSG is quickly induced in Mos that infiltrate the subretinal space (Combadiere et al., 2007; Ng and Streilein, 2001; Ng et al., 2009). This is also the case in spinal cord injury, but not in autoimmune neuritis (Jones and Tuszynski, 2002; Matsui et al., 2013). CFH402H might therefore have a particularly strong influence on the subretinal inflammation observed in AMD but not necessarily in other chronic inflammatory diseases.

Our findings also accommodate several observations that perturbed the concept of insufficient complement inactivation by CFH as the reason for its link to AMD: (i) CFH immunoreactivity in the eye is invariably stronger, not weaker, in AMD donor tissues (Hageman et al., 2005; Johnson et al., 2006; Shaw et al., 2012; Weismann et al., 2011), (ii)

there is no evidence to suggest that CFH402H is less efficient in inhibiting the alternative complement cascade and contrary to CFH-deficient patients, CFH402H carriers are not characterized by plasma C3 and CFB depletion (Harris et al., 2012), (iii) contrary to aHUS, CFH autoantibodies have a protective, not harmful, effect in AMD (Dhillon et al., 2010), (iv) the recipient, but not the liver Cfh genotype confers AMD-risk in liver transplant patients (Khandhadia et al., 2013), suggesting that plasma CFH is not involved in AMD pathogenesis and points to the possible importance of MP-derived CFH in the disease, and (v) preliminary data from the MAHALO phase II study suggests that an anti-CFD antibody that inhibits the alternative complement cascade has no effect on CFH402H carriers, but on GA patients with the disease-associated CFI variant (Rhoades et al., 2015).

Our results suggest that a main reason for the association bewteen CFH402H and AMD is its capacity to inhibit inflammation resolution, promoting the pathological chronification of subretinal inflammation. This mechanism should not be seen in opposition but rather as an additional trait of CFH402H, adding to its previously described decreased capacity to (i) inhibit oxidative stress (Shaw et al., 2012; Weismann et al., 2011), and (ii) to bind to Bruchs membrane (Clark et al., 2010), which might protect the RPE against uncontrolled complement activation (Coffey et al., 2007; Toomey et al., 2015).

6.1.3. 10q26

Numerous genetic association studies have shown that chromosome 10q26, that contains the age-related maculopathy susceptibility 2 (ARMS2), and high-temperature requirement A serine peptidase 1 (HTRA1) is a major candidate region associated with AMD (Fritsche et al., 2016; Swaroop et al., 2007; Yang et al., 2006). The risk haplotype contains a number of SNPs (rs10490924, rs3750848, rs3750847, 372_815del443ins54, rs11200638, rs3793917 and rs932275) and it is not yet clear how the SNPs affect the gene expression or function of the

genes in 10q26 and how they translate into AMD pathogenesis (Friedrich et al., 2011; Wang, 2014).

It has recently been reported, that the 10q26 risk haplotype is associated with a significant reduction of ARMS2 gene expression, yet with an increase of HTRA1 mRNA levels in several tissues including testis (Sha-Mei et al., 2016). The risk haplotype was correlated with higher levels of HTRA1 mRNA in lymphocytes (Yang et al., 2006) and RPE (An et al., 2010) in some but not all studies (Yang et al., 2006). The increased HTRA1 transcription observed in 10q26 carriers was suggested to be due to the SNP rs11200638 that disrupts the CG pattern in a conserved CpG Island (sites of DNA methylation) of the HTRA1 promoter (Wang, 2013), but could also be due to more complex interference of one or several SNPs of the risk haplotype. HTRA1 has been suggested to deposit in drusen (Chan et al., 2007; Yang et al., 2006). Additionally, in MPs LPS induces an increase in HTRA-1 (Hou et al., 2013). In LPS-induced experimental endotoxin-induced uveitis, HTRA1-deficiency protected and systemic inducible HTRA1-overexpression exaggerated subretinal MP accumulation. Furthermore, HTRA1-deficiency inhibited the MP accumulation observed in aged mice (Liao et al., 2013). Together these results suggest that HTRA1 expression in MPs promotes their subretinal accumulation, but the molecular mechanism remains elusive.

On the other hand, a recent publication suggests that ARMS2 is expressed in MPs with the common-, but not the risk-10q26 allele. The authors show that ARMS2 binds to cell debris, where it promotes C3b generation and opsonization for phagocytosis by MPs. 10q26 risk-allele carriers would therefore lack necessary opsonization, promoting debris accumulation and AMD (Micklisch et al., 2017).

6.1.4. General considerations on genetic risk and mononuclear phagocytes

Taken together, these studies show that the genetic-risk of AMD that is due to the APOE isoforms and CFH402H (and possibly the 10q26 risk allele) profoundly promotes

pathogenic subretinal inflammation (Calippe et al., 2017; Levy et al., 2015a; Levy et al., 2015b; Liao et al., 2013; Micklisch et al., 2017). Importantly, the affected genes are all expressed in MPs and the consequences of the disease-associated variants directly affect MP function. The genetic risks for AMD therefore directly influence inflammation, rather than increasing inflammation secondarily to choroid, RPE, or photoreceptor dysfunction.

In evolution, a common polymorphism or isoform appears in a population, when a germline mutation in the ancestral version of an allele generates an advantage in the "fitness" (survival, reproduction, etc.) of the carrier. The frequency of the allele thereby increases over the generations in the population under evolutionary pressure. Infectious diseases exert a constant, strong, evolutionary pressure on the genetic makeup of a population. The AMD-associated variants of CFH and the APOE isoforms might have appeared in human populations because they lead to a stronger inflammatory response and better defense against infectious disease. In that way, the limited elimination of MPs and the increased inflammatory reaction associated with CFH402H (Calippe et al., 2017), might have increased our survival to certain infectious diseases in our evolutionary past. Similarly, APOE has a role in the susceptibility to infection (de Bont et al., 1999; Roselaar and Daugherty, 1998; Sinnis et al., 1996). Moreover, the *APOE3* allele, that increases risk for AMD compared to the ancestral *APOE4*-allele (McKay et al., 2011), significantly delays progression and death from HIV infection (Burt et al., 2008) compared to the ancestral APOE4 genotype (Raichlen and Alexander, 2014). Well beyond the reproductive age, the increased inflammatory reaction that these genetic risk factors might have once been selected for, tip the balance from beneficial subretinal inflammation that helps control drusen accumulation to the disproportionate chronic inflammation that contributes to destructive late AMD.

6.2. Aging and environmental risk factors

Smoking, obesity, and light-exposure are the main environmental factors (Adams et al., 2011; Chakravarthy et al., 2010; Schick et al., 2016). Aging and the main environmental factors likely have multiple local and systemic effects and their involvement in the promotion of subretinal MP infiltration is becoming increasingly clear.

6.2.1. Age

Advanced age is the defining risk factor for AMD. Advanced age impacts homeostasis through a multitude of mechanisms that include genetic programs and environmental factors that are yet ill defined. In mice, age has been shown to be sufficient to induce subretinal accumulation of MPs in wildtype mice (Damani et al., 2011; Xu et al., 2008) and this age dependent accumulation is amplified and shifted to younger ages in in Cx3cr1-, TSP-1-, and CD47-deficient mice (Calippe et al., 2017; Combadiere et al., 2007; Levy et al., 2015a; Sennlaub et al., 2013). Mice that express the AMD-risk APOE2 allele display a similar age-dependent increase of subretinal MPs and the APOE4 allele protects Cx3cr1-deficient mice against the accumulation and associated degeneration in aged mice (Levy et al., 2015b). Aging is associated with an increase in the production of chemokines such as CCL2 in the eye (Chen et al., 2008; Sennlaub et al., 2013) and also decreases the immunosuppressive properties of the subretinal space, as the resolution of acute, laser-induced subretinal MP acuumulation is significantly slowed in aged mice (Damani et al., 2011). The increased infiltration of MPs could be due to the aging and senescence-like phenotype in a sub-population of RPE cells and photoreceptors, but also be influenced by the aging of the MPs themselves. Indeed, the number of MCs is increased in aged mice and they are significantly smaller, have less branched arborizations (Damani et al., 2011), and are characterized by profound transcriptional changes (Ma et al., 2013). Additionally, circulating monocytes from aged subjects are characterized by increased basal and induced cytokine production (Agrawal et al., 2007; Hearps et al., 2012). The importance of changes within the circulating leukocytes

themselves in subretinal inflammation and AMD pathogenesis was demonstrates using bone marrow chimeras between young and aged mice. Indeed, the heightened CNV response observed with aging is dependent on the age of bone marrow-derived cells, rather than resident ocular cells (Zhao et al., 2013). These results suggest that the effect of aging on Mo and Mo-derived Mφs is of far greater importance than the aging of the eye itself in the pathogenesis of AMD.

6.2.2. Light

Excessive sunlight exposure, although difficult to quantify, has been suggested to be associated with both early and late AMD (Fletcher et al., 2008; Schick et al., 2016). Previous cataract surgery, which increases retinal light exposure, is also a strong risk factor for AMD (Chakravarthy et al., 2010). In animal models, light conditions in conventional animal facilities (250lux) are sufficient to induce subretinal MP accumulation in young adult albino mouse strains, but not in pigmented mice (Ng and Streilein, 2001). Subretinal MPs do not accumulate when the albino mice are raised in darkness and are eliminated when placed into darkness, which clearly shows the light dependence of the accumulation in these mice. Similarly, light exposure to intensities that are harmless to wildtype mice can trigger the accumulation of subretinal MPs in young, adult Cx3cr1-deficient mice that lack a tonic retinal inhibitory signal (Combadiere et al., 2007; Sennlaub et al., 2013), and raising albino $Cx3cr1^{-/-}$ mice in darkness prevents this accumulation (Combadiere et al., 2007) (see 3.2.). Interestingly, young, adult mice that are deficient in factors that mediate subretinal immune-suppression, such as $FasL^{gld/gld}$, $Fas^{lpr/lpr}$, $Tsp1^{-/-}$ and $CD47^{-/-}$-mice are equally susceptible and accumulate subretinal MPs in an exaggerated manner (Calippe et al., 2017; Levy et al., 2015a) (see 3.3.). These results suggest that the accumulation of subretinal MPs in young wildtype animals does not become apparent in part because they were efficiently eliminated, rather than not recruited in the first place. In this context, it is interesting to note that TRE2 mice, a mouse strain that

expresses the AMD-risk APOE2 allele, is also more susceptible to light exposure that is harmless in mice expressing the APOE3- and APOE4-allel (Calippe et al., 2017; Levy et al., 2015b). To our knowledge there is currently no evidence that light directly activates MPs. The increased subretinal accumulation of MPs is therefore likely due to the photo-oxydative stress that light exposure induces in the photoreceptor segments and the RPE, which indirectly triggers the production of chemokines and inhibits RPE-mediated elimination of MPs.

In analogy, the exposure to intense light, such as a sunny day on the beach or on the snow, might be sufficient to trigger some degree of transient central subretinal MP accumulation in humans, which is quickly suppressed. In aged subjects, who accumulate several additional risk factors that promote subretinal inflammation, a normally harmless light intensity might be sufficient to trigger and maintain pathogenic subretinal MP infiltrates.

6.2.3.Smoking

Current smoking increases the risk of AMD between 1.7 and 3.4 fold (Chakravarthy et al., 2010) and is one of the main environmental risk factors. To our knowledge there are no studies investigating subretinal inflammation in experimental chronic cigarette smoke exposure to date, but smoking has been shown to exacerbate a variety of auto-inflammatory and autoimmune diseases (Arnson et al., 2010), suggesting that it affects common inflammatory pathways. It is to date unclear if smoking-induced lesions are primarily through ocular- or inflammatory-cells and how the dysregulation plays into AMD pathogenesis.

6.2.4. Obesity and diet

The incidence of obesity has more than doubled worldwide since 1980, and estimates suggest that in 2014, more than 1.9 billion adults were overweight and over 600 million obese (World Health Organisation, 2015). Consequently, complications of obesity such as metabolic syndrome, type 2 diabetes mellitus and cardiovascular disease have become a global pandemic (Taubes, 2009). In addition to well documented associations of obesity with insulin

resistance and cardio-vascular disease, it is becoming increasingly clear that being overweight is also a risk factor for neurodegenerative diseases such as Parkinson's disease and multiple sclerosis (Hu et al., 2006; Marrie and Beck, 2014), introducing the idea that neuro-inflammatory conditions such as AMD may be associated with obesity.

Obesity has been suggested as a risk factor for AMD, yet associations between increased body-mass index (BMI) and AMD were inconclusive. However, the BMI has been criticized previously as a poor measure of adiposity (Garn et al., 1986), particularly for the elderly where it lacks sensitivity. This might partly explain the paradoxical beneficial effect from excess weight in mortality studies of elderly populations (Romero-Corral et al., 2008).

Compelling evidence for a link between obesity and AMD has been provided in a series of epidemiological studies using waist/hip ratio as an approximate measure for abdominal obesity, which mainly reflects visceral adipose tissue increase. For example, the Melbourne Collaborative Cohort Study, demonstrated in a cohort of 21,287 participants that in men, each increase of 0.1 in waist/hip ratio was associated with a 13% increase in the odds of early AMD and a 75% increase in the odds of late AMD (Adams et al., 2011). Similarly, a population-based cohort study of 12 515 middle-aged participants demonstrated that a decrease in waste-hip ratios of 3% or more was associated with 29% lower odds of developing AMD after 6 years of follow-up. This effect was particularly striking among participants that were already obese at baseline, where a decrease in waste-hip ratio was associated with 59% lower odds of developing AMD (Peeters et al., 2008). Together, these studies suggest that obesity in men, and possibly particularly visceral obesity, would the second most important environmental risk factor for late AMD after smoking.

In obesity, a significant number of adipocytes undergo necrotic-like adipocyte death, which attracts Mφs that phagocyte the cell debris and leaked lipid droplets (Cinti et al., 2005).

Thereby, the numbers of Mφs increase in adipose tissues, in particular in the visceral fat that surrounds internal organs (Greenberg and Obin, 2006; Odegaard and Chawla, 2013). In fact, in extremely obese mice and humans, Mφs constitute up to 40-50% of the cells of the adipose tissue compared to below 5% in lean individuals (Weisberg et al., 2003). Additionally to their increased numbers the Mφs are activated and secrete inflammatory cytokines, such as CCL2, TNF-α□, IL-6, and IL-1β, which increase in fat tissue and blood of obese individuals (Li et al., 2010; Park et al., 2005) and can induce insulin resistance and type 2 diabetes (Shoelson et al., 2006). In mice, during diet-induced weight gain, over 50% of total upregulated mRNA transcripts in adipose tissue are inflammatory-related genes (Xu et al., 2003). Interestingly, a short-term diet switch in obese mice from high-fat diet to normal diet results in strongly reduced expression of inflammatory cytokines without altering the numbers of necrotic adipocytes or macrophages (Li et al., 2010). These results suggest that components of diet, or their effect on the microbiota of the gut, polarize macrophages towards a pro-inflammatory state in obesity.

The human intestinal tract hosts up to 100 trillion microorganisms, which far outnumber human cells. The gut microbiome, which encompasses the genomes of all microorganisms, is though to be 150 times more extensive than the human genome (Qin et al., 2010). In the first years of life, colonization of the gastrointestinal track is critical for the development of a functional and efficient host immune system (Sommer and Backhed, 2013). Misbalance of certain species of gut microbes at the expense of others (dysbiosis) is particularly concerning for the aging population given that they modulate aging-related changes in innate immunity, sarcopaenia, and cognitive function (O'Toole and Jeffery, 2015). Several studies have associated dysbiosis of the gut microbiota with the onset of chronic inflammatory diseases such as obesity, diabetes and metabolic syndrome (Caesar et al., 2010; Caesar et al., 2015; Cani et al., 2007a; Ley et al., 2006; Turnbaugh et al., 2006) and a recent

study has suggested that modifications in intestinal microbiome are associated with AMD (Zinkernagel et al., 2017). Given that commensal gut microbiota exert profound influence on digestion, dietary metabolism and endotoxemia, they are prime candidates to impact chronic low-grade inflammation (Cani et al., 2008; Spanogiannopoulos et al., 2016) such as that associated with obesity and AMD.

Diet has a profound impact on modifying gut microbial composition by shifting the ratio of the two dominant phyla in the distal gut the *Bacteroidetes* and *Firmicutes* (David et al., 2014; Scott et al., 2013; Turnbaugh et al., 2009). Together, these phyla make up over 90% of bacterial phylogenetic types in the distal gut (Ley et al., 2006) and the relative proportion of *Bacteroidetes* is reproducibly decreased in both obese humans (David et al., 2014; Turnbaugh et al., 2006) and obese mice (Hildebrandt et al., 2009; Ley et al., 2006; Turnbaugh et al., 2006) but also in AMD patients (Zinkernagel et al., 2017). We recently demonstrated that high-fat diets exacerbate subretinal inflammation and choroidal neovascularisation by causing dysbiosis by increasing proportions of gut *Firmicutes* populations at the expense of *Bacteroidetes (Andriessen et al., 2016).* Microbiotal transplants (fecal-oral) and other paradigms that modify the gut microbiome such as gut-impermeable oral antibiotics collectively showed that gut dysbiosis provoked heightened intestinal permeability, which increases the diffusion and contamination of the blood by bacterial components (pathogen associated molecular patterns, PAMPs) *(Andriessen et al., 2016),* as previously shown in other studies (Caesar et al., 2010; Cani et al., 2007a; Cani et al., 2007b; Henao-Mejia et al., 2012). These PAMPs activate the IIRC and Nod-like receptors (NLRs) on Mos that pass the intestinal circulation, resident MPs, and likely other cells and induce elevated production of IL-6, IL-1β TNF-α and VEGF-A that promote chronic low-grade inflammation and exacerbate pathological angiogenesis (Figure 10). Interestingly, these results also suggest that

using LPS as a mean to stimulate MPs in AMD culture models (Mathis et al., 2017), often considered as being non-related to AMD pathogenesis, might actually not be that far fetched.

Taken together, these studies suggest that dysbiosis of the gut microbiota, compromises the gut's barrier function, leading to the release of gut-born microbial particles into the blood stream that activate the innate immune system, which participates in metabolic diseases, but also exacerbates neuro-inflammation, including that seen in AMD. In our study, dysbiosis was induced by high fat diet, but a similar dysbiosis was recently reported in AMD patients, apparently independently of obesity (Zinkernagel et al., 2017), suggesting that this pathogenic mechanism is not restricted to over-weight patients.

6.2.5. General considerations on aging and environmental risk and mononuclear phagocyte function

In summary, the consequences of at least two of the major risk factors, aging and obesity/diet, directly induce an inflammatory activation state in MPs and thereby exacerbate pathogenic subretinal inflammation. In mouse models of CNV, the increased neovascularization observed in aged- and high-fat-diet-mice is the consequence of a systemically pre-activated innate immune system and heightened local tissue inflammatory reaction induced by laser injury. A similar phenomenon of age- and "western-diet"-induced increased Mo excitability might confer the increased AMD-risk in humans. Non-toxic light-exposure, on the other hand, is likely an example of a local trigger for subretinal MP infiltration that only leads to chronic, possibly pathogenic, inflammation if combined with sufficient other AMD-risk factors, such as homozygosity for APOE2 and age.

7. Patho-mechanistic conclusions

The photoreceptor cell layer is anatomically and immunologically peculiar as it is physiological devoid of resident MPs, vasculature and lymphatic vessels, which possibly reflects its particular vulnerability to immune-cell and plasma-induced collateral damage (3.1.). In healthy individuals the leukocytes and MPs that are likely occasionally recruited to

the subretinal space are quickly eliminated by the immune-suppressive capacities of the RPE that efficiently induces death of the immune cells (3.3.). With age, dysfunction of the interactions between RPE and the choriocapillaries lead to the benign deposition of debris in most individuals that can take the form of hard drusen or basal laminar deposits (BlamD) that are not associated with AMD. In some individuals these accumulations become bigger and form BlinD and large drusen that define ARM (1.). These accumulations attract MPs (2.1.) that likely help control an excessive debris accumulation in the majority of subjects as only ~15% -~30% of patients with small numbers of large drusen progress to late AMD (5.1. and 1.).

In some patients however, the unfortunate combination of AMD-risk factors likely leads to particularly inflammatory Mo and MP characteristics that increase MP resistance to RPE-induced elimination and lead to excessive MP recruitment (6.). The chronic presence of activated MPs on the apical side of the RPE and in the photoreceptor cell layer, and in particular the continuous recruitment of neurotoxic inflammatory Mos from the blood (2.2.1. and 4.), induce significant collateral damage and dysfunction in the RPE, choroid and photoreceptors (5.2.). The collateral damage further fuels chronic, pathogenic inflammation, degeneration and finally, late AMD. Chronic inflammation can be a part of the problem, or constitute the main pathomechanism in patients with a particularly unfavorable selection of pro-inflammatory risk factors. To what extent subretinal inflammation affects chorioretinal homeostasis, depends on the amplitude of the infiltrate and on the production of pathogenic mediators in the individual patient. The fact that all major genetic-risk factors, aging and obesity/diet directly influence MP characteristics towards a more inflammatory phenotype strongly suggest that subretinal MP infiltration is likely a leading pathogenic factor that pushes the balance from healthy aging to degenerative late AMD in many patients and could be potentially exploited when developing future therapies. Therapeutically, it is likely not

important whether a drug targets the primary reason of a disease, but whether it inhibits the mechanisms that cause the debilitating symptoms, which can be downstream secondary events, as seen in many chronic diseases. Anti-VEGF therapies for wet AMD for example clearly do not inhibit the origin, but a symptom-causing late complication of AMD.

8. Therapeutic considerations

Life style adaptations

AMD is often perceived as the result of a slow, life long accumulation of cellular damages that lead to the degenerative changes. To adapt a healthier life style once the disease manifests itself would therefore appear too late to counterbalance the overall load of disease-induced damages that occurred over the years. However, the recent advances described above suggest that the continuous recruitment of Mo and MPs to the subretinal space is a highly dynamic process that is greatly influenced by modifiable environmental factors during early and even late AMD. Excessive light avoidance, in particular after cataract surgery, likely reduces one of the triggers of local MP recruitment. Our studies on high fat diet in mice (6.2.4.) suggest that healthier diets low in processed fats or high fiber diet, can correct gut microbiota dysbiosis and reduce subretinal inflammation (Andriessen et al., 2016). The recent report of a similar dysbiosis in AMD patients (Zinkernagel et al., 2017), and the observation that healthier diet reduces the incidence of AMD (Klein and Klein, 2007) suggest that similar mechanisms are at play in humans. Another means to attenuate neuroinflammation with life style adaptations is regular physical activity, that has been shown to be beneficial for a number of neurological disorders that are characterized among others by chronic pathogenic neuroinflammation (Spielman et al., 2016). Physical activity lowers the quantity of circulating Mo, inhibits the expression of TLRs on MPs, and reduces levels of pro-inflammatory cytokines in the blood and the secretion from activated leukocytes (Gleeson et al., 2011). Indeed, a high level of physical activity has been shown to be protective in AMD in a number of studies, but might not be independent of other factors that are influenced by physical

activity, such as obesity and leukocyte numbers (Gopinath et al., 2014; Klein and Klein, 2007; McGuinness et al., 2016). Although the effect of smoking on subretinal inflammation has not yet been formally investigated, cessation of smoking would likely also diminish inflammation, as it exacerbates a variety of auto-inflammatory and autoimmune diseases (Arnson et al., 2010).

"Anti-inflammatory" pharmaceutical therapies

As outlined above, inflammation is a complex process involving innate and adaptive immunity, the interplay of several cell types, and numerous distinct signaling pathways that span from eicosanoid mediators, to dozens of distinct interleukins and cytokines. Therapeutics such as glucocorticoids, non-steroidal anti-inflammatory drugs (NSAID) such as cyclooxygenase inhibitors, and immunosuppressants such as ciclosporine are often referred to as "anti-inflammatory" drugs, because they inhibit different aspects of inflammation. However they do not inhibit inflammation as a whole. Ciclosporine inhibits calcinurin-induced transcription of cytokine genes mainly in activated T cells (Matsuda and Koyasu, 2000), which impacts the function of lymphocytes, but it also upregulates toll-like receptors on Mφs (Tedesco and Haragsim, 2012). Glucocorticoids affect carbohydrate, fat, and protein metabolism, and are possibly best known for their ability to repress delayed hypersensitivity reactions by a direct action on T cells (Liu et al., 2013a), but exert opposing effects on Mφ function depending on their concentration (Lim et al., 2007). NSAID are cyclooxygenase inhibitors that inhibit the production of prostaglandins, but increase the synthesis of leukotriens (Robinson, 1989) that activate MPs (Gagnon et al., 1989) and can prolong MP infiltration (Gilroy et al., 1999). This lack of efficiency to inhibit MP-mediated subretinal inflammation might also explain why widely used "anti-inflammatory" therapies, such as treatments with systemic NSAIDs, have not slowed AMD progression. Together, these considerations show the need for a specifically adapted "anti-inflammatory" therapy to inhibit

the mechanisms of subretinal MP accumulation and their activation in AMD.

The recent advances in our understanding of the mechanisms of subretinal inflammation summarized above outline several possible avenues to inhibit pathogenic MP accumulation locally and specifically: 1) reinforce retinal tonic inhibitory signals, 2) restore subretinal immune suppression, 3) inhibit the recruitment of pathogenic Mos, or 4) inhibit the production of MP-derived inflammatory cytokines. In animal models, the pharmaceutical reinforcement of retinal tonic inhibitory signals using soluble CX3CL1 (Mendiola et al., 2016; Zabel and Kirsch, 2013) or CD200R agonists (Horie et al., 2013) have been shown to curb pathogenic inflammation. We recently showed that IL-6 inhibition can restore RPE immune suppression and that CD47-agonists accelerate subretinal MP elimination *in vivo* (Calippe et al., 2017; Levy et al., 2015a). Pharmacological inhibition of CCR2-dependent Mo recruitment also significantly inhibited the pathogenic accumulation of Mo-derived MPs (Sennlaub et al., 2013) and inhibition of IL-1β or inhibition of IL-1β maturation significantly protected against CNV and photoreceptor degeneration in mice (Eandi et al., 2016; Hu et al., 2015; Lavalette et al., 2011).

The studies in human patients also suggest that improved definition and understanding of retinal imaging in patients with AMD, using adoptive optics and optical coherence tomography, as well as systemic biomarkes in the plasma and circulating Mos, might help identify AMD patients in whom inflammation-mediated mechanisms play a decisive role and hence appropriately segment target populations. Pharmacologically-induced resolution of chronic pathogenic inflammation in the eye of these patients might help diminish the need for anti-angiogenic therapies in neovascular AMD and stop GA lesion growth before it destroys the fovea and high acuity vision.

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Figure Legends

Figure 1: Subretinal MPs accumulate on the retinal pigment epithelium in AMD

Confocal microscopy of IBA-1 (green staining) immunohistochemistry of RPE flatmounts (RPE autofluorescence visible as orange due to its autofluorescence in the red and green channel) from a healthy donor (A), a donor with small drusen (B), a donor with large drusen (C) and a donor with geographic atrophy lesions. AZ: atrophic zone; TZ: transitional zone.

Figure 2: Mononuclear phagocyte accumulation in AMD

The healthy photoreceptor cell layer and subretinal space is devoid of mononuclear phagocytes. However, mononuclear phagocytes (MPs), derived from circulating monocytes (Mos) and resident macrophages (Mφs) and microglial cells (MCs), infiltrate the subretinal space around chroidal neovascularizations of neovascular AMD patients and in the atrophic zone of geographic atrophy patients (GA), and phagocyte cell debris and pigment from but dying retinal pigment epithelium (RPE) and photoreceptors. They are also present on the apical side of RPE of the transitional zone of GA patients and in and around large drusen.

Figure 3: Immunosupressive retinal environment

Physiologically, the retina constitutes an immunosuppressive environment that is mediated by tonic inhibitory signals from neurons and leukocyte-death inducing signals of the RPE. Retinal tonic inhibitory signals include the expression of neuronal CX3CL1 expression, which inhibits the induction of pro-inflammatory cytokines by reducing the expression of Apolipoprotein E and PR2X7 among others. In the subretinal space, activation of CD47 on infiltrating leukocytes by thrombospondin-1 (TSP-1) sensitizes the inflammatory cells to FasL-induced cell death and elimination.

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Figure 4: Schematic representation of a generic TSP1 monomer and its receptors.

The type 1 repeats bind to the CD36 receptor and the latency-associated peptide (LAP) of the latent TGFβ binding protein. The carboxy-terminal cell-binding domain (CBD) contains two valine-valine-methionine (VVM) sequences that interact with CD47 receptors, notably at the surface of leukocytes, such as macrophages (Mφ). CD47, also called integrin associated protein clusters with the integrin CD11b/CD18 among others. The N-terminal domaine of CD47 on stromal cells also functions as the ligand of the Mφ receptor Sirpα and their interaction inhibits the phagocytosis of the CD47 expressing cell ("don't eat me" signal).

Figure 5: Monocyte derived inflammatory macrophages drive photoreceptor degeneration and choroidal neovascularisation.

The chemokine CCL2 that is increased in eyes from neovascular and atrophic AMD, is produced by infiltrating activated MPs and recruits further inflammatory CCR2+monocytes (Mo) from the circulation that differentiate into angiogenic, photoreceptor-toxic inflammatory macrophages (Mφ) in the subretinal space. Mo-derived Mφs are represented with the typical bean shaped nuclei of Mos in this graphic representation, although their nuclei quickly adapt the globular shape of nuclei from resident Mφs once they infiltrate the tissue and are generally morphologically indistinguishable from MPs of other origins.

Figure 6: Pathogenic inflammatory cytokines released from infiltrating mononuclear phagocytes.

Activation of the innate inflammatory receptor cluster (IIRC) that is formed by toll like receptors 2 and 4 (TLR) and their obligate co-receptor CD14 on activated MPs, derived from MCs and Mos, produce inflammatory cytokines that disturb chorioretinal homeostasis at different levels.

Figure 7: Schematic model of the activation of the innate immunity receptor cluster by apolipoprotein E

Under unstimulated conditions, the different components of the IIRC of Mφs are separated from each other, as some elements cluster to cholesterol-rich membrane domains called lipid rafts (eg. CD14) while others are located in the non-raft plasma membrane (eg. TLR2 and TLR4). IIRC-ligands, such as bacterial lipopolysaccharides overcome this separation, as they bind to the extracellular domains of both co-receptors (CD14 and TLRs), bringing the receptors and their intracellular domains closely together, which activates Myd88, NFκB and induces the transcription of inflammatory cytokines. Excessive APOE and/or APOA-I can destabilize the lipid raft by extracting cholesterol, lift the physiological separation of the receptors and trigger the intracellular signaling in the absence of IIRC ligands.

Figure 8: Schematic model of the activation of the innate immunity receptor cluster by apolipoprotein E

The alternative complement cascade is initiated by the spontaneous hydrolysis of C3, the most abundant complement factor, that forms C3a and C3b. C3b can attach itself to the bacterial cell surfaces and assemble with the complement Factor Bb (a fragment that results from the activation of Factor B by Factor D) to form the C3 converting enzyme C3bBb. C3bBb catalyzes an avalanche of C3a and C3b from C3. C3b opsonization of bacteria triggers their phagocytosis and neutralization by neutrophils and MPs. The recruitment of an additional C3b molecule to C3bBb leads to the formation of the C3bBbC3b complex, the C5 convertase that cleaves C5 into C5a and C5b. C5b recruits C6, C7, C8, and C9 to form a pore (MAC) that can induce lytic pathogen death. The C3a and C5a fragments are potent chemotactic agents that recruit neutrophils and Mos. Complement factor H (CFH) binds to glycoaminoglycans (GAG) on host cells and apoptotic bodies and acts as a cofactor of Complement factor I (CFI) that cleaves C3b into iC3b, which has no hemolytic or

amplification potential and opsinizes apoptotic bodies together with CFH, as they mediate the their phagocytosis by the integrin CD11b/CD18 expressed on mononuclear phagocytes. Mo express high amounts of C3, which might reflect their bactericidal functions, while long lived MCs express high amounts of CFH, which is involved in the phagocytosis of apoptotic cells.

Figure 9: Schematic model of the implication of Apolipoprotein E and Complement factor H in subretinal mononuclear phagocyte accumulation

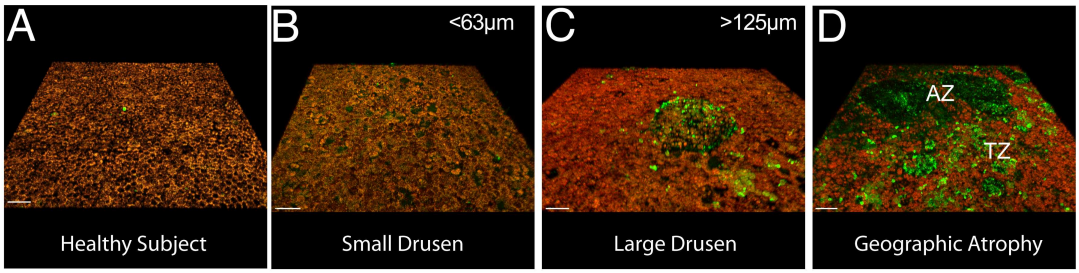
Physiologically activation of CD47 on infiltrating mononuclear phagocytes (MPs) by thrombospondin-1 (TSP-1) sensitizes the cells to cell death and elimination induced by FasL expressed by the retinal pigment epithelium (RPE). Excessive Apolipoprotein E destabilizes MP lipid rafts, which activates the innate immunity receptor cluster (IIRC) and induces inflammatory cytokines such as IL-6 that inhibits FasL expression and elimination by the RPE. The AMD-associated APOE2 isoform leads to excessive intracellular APOE concentrations and the AMD-protective APOE4 isoform is associated with lower concentrations and an impaired capacity to activate the IIRC. Complement factor H (CFH) binds to the integrin CD11b/CD18 and sterically inhibits the activation of the integrin associated protein CD47 by thrombospondine 1 (TSP-1), that is necessary to sensitize subretinal MPs to FasL-induced elimination. The AMD-associated CFH402H variant has an increased capacity to inhibit TSP-1 mediated subretinal MP elimination.

Figure 10: Schematic model of the implication of obesity and high fat diet on subretinal mononuclear phagocyte accumulation

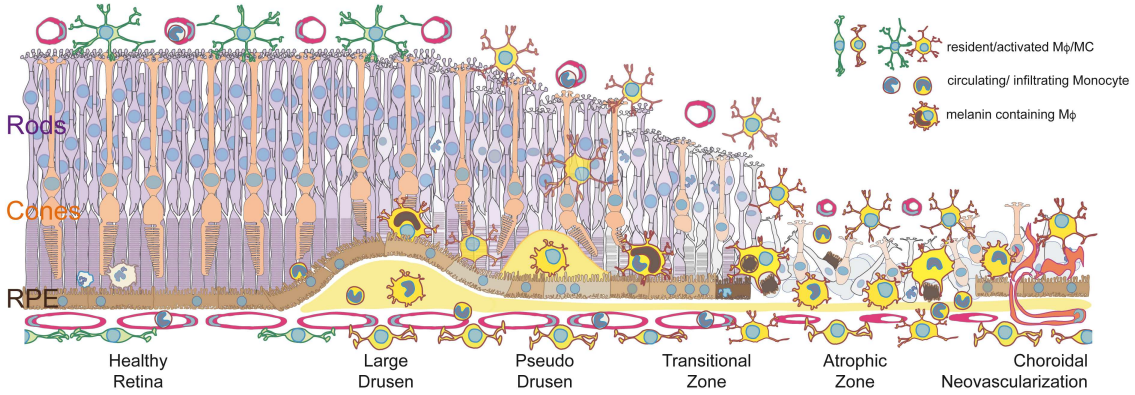
High fat diets (processed fats) provoke intestinal dysbiosis of the gut microbiota with increased proportions of gut *Firmicutes* populations at the expense of *Bacteroidetes.* This is accompanied by compromised gut barrier function and leads to the release of gut-born microbial particles (pathogen associated molecular patterns, PAMPs) into the blood stream.

These PAMPs activate the IIRC on Mos that pass the intestinal circulation, resident MPs, and likely other cells and induce elevated production of inflammatory cytokines that promote chronic low-grade inflammation and exacerbate pathological angiogenesis.

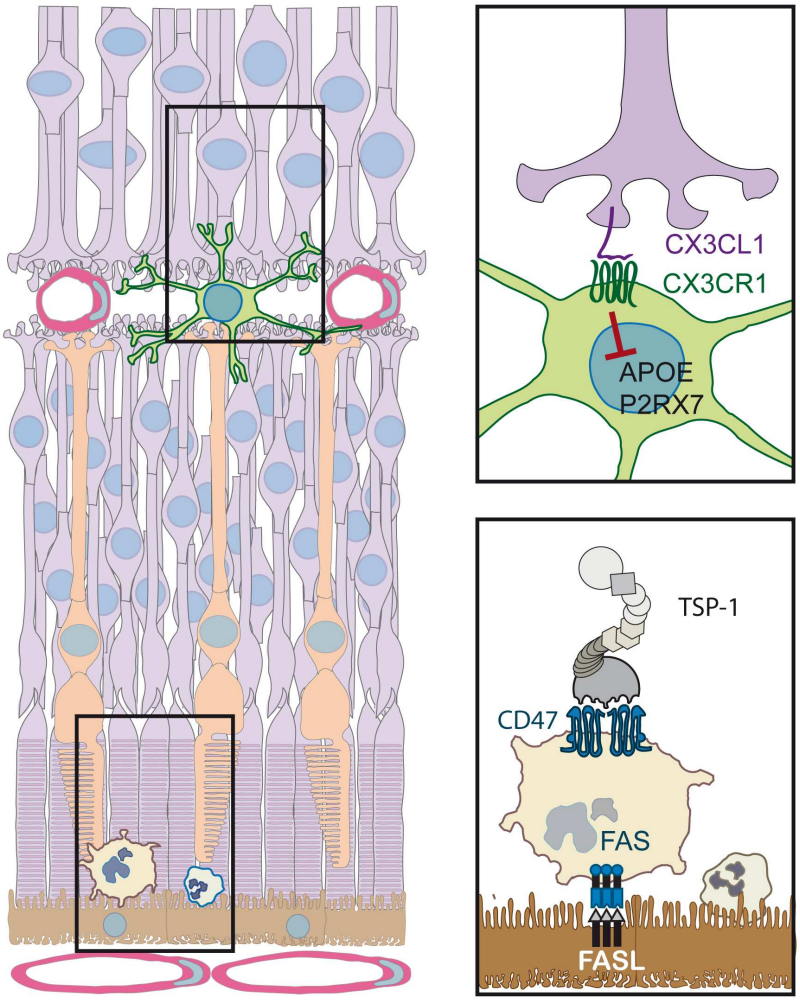
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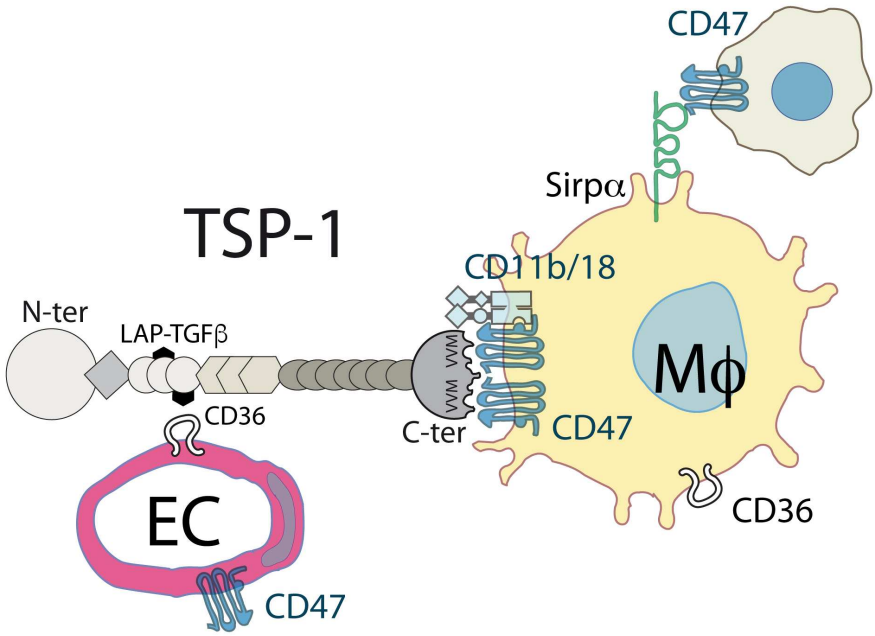


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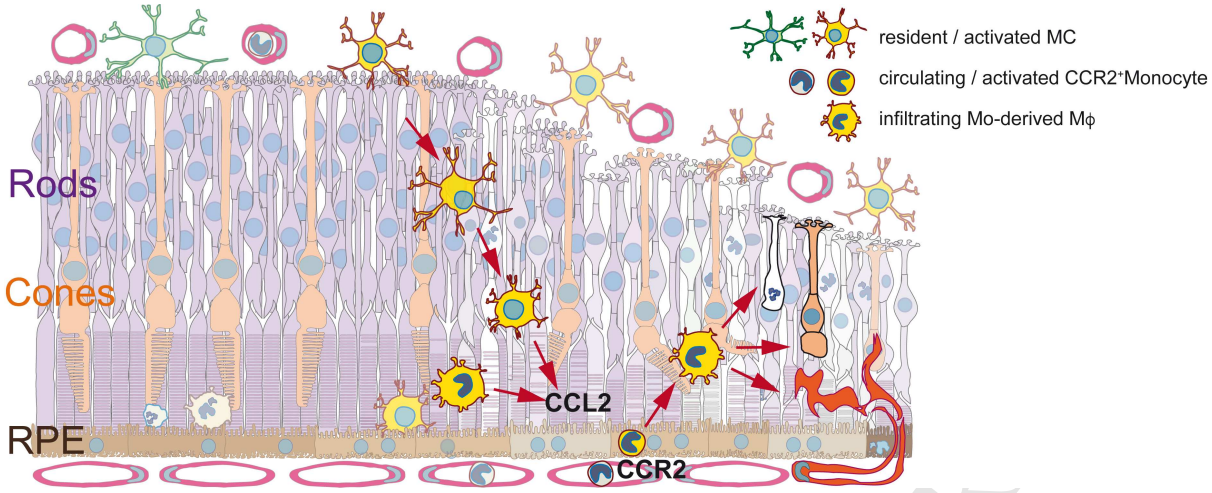


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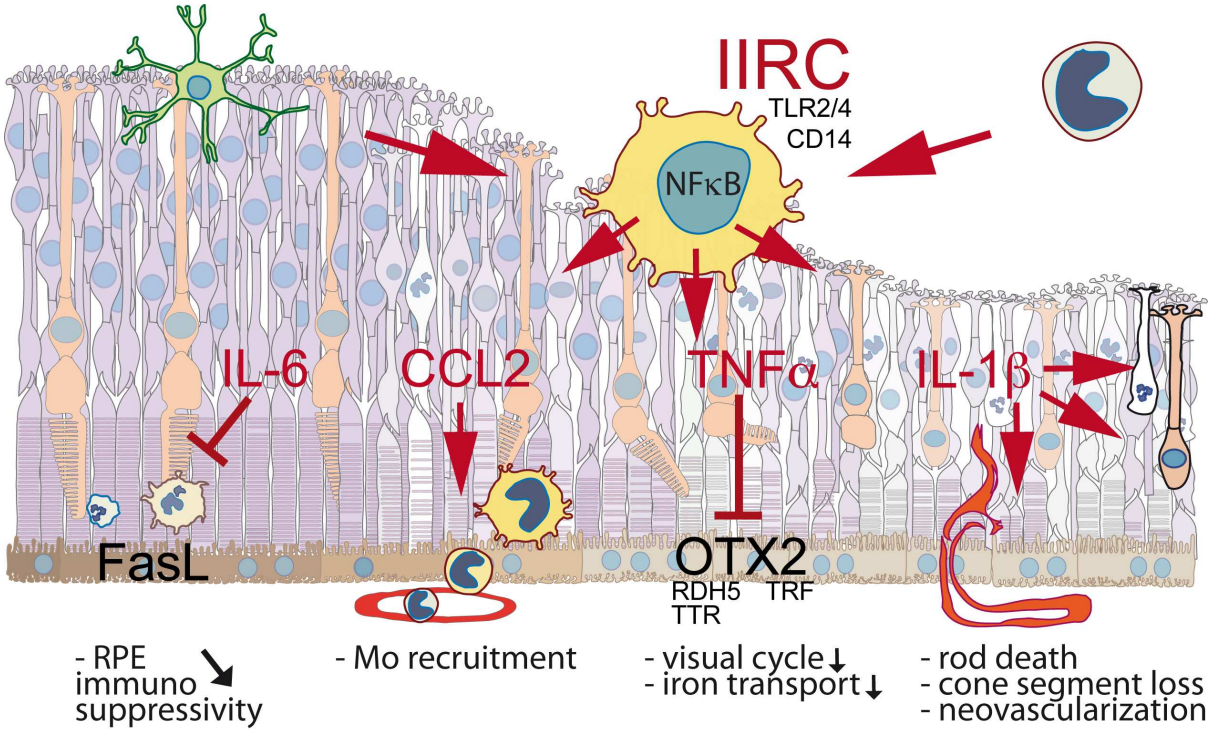

CX3CL1
CX3CR1
APOE
P2RX7
TSP-1
CD47
FAS
FASL


CD47
Sirpα
TSP-1
CD11b/18
N-ter
LAP-TGFβ
Mϕ
CD36
C-ter
CD47
EC
CD36
CD47

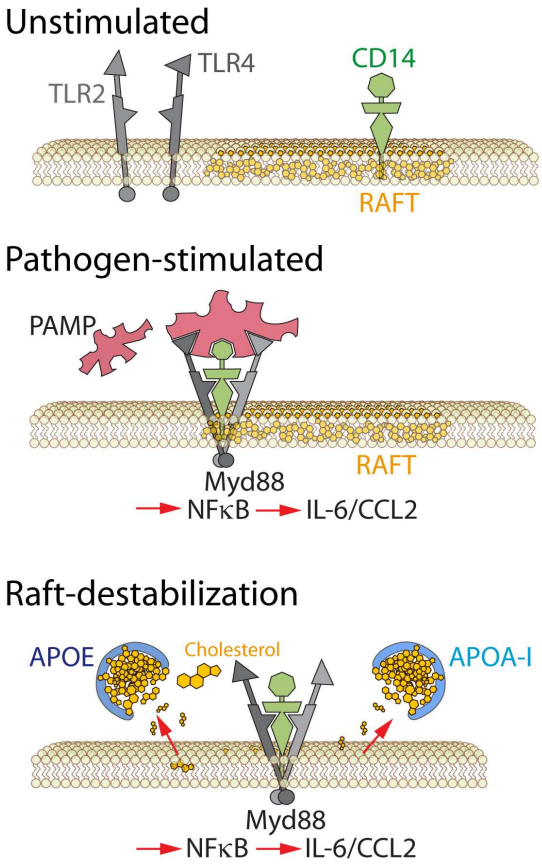
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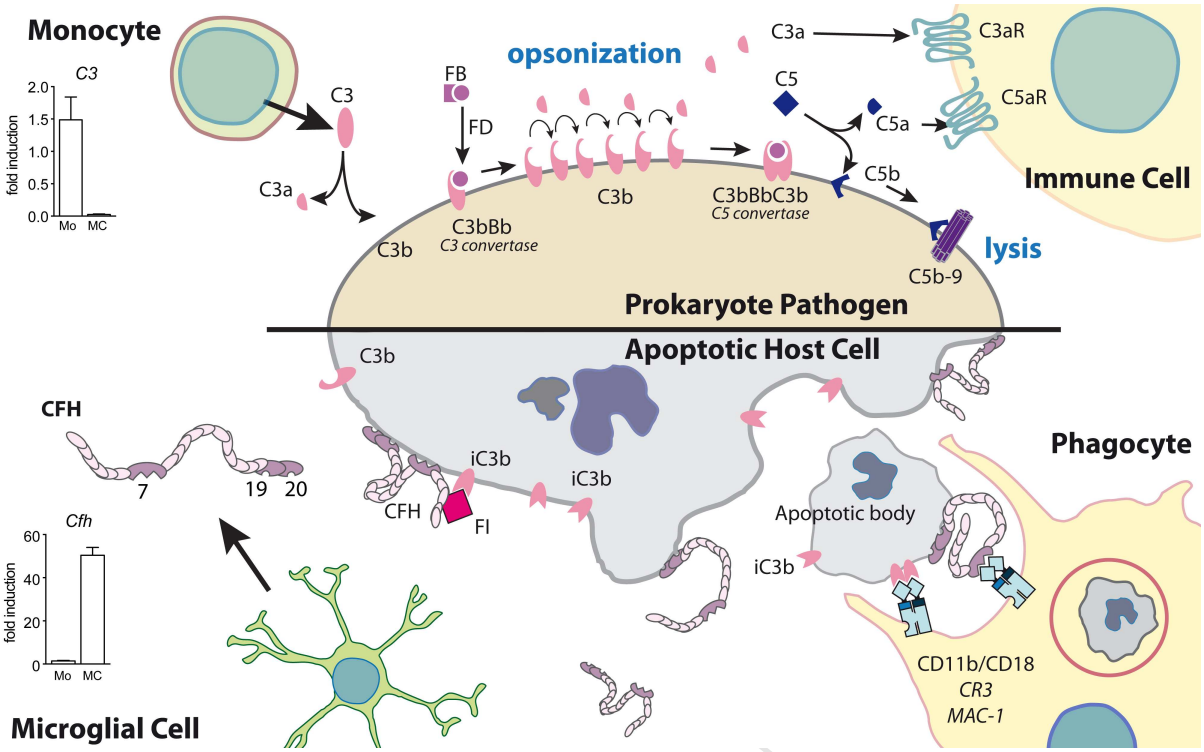
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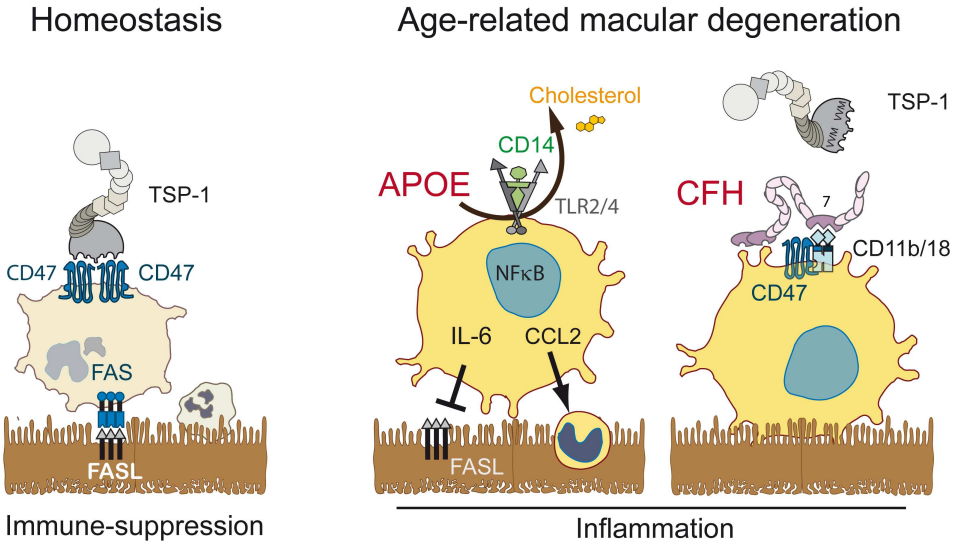
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Unstimulated
TLR2
TLR4
CD14
RAFT
Pathogen-stimulated
PAMP
RAFT
Myd88
NFκB
IL-6/CCL2
Raft-destabilization
APOE
Cholesterol
APOA-I
Myd88
NFκB
IL-6/CCL2

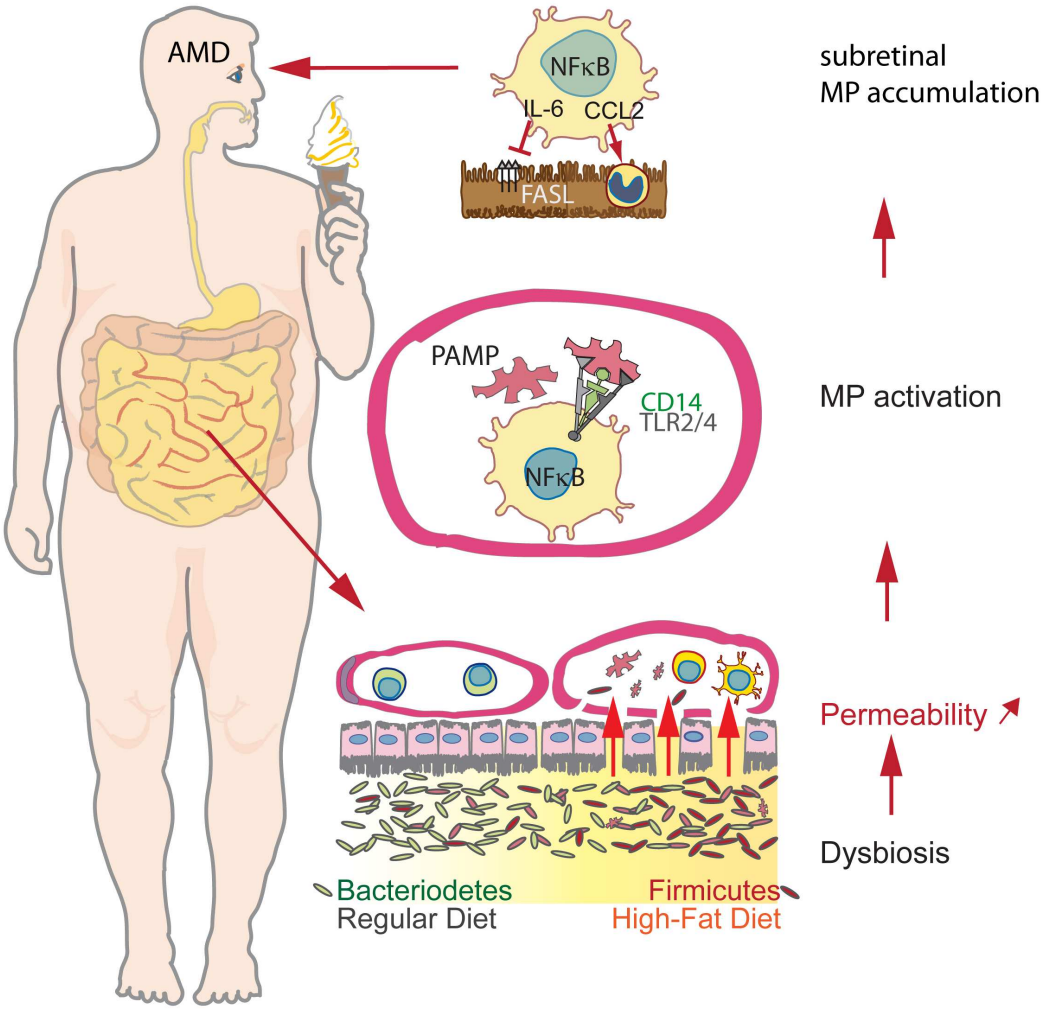
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AMD
NFκB
IL-6
CCL2
FASL
subretinal
MP accumulation
PAMP
CD14
TLR2/4
NFκB
MP activation
Permeability
Dysbiosis
Bacteriodetes
Regular Diet
Firmicutes
High-Fat Diet