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MACROPHAGES IN INFLAMMATION AND ITS RESOLUTION

Hosted by
Heiko Mühl, Patrizia Rovere-Querini,
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Isabelle Maridonneau-Parini

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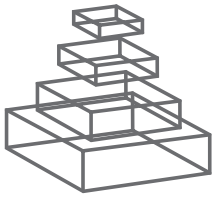

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MACROPHAGES IN INFLAMMATION AND ITS RESOLUTION

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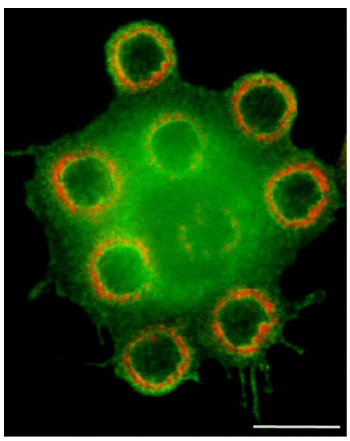

Formation of frustrated phagosomes by a human monocyte-derived macrophage layered on micro-patterned antigen-IgG complexes. Macrophages were incubated for 15 min on 4-μm square Ag-IgG patterns before being fixed and stained for F-actin (red) and Paxillin (green). Frustrated phagosomes form on immune complex patterns, rings of F-actin dots are surrounded by Paxillin. Bar = 5 μm. Image provided by Arnaud Labrousse.

Macrophages were initially identified as a key element in the innate host response to infection and injury due to their phagocytic clearance and elimination of pathogenic and non-pathogenic entities. However, as macrophage research advanced it became clear that not only are these cells amenable to the acquisition of multiple plastic phenotypes during inflammatory responses to different pathogens, they also play a paramount role in the termination of inflammation and acquired immune responses. In addition, macrophages profoundly affect host physiology when they migrate to distant sites and differentiate to specialized cells, like foam cells, osteoclasts, adipose tissue- and tumor -associated macrophages and other macrophage-derived cell types. These processes are affected by the inflammation-resolution axis and can result in health threats, such as atherosclerosis, bone loss, obesity, fibrosis and cancer.

This Research Topic issue will cover a wide range of topics in macrophage biology:
1. Macrophages in immune responses to pathogens
2. Macrophages in the termination of acute and acquired immunity.
3. The role of macrophages and their descendents in inflammation-associated pathologies.
4. Macrophage polarization and differentiation.

Particular focus will be given to the modulation of macrophage phenotype and function following their encounter with apoptotic cells and the signaling cascades that govern these changes.

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EDITORIAL
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Macrophages in inflammation and its resolution

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Macrophages are highly plastic leukocytes that differentiate from monocytes following their entry into extravascular tissues. Macrophages can enter various tissues under inflammatory or non-inflammatory conditions and assume different functions and phenotypes according to the cues they receive from the environment. The notion that inflammation in general and macrophage responses in particular affect physiological phenomena that were previously considered to be not immune-related has enhanced and broadened our understanding of macrophage function during inflammation and its resolution.

This volume brings together 14 manuscripts that cover various aspects of macrophage function during inflammation and its resolution, as well as in several pathologic states for which a significant, long-lasting, macrophage-mediated immune response plays a significant role. Two of the manuscripts present original research on macrophage phagocytosis and its implications. Five provide an overview of macrophage function during inflammation and its resolution, with an emphasis on the modulatory role of particular elements in this response, such as apoptotic leukocytes, specific pathogens, hypoxia, and hormone receptors. The remaining seven manuscripts outline the role of macrophages during inflammation and its resolution in different tissues, including the lung, cardiovascular and adipose tissues, injured skeletal muscle and neuronal tissues, and synovial and oral cavities.

The two original research articles are devoted to the consequences of particle engulfment by macrophages. Labrousse et al. (2011) describe a novel experimental strategy in which they use micro-patterned immune complexes to trigger frustrated phagocytosis and thereby determine spatial parameters in lysosomal movement and fusion. Janko et al. (2011) report on the cumulative binding of CRP and anti-CRP antibodies to the surface of secondary necrotic cells. This binding leads to a pro-inflammatory cytokine response following engulfment by macrophages, implying a potential role for these elements in the etiology of systemic lupus erythematosus.

Of the review articles that discuss the regulation of macrophage differentiation and function by discrete events, two cover the interaction between macrophages and apoptotic leukocytes during the resolution of inflammation. Korns et al. (2011) outline the regulation of apoptotic cell clearance by macrophages (efferocytosis) and the environmental cues that promote the efferocytic capabilities of macrophages. The second manuscript by Ariel and Serhan (2012) reviews the impact of apoptotic cell sensing and disposal by macrophages on the switches in functional phenotypes displayed by these cells. The effect of another environmental factor, hypoxia, on monocyte/macrophage activation, and differentiation through transcriptional and translational modulation is covered by Rahat et al. (2011). Lugo-Villarino et al. (2011) discuss the pathogenesis and co-mortality displayed by two macrophage-inhabiting microbes (HIV and Mycobacterium Tuberculosis) and their influence on macrophage polarization. Patel et al. (2011) review the role of melanocortin receptor expression by macrophages in anti-inflammation and the resolution of inflammation, with attention given to melanocortin receptor agonists as therapeutic agents.

Several review articles discuss the function of macrophages during inflammation and/or its resolution within distinct anatomical sites, taking into account the unique features of these tissue-specific macrophages, in particular the distinct environments in which they reside and their interactions with neighboring cells. Clària et al. (2011) review current knowledge on the contribution of macrophages to the inflammatory state characterizing adipose tissue and the phenotypic changes observed in macrophages during obesity. Kennedy et al. (2011) discuss macrophage polarization occurring within the synovial space of arthritic joints and its modulation by cytokines, transcription factors, and pro-resolving lipid mediators. The article from Bosurgi et al. (2011) describes the multiple actions of macrophages in injured skeletal muscle, where the effects of these cells are a double-edged sword and can either promote healing and repair or lead to fibrosis and fat replacement. Herold et al. (2011) survey the indispensable role of macrophages in the resolution and termination of inflammation in lung infection and injury as well as the molecular pathways involved in these processes. Proper termination of inflammatory events and clearance of apoptotic cells are also critical to the cardiovascular system, as reviewed by Thorp (2012), and defects in macrophage efferocytosis can lead to atherosclerosis and myocardial infarction. While monocyte-derived macrophages

and resident microglia were previously considered to be detrimental in brain inflammation and injury, recent advances reviewed by Jung and Schwartz (2012) suggest an opposite role for these macrophage-like cells, with a positive impact on brain maintenance and repair. Finally, Hasturk et al. (2012) outline the reciprocal interaction between periodontal disease and chronic inflammatory illnesses and the role that macrophages play in mediating these chronic inflammatory diseases.

Altogether, the articles in this volume portray the complexity of the multiple roles played by macrophages and members of their lineage during inflammation and its resolution, and their manipulation by the injured milieu. These topics are currently heavily studied, and advances in the field, facilitated by state-of-the-art genetics and optical technologies, will undoubtedly continue to contribute to our understanding of the immune system's response to foreign insults, trauma, and inflammatory disorders.

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Melanocortin receptors as novel effectors of macrophage responses in inflammation

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Macrophages have crucial functions in initiating the inflammatory reaction in a strict temporal and spatial manner to provide a "clear-up" response required for resolution. Hormonal peptides such as melanocortins modulate macrophage reactivity and attenuate inflammation ranging from skin inflammation to joint disease and reperfusion injury. The melanocortins (e.g., adrenocorticotrophin, ACTH and αMSH) elicit regulatory properties through activation of a family of GPCRs, the melanocortin (MC) receptors; MC_1–MC_5. Several studies have focused on MC_1 and MC_3 as anti-inflammatory receptors expressed on cells of the macrophage lineage. We review here elements of the melanocortin pathway with particular attention to macrophage function in anti-inflammatory and pro-resolving inflammatory settings. Evidence shows that ACTH, αMSH, and other MC agonists can activate MC_1 and MC_3 on macrophage through cAMP and/or NFκB-dependent mechanisms to abrogate pro-inflammatory cytokines, chemokines, and NO and enhance anti-inflammatory mediators such as IL-10 and HO-1. Melanocortins and their receptors regulate inflammation by inhibiting leukocyte recruitment to and interaction with inflamed tissue. An intensely exciting addition to this field of research has been the ability of an αMSH analog; AP214 to activate MC_3 expressed on macrophage to enhance their clearance of both zymosan particles and apoptotic neutrophils thus putting melanocortins in line with other pro-resolving mediators. The use of mouse colonies mutated or nullified for MC_1 or MC_3, respectively as well as availability of selective MC receptor agonist/antagonists have been key to deciphering mechanisms by which elements of the melanocortin system play a role in these phenomena. We review here melanocortin pathway components with attention to the macrophage, reiterating receptor targets required for pro-resolving properties. The overall outcome will be identification of selective MC agonists as a strategy for innovative anti-inflammatory therapeutics.

Keywords: inflammation, resolution, macrophage, melanocortins, melanocortin receptor, melanocyte stimulating hormone, G-protein coupled receptor, anti-inflammatory therapeutics

INTRODUCTION

An inflammatory reaction is characterized by cellular recruitment within a tissue that firstly involves an acute phase whereby neutrophils act as invaders to release toxins to kill and eliminate foreign encounters. Macrophages play a crucial role in the latter phase of this inflammatory reaction. At the site of injury their ultimate goal is counterbalance the acute phase to cease inflammation and clear-up detrimental artifacts including pathogens and debris as well as apoptotic cells from the environment. To disperse inflammation macrophage must complete complex reactions, including chemotaxis to move to the site of inflammation, phagocytose particles or apoptotic cells (process of efferocytosis) and secrete cytokines/chemokines, lipid mediators, reactive oxygen species, and other factors to underpin an immune response facilitating healing/repair and return to normal homeostatic physiology. Pathological conditions can arise as a consequence of disruption to the sequence of events leading to resolution of inflammation. There has been a recent spate in research to identify and understand these biochemical pathways and mediators enhancing the resolution of inflammation by macrophage in the hope to discover new therapeutics that "switch on" this protective arm of inflammation. Within the umbrella of anti-inflammatory and pro-resolution mediators being investigated are the omega-3 fatty acid derived resolvins, aspirin-related lipoxins, and the glucocorticoid induced protein Annexin A1. One other such pathway gaining attention within this field includes the melanocortin peptides and their counterpart melanocortin receptors.

THE MELANOCORTIN SYSTEM

Melanocortins are derived from proteolytic cleavage of pro-opiomelanocortin (POMC), a hormone first thought to be expressed only within the pituitary. Evidence now shows that POMC is also expressed within peripheral cells and tissues (Blalock, 1985). The enzymes proprotein convertase 1 (PC1) and 2 (PC2) belong to a conserved family of serine proteinases of the subtilisin/kexin-type that also include furin, PC4, PACE4, PC5/6, and PC7/LPC/PC8. Their action upon single and/or pairs of dibasic residues within the POMC sequence generate the melanocortin peptides; adrenocorticotrophin (ACTH), α-, β-, γ-melanocyte stimulating hormone. PC1 leads to generation of full-length

pro-ACTH$_{1–39}$ and β-lipotropin. PC1 then further cleaves β-lipotropin to generate γ-lipotropin and β-endorphin, and pro-ACTH to generate *N*-pro-opiocortin (POC), joining peptide (JP), and ACTH. The down-stream actions of PC2 result in production of ACTH$_{1–17}$ and corticotrophin-like intermediate lobe peptide (from ACTH), γMSH (from *N*-POC), and β-MSH (from β-lipotropin). Alpha-MSH (αMSH) is generated by the combined actions of carboxypeptidase (CPE), peptidylglycine alpha-amidating mono-oxygenase (PAM), and *N*-acetyltransferase (*N*-AT) on ACTH$_{1–17}$ (Mountjoy, 2010; **Figure 1**).

The biological activity of the ACTH and MSH species occurs through activation of melanocortin receptors (MC) of which five have been cloned so far. All are seven transmembrane coupled to s-type G-proteins thus positively coupled to adenylate cyclase; their activation leading to increases in intracellular cAMP and, possibly partly independently from it, down-regulation of nuclear factor-kappa beta activation (Wikberg et al., 2000; Gantz and Fong, 2003). MC$_1$ is the receptor expressed on melanocytes, responsible for MSH control over skin pigmentation, whereas MC$_2$ is the canonical ACTH receptor, expressed on adrenal cells and responsible for glucocorticoid synthesis and release. The other MCs have less clear-cut biological functions. Within the central nervous system, MC$_4$ is actively studied for its role in feed control, pain, and sexual health; MC$_5$ might modulate exocrine gland activity (Gantz and Fong, 2003). MC$_3$ might exert a control over energy metabolism (Butler et al., 2000), but our own work has indicated an important modulatory role for this receptor in the control of the host inflammatory response (Getting et al., 2002). Of interest here, there is now ample evidence that MC, especially MC$_1$, MC$_3$, and MC$_5$ are distributed on peripheral cells (Catania et al., 2010).

PRODUCTION OF αMSH AND EXPRESSION OF MC RECEPTORS BY MACROPHAGE

Melanocortins are expressed and functionally active on the macrophage. Star et al. (1995) reported increases in cAMP accumulation in RAW 264.7 macrophage (shown to express MC$_1$ and MC$_3$ but not MC$_5$) upon αMSH (pan agonist) incubation, suggestive of the presence of functionally active MCs on the cell surface (Star et al., 1995; Li and Taylor, 2008). Production of αMSH by these cells was also noted with further augmentation of the response upon TNFα stimulation. These two lines of evidence along with the anti-inflammatory action of αMSH to reduce nitric oxide release by RAW 264.7 macrophage suggest that αMSH may act in an autocrine/paracrine manner by counteracting pro-inflammatory effect of cytokines (Star et al., 1995). A similar autocrine circuit emerged in a latter study with human monocyte/macrophage THP-1 cells. Here, incubation of resting macrophage with an antibody recognizing MC$_1$ on its own increased TNFα release, with the addition of αMSH reducing this response. Furthermore the presence of the anti-MC$_1$ attenuated this inhibitory signal caused by αMSH. Collectively, these data indicate that MC receptor agonists limit pro-inflammatory cytokine production from macrophages, possibly being pivotal also in peripheral autocrine circuits (Taherzadeh et al., 1999).

Expression of melanocortin receptors is not only restricted to macrophage cell lines, as MC receptor mRNA has been detected in primary rodent macrophage of alveolar (MC$_1$ and MC$_3$), knee joint (MC$_3$), mesentery (MC$_1$ and MC$_3$), and peritoneal (MC$_1$, MC$_3$, and MC$_5$) origin and human macrophage blood derived macrophage (MC$_1$, MC$_3$, and MC$_5$; Getting et al., 2002, 2003, 2008; Andersen et al., 2005; Leoni et al., 2008; Montero-Melendez et al., 2011).

MELANOCORTINS RECEPTOR ACTIVATION ON MACROPHAGE AUGMENT ANTI-INFLAMMATORY RESPONSES

In inflammatory/tissue injury settings, activation of MCs can influence a number of fundamental macrophage functions including:

(1) *antagonizing* the release of pro-inflammatory cytokines, chemokines, nitric oxide
(2) *inhibiting* leukocyte chemoattraction
(3) *inducing* release of anti-inflammatory cytokines and expression of heme oxygenase-1
(4) *augmenting* phagocytosis and efferocytosis.

It should be noted that MC receptor activation can also affect macrophage responsiveness indirectly. For instance, the existence of a circuit involving MC$_3$ and MC$_4$ within the CNS that activates descending cholinergic fibers to activate anti-inflammatory nicotinic receptors have been described (Catania, 2007). Here we will restrict our overview on the direct effects of MC receptor on this cell type and discuss now some of the properties listed above in more detail.

MELANOCORTINS INHIBIT CYTOKINE PRODUCTION

The ability of melanocortin peptides to reduce macrophage release of pro-inflammatory mediators and increase anti-inflammatory mediators is a well-understood phenomenon with a number of

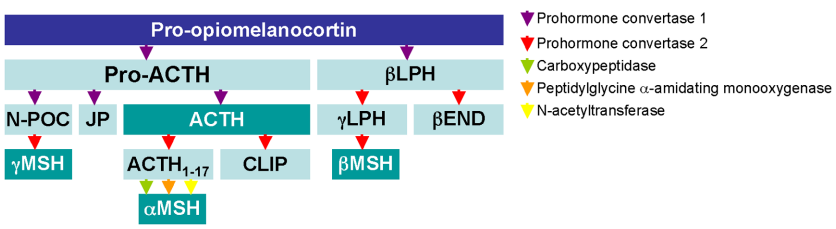

FIGURE 1 | Biochemical processing of the POMC gene product as detailed in the pituitary. The processing of POMC and products by posttranslational modification enzymes is depicted by arrows. Agonists of the melanocortin receptors MC1–MC5 are highlighted.

groups contributing to this field. For example Capsoni et al. (2009) stimulated peripheral blood derived monocytes *in vitro* with monosodium urate crystals in the presence of αMSH (pan agonist) or $(CKPV)_2$ (a biologically potent Lys-Pro-Val peptide linked by Cys–Cys residues) and found significant reductions in IL-1β, IL-8, and TNFα release into supernatants (Capsoni et al., 2009). We treated the RAW264.7 mouse macrophage cell line with $ACTH_{1–39}$ (pan agonist) and MTII, amore stable αMSH derivative with higher affinity for MC_3 and MC_4 promoted an increase in cAMP accumulation and release of anti-inflammatory cytokine IL-10, the later effect being abrogated when cells were treated with H-89 (protein kinase A [PKA] inhibitor) suggesting signaling down-stream of MC_3 and not MC_4 (as RAW264.7 did not express MC_4) was through the cAMP–PKA pathway (Lam et al., 2006). In similar experiments both ACTH and MTII elicited in a cAMP–PKA dependent manner cytoprotective and anti-inflammatory heme oxygenase-1 (HO-1) but not heme oxygenase-2, heat shock protein 70 and 90 in RAW267.4 cells (Lam et al., 2005). Mandrika et al. (2001) showed dual signaling mechanism by which αMSH inhibited nitric oxide production by RAW267.4 macrophage. One pathway dependent of MC_1/cAMP activation and the other causing inhibition of NFκB translocation in a cAMP-independent manner. Other specialized cells of the macrophage lineage such as microglia treated with αMSH or ACTH substantially reduced TNFα, IL-6, and nitric oxide increases caused by LPS + IFNγ activation, suggesting that MC peptides exert their anti-inflammatory actions on peripheral as well as central cells of the phagocytic lineage. There is evidence of active MC receptors because the same study reported that αMSH could induce cAMP accumulation in both resting and activated microglia (Delgado et al., 1998).

Rat macrophage isolated from gouty knee joints also accumulated intracellular cAMP upon treatment with melanocortin peptide; ACTH (Getting et al., 2002). More recently functionality of MC receptors by cAMP readout on murine alveolar macrophage was confirmed after incubation with αMSH, [D-Trp^8]γMSH (MC_3/MC_5 agonist), and MSO5 (MC_1 agonist; Getting et al., 2008; Joseph et al., 2010). Together these studies suggest that the cAMP pathway represents a common underlying mechanism for melanocortin to deliver anti-inflammatory effects. Manna and Aggarwal stimulated human monocytes with various inflammatory agents including TNFα, LPS, ceramide, and okadaic acid to obtain NFκB activation, which was diminished in cells treated with αMSH (Manna and Aggarwal, 1998). The agonistic effect appeared to be cAMP-dependent such that inhibitors of adenylate cyclase and PKA reversed the inhibitory effect of αMSH. Furthermore αMSH inhibition of degradation of the NFκB inhibitory subunit IκBα and nuclear translocation of p65 subunit were also noted (Manna and Aggarwal, 1998).

Collectively these data appear to show that the anti-inflammatory actions of αMSH are dependent on MC_1, MC_3, and/or MC_5 receptor activation and triggers inhibition of NFκB in a cAMP-dependent and independent manner through molecular links which are yet to be deciphered.

MELANOCORTINS INHIBIT CHEMOTAXIS OF LEUKOCYTES

As discussed so far, melanocortins are able to dampen macrophage release of pro-inflammatory mediators in inflammatory settings. These phenomena were exploited further by Capsoni et al. (2009) who showed that melanocortins could influence the monocytes ability to recruit and prime neutrophils. Chemotaxis of neutrophils treated with αMSH or $(CKPV)_2$ was examined using a boyden chamber assay and supernatants collected from monosodium urate crystal-stimulated monocytes as the source of chemoattractants. Chemotaxis was significantly reduced in neutrophils treated with agonist compared to controls. Furthermore reduced chemiluminescence (as a marker of reactive oxygen intermediates) production by pre-treated primed neutrophils was observed, in line with other studies showing melanocortin peptides can inhibit chemotaxis and generation of reactive oxygen species (Catania et al., 1996; Getting et al., 1999a; Capsoni et al., 2009).

To decipher what role MC_3 has on leukocyte interaction with the inflamed mesentery, experiments were performed using pharmacological agents and mice nullified for MC_3 where the superior mesenteric artery was occluded for 35 min followed by reopening to allow for blood reperfusion. Treatment with [D-Trp^8]γMSH, a MC_3/MC_5 agonist did not alter cell rolling but decreased cell adhesion and emigration compared to vehicle control an effect not observed in *Mc3r*−/− mice. These data were mirrored by augmented KC and MCP-1 responses in mesenteric tissues of *Mc3r*−/− mice suggesting MC_3 is able to regulate leukocyte interaction with postcapillary venules and regulate levels of pro-inflammatory molecules in ischemia reperfusion injured tissues (Leoni et al., 2008). Moreover, whilst agonizing more than one MC receptor, the vasculoprotective properties of [D-Trp^8]γMSH are solely transduced by MC_3.

Of interest, the modulation of chemokines occurred in the tissue and not with respect to plasma levels, moreover, levels of the cytokines IL-1β and TNFα were not affected by pharmacological treatment with [D-Trp^8]γMSH or absence of the MC_3 gene. The "selective" effect on tissue chemokine levels may be secondary to inhibition of activation of resident cells, such as tissue macrophages and connective tissue type mast cells (Kubes and Granger, 1996; Tailor et al., 1999).

In similar experimental settings, the effects of a small molecule MC_1 agonist, compound BMS-470539 (Kang et al., 2006) inhibited cell adhesion, and emigration, an effect that was lacking in $yellow^{e/e}$ mice. Interestingly, unlike *Mc3r*−/− mice, the $yellow^{e/e}$ mice (expressing non-functional MC_1) displayed a comparable leukocyte adhesion and emigration response to wild types. Collectively these two studies identify a complex scenario whereby although both MC_1 and MC_3 are expressed in the inflamed mesentery, MC_1 may not be endogenously activated in ischemia reperfusion injury whereas MC_3 is both exogenously and endogenously activated in this setting (Leoni et al., 2010). Future studies addressing the hypothetical generation of selective agonists post-ischemia reperfusion might help deciphering this apparent discrepancy.

ACTIVATION OF MC_3 IS A KEY MEDIATOR OF ANTI-INFLAMMATORY EFFECTS IN MACROPHAGES

Until a decade ago, the mainstream school of thought was that the anti-inflammatory actions of melanocortin agonists were *solely* through MC_1 activation (Lipton and Catania, 1997; Luger et al., 2003). We used two mouse colonies, one bearing a non-functional

MC_1 (yellow$^{e/e}$ mice) and the other knocked out for MC_3 (*Mc3r*−/− mice), to conclude that MC_3 can be engaged by agonists with anti-inflammatory properties. Cultured primary peritoneal macrophage from yellow$^{e/e}$ mice were treated with [D-Trp^8]γMSH displaying a dose-dependent increase in cAMP accumulation, an effect reversed in the presence of an antibody to MC_3. The release of the chemokine KC was abrogated in the presence of [D-Trp^8]γMSH with the antagonist agouti related protein (AGRP) abolishing the inhibitory effect.

In another study mice pre-treated with ACTH were injected with MSU crystals to induce peritonitis. A reduced accumulation of PMN was observed in the peritoneal cavity of mice treated with ACTH compared to vehicle control, data that was mirrored by decreased levels of KC also within the cavity. Interestingly co-administration of SHU9119 (an MC_3/MC_4 antagonist) with ACTH inhibited the agonists effects suggesting ACTH was acting through MC_3 and further confirmed by the detection of MC_3 but not MC_4 at the mRNA transcript level on peritoneal macrophage (Getting et al., 1999b). At a latter date, Getting et al. (2003) in the same peritonitis model using more selective MC_3 agonists and yellow$^{e/e}$ mice confirmed that indeed agonism at MC_3 inhibited pro-inflammatory cytokines (IL-1) and chemokines (KC) and accumulation of neutrophils in the inflamed peritoneal cavity (Getting et al., 2003). Collectively these data suggest MC_3 more than MC_1 (at least in mouse) to be pivotal in bringing about the anti-inflammatory effects observed following treatment with these agonists (Getting et al., 1999b, 2003, 2006).

MC_3 REGULATES MACROPHAGE DIFFERENTIATION TO OSTEOCLASTS DURING INFLAMMATORY ARTHRITIS

More recently MC_3 has been implicated in the regulation of macrophage precursor differentiation to osteoclasts. Inflammatory arthritis was induced by injection of an arthritogenic serum into wild type and *Mc3r*−/−, observing a higher prevalence and severity of disease observed in the latter genotype (Patel et al., 2010a). In a series of real time PCR analyses of extracts from the mouse ankle joint, a discrete set of inflammatory genes (13 out of 96) were upregulated in *Mc3r*−/− including IL-1β, IL-6, NOS2, CCR4, CXCR3, CCL2 as compared to wild types. It is worthy to note here that major macrophage secretory products include IL-1β and IL-6 (Gordon, 2003).

Upon microscopic analyses of the ankle joints, a significantly higher number of joints were affected by bone erosion, as indicated by histological scores and number of TRAP-positive osteoclasts within the *Mc3r*−/− joint. This observation was corroborated by the higher levels of RANKL (a key driver of osteoclast formation) mRNA in *Mc3r*−/− ankle joints (Lacey et al., 1998). Although the exact mechanism was not characterized, some conclusion could be reached by the study of osteoclast formation *in vitro*. Bone marrow-derived macrophage from wild type and *Mc3r*−/− mice were differentiated to osteoclasts in the presence of M-CSF and RANKL. A defect was noted such that a higher number of osteoclasts were generated from macrophage absent of the MC_3 gene compared to MC_3 sufficient cells. The *Mc3r*−/− osteoclasts displayed an increased "eating" ability such that when cultured on calcium phosphate coated wells significantly more resorption pits could be observed and quantified. Interestingly *Mc3r*−/− cells had increased and sustained RANKL-mediated NFκB signaling compared to wild types; this finding could provide mechanistic support to the increased CCL2 synthesis observed by these cells (Patel et al., 2010a). Together with the study by Cornish et al. (2003), our work implicates a role for melanocortins and their melanocortin receptors in the regulation of macrophage differentiation to specialized cells, example being here the osteoclast. It is plausible that MCs may modulate macrophage differentiation in other specialized cells such as the microglia or Kupffer cells, with further implications for their therapeutic potential.

MC_3 ACTIVATION ENHANCES RESOLUTION OF INFLAMMATION

Resolution of inflammation is an important process required to reset tissue/cells to a state of normalization after insult/injury. During this process a number of endogenous pathways are activated in order to regain homeostatic balance after inflammation. An exciting enhancement to the field of melanocortin biology in inflammation has been the recent finding that AP214, a modified αMSH analog, possesses prophagocytic and pro-resolving effects (Montero-Melendez et al., 2011), in line with the profile reported for resolvins and lipoxins (Schwab et al., 2007).

AP214 inhibits neutrophil recruitment in the zymosan peritonitis model, an effect that could be due, at least in part, to a modulation of macrophage phagocytic abilities of the particles. *In vitro*, AP214-treated biogel-elicited macrophages were incubated with zymosan particles to determine the percentage of phagocytic cells and number of ingested particles, observing an increment in both parameters. Such an effect of AP214 was also observed with respect to efferocytosis since this MSH analog augmented phagocytosis of human apoptotic neutrophils by mouse macrophages, an effect that was absent when *Mc3r*−/− macrophages were used. In *in vivo* settings, injection of apoptotic neutrophils into murine peritoneal cavities pre-treated with AP214 led to an increase in macrophage ingestion of neutrophils compared to vehicle control. This recent study has uncovered a new angle in which melanocortins and their receptors can affect the inflammatory reaction providing strong evidence for genuine pro-resolving activities centered on tight regulation of macrophage functions.

CONCLUSION

In this review we have highlighted the importance of melanocortins and their receptors in modulating the macrophage function in inflammation and tissue injury (see **Figure 2**). We have seen that MC activation does not merely reduce production of pro-inflammatory mediators, but can regulate cell differentiation as well as chemotaxis of leukocytes. Excitingly, melanocortins can now be added to the plethora of pro-resolution mediators by way of their ability to augment phagocytosis and efferocytosis; we predict these *portfolio of properties* will define other functions/biological properties of melanocortins in other aspects of resolution of inflammation in the years to come.

What does this mean for the development of melanocortin-based therapies? Noteworthy, ACTH has been used as early as the 1940s for the treatment of gouty and rheumatoid arthritis, yet as discussed above never were the mechanisms of actions deciphered until the recent decade (Hench et al., 1949; Gutman and Yu, 1950). What does remain in its infancy is the pattern in which MCs may

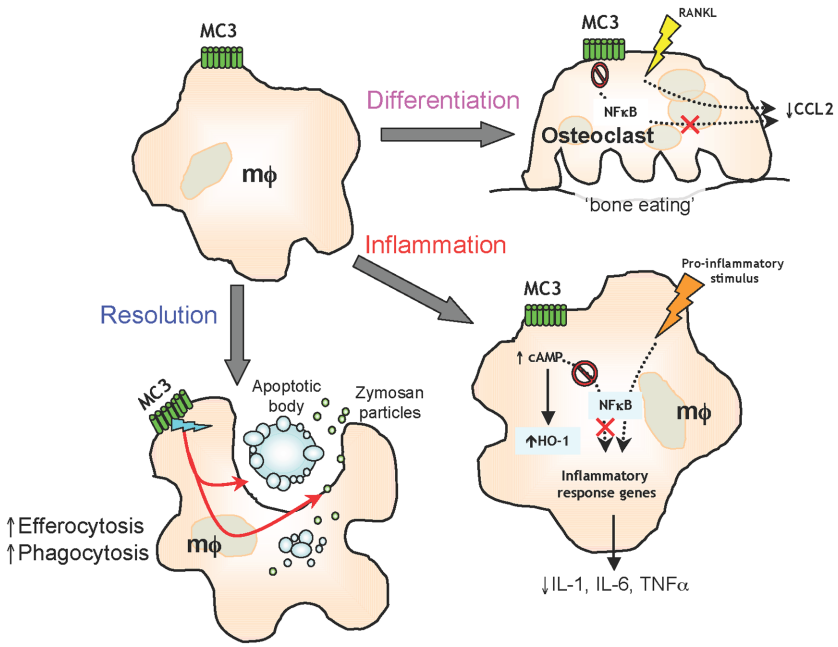

FIGURE 2 | Melanocortin receptor type 3 activation on macrophage (mϕ) functions Activation of MC_3 by endogenous or selective synthetic agonists leads to regulation of osteoclast generation, control of pro-inflammatory and anti-inflammatory mediators and resolution of inflammation by efferocytosis and phagocytosis.

be expressed in specific disease, nevertheless this area of research and drug development has become particularly eye-catching as seen with the development of AP214 for the treatment of post-surgical kidney injury following cardiac heart surgery in phase II trials (www.clinicaltrials.gov). Other melanocortin-based therapies are in the development pipeline for biotechnology companies with emphasis on obesity and sexual dysfunction as disease targets (Patel et al., 2010b).

We envisage over the next decade new drugs based on the melanocortin peptides – specifically targeting MC_3 – to be developed and designed for the treatment of inflammatory conditions. These drugs would be better tolerated with a lower burden of side-effects as they would mimic the body's way of naturally abating inflammation to promote pro-resolving and anti-inflammatory mediators.

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Macrophage polarization: convergence point targeted by *Mycobacterium tuberculosis* and HIV

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In the arms race of host–microbe co-evolution, macrophages (Mϕs) have been endowed with strategies to neutralize pathogenic challenge while preserving host integrity. During steady-states conditions, Mϕs perform multiple house-keeping functions governed by their differentiation state, tissue distribution, and signals from the microenvironment. In response to pathogenic challenge and host mediators, however, Mϕs undergo different programs of activation rendering them either pro-inflammatory and microbicidal (M1), or immunosuppressants and tissue repairers (M2). An excessive or prolonged polarization of either program may be detrimental to the host due to potential tissue injury or contribution to pathogenesis. Conversely, intracellular microbes that cause chronic diseases such as tuberculosis and acquired immunodeficiency syndrome exemplify strategies for survival in the host. Indeed, both *Mycobacterium tuberculosis* (Mtb) and human immunodeficiency virus (HIV-1) are successful intracellular microbes that thrive in Mϕs. Given these microbes not only co-circulate throughout the developing world but each has contributed to prevalence and mortality caused by the other, substantial insights into microbe physiology and host defenses then rest in the attempt to fully understand their influence on Mϕ polarization. This review addresses the role of Mϕ polarization in the immune response to, and pathogenesis of, Mtb and HIV.

Keywords: macrophage, *Mycobacteria*, tuberculosis, HIV, AIDS, polarization

INTRODUCTION

Pathogens have evolved ingenious strategies to circumvent the host immune response as part of the constant evolutionary process-taking place in all living organisms. Chief among these strategies is the prevention of the inflammatory response or seizure of the anti-inflammatory mechanism in place to protect tissue integrity. The manipulation of macrophage (Mϕ) polarization is one of the main targets to accomplish this, since this antigen presenting cell represents the first line of an active defense system in the host, and if successfully done, it can then undermine adaptive immunity (Benoit et al., 2008). Mϕ polarization is a dynamic process governed by mechanisms dictating their tissue distribution and functional capacities in response to endogenous and exogenous signals (Martinez et al., 2009). Polarized Mϕs are broadly classified into two groups: classical (M1) and alternative (M2) activated. On one hand, M1 program is a direct response to type-1 inflammatory conditions (e.g., IFN-γ) and pathogen challenge, and it has been associated to resistance to intracellular pathogens and to some form of tumors. On the other hand, the M2 program is driven by type-2 inflammatory signals such as IL-4 and IL-13 (M2a); immune complexes, toll-like receptors (TLRs) agonists, or IL-1 receptors (M2b); and immunosuppressants including IL-10, transforming growth factor-β (TGF-β) or glucocorticoids (M2c; **Table 1**). M2 Mϕs participate in diverse activities including the suppression of inflammation, enhancement of phagocytosis, promotion of tissue remodeling and repair, elimination of parasites, and unwanted tumor angiogenesis (Sica et al., 2008; Martinez et al., 2009; Murray and Wynn, 2011). Furthermore, it is becoming clear that Mϕ polarization supports different, and in some cases, opposing biological functions, that influences tissue homeostasis, and numerous pathological situations, including infectious diseases (Benoit et al., 2008; Cairo et al., 2011). Given the pivotal role Mϕs play as sentinels of the immune system, they represent ideal cell targets for subversion by successful intracellular pathogens.

The purpose of this short review is not to provide a comprehensive summary of Mϕ polarization; others have recently reviewed this growing research area (Martinez et al., 2009; Murray and Wynn, 2011). Also, we will not address the multiple ways by which the pathogens in question circumvent the immune system, as there are excellent reviews covering this subject (Deretic et al., 2004; Carter and Ehrlich, 2008; Meena and Rajni, 2010; Hajishengallis and Lambris, 2011). Instead, we will focus exclusively on the significance of Mϕ polarization in the context of pathophysiology caused by *Mycobacterium tuberculosis* (Mtb) and human immunodeficiency virus (HIV).

MACROPHAGE POLARIZATION IN Mtb INFECTION

The world health organization reports tuberculosis (TB) is still one of the leading causes of death due to a single infectious agent (Mtb) with 1.7 million deaths and 9.4 million new cases in 2009, and estimates that about one-third of the human population may be latently infected (WHO Global Tuberculosis Control Report 2010, 2010). Active TB may occur directly after infection or through the reactivation of latent infection that is confined in granulomas. The

Table 1 | Priming stimulus for the classical (M1) and alternative (M2a-c) activation of macrophages.

	M1 program	M2 program		
		M2a	M2b	M2c
Priming stimulus	IFN-γ+ LPS or TNF	IL-4	Immune complexes	IL-10
		IL-13	TLR ligands IL-1R ligands	TGF-β Glucocorticoids MCSF

elaboration and maintenance of granulomas depends on a dedicated immune response, which is not fully understood. Recently, however, it was demonstrated mycobacteria exploits Mϕ activation to turn the granuloma into an effective tool for pathogenesis (Davis and Ramakrishnan, 2009; Volkman et al., 2010). Therefore, a better understanding of Mϕ polarization during Mtb infection might yield further clues about how Mtb circumvents the immune system.

As aforementioned, Mϕ polarization is mainly driven by type-1 and type-2 inflammatory signals (**Table 1**). Type-1 inflammatory cytokines are essential in the defense against Mtb since their expression often correlates with efficient anti-Mtb immune responses, and genetic deficiencies of these factors lead to increased TB susceptibility (Quintana-Murci et al., 2007). IFN-γ drives the M1 program characterized by Mϕ capacity to kill most mycobacteria and restrict the replication of the remainder (Ehrt et al., 2001). The early phase of the anti-Mtb immune response is marked by M1 Mϕ polarization in multiple animal models and reminiscent of the clinical data collected from patients with active TB (**Figure 1**; Benoit et al., 2008). At the transcriptome level, the gene modulation induced by Mtb in Mϕs highly overlaps, and in some cases synergizes, with that induced by IFN-γ to establish the M1 phenotype (Ehrt et al., 2001). At the granuloma level in mice, M1 Mϕ polarization is evident in mice between 7 and 30 days after Mtb infection when high levels of IFN-γ and iNOS are also detected within this structure and around the alveolar compartment (Redente et al., 2010). All in all, polarization of M1 Mϕs is part of the "common host response" against intracellular bacteria characterized by high expression of iNOS and consequent nitric oxide (NO) production (characteristic of murine models), secretion of pro-inflammatory cytokines and chemokines, release of proteolytic enzymes and anti-microbial peptides, enhanced phagocytosis, and development of a toxic intracellular environment reflected in the fusion of microbial phagosomes with acidic and hydrolase-rich lysosomes (Ehrt et al., 2001; Deretic et al., 2004; Martinez et al., 2009; Cairo et al., 2011; Murray and Wynn, 2011). It remains to be demonstrated whether transcription factors [e.g., p65 and interferon regulatory factor (IRF5)] or regulators (e.g., SHIP1) that dictate the M1 program of macrophage polarization also play a role in TB infection (Martinez, 2011). Considering this hostile environment created by M1 Mϕs, it is not surprising Mtb has evolved strategies to interfere with M1 polarization. Indeed, Mtb inhibits IFN-γ activation of Mϕs by secreting virulence factors such as lipoarabinomannan that halters phagosome maturation, or early secretory antigenic target-6 (ESAT-6) that prevents the activation of NF-κB and IFN-γ regulatory factors downstream of TLR-2 (Deretic et al., 2004; Benoit et al., 2008). Indirectly, Mtb blocks M1 polarization by the transcriptional inhibition of IFN-γ-responsive genes through a bystander effect involving IL-6 (Sibley et al., 1990; Benoit et al., 2008).

Perhaps the best strategy to avoid the challenges posed by M1 Mϕs is to shift their program into M2 Mϕs. TB susceptibility parallels with elevated levels of type-2 inflammatory signals (e.g., IL-4, IL-13; Kahnert et al., 2006; Raju et al., 2008; Almeida et al., 2009; Schreiber et al., 2009). Likewise, high levels of IL-10 (mostly derived from Mϕs) correlate with active TB patients (Barnes et al., 1993; Verbon et al., 1999). Interestingly, the predominant type-2 inflammatory environment shifts back to type-1 after successful treatment of pulmonary TB in infected patients (Verbon et al., 1999; Raju et al., 2008). These observations in humans parallel with those reported in Mtb-infected mice; that is, there is an early type-1 immune response characterized by IFN-γ during the first 3 weeks after infection, followed by a type-2 immune response that contains high levels of IL-4 (**Figure 1**; Orme et al., 1993). A type-2 inflammatory environment drives the M2 program that renders Mϕs immunomodulatory and poorly microbicidal (Raju et al., 2008; Martinez et al., 2009). At the transcriptome level, this seems to be the case in mice since M2 Mϕs displayed a diminished inflammatory response to Mtb as reflected by a reduced NO production and increased of iron availability, suggesting these phagocytes offer a permissible intracellular environment for bacterial replication (Kahnert et al., 2006). Indeed, IFN-γ-induced NO production is essential for host survival with respect of experimental TB, while iron-starvation is key to bacteriostasis (Ehrt et al., 2001; Forbes and Gros, 2001; Cairo et al., 2011). It remains to be seen if Mtb also influences the expression level of Kruppel-like factor 4 (KLF4) or any other transcription factor/regulator recently shown to be critical for both the establishment of the M2 program and the inhibition of M1 polarization (e.g., STAT6, Cot/tpl2; Liao et al., 2011; Martinez, 2011). At the functional level, it has been demonstrated that both IL-4 and IL-13 inhibit autophagy in M1 Mϕs resulting in enhanced survival of Mtb, an impairment that might also extend to M2a Mϕs (Harris et al., 2007). At the granuloma level in mice, iNOS continues to be expressed within this structure but a significant shift from M1 toward M2 Mϕs [$iNOS^{neg}$ Arginase-1 $(ARG1)^{hi}$] occurs around the alveolar compartment starting at day 35 and continuing up to day 60 after Mtb infection, accompanied by high levels of type-2 inflammatory signals (Ly et al., 2007; Redente et al., 2010). Given the development of fibrosis is a key characteristic of caseous granulomas during Mtb dissemination, and that M2 Mϕs have been implicated in the inhibition of fibrosis development, the shift from M1 into M2 program might represent an attempt by the host to halter the pathophysiology caused by Mtb or a microbial strategy to shield from immune attack (Dorhoi et al., 2011).

Mycobacterium tuberculosis also reprograms M2 Mϕs through secretion of immunosuppressants such as IL-10. For instance, Mtb might influence all TLR-dependent signaling by targeting DC-SIGN to induce IL-10 and counteract the pro-inflammatory response, as shown in dendritic cells (Geijtenbeek et al., 2003; Hajishengallis and Lambris, 2011). Likewise, the mannosylated

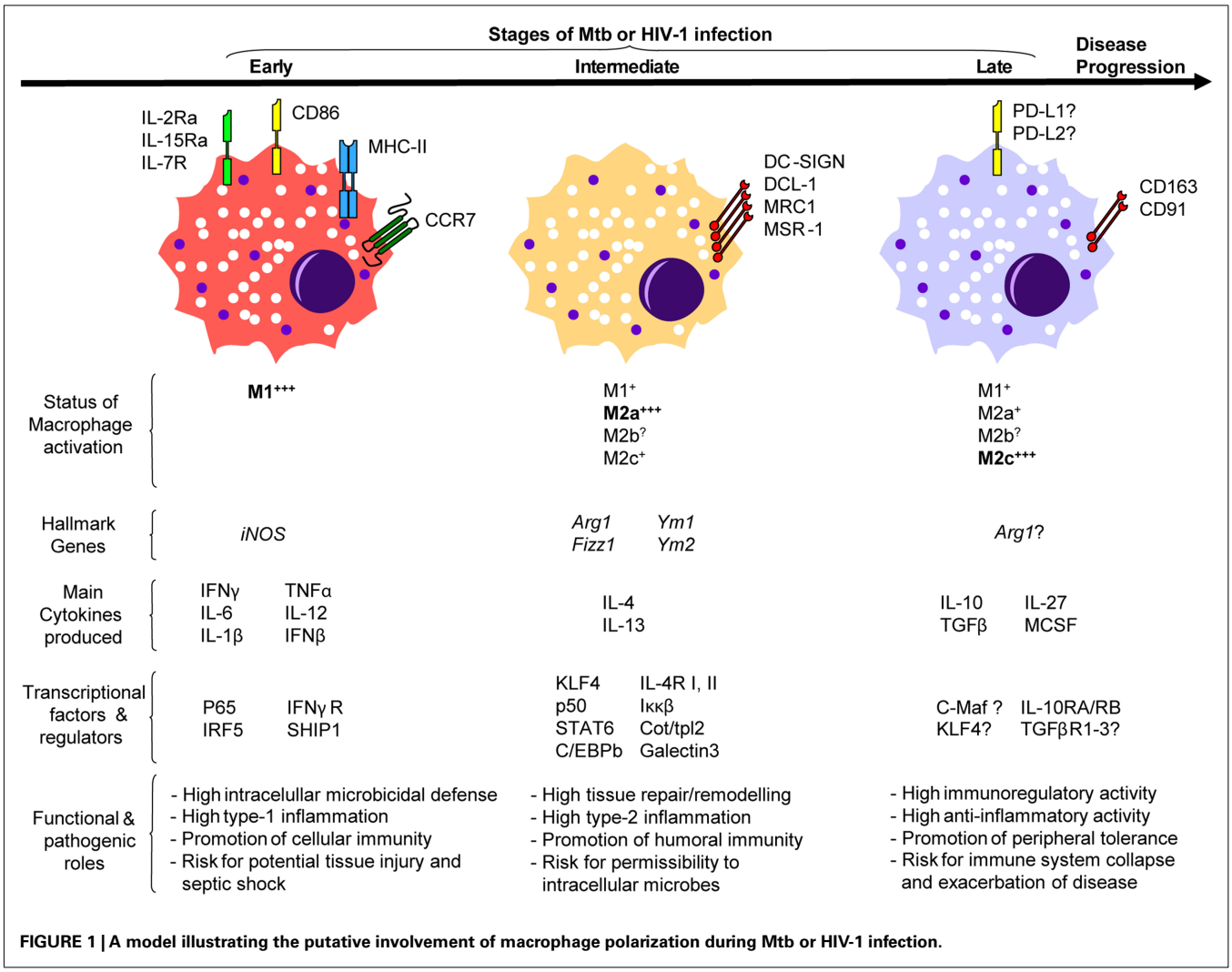

FIGURE 1 | A model illustrating the putative involvement of macrophage polarization during Mtb or HIV-1 infection.

lipoarabinomannan from Mtb enhances the production of IL-10 and other immunosuppressants through recognition by the mannose receptor (MR) in immature dendritic cells (Chieppa et al., 2003). Although alveolar Mϕs express DC-SIGN and MR, their role in M2 Mϕs has yet to be demonstrated (Chroneos and Shepherd, 1995; Tailleux et al., 2005). Nevertheless, Schreiber et al. (2009) reported Mtb-induced IL-10 in Mϕs promotes the M2 polarization program displaying diminished anti-mycobacterial effector mechanisms. Indeed, Mϕ-specific overexpressing IL-10 transgenic mice were indeed susceptible to Mtb infection, displayed a specifically suppressed IL-12 in infected tissues, and were characterized by lung Mϕs with a M2 phenotype permissive to Mtb infection (Schreiber et al., 2009). These observations correlate well with another study in mice where Mtb was shown to promote its survival and ability to cause disease through a MyD88-dependent induction of ARG1. ARG1 inhibits NO production by Mϕs by competing with iNOS for arginine (the common substrate), thus rendering these cells permissive to Mtb infection (El Kasmi et al., 2008; Hajishengallis and Lambris, 2011). Taken together, these observations suggest the reprogramming toward M2 Mϕs by IL-10, and other immunosuppressants such TGF-β and glucocorticoids (Hernandez-Pando et al., 2006), might be yet another adaptation by Mtb to survive and thrive inside of Mϕs (**Figure 1**). However, it should be noticed that this phenomenon might also represent a control mechanism by the host to preserve the integrity of mucosal sites as uncontrolled type-1 inflammatory responses against Mtb result into lung immunopathology (Hernandez-Pando et al., 2006; Ordway et al., 2006).

MACROPHAGE POLARIZATION IN HIV INFECTION

Human immunodeficiency virus-1 is another successful intracellular pathogen responsible for a worldwide pandemic. According to 2009 estimates by the United Nations, there were about 33.2 million people worldwide living with HIV-1 infection and 2.6 million individuals had been newly infected (Cohen et al., 2011). In the absence of antiviral therapy, HIV-1 infection progresses through acute and asymptomatic stages leading to the eventual failure of the host immunological functions and acquired immunodeficiency syndrome (AIDS). A reason is that HIV-1 targets cells from the mononuclear phagocyte lineage that drive an effective antiviral response and simultaneously serve as reservoirs of latent or productive infection (Goodenow et al., 2003). Among these cells,

Mϕs are critical to pathogenesis because they contribute to early transmission, systemic dissemination, and persistence of HIV-1. Indeed, HIV-1 evades immune surveillance by hiding and thriving inside Mϕs despite anti-retroviral treatment, and when infected, they persist for months displaying insensitivity to viral cytopathic effects. In addition, Mϕs continuously secrete high level of viral particles over prolonged time periods by storing assembled virus in specialized endosomal compartments (Orenstein et al., 1988; Benaroch et al., 2010). Thus, they represent powerful long-term viral reservoirs (Goodenow et al., 2003; Carter and Ehrlich, 2008; Herbein and Varin, 2010; Cohen et al., 2011). In light of recent evidence suggesting that M1 and M2 Mϕs influence HIV-1 pathogenesis, there is a surging interest to study the viral effects in Mϕ polarization.

In vitro, HIV-1 infection drives Mϕs toward a M1 program. This Mϕ response includes production of type-1 pro-inflammatory cytokines (IFN-γ, IL-2, IL-12, TNFα, IL-1β, IL-6, IL-18) and chemokines (CCL3, CCL4, MIP-α, MIP-β, RANTES), increased NO and respiratory burst, up-regulation of MHC-II molecules, and down-regulation of HIV-entry receptors (e.g., CD4, CCR5, CXCR4), and endocytic receptors (e.g., CD163, CD206; Swingler et al., 1999; Cassol et al., 2009, 2010; Herbein and Varin, 2010). Although few studies have examined thoroughly HIV-induced polarization of Mϕs *in vivo,* there is a predominance of Mϕs displaying a M1 phenotype during the acute stage (**Figure 1**; Cassol et al., 2010; Herbein and Varin, 2010; Cohen et al., 2011). Whether M1 Mϕs are beneficial to the host during HIV-1 infection remains an open question since Mϕ functions vary according to the experimental context. For instance, *in vitro* infection of M1 Mϕs in the presence of IFN-γ and TNFα is associated with a suppression of HIV-1 replication, a sharp decrease in HIV-1 DNA synthesis at 48 h, and a decrease in the accumulation of HIV-1 proteins (Cassol et al., 2009). In addition, other studies demonstrate that M1 Mϕs inhibit viral entry, assembly, and budding, suggesting the M1 program can be beneficial to the host (Cassol et al., 2010; Herbein and Varin, 2010). However, it is also known that pro-inflammatory signals deriving from M1 Mϕs favor the formation of viral reservoirs with increased transcription of HIV-1 LTR (long terminal repeat), alluding M1 Mϕs might benefit HIV pathogenesis (Cassol et al., 2010; Herbein and Varin, 2010). This is supported by multiple observations that immune activation driven by Mϕs correlates with HIV-1 pathogenesis (Goodenow et al., 2003; Lamers et al., 2009; Cohen et al., 2011). Recently, Brown et al. (2008) characterized the HIV-1-induced polarization of Mϕs as "$M1_{HIV}$" since it displays a pro-inflammatory state with increased production of cytokines independently of TLR-pathway. The authors argue that while HIV-1 stimulates Mϕs through a variety of signaling pathways to promote a "tailored" inflammation in its favor, the TLR recognition of viral replication is impaired and could serve as a viral evasion strategy. Given that prolonged pro-inflammatory Mϕ activation during chronic HIV-infection contributes, not only to a permissive environment for the formation of viral reservoirs with strong transcriptional activity, but also to disease progression and HIV-induced tissue damage, the proposed $M1_{HIV}$ polarized state may render Mϕs detrimental to the host (Goodenow et al., 2003; Brown et al., 2008; Lamers et al., 2009).

As HIV-disease progresses from the acute to asymptomatic stage, there is a switch from a type-1 toward a type-2 inflammatory environment (**Figure 1**; Vasilescu et al., 2003; Becker, 2004). At the transcriptional level, lymphatic tissue microarray analyses from HIV-1-infected subjects at different clinical stages revealed that each stage has a unique gene profile (Li et al., 2009). The acute phase is characterized by gene expression involved in innate and adaptive immunity. The asymptotic phase, however, down-regulates the acute phase gene profile to baseline level while it displays an increased expression of immunosuppressive genes (Li et al., 2009). Based on these immunological systemic changes, it is likely that a polarization switch occurs in Mϕs from a M1 program during the acute phase to the M2 programs through later stages. Although there is no overwhelming evidence confirming the abundance of M2 Mϕs in either the asymptotic or AIDS phase *in vivo*, the fact CD163 (a M2 Mϕ cell surface marker) is considered as a potential biomarker for HIV-1 disease progression may allude to the presence of M2 Mϕs in HIV-1-infected individuals (Burdo et al., 2011; Tippett et al., 2011). Similar to M1 Mϕs, it is not known whether M2 Mϕs benefits the host during HIV-1 infection. *In vitro* activation of M2a (IL-4-treated) Mϕs results in inhibition of virus replication (Cassol et al., 2009). Other studies have demonstrated that both IL-4 and IL-13 down-regulate viral entry receptors and HIV-1 reverse transcription in Mϕs (Cassol et al., 2010). Furthermore, activation of M2c (IL-10-treated) Mϕs strongly inhibits reverse transcription, transcription of HIV-1 LTR and viral assembly (Herbein and Varin, 2010). Based on these observations, it might be tempting to conclude that M2 Mϕs are beneficial to host immunity against HIV. However, the progression of AIDS is characterized by the loss of IL-2 and increase of IL-10 correlating with HIV viremia (Brockman et al., 2009; Sandanger et al., 2009). Moreover, the haplotypes of both IL-4 and IL-10 genes have been associated recently with AIDS progression (Vasilescu et al., 2003). Therefore, the switch toward a M2 Mϕ program might simply be part of a defensive mechanism by the host to control HIV-induced tissue damage since they participate in suppression of inflammation and promotion of tissue repair (**Figure 1**; Martinez et al., 2009; Murray and Wynn, 2011). Recently, a functional proteomic analysis of HIV-infected Mϕs in the presence of regulatory T cells showed that a deviation of M1 to M2 Mϕ program is associated with neuroprotection in the case of HIV-associated neurocognitive disorders, suggesting M2 Mϕs may curtail the $M1_{HIV}$ polarized activity resulting in tissue damage (Huang et al., 2010). Conversely, the switch toward the M2 Mϕ program might also occur as an evasion strategy by HIV to promote its own survival. A recent study demonstrated that HIV up-regulates both programmed cell death ligand 1 (PD-L1) and PD-L2 expression, members of the B7:CD28 family, and PD-1 ligands, in Mϕs (Porichis et al., 2011). Given the importance of these molecules in T cell exhaustion during HIV infection, the ability of IL-10 to both activate the M2c Mϕ program and induce PD-L1, and the fact that IL-10 production and increased expression of PD-L1 correlate in HIV-infected patients, the authors propose the manipulation of PDL expression in Mϕs as a strategy to evade immune responses (Trabattoni et al., 2003; Porichis et al., 2011). Whatever the true role of M2 Mϕs in HIV infection, it is clear they

influence the establishment of HIV pathogenesis, and more studies are needed to examine thoroughly HIV-induced polarization of Mϕs *in vivo*.

CONCLUSION

Tuberculosis is the most common opportunistic infection in AIDS and often used as a clinical parameter for undiagnosed AIDS cases (Deretic et al., 2004). While the synergy between Mtb and HIV is evident at the clinical level, the mechanisms accounting for it are poorly understood. Deretic et al. (2004) proposed the interference with endosomal sorting machine as a molecular mechanism contributing to the synergy between these two pathogens. Likewise, we envision the pathogenic modulation of Mϕ polarization as a cellular mechanism that might influence this synergism. As aforementioned, it is estimated that about one-third of the human population may be latently infected with Mtb (WHO Global Tuberculosis Control Report 2010, 2010), suggesting that one in three of the 2.6 million people newly infected with HIV-1 in 2009 (Cohen et al., 2011), for example, would also be coinfected with Mtb. Latent Mtb is confined in solid granulomas composed of mainly by Mϕs and T cells that maintain their stability. The coinfection with HIV-1 results in a dramatic increase in the odds of latently infected people progressing into overt TB to a staggering annual risk of 10% (Deretic et al., 2004; Swaminathan et al., 2010). HIV-driven immune perturbation, reflected in the loss of $CD4^+$ T cells and abnormal low levels of TNFα causes the loss of granuloma integrity and efficiency in anti-microbial containment leading to post-primary reactivation state (Paige and Bishai, 2010). These events may increase both Mϕ necrosis and release of intracellular bacilli accounting for the extrapulmonary TB manifestation diagnosed in patients with HIV-driven immunosuppression (Swaminathan et al., 2010). The awakened Mtb then might induce M1 Mϕs to drive an excessive TNFα response (together with other mechanisms such as MMP secretion) to deliberately promote parasitic granuloma formation, resulting in the recruitment of additional naïve Mϕs and the tissue pathology (Davis and Ramakrishnan, 2009; Paige and Bishai, 2010; Volkman et al., 2010). Excessive levels of TNFα, may not only contribute to the classical symptoms of cachexia in TB, but also to the augmentation of HIV-1 transcription and accelerated formation of viral reservoirs (Deretic et al., 2004). In the absence of an efficient adaptive immune response due to HIV-driven impairment, uncontrolled inflammation can result in lung immunopathology, and consequently, the host may induce tissue repair responses. The shift from M1 to M2 Mϕ program may become pronounced and prolonged in the sterile attempt to restore tissue integrity, elevating the level of IL-10 that is typical of disease progression by both pathogens, and thus contributing to the failure of all immunological functions and clinical collapse. While highly speculative, this scenario highlights the importance to understand Mϕ polarization in the context of immune activation and pathogen-driven disease, and its potential to be yet another convergence point targeted by Mtb and HIV to circumvent the host immune system.

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Molecular mechanisms regulating macrophage response to hypoxia

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Monocytes and Macrophages (Mo/Mϕ) exhibit great plasticity, as they can shift between different modes of activation and, driven by their immediate microenvironment, perform divergent functions. These include, among others, patrolling their surroundings and maintaining homeostasis (resident Mo/Mϕ), combating invading pathogens and tumor cells (classically activated or M1 Mo/Mϕ), orchestrating wound healing (alternatively activated or M2 Mo/Mϕ), and restoring homeostasis after an inflammatory response (resolution Mϕ). Hypoxia is an important factor in the Mϕ microenvironment, is prevalent in many physiological and pathological conditions, and is interdependent with the inflammatory response. Although Mo/Mϕ have been studied in hypoxia, the mechanisms by which hypoxia influences the different modes of their activation, and how it regulates the shift between them, remain unclear. Here we review the current knowledge about the molecular mechanisms that mediate this hypoxic regulation of Mϕ activation. Much is known about the hypoxic transcriptional regulatory network, which includes the master regulators hypoxia-induced factor-1 and NF-κB, as well as other transcription factors (e.g., AP-1, Erg-1), but we also highlight the role of post-transcriptional and post-translational mechanisms. These mechanisms mediate hypoxic induction of Mϕ pro-angiogenic mediators, suppress M1 Mϕ by post-transcriptionally inhibiting pro-inflammatory mediators, and help shift the classically activated Mϕ into an activation state which approximate the alternatively activated or resolution Mϕ.

Keywords: low oxygen tension, inflammation, post-transcriptional regulation, post-translational regulation, M1 macrophages, M2 macrophages

INTRODUCTION

Many physiological and pathological processes (e.g., inflammation, wound healing, acute myocardial infarction, retinopathies, atherosclerosis, solid tumors, and more) are characterized by both low oxygen tensions (hypoxia) and presence of monocytes/macrophages (Mo/Mϕ). Mo/Mϕ are essential regulators of inflammation and central participants in hypoxia-driven processes, and the mediators they express and secrete recruit other cells and orchestrate their activity. The role of Mo/Mϕ and the effects hypoxia exerts on them were studied mostly in solid tumors, but the same molecular mechanisms apply in other clinical scenarios. We review here the different molecular mechanisms exerted by hypoxia, which regulate Mo/Mϕ functions. We elaborate on the hypoxia-induced transcriptional network in Mϕ, which is driven mostly by the hypoxia-induced factors (HIFs) and NF-κB, the two master regulators of the hypoxic response. We also draw attention to additional post-transcriptional, translational, and post-translational mechanisms that enable hypoxia to activate or suppress gene and protein expression. Finally, we review the relatively few studies on the mechanisms and effects of hypoxia on differently activated Mo/Mϕ, and discuss how these mechanisms help shift the pro-inflammatory activated Mϕ toward an anti-inflammatory, pro-angiogenic phenotype.

We do not expand on how hypoxia-induced mechanisms affect the different Mϕ functions, as these aspects were extensively reviewed before (Lewis et al., 1999; Murdoch et al., 2004, 2008; Bosco et al., 2008; Martinez et al., 2008; Coffelt et al., 2009; Walmsley et al., 2009; Qian and Pollard, 2010). Because the investigation of mechanisms is most often performed *in vitro*, we limit our discussion to these studies, and only briefly mention the increasing number of evidences pointing to the crucial role hypoxia plays *in vivo* in the pathophysiology of many diseases. Moreover, although hypoxia is physiologically and pathologically followed by re-oxygenation, we focus only on the isolated effects of hypoxic stress on Mo/Mϕ. Likewise, the effects of reduced pH, low glucose levels, or increased lactate, conditions that also accompany hypoxia and ischemia, are not discussed.

DIFFERENT MODES OF MACROPHAGE ACTIVATION

Monocytes (Mo) migrate into tissues and differentiate into Macrophages (Mϕ) to perform many functions needed in the tissue. These include resident Mϕ that patrol their surroundings and maintain homeostasis, Mϕ that combat invading pathogens and tumor cells and protect the tissue, Mϕ that orchestrate the process of wound healing, and Mϕ that resolve inflammation. To perform these multiple tasks, Mϕ phagocytose pathogens, secrete cytokines, chemokines, and growth factors, present antigenic determinants to

T cells and activate them, scavenge dead cells and necrotic debris and deposit matrix proteins. Since one cell cannot perform all these divergent tasks simultaneously, it was suggested that Mϕ can be differently activated, depending on the signals received from the microenvironment. Thus, Mϕ exhibit an enormous plasticity (Stout and Suttles, 2004), and dynamically shift from one form of activation to another according to the conditions in the changing microenvironment (Stout et al., 2009). This concept, as well as the evidences supporting it and the markers that characterize each Mϕ phenotype, has been extensively reviewed (Mosser, 2003; Martinez et al., 2008; Mosser and Edwards, 2008; Murdoch et al., 2008; Gordon and Martinez, 2010; Qian and Pollard, 2010), and will be only briefly mentioned here.

In the continuum of Mϕ phenotypes, two main sub-populations have been described. In one extreme, the classically activated Mϕ, or M1 Mϕ, is responsible for the multi-stage process of recognizing pathogens, phagocytosing, and degrading them to present their antigenic determinant to helper T cells in the context of the MHC class II molecules, whose expression is elevated (Mosser, 2003; Martinez et al., 2008). This process is accompanied by secretion of IL-12, which is necessary to support a Th1 response. M1 Mϕ are activated by ligands of different receptor families such as the toll-like receptor (TLR) ligands (e.g., lipopolysaccharides – LPS), and pro-inflammatory cytokines (e.g., interferon-γ – IFNγ, tumor necrosis factor-α – TNFα, and interleukin-1β – IL-1β). They secrete high amounts of pro-inflammatory mediators that kill the invading pathogens or tumor cells, such as the cytotoxic TNFα and nitric oxide (NO), the latter (and the enzyme producing it, inducible nitric oxide synthase – iNOS) serving as the hallmark of this Mϕ subset. They also secrete chemokines, which attract more neutrophils and Mo/Mϕ to the inflamed site, thus amplifying the pro-inflammatory response. Secretion of proteolytic enzymes, such as matrix metalloproteinases (MMPs) helps degrade components of the extracellular matrix (ECM) and allows migration of leukocytes to the inflamed tissue.

In the other extreme we find Mϕ that are activated by and secrete anti-inflammatory mediators (e.g., IL-10, IL-13, tumor growth factor beta – TGFβ, and prostaglandin E_2 – PGE_2), which generate a microenvironment that suppresses the activity of M1 macrophage. They remove cellular debris and perform phagocytosis which is mediated by receptors different than those expressed by M1 Mϕ, such as the mannose receptor CD206; contribute to tissue remodeling by depositing ECM proteins; and express high levels of arginase-1, which produces ornithine, a precursor for the synthesis of the ECM protein collagen. Arginase-1 also competes with iNOS for their common substrate L-arginine and thus prevents NO production. Thus, these alternatively activated or M2 Mϕ are involved mainly in homeostasis and wound healing. M2 Mϕ also secrete proteolytic enzymes that help them move about, and as they are activated by IL-4 and IL-13, they are particularly suited to present helminthes antigens to helper T cells and initiate a Th2 response (Martinez et al., 2009), so that the term "anti-inflammatory" refers, in fact, to a different type of inflammation.

A third subset of Mϕ is responsible for immune regulation, and may include several subtypes. Some of these cells are activated by ligands of TLRs in combination with immune complexes, and some are activated by anti-inflammatory signals, such as adenosine or phagocytosed apoptotic cells (Mosser, 2003; Mosser and Edwards, 2008). Immature Mϕ, which compose some of the myeloid-derived suppressor cells (MDSCs) population, also belong to regulatory Mϕ. MDSCs are triggered by a combination of IFNγ and IL-13, and secrete IFNγ, IL-13, IL-10, and TGFβ, which help them suppress Th1 cell-mediated immune response, induce regulatory T cells and inhibit M1 Mϕ (Bronte, 2009; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009).

A different subset of Mϕ isolated at the resolution-phase of inflammation (resolution or rMϕ) is often activated by mediators released from apoptotic cells, such as sphingosine-1-phosphate (S1P) or TGFβ. These rMϕ secrete predominantly the anti-inflammatory IL-10, very low levels of pro-inflammatory cytokines and chemokines and express arginase-1 and CD206, as well as the pro-inflammatory iNOS and COX-2 (Bystrom et al., 2008). Moreover, in a model of self-resolving peritonitis, rMϕ were further divided into $CD11b^{high}$ and $CD11b^{low}$ cells (Schif-Zuck et al., 2011). $CD11b^{high}$ rMϕ engulf apoptotic neutrophils and stay at the inflammatory site, where they express markers of both M1 and M2 Mϕ (e.g., iNOS, arginase-1, COX-2, and MMP-9). However, once they engulf enough apoptotic cells (threshold determined at 7 cells), they convert into $CD11b^{low}$ cells, induce the expression of the enzyme 12/15-lipoxygenase which produces pro-resolving lipid mediators, and emigrate to lymphoid organs to convey resolution signals to lymphocytes (Schif-Zuck et al., 2011).

Collectively, all these regulatory Mϕ, which secrete anti-inflammatory mediators designed to resolve inflammation, are positioned between M1 and M2 Mϕ, and share markers with these two sub-populations.

Malignant tumors are characterized by repeated cycles of hypoxia and reoxygenation, and several Mϕ subsets have been found located in different regions of the tumor (Lewis and Pollard, 2006). These include the tumor-associated macrophages (TAMs) and the Tie-2 expressing macrophages (TEMs), which support tumor growth and metastasis, and are both an important source of pro-angiogenic factors. Whereas some TEMs reside close to blood vessels (peri-endothelial; Venneri et al., 2007), TAMs infiltrate deeper into the tumor and are located in perinecrotic areas where oxygen tensions are very low. Regulatory MDSCs are present within tumors, as well as in lymphoid organs, such as the bone marrow and spleen, and expand proportionally to the tumor burden. All these subtypes share activation markers with both M2 and regulatory Mϕ, thus placing them between these two subsets (De Palma et al., 2007; Mosser and Edwards, 2008; Murdoch et al., 2008). Of note, the principle of Mϕ plasticity extends even to TAMs subset, as they exhibit divergent phenotypes (Lewis and Pollard, 2006), depending on the type of the tumor, the stage of its development, the interactions with both the ECM and other neighboring cells, and the level of oxygen tensions they are exposed to.

Moving from Mϕ to Mo, we find the monocytes to have similar but not identical classifications. Circulatory Mo are divided into three subsets according to their CD14 and CD16 expression (in humans) or Ly6C expression (in mice). The Classical or M1 Mo express high levels of CD14 and no CD16 (denoted either $CD14^{++}CD16^{-}$ or $CD14^{+}CD16^{-}$ in human, and $Ly6C^{high}$ in

mice), intermediate Mo, in humans only, express intermediate levels of CD14 and CD16 ($CD14^{++}CD16^{+}$ or $CD14^{+}CD16^{+}$), and resident, M2 or non-classical monocytes express very low levels of CD14 and high levels of CD16 ($CD14^{dim}CD16^{+}$ or $CD14^{-}CD16^{+}$ in human, and $Ly6C^{low}$ in mice) (Ziegler-Heitbrock, 2007; van de Veerdonk and Netea, 2010). However, the function of these subsets as cells secreting either pro- or anti-inflammatory cytokines is still controversial. It is assumed that M1 and M2 Mo are the counterparts of M1 and M2 Mϕ, but this is not always accurate. For example, sorted Mo subsets that were stimulated *ex vivo* with LPS increased IL-10 secretion from $CD14^{+}CD16^{+}$ cells, whereas LPS-stimulated $CD14^{dim}CD16^{+}$ cells showed increased TNFα with little IL-10 secretion (Skrzeczynska-Moncznik et al., 2008). In a different study, $CD14^{-}CD16^{+}$ cells showed patrolling characteristics with weak ability for phagocytosis and low production of ROS and cytokines when challenged by bacterial ligands of TLRs, but secreted high amounts of the pro-inflammatory TNFα and IL-1β cytokines upon stimulation with viral ligands or nucleic acids (Cros et al., 2010). These results suggest that non-classical or M2 Mo can behave as M1 Mo under certain circumstances. Thus, Mo classification does not fully match the M1-M2 Mϕ taxonomy.

During inflammation differently activated Mo/Mϕ enter the tissue, first in order to eliminate pathogens or tumor cells (M1 Mo/Mϕ), and later to heal the damaged tissue and to restore homeostasis (M2 Mo/Mϕ and rMϕ; Nahrendorf et al., 2010). This requires precise signals that regulate this sequential migration. For example, the chemokines CCL2 and CX_3CL1 and their receptors, expressed on $CD14^{+}CD16^{-}$ and both $CD16^{+}$ monocytes, respectively, were shown to mediate these responses (Geissmann et al., 2003; Auffray et al., 2009). Of note, this specific sequence of events may not always apply, and clinical scenarios which favor a Th2 response may differentially recruit non-classical Mo. Furthermore, whether classically activated Mo mature to M1 Mϕ in the tissue and are later skewed to become M2 Mϕ, or whether they die on site and are replaced by newly recruited M2 Mo, remains unclear, and the effects of the hypoxic microenvironment in this context must be further explored.

According to the danger model (Matzinger, 2002), the direction and magnitude of the inflammatory response depend not only on the nature of the stimulus (e.g., types of bacteria, site of entry, size of the bacterial inoculation), but also on the type and extent of expression of co-stimulatory molecules (e.g., CD80 vs. CD86) and specific signals received from the microenvironment, such as danger-associated molecular patterns (DAMPs). Necrotic and apoptotic cells that release their content into the microenvironment, are picked up by Mo/Mϕ, processed and presented to T cells in the context of MHC class II and co-stimulatory molecules. Thus, microenvironmental stimuli can affect Mo/Mϕ functions, directly or via their effect on other cells.

These examples reveal the high plasticity of Mo/Mϕ, and emphasize that their heterogeneity ranges beyond the M1–M2 polarization models, and extends to new phenotypes we may yet discover. Thus, Mo/Mϕ classification and the distinction between their different activation modes and differentiation status is an active area of study.

INFLAMMATION AND HYPOXIA

Low oxygen tensions (hypoxia) are relevant in many physiological and pathological conditions. For example, hypoxia may arise due to occlusion of a blood vessel (e.g., during myocardial infarction or pulmonary embolism), significant blood loss or dilated blood vessels which instigate reduced blood flow and lack of oxygen (e.g., in burns, trauma, and sepsis), or simply when oxygen demands are not met (e.g., physical exercise, high altitudes, tumors). Hypoxia has been demonstrated *in vivo* in a variety of acute and chronic inflammatory sites, including the synovium in RA patients, the arterial intima in atherosclerotic lesions, myocardial infarcts, wounds, and sites of bacterial infection (reviewed in Murdoch et al., 2005). Since even healthy tissues exhibit a wide range of oxygen tensions, no one value can represent hypoxia, and hypoxia is, therefore, functionally defined as the inability of oxygen delivery to meet oxygen demands of the tissue (Papandreou et al., 2005). According to this definition, hypoxia occurs during inflammation, as more cells that require oxygen infiltrate the site and increase oxygen consumption, but only few capillaries can supply it. Thus, different oxygen tensions can be measured in different areas of tumors, wounds, or in tissues affected by chronic inflammation, generating a gradient of hypoxia, where levels as low as 5–10 mmHg were measured (Crowther et al., 2001). Of note, other microenvironmental factors, such as increased lactate concentrations and reduced pH, are linked to hypoxia, as they result from the shift to anaerobic metabolism. Therefore, investigating the role of prolonged hypoxia actually looks into the combined effect of all of these stress factors. Most *in vitro* studies measure the percent oxygen in the atmosphere flowing into the hypoxic chamber, but usually even in anoxic atmosphere (0% O_2) some oxygen remains dissolved in the medium (20–30 mmHg).

Depending on its duration and severity, hypoxia can drive inflammation and aggravate cellular and tissue injury. For example, humans exposed to the scarce oxygen available in high altitudes or mice exposed to ambient hypoxia experience reduced arterial partial oxygen tensions, and develop pulmonary edema and increased release of pro-inflammatory cytokines (Hartmann et al., 2000; Grocott et al., 2009; Rosenberger et al., 2009). Hypoxia shifts cells toward anaerobic metabolism, leading to exclusive use of glycolysis as the means for ATP production, instead of Krebs cycle. As a result, lactate accumulates in the cells, causing cellular acidosis, production of ROS is increased, and lipids are peroxidated leading to membranal damage. This, combined with the lack of ATP, impairs the function of ion channels, increases Ca^{+2} influx, and membrane permeability, leading to spillage of cellular content (including proteolytic enzymes, hydrolases, and increased lactate), thereby increasing acidosis and tissue damage (Minko et al., 2005; Behn et al., 2007). Necrotic cells and increased tissue acidosis recruit more leukocytes into the area, and trigger inflammation. Hence, hypoxia and inflammation are interdependent, as chronic inflammation is accompanied by hypoxia and prolonged hypoxia leads to inflammation (Eltzschig, 2011).

ROLE OF HIFs

Like other cell types, the macrophage response depends on the severity and the duration of the hypoxic insult. In recent years, the search for the way cells sense differences in oxygen tensions was

focused primarily on the prolyl hydroxylases (PHD)–HIF pathway outlined below.

Hypoxia-induced factors are heterodimeric transcription factors consisting of a constitutively expressed β subunit (aryl hydrocarbon nuclear translocator – ARNT), and an α subunit, which is constitutively transcribed but immediately degraded in normoxia. Three α subunits (HIF-1α, HIF-2α, and HIF-3α) have been identified, which together with HIF-1β compose three isoforms that are differently expressed in various tissues. HIF-3α (also called inhibitory PAS domain protein – IPAS) is a transcriptional inhibitor and functions as a negative regulator of HIF-1α and HIF-2α, although its biology is still unclear (Heikkila et al., 2011), whereas HIF-1α and HIF-2α were more extensively studied. HIF-1α and HIF-2α are homologous, especially in their DNA binding domains, allowing their binding to the same hypoxia response element (HRE).

The binding of HIF-1 and HIF-2 to many HRE-containing promoters can induce the expression of a myriad of genes, some with opposing effects. By means of over-expressing the HIF-α subunits, or by ablating them in knockouts or by siRNA knockdown, the role of each subunit in determining Mϕ phenotypes and response to hypoxic stress was established. Thus, HIF proteins were shown to regulate all aspects of Mϕ functions in response to hypoxia, including the shift to anaerobic glycolysis, mitochondrial impairment, angiogenesis, invasion, and immune suppression (Coffelt et al., 2009; Walmsley et al., 2009; Werno et al., 2010a). Moreover, HIFs are also induced in normoxia, and play a central role in immune recognition, phagocytosis, bacterial killing, and pro-inflammatory cytokine production (Cramer et al., 2003; Peyssonnaux et al., 2007).

HIFs EXPRESSION IN DIFFERENT Mϕ SUBSETS

The two HIF isoforms, HIF-1α and HIF-2α are differently expressed in Mo and Mϕ, as hypoxia could not induce the expression of HIF-1α or HIF-2α in human Mo, but did induce them in human monocyte-derived macrophages (hMDM; Elbarghati et al., 2008). Differentiated human and mouse MDM show different kinetics of HIFs expression in response to hypoxia, with higher stability for HIF-2α expression (Elbarghati et al., 2008; Takeda et al., 2010). This suggests that HIF-α subunits could, at least partially, affect Mϕ activation modes. Furthermore, arginine metabolism was enhanced toward NO production in a HIF-1α-dependent manner upon LPS or IFNγ stimulation, but NO production was reduced by IL-4 due to increase in HIF-2α-induced arginase-1 activity (Takeda et al., 2010). Conflicting evidences show higher accumulation of HIF-1α in hypoxic hMDM (Burke et al., 2002), or higher expression of HIF-2α under similar conditions (Griffiths et al., 2000). One study showed almost undetectable HIF-2α mRNA after prolonged exposure to hypoxia, but induced HIF-1α mRNA that was even further elevated by LPS in thioglycollate (TG)-elicited peritoneal Mϕ (Acosta-Iborra et al., 2009), whereas another study showed constitutive HIF-2α mRNA levels in these cells (Takeda et al., 2010). TAMs present in human breast carcinomas show high expression of HIF-2α, which correlates with increased micro-vessel density and high tumor grade (Patel and Simon, 2008). TAMs in different tumors *in vivo* show high expression of both HIF-1α and HIF-2α (Lewis and Pollard, 2006). The importance of exposure time is demonstrated in a study that compared acute (24 h) and chronic (5 days) exposures to hypoxia, and reported that hMDM subjected to chronic hypoxia increased HIF-1α and HIF-2α proteins relative to hMDM that were subjected to normoxia or to acute hypoxia (Staples et al., 2010). However, there is still no clear perspective regarding the role of the different HIFs in hypoxic Mϕ, and different periods of time of hypoxic exposure or different oxygen levels may have different effects on HIFs' expression.

REGULATION OF HIFs IN NORMOXIA AND HYPOXIA

Protein stability

Hypoxia-induced factor proteins are regulated primarily by protein stability. HIF-α subunits are constitutively transcribed and translated, but immediately directed for degradation in normoxia. This is achieved by the hydroxylation of proline residues (402 and 564 on HIF-1α and 405 and 532 on HIF-2α) by the three PHDs, which depend on oxygen, 2-oxoglutarate, Fe^{+2}, and ascorbate as substrates and cofactors for their activity. Hydroxylation recruits the von Hippel Lindau (VHL), which forms a complex together with elongin B, elongin C, cullin-2, and ring-box 1 that has an E3 ubiquitin ligase activity. This complex ubiquinates HIF-α subunits and targets them for proteosomal degradation (Nizet and Johnson, 2009; Walmsley et al., 2009). Hypoxia inactivates PHDs due to the limited oxygen substrate, and therefore stabilizes the HIF-α subunits, allowing their heterodimerization with the HIF-1β subunit. Hypoxia also impairs electron transport in the mitochondria, leading to increased production of ROS, which oxidizes Fe^{+2} to Fe^{+3} and further inhibit PHDs. Additional proteins contribute to this regulation, and details on their activity are reviewed elsewhere (Yee Koh et al., 2008).

Although HIF-α subunits are typically induced in hypoxia, they can also be expressed in normoxia upon inflammatory stimulation. For example, high levels of succinate (the product of 2-oxoglutarate) or increased ROS production that oxidizes Fe^{+2} may cause PHDs inactivation and HIF-1α stabilization (Denko, 2008). Likewise, high concentrations of NO in normoxia can stabilize HIF-1α by inhibiting the activity PHDs, probably by targeting their Fe^{+2} catalytic site (Weigert and Brune, 2008). Ligation of TLRs, such as the binding of LPS to TLR4, stimulate HIF-1α expression in normoxia through the activity of NF-κB, and hypoxia in the presence of LPS shows a synergistic effect (Frede et al., 2006). Additional pathways can contribute to HIF-1α expression, including the Phosphatidylinositol 3-kinase (PI3K), Mitogen-activated protein kinases (MAPK) and the Ca^{2+}/calmodulin-dependent protein kinases (CaMKII) pathways, and their inhibitors reduce HIF-1α accumulation as well as the expression of its targets (Westra et al., 2010). Thus, in addition to hypoxia, other stimuli, including bacterial infection, hormones, growth factors, cytokines, or RNS can stabilize and activate HIF-1α in normoxia.

Translation and transcription of HIFs

The main known level of HIFs regulation is post-translational, as described above. However other possible regulatory checkpoints, such as transcription and translation, are under-investigated. Although HIF-α subunits are constitutively transcribed in many cell types, their mRNA levels are increased in hypoxia. HIF-1α

promoter consists of Sp1 sites, several HRE sites (suggesting a positive feedback loop), as well as NF-κB, Egr-1, and Stat3 binding sites that mediate increased transcription in hypoxia (Niu et al., 2008; Galban and Gorospe, 2009). Evidences show differences between Mo and Mϕ, as hypoxia inhibits HIF-1α transcription in human Mo (Bosco et al., 2006), but increased HIF-1α mRNA accumulation in hMDM by mechanisms other than increasing mRNA stability (Staples et al., 2010). Likewise, HIF-1α mRNA was increased during differentiation of THP-1 cells or peripheral blood Mo into Mϕ (Oda et al., 2006). This suggests a negative feedback designed to control the systemic hypoxic response.

Translational regulation of HIFs is an important checkpoint in both normoxia and hypoxia, which was investigated mainly in cancer cells. Increased HIF-1α protein levels in hypoxia can be observed without a parallel increase in the mRNA levels. This is mediated by the binding of the two RNA-binding proteins (RBP) polypyrimidine tract-binding protein (PTB) and human antigen R (HuR) to the 3′-UTR and 5′-UTR, respectively, as their knockdown through RNA interference inhibited HIF-1α accumulation (Galban et al., 2008). Iron response proteins (IRP) bind to their elements located in the 5′-UTR region of the HIF-2α mRNA and inhibit translation of the mRNA in normoxia, while hypoxia releases this binding and enhances HIF-2α translation (Galban and Gorospe, 2009). Furthermore, specific microRNAs (the miR-17-92 cluster and miR-199a) target HIF-1α mRNA in normoxia and inhibit its translation, whereas hypoxia reduces their levels and release HIF-1α mRNA translation (Galban and Gorospe, 2009). The mechanisms for translational controls have not yet been described in Mo/Mϕ, and this warrants more investigation.

Regulation of HIFs' activity

Hypoxia-induced factors activity as transcription factors that bind to HREs and to the co-activators CBP/p300 is regulated by oxygen-dependent protein–protein interactions. Factor-inhibiting HIF-1 (FIH-1), another oxygen-dependent hydroxylase, can hydroxylate asparagine residues on HIF-α subunits (803 on HIF-1α and 847 on HIF-2α), resulting in proteins that cannot bind to the co-activators CBP/p300 (Walmsley et al., 2008; Nizet and Johnson, 2009). Additionally, VHL also acts as an adapter that facilitates the binding of FIH to HIF-α subunits in normoxia, while hypoxia disrupts this interaction (Li et al., 2011), allowing for the accumulation of HIFs and their binding to HRE-containing promoters. FIH-1 also plays a role in normoxia, as the cytoplasmic tail of MT1-MMP/MMP-14 provides a platform to which both FIH-1 and its inhibitor mint3/APBA3 bind. This limits the complex localization to the Golgi apparatus and allows mint3 to inhibit FIH-1 ability to hydroxylate HIF-1α, resulting in enhanced accumulation of HIF-1α in normoxia (Sakamoto and Seiki, 2010). It is yet unclear whether a similar mechanism operates on PHDs localization to render them inactive and inhibit HIFs degradation in normoxic Mϕ.

Post-translational modifications regulate stabilization, degradation, and activity of HIF-α subunits by affecting their protein–protein interactions, especially other transcription factors or components of the transcriptional machinery. These were investigated mostly in cancer cell lines and fibroblasts. For example, HIF-α is directly phosphorylated by the ERK1/2 MAPK, leading to its transport to the nucleus and increased activity (Mylonis et al., 2006). Two components of the PI3K/Akt pathway were shown to oppositely regulate HIF-1α. The mTOR kinase phosphorylates HIF-1α in its oxygen-dependent degradation domain, leading to its increased stabilization and enhanced transactivation (Hudson et al., 2002), whereas GSK-3-mediated phosphorylation induces HIF-1α destabilization and proteosomal degradation (Flugel et al., 2007). Recently, PHD3 was found to hydroxylate pyruvate kinase M2, leading to its enhanced interaction with both HIF-1α and p300 in hypoxia and promoting their transactivation of HRE-containing target genes (Luo et al., 2011).

Effects of HIF-1α S-nitrosylation are controversial, as it could increase or decrease its ability to interact with its co-activator p300 (Sumbayev et al., 2003; Cho et al., 2007; Li et al., 2007). However, this mechanism could be relevant only in normoxic induction of HIF-1α, as iNOS is inactivated in hypoxia (see Intracellular Trafficking and Protein–Protein Interactions). Hypoxia was shown to induce SUMOylation of HIF-1α, either promoting its binding to VHL and degradation (Cheng et al., 2007) or promoting its stabilization and transcriptional activity (Carbia-Nagashima et al., 2007). These apparent contradictions may reflect use of different cell types or different hypoxic regimes, and the effects of such post-translational modifications in hypoxic Mϕ should be studied.

ROLE OF NF-κB

NF-κB are a family of proteins consisting of NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel that can heterodimerize. The role of the NF-κB family members as master regulators of many pro-inflammatory associated gene is widely accepted (Vallabhapurapu and Karin, 2009).

NF-κB in different Mϕ subsets

Canonical NF-κB activation is indispensible for M1 activation, although cooperation with other transcription factors such as Stat1 or the MAPK/AP-1 pathway is essential (Biswas and Lewis, 2010; Lawrence and Fong, 2010). NF-κB also regulates anti-inflammatory and pro-angiogenic gene expression (e.g., IL-10, VEGF, COX-2, TGFβ, and MMPs) and inhibits M1 Mϕ (Lawrence and Fong, 2010). In part, this is associated with over-expression of p50 and formation of p50:p50 homodimers, which inhibit transcription of pro-inflammatory cytokines while enhancing transcription of anti-inflammatory genes (Saccani et al., 2006; Biswas and Lewis, 2010; Mancino and Lawrence, 2010). Furthermore, deletion of IKKβ in myeloid cells shifts Mϕ toward M1 activation, increasing the expression of IL-12, MHC class II and iNOS, and revealing the inhibitory role of IKKβ on Stat1 (Fong et al., 2008).

Tumor-associated macrophages derived from human and murine tumors and cultured in normoxia demonstrate defective NF-κB activation, high IL-10 expression and reduced IL-12 and TNFα production (Sica et al., 2000). However, as distinct regions can be identified within solid tumors, TAMs could be activated both as M1 and M2 Mϕ and fluctuate between them, depending on the type of the tumor, its stage, Mϕ localization within the tumor, and the signal received from the microenvironment, including hypoxia (Mancino and Lawrence, 2010). Therefore, the role of NF-κB in specific Mϕ subsets is very complex.

Regulation of NF-κB in normoxia and hypoxia – links to the HIF pathway

Activation of NF-κB occurs in normoxia when TLRs or receptors for pro-inflammatory cytokines recruit the MyD88 adapter protein, which activates a complex signaling cascade that is based on protein–protein interactions and culminates in the phosphorylation, ubiquitination, and degradation of IκB proteins and translocation of NF-κB to the nucleus.

Hypoxia has long been shown to simulate NF-κB signaling. *In vivo*, hypoxia activates NF-κB expression in a tissue-specific manner, as transgenic mice harboring the luciferase gene under the control of NF-κB expressed it in the hearts and lungs (Fitzpatrick et al., 2011). *In vitro* there are many examples for hypoxia-induced expression of pro-inflammatory genes. Hypoxic RAW 264.7 cells induced MIP-2 expression, and inhibition of the ERK1/2 or PI3K pathways decreased p65 activity (Zampetaki et al., 2004). Increased production of ROS due to hypoxia was demonstrated in Mo/Mϕ to be responsible for NF-κB activation and for the synergetic effects of hypoxia and LPS (Chandel et al., 2000; Kim et al., 2010). Recently, PDHs were demonstrated as regulators of NF-κB signaling, as their inhibition suppressed LPS-induced expression of TNFα in Mϕ (Takeda et al., 2009). IKKβ was discovered as an additional substrate that PDHs can hydroxylate, leading to its activation and to IkB phosphorylation (Cummins et al., 2006). Additionally, ankyrin repeats on both IκBα and the p105 subunit of NF-κB can be hydroxylated by FIH-1 (Cockman et al., 2006; Cummins et al., 2006; Frede et al., 2006), explaining the effect of ROS on NF-κB activation. In fact, PDHs and FIH-1 involvement in NF-κB regulation suggests that both HIF and NF-κB proteins are redox-sensitive proteins regulated by the same oxygen sensors, in addition to their role as master regulators of inflammation and hypoxia.

Many hypoxia-induced genes are targeted by both NF-κB and HIF proteins. Indeed, a link between the HIF and NF-κB pathways is also provided by the presence of an NF-κB binding site in the HIF-1α promoter (but not HIF-2α), and the ability of p50:p65 heterodimers to bind to it and drive its expression in hypoxia (Belaiba et al., 2007), while siRNA for p65 inhibits this expression (Fitzpatrick et al., 2011). For example, COX-2 was induced by hypoxia in a manner dependent on both HIF-1 and NF-κB (Fitzpatrick et al., 2011), suggesting that the pro-inflammatory response to hypoxia is regulated by NF-κB both by direct binding to promoters of relevant genes and by inducing HIF-1α expression (Fitzpatrick et al., 2011).

Mice lacking IKKβ show impaired accumulation of HIF-1α both in hypoxia and upon bacterial infection (Rius et al., 2008), demonstrating that NF-κB transcriptionally regulates HIF-1α in both hypoxia and normoxia. In addition, a physical interaction between IKKγ/NEMO and HIF-2α, but not HIF-1α, was described to enhance HIF-2α transcriptional activity in reporter assays (Patel and Simon, 2008), but the specific gene targets remain unknown. This link may be bidirectional, as prolonged hypoxia of hMDM increased the mRNA expression and phosphorylation of the several components in the NF-κB pathway (e.g., IKKβ, IKKγ, IkBα, RelA/p65) in a manner that was dependent on both HIF-1α and HIF-2α, as demonstrated by knocking down their expression using siRNA (Fang et al., 2009).

INTERACTIONS WITH OTHER TRANSCRIPTION FACTORS

Hypoxia-induced factor and NF-κB can cooperate not only with each other, but also with additional transcription factors in normoxia and hypoxia, to achieve better target specificity or maximal activity while forming a large protein complex at the promoters of target genes. Activation of many transcription factors is managed by the crosstalk between signaling pathways. For example, ligation of TLRs or pro-inflammatory cytokines can recruit the adapter protein MyD88 that activates both the IKK–NF-κB and the MAPK pathways, leading to activation of NF-κB and AP-1. The TRIF adapter protein may activate IRF3 and initiate delayed expression of IFNβ, which activates Stat1 in an autocrine manner. Cooperation between NF-κB, AP-1, Stat1, HIF-1, and HIF-2 is required for M1 Mϕ activation (Biswas and Lewis, 2010; Lawrence and Fong, 2010). In contrast, anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13) activate Stat3 and Stat6, which are needed to induce anti-inflammatory gene expression (e.g., arginase-1, suppressor of cytokine signaling 3 – SOCS-3), and inhibit Stat1 and NF-κB.

The integration of all the different microenvironmental signals determines the balance between M1 and M2 activation modes, suggesting a complex interplay between the transcription factors. Thus, different combinations of transcription factors that cooperate on specific target promoters may determine the activation phenotype of the Mϕ (Biswas and Lewis, 2010; Lawrence and Fong, 2010). Signal transduction is transient, and different pathways can be activated with different kinetics, and may influence the complexity of the transcriptional network. This aspect warrants further study in differently activated Mϕ. **Table 1** details some of the interactions between HIFs/NF-κB and several of the central transcription factors that are induced in hypoxia.

TRANSCRIPTIONAL INHIBITION

Generally, HIF and NF-κB are considered activators of gene transcription, although the NF-κB p50:p50 homodimers can be negative regulators, as mentioned above. Hypoxia can also selectively inhibit gene transcription by other means. For example, in human Mo hypoxia inhibited the constitutive expression of MCP-1/CCL2 mRNA and its induction by IFNγ and LPS by a dual mechanism that included inhibition of transcription and enhancement of mRNA destabilization (Bosco et al., 2004). As MCP-1/CCL2 is a central chemoattractant for Mo, its inhibition may represent a negative regulatory mechanism that controls recruitment of M1-activated Mo/Mϕ into inflamed tissues. In support of this finding, hypoxia can also inhibit gene expression of CCR5, cathepsin C, 2.5-oligoadenylate synthetase, and the Ras family member Rab7 (Bosco et al., 2006). TIMP-2, the endogenous inhibitor of MMPs, is constitutively expressed in Mo, and hypoxia down-regulates it through the involvement of Sp1 phosphorylation (Lahat et al., 2011).

Surprisingly, in several cases HIF-1 plays a role as a suppressor. In fibroblasts and epithelial cells HIF-1α was implicated in the down-regulation of anti-angiogenic targets such as thrombospondin-1 (Laderoute et al., 2000) and the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) together with histone deacetylase 1 (HDAC1; Lee et al., 2010). In Mϕ, the scavenger receptor MRS1 was down-regulated in hypoxia.

Table 1 | Transcription factors other than HIFs and NF-κB, which are involved in hypoxic regulation of gene expression.

TF	General, function	Induction/activation	Hypoxia stimulates	Cooperation with
AP-1	Heterodimers of the c-Jun, c-Fos, and ATF families	TLR ligation via MAPK activation	Increased Fos-2, Fra-2. Activated by JNK	HIF-1, NF-κB
Egr-1	Zinc-finger factor, regulates Mo differentiation and mitogenic responses	Growth factors	Ligation of RAGE receptor, involving PKCβII and JNK activation	HIF-1 and C/EBPα. Also binds to HIF-1α promoter
Stat1	Regulates immune recognition (e.g., MHC class II, co-stimulatory molecules)	IFNα/β, IFNγ	Both increased and repressed expression were observed	NF-κB and C/EBPβ
Stat3	Regulates typical M2-Mϕ activation genes (e.g., arginase-1 and -2, SOCS-3)	IL-6, IL-10	Increased phosphorylation and activation	NF-κB and HIF-1, but also inhibits IKKβ and opposes NF-κB; key player in M1 to M2-Mϕ shift
Stat6	Regulates typical M2-Mϕ activation genes (e.g., arginase-1, arginase-2, SOCS-3)	IL-4, IL-13	ND	ND
C/EBPβ (NF-IL-6)	Heterodimer with C/EBPα/γ/δ. Regulates genes of acute phase response, Mo differentiation, IL-12, iNOS, and arginase-1	LPS, IL-6, IFNγ. Constitutively expressed in Mϕ	ND	NF-κB for IL-6 production
C/EBPα	Homodimer or heterodimer with C/EBPβ/γ	Growth hormone, IGF-1 via ERK1/2 and GSK3	Suppressed expression by HIF-1α	HIF-1α and Egr-1. Also opposes HIF-1α by competing with HIF-1β for its binding

Studies involving the transcription factors indicated were conducted mostly in tumor cell lines and require confirmation in Mo/Mϕ.
References pertaining to the transcription factors mentioned are: AP-1 (Bandyopadhyay et al., 1995; Alfranca et al., 2002; Laderoute, 2005; Bosco et al., 2006; Mancino and Lawrence, 2010); Egr-1 (Liao et al., 2007; Sperandio et al., 2009; Xu et al., 2010); Stat1 (Lee et al., 2006; Ivanov et al., 2007; Sow et al., 2009); Stat3 (Sica et al., 2008; Hagemann et al., 2009; Noman et al., 2009; Grivennikov and Karin, 2010; Kang et al., 2010); Stat6 (Hagemann et al., 2009); C/EBPβ (Dlaska and Weiss, 1999; Gorgoni et al., 2002; Albina et al., 2005; Elbarghati et al., 2008); C/EBPα (Liao et al., 2007; Janardhan, 2008; Yang et al., 2008).
TF, transcription factor; ND, not determined.

Over-expression of HIF-1α suppressed MSR1 mRNA expression, and or its depletion by siRNA restored MSR1 expression, demonstrating the ability of HIF-1 to convey transcriptional inhibition (Shirato et al., 2009). Furthermore, hypoxia was able to suppress gene expression indirectly through HIF-1α, as it induced the expression of the transcription repressor BACH1, which regulates the expression of heme oxygenase-1 in human primary monocytes (Bosco et al., 2006). However, the mechanisms that allow HIF-1α to play a dual role as both an activator and suppressor are not yet known, and this needs to be further explored.

POST-TRANSCRIPTIONAL MECHANISMS OF HYPOXIC REGULATION

ALTERNATIVE SPLICING AND mRNA STABILITY

Like many other genes, the murine HIF-1α mRNA is alternatively spliced, generating the HIF-1αI.1 and HIF-1αI.2 isoforms, which have alternative first exons and promoters. Stimulation of TG-elicited peritoneal Mϕ and RAW 264.7 cells with TLR4 and $A_{2A}R$ agonists synergistically increased the expression of both isoforms with different kinetics. However, only HIF-1-αI.1 was involved in cytokine production and down-regulated the LPS-induced production of pro-inflammatory cytokines (e.g., TNFα, IL-1β, IL-6, IL-12p40, MIP-1α, MCP-1, and MIP-2), but not IL-1 or VEGF, as was demonstrated in HIF-1αI.$1^{-/-}$ cells. Hypoxia had no effect on the expression of both isoforms (Ramanathan et al., 2009).

The only case that we are aware of hypoxia-induced alternative splicing is of CD80, where hypoxia reduced its surface expression on RAW 264.7 cells while increasing the concentrations of the soluble protein in the supernatants. As amplification of the total mRNA coding for CD80 remained unchanged, but the mRNA coding for the transmembranal region was reduced, it was concluded that hypoxia triggers alternative splicing to generate soluble CD80 (Lahat et al., 2003).

Stability of mRNAs is mediated through the AU-rich elements (AREs) located on the 3′-UTRs of many labile mRNAs and their interaction with different RBP. Presence of AREs is often correlated with rapid mRNA degradation, as occurs with many pro-inflammatory genes such as cytokines (e.g., TNFα, GM-CSF, IL-8), growth factors (e.g., VEGF), transcription factors (e.g., HIF-1α), and other genes (e.g., iNOS, COX-2, uPA, MMPs; Khabar, 2010). Efficient AREs consist of overlapping repetitions of the pentamer AUUUA, giving rise to one or several UUAUUUAUU nonamers, and can assume either a linear or a stem-and-loop conformation. The length of the 3′-UTR and of the ARE itself affects mRNA stability, and longer AREs are associated with shorter half-lives of the mRNAs.

Several RBPs bind to 3′-UTR AREs and target the mRNAs for degradation, including tristetraprolin (TTP) and K-homology splicing-regulatory protein (KSRP), while the HuR promote their stabilization. HuR is mostly a nuclear protein, which upon stimulation translocates to the cytoplasm where it binds to selected mRNAs and stabilizes them. Alternatively, phosphorylation of TTP and KSRP reduce their affinity to AREs and thus stabilize the mRNA. In its unphosphorylated form, TTP recruits factors that mediate decapping, deadenylation, and mRNA destruction. Thus, there is a complex interplay between RBPs that bind to the AREs and determine the mRNA half-life (Khabar, 2010).

The 3′-UTR of the HIF-1α mRNA contains six scattered pentamer AREs and one nonamer ARE, suggesting that TTP could bind to the HIF-1α 3′-UTR and regulate its stability. In several cancer cell lines, hypoxia increased TTP expression, and the protein directly bound to the HIF-1α mRNA AREs. Consistently, overexpression of TTP resulted in decreased HIF-1α mRNA, suggesting that hypoxic cells use TTP to modulate HIF-1α expression (Shin et al., 2010). Although this was not tested in Mo/Mϕ, another study demonstrated that prolonged hypoxia and LPS increased the expression of the dephosphorylated form of TTP in RAW 264.7 cells, and reduced the stability of TNFα, IL-6, MIP-2, and GM-CSF. Knock-down of TTP by siRNA abolished this destabilization (Werno et al., 2010b). On the other hand, the expression of CXCR4 mRNA was stabilized in hypoxia, although the mechanism was not studied (Schioppa et al., 2003).

TRANSLATIONAL REGULATION: RIBOSWITCHES AND microRNAs

Hypoxia leads to immediate shut-down of general protein translation. However, selected proteins, including the HIF proteins and their targets, are nonetheless translated. How these proteins escape global inhibition of translation remains unclear.

Translational regulation is achieved by several mechanisms, and structural elements in the non-coding regions of messenger RNA were shown to modulate gene expression. An example of such an element is the riboswitch, a regulatory element that binds small molecules or ribonucleicprotein (RNP) complexes that induce its conformational change. Riboswitches are usually identified in bacteria, but recently a similar element was identified in the VEGF-A mRNA. This transcript is equally transcribed in IFNγ-induced U937 cells in normoxia and hypoxia, but the protein translation is inhibited after 24 h in normoxia, whereas hypoxia abolishes this effect (Ray et al., 2009). The VEGF-A transcript contains a 126-nt ARE called hypoxia stability region (HSR) that mediates the mRNA stability in hypoxia. It includes two cis-elements, one is a 21-nt CA-rich element (CARE) that binds with high affinity to the heterogeneous nuclear ribonucleoprotein L (hnRNP L), and the other is a 29-nt IFNγ-activated inhibitor of translation (GAIT) element that binds the GAIT complex and silences inflammatory gene expression. The binding to these two elements is mutually exclusive. Thus, in normoxia IFNγ stimulates the binding of GAIT complex to its element, thereby silencing VEGF-A gene expression, whereas in hypoxia the HSR undergoes a conformational change, which favors binding of hnRNP L and overrides the suppressive effects of IFNγ and the GAIT complex (Ray et al., 2009).

Additional translational regulation is exerted by microRNAs (miRNAs), small non-coding RNA molecules that bind with imperfect complementarity to specific mRNA target sequences, usually located in the 3′-UTR of target mRNA, and rapidly and reversibly inhibit their translation. As miRNA are usually independently transcribed, and some contain HRE in their promoter, hypoxic stress can stimulate their transcription, as was shown in different cell types (Pocock, 2011). For example, miR-210 whose promoter contains an HRE is induced in hypoxic tumor and endothelial cells, and can bind to target genes that are crucial in the mitochondria electron transport, thus diverting the cells away from oxidative phosphorylation and toward glycolysis. Alternatively, miR-210 can repress normoxic target genes that are not required for the hypoxic response (Pocock, 2011).

In Mo/Mϕ several miRNAs were identified as regulators of the inflammatory response, in particular miR-155 (O'Connell et al., 2007), miR-146a, and miR-132 (Taganov et al., 2006). LPS markedly induced miR-146a expression in Mo/Mϕ in a prolonged kinetics, and negatively regulated IRAK-1 and TRAF-6 expression leading to down-regulation of the NF-κB pathway, as well as to LPS tolerance and cross tolerance (activation by other TLR ligands) in LPS-primed Mo/Mϕ (Nahid et al., 2009, 2011).

H_2O_2-induced oxidative stress in RAW 264.7 Mϕ activated the NF-κB pathway and down-regulated the expression of a unique set of miRNA*s (miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin; i.e., miR-27a*, miR-27b*, miR-29b*, miR-24-2*, and miR-21*), implicating them, and miR-27b* in particular, in NF-κB regulation (Thulasingam et al., 2011). Regulation by both miRNA and RBPs is primarily executed on the 3′-UTR region in overlapping sequences, indicating possible crosstalk. Indeed, the translation of VEGF-A mRNA is inhibited by the binding of several miRNAs to the CARE, particularly miR-297 and miR-299 in normoxic monocytic cells (even without additional stimulation with IFNγ). However, hypoxia induces translocation of hnRNP L to the cytoplasm, where it binds the VEGF-A mRNA and prevents miRNA silencing (Jafarifar et al., 2011).

Regulation of protein translation by miRNAs is even further complicated by the finding that miRNA can oscillate between translational repression and activation (which is distinct from alleviating translational repression), depending on their binding location and/or on the conditions in the microenvironment. For example, in proliferating cells TNFα translation is inhibited by the binding of miRNAs to its ARE located in the 3′-UTR. However, upon serum-starvation and cell cycle arrest, HEK293 cells up-regulated TNFα-ARE expression in a manner that depended on miR-369-3. This was mediated by the increased expression of miR-369-3, which recruited the fragile X mental retardation-related protein 1 (FXR1), found exclusively in the activating complex, and Ago2, found in both repressing and activating complexes, into a complex that executed translation activation (Vasudevan et al., 2007). However, as TNFα is mainly produced in Mo/Mϕ, and its 3′-UTR ARE is representative of other pro-inflammatory cytokines with short half-life, it is very important that these results be confirmed in Mo/Mϕ cells, and the precise microenvironmental conditions that activate this switch be defined. These results suggest a new multi-input signal integration mechanism, which could be advantageous particularly in Mo/Mϕ that must adapt to the changing microenvironment.

INTRACELLULAR TRAFFICKING AND PROTEIN–PROTEIN INTERACTIONS

The ability of Mo/Mϕ to migrate through the ECM is mediated by the balanced secretion of different MMPs, particularly MMP-9, and their endogenous inhibitors TIMPs. Hypoxia inhibits migration of human monocyte-derived dendritic cells by disrupting this balance, as TIMP-1 was increased whereas MMP-9 and MT1-MMP were decreased (Qu et al., 2005). These inhibitory effects of hypoxia may have been mediated by the adenosine receptor A_{2B} in a cAMP and PKC dependent manner (Zhao et al., 2008). Hypoxia also inhibited the TNFα-induced secretion of MMP-9 from U937 cells and primary Mo, thus markedly inhibiting their migration (Rahat et al., 2006). This was not regulated at the transcriptional level, as MMP-9 mRNA was unchanged, or at the translational levels, as the amounts of the intracellular enzyme were increased in hypoxia. Instead, confocal microscopy revealed that in hypoxia MMP-9 was attenuated in secretory vesicles due to the effects of hypoxia on the cytoskeleton. Indeed, secretion of proMMP-9 was reduced by the addition of cytochalasin B or nocodazole, which inhibits the polymerization of actin and tubulin fibers, or by the addition of the Rho kinase inhibitor Y27632, suggesting the involvement of the cytoskeleton and the Rho GTPases in the process of enzyme secretion (Rahat et al., 2006).

Reduced MMP-9 in hypoxia could explain the immobilization of Mo/Mϕ in hypoxic regions, in addition to other mechanisms that were reviewed elsewhere (Murdoch et al., 2004; Bosco et al., 2008). Another example of intracellular trafficking is mentioned later (see TNFa Trafficking).

The hallmark of M1-activated Mϕ is iNOS expression and high NO production, which is crucial for the killing abilities of the Mϕ, whereas low levels are considered pro-angiogenic (Weigert and Brune, 2008). The main regulatory checkpoint for iNOS is transcriptional, and NF-κB, IRF-1, Stat1, HIF-1α, and C/EBPβ are implicated in its regulation (Pautz et al., 2010). In hypoxia, induction of iNOS transcription and protein expression is demonstrated in differently activated Mϕ (e.g., peritoneal TG-elicited Mϕ, wound Mϕ, spleen Mϕ). However, the enzyme is inactivated and may produce only very low amounts of NO (Melillo et al., 1996; Daniliuc et al., 2003). This inactivation was attributed to the lack of the oxygen substrate during hypoxic stress, however, hypoxic Mϕ lysates failed to produce NO in an a-cellular system in normoxia, where all cofactors and substrates were abundantly present, suggesting a more intricate regulation. Further investigation revealed that in normoxia iNOS is associated with α-actinin-4, a protein responsible for the cross-linking of actin fibers. This interaction recruits iNOS to the sub-membranal area and ensures its activity. However, hypoxia disrupts these interactions, interferes with iNOS localization and causes its inactivation (Daniliuc et al., 2003).

These two examples highlight a special role for the cytoskeleton in regulating enzyme activity and Mϕ phenotype. As cytoskeleton fibers are responsible for the cellular protein trafficking, their disruption may attenuate vesicles within the Mϕ and prevent secretion of their content, or inhibit delivery of proteins to their destination. For example, hypoxia increased tubulin stabilization and changed vesicle trafficking in breast carcinoma cells (Yoon et al., 2005). This is further supported by recent findings linking actin cytoskeleton to HIF-1α stability (Shin et al., 2010).

THE EFFECTS OF PREVIOUS Mϕ ACTIVATION ON THE HYPOXIC STIMULATION – THE TNFα EXAMPLE

TNFα TRAFFICKING

In addition to the MMP-9 example, where hypoxia attenuated secretory vesicles, hypoxia also affects TNFα transport. In TG-elicited peritoneal Mϕ and in RAW 264.7 cells, hypoxia did not change the LPS-induced TNFα mRNA levels relative to normoxia, whereas the membranal and intracellular amounts of the protein were reduced, suggesting degradation of TNFα. The proteososmal inhibitor MG132 did not change the hypoxic down-regulation of TNFα, whereas the lysosome inhibitor bafilomycin A1 dose-dependently inhibited degradation of intracellular TNFα, suggesting that TNFα is degraded at the lysosome. Using several Rab proteins as markers for different vesicles, TNFα trafficking was mapped to the endosomal pathway, and its presence was demonstrated in recycling endosomes, early endosomes, lysosome, and secretory lysosomes. The increased hypoxic co-localization of TNFα and LAMP-1, the lysosome marker, and the ability of bafilomycin A1 to increase intracellular TNFα to normoxic values, suggested that TNFα is directed to the lysosome and that hypoxia enhances its degradation (Lahat et al., 2008).

EFFECTS OF PREVIOUS Mϕ ACTIVATION ON THE HYPOXIC STIMULATION

The overall effects of hypoxia on the expression of TNFα are controversial. It is widely accepted that hypoxia and LPS increase Mϕ TNFα secretion (**Table 2**). However, in some cases hypoxia can inhibit TNFα secretion. In our own hands hypoxia exerted opposing effects on LPS-induced secretion of TNFα, as it enhanced it from human monocytes (Lahat et al., 2003), but down-regulated its secretion from peritoneal TG-elicited Mϕ and RAW 264.7 cells (Lahat et al., 2008). We have demonstrated that hypoxia enhances the lysosomal degradation of TNFα, thus causing its reduction in pro-inflammatory induced RAW 264.7 cells (Lahat et al., 2008). Werno et al. (2010b) have also shown that hypoxia reduced TNFα secretion from pro-inflammatory RAW 264.7 cells, although they suggested a mechanism of reduced mRNA stability, which also affected other pro-inflammatory mediators such as MIP-2 and IL-6, and is not mutually exclusive with lysosomal degradation.

This apparent contradiction between the enhancing and inhibiting effects of hypoxia on TNFα secretion may be resolved by taking into account the priming state of the Mϕ. Priming signals may by themselves be insufficient to stimulate Mϕ, but they sensitize it for further triggering. Traditionally, IFNγ is used to prime Mϕ that consequently encounter TLR ligands (such as LPS) and become fully M1 activated, although the sequence of these events is insignificant (Schroder et al., 2006). We suggest that "naïve" Mϕ that were not previously primed respond to a combination of hypoxia and LPS by elevating TNFα secretion, whereas Mϕ that are already primed or M1-activated react by down-regulating TNFα secretion, relative to activation with LPS alone. In this sense, RAW 264.7 cells behaved like primed cells, and we speculate that as the cell line was obtained from a tumor induced by the Abelson murine leukemia virus, the viral transformation primes the macrophage.

To further make the point, we compared resident peritoneal Mϕ with TG-elicited Mϕ, by exposing them for 48 h to hypoxia,

Table 2 | Controversial effects of hypoxia on TNFα secretion from Mϕ.

Mϕ type	Priming	Hyp. (%)	Time (h)	Stim.	Mechanism	Effect	Reference
NAïVE MO/Mϕ							
Human Mo	None	<0.3	24	LPS	Transcription	Up-regulation	Lahat et al. (2003)
	None	3	16	LPS	Transcription	Up-regulation	Guida and Stewart (1998)
	None	1		None	Transcription	Up-regulation	Demasi et al. (2003)
Resident peritoneal Mϕ	None	<0.3	24	LPS	Transcription	Up-regulation	Lahat et al. (2003)
	None	0	18	LPS	Transcription	Up-regulation	Meng et al. (2001)
	None	0	24	LPS + IFN	Transcription	Up-regulation	Albina et al. (1995)
Alveolar Mϕ	None	2	2	LPS	Transcription	Up-regulation	Leeper-Woodford and Detmer (1999)
Wound Mϕ	None	0	24	LPS + IFN	Transcription	Up-regulation	Albina et al. (1995)
BV-2 microglial cells	None	3	12	None	Transcription	Up-regulation	Li et al. (2009)
THP-1	None	1	18	None	Transcription	Up-regulation	Scannell et al. (1993)
PRIMED MO/Mϕ							
RAW 264.7	(Viral) trans-formation?		16	LPS	mRNA stability	Down-regulation	Werno et al. (2010b)
			24	None	Not specified	Down-regulation	Yun et al. (1997)
		<0.3	24	LPS	Enhanced lysosomal degradation	Down-regulation	Lahat et al. (2008)
TG-elicited peritoneal Mϕ	TG	<0.3	24	LPS	Enhanced lysosomal degradation	Down-regulation	Lahat et al. (2008)

Stim., stimulus; Hyp., hypoxia.

with or without addition of LPS. **Figure 1** shows the opposing effects of hypoxia on these Mϕ, although they originate from the same organ. Given the wide use of TG-elicited Mϕ, it is important to note that these cells are derived from a site of ongoing inflammation, and are therefore at least partially activated and should be considered primed in comparison to the "naïve" resident peritoneal Mϕ (as stated in Takeda et al., 2010).

Likewise, when we primed U937 or THP-1 cells by incubating them or 48 h with LPS (simulating gram negative bacterial activation) or with IFNβ (simulating viral activation), and then exposed them for additional 24 h to LPS (at a higher concentration) and hypoxia, we again observed the inhibitory effects of hypoxia on IFNβ-primed cells, but not on "naïve" cells (**Figure 1**). Note that the lack of response when cells were primed with LPS suggests the involvement of LPS tolerance, which is mediated by the NF-κB p50:p50 homodimers (Frede et al., 2009). It may also imply that IFNβ-priming is mediated by a different mechanism. Thus, the inhibited secretion of TNFα, a typical pro-inflammatory marker, from primed M1-activated Mϕ is inhibited by hypoxia, suggesting that hypoxia may act to restrain the pro-inflammatory Mϕ.

CONCLUDING REMARKS AND PERSPECTIVES

Generally, the hypoxic response depends on cell type, duration of exposure, severity of the hypoxic insult, and as demonstrated for TNFα, previous priming of the Mϕ. Thus, hypoxia can activate Mϕ in a pro-inflammatory way, or skew the pro-inflammatory Mϕ toward a phenotype approximating the M2 or resolution phenotypes. Since hypoxic Mϕ display a mixture of markers of both M1 and M2 activation, classifying them is not straightforward, and they may represent a unique mode of activation which has several common markers with both M1 and M2 activation. In this respect, hypoxia may serve to restrain the pro-inflammatory Mϕ and prevent exacerbation of tissue damage, or alternatively ensure that Mϕ retained in the hypoxic tissue for long periods of time can gradually become M2 activated and engage in processes such as wound healing and/or resolution. In physiological scenarios (e.g., acute inflammation) this has a favorable outcome, whereas in chronic inflammation (e.g., in autoimmune diseases or solid tumors) the inhibitory effects of hypoxia may have devastating results.

Hypoxia profoundly changes Mo/Mϕ activation mode by regulating gene expression. Most of the known regulatory mechanisms are transcriptional, and rely on the cooperation between the master regulator NF-κB and HIFs to mediate this induction of gene expression. However, in some cases HIF can also act as a transcriptional suppressor. Determination of Mϕ activation mode further rely on the cooperation with additional transcriptional factors that are expressed or modified according to the signals received by the microenvironment. The effects of hypoxia on differently activated Mϕ and the molecular mechanisms it exerts (summarized in **Figure 2**) were not investigated enough. For example, it seems reasonable to assume that differently activated Mϕ located in divergent microenvironments would express different HIF isoforms in different proportions and with different kinetics. However, this assumption merits further study.

Additional levels of post-transcriptional and post-translational regulation are gradually revealed. These include regulation of

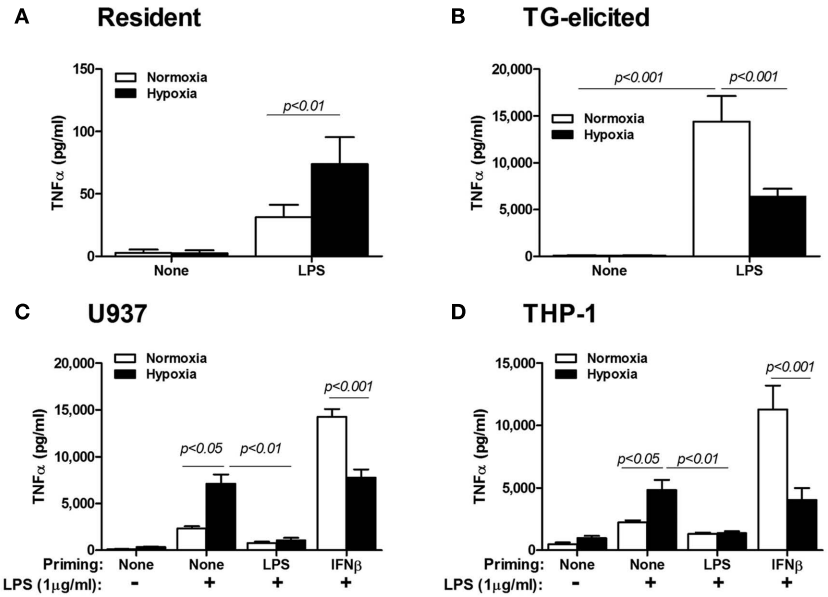

FIGURE 1 | Priming of Mϕ affects the hypoxic response. (A) Resident peritoneal Mϕ ($n = 14$) or **(B)** TG-elicited peritoneal Mϕ ($n = 16$) were subjected to hypoxia (<0.3% O_2) for 24 h, with or without stimulation with LPS (1 μg/ml). Hypoxia inhibited the secretion of high amounts of TNFα that were produced in normoxia in primed TG-elicited Mϕ, while an opposite response to hypoxia was observed for the naïve resident Mϕ. The human monocytic cell lines **(C)** U937 ($n = 12$) and **(D)** THP-1 ($n = 18$) were primed with either LPS (100 ng/ml) or IFNβ (10 U/ml) for 48 h in normoxia, before LPS (1 μg/ml) was added and cells were incubated for additional 24 h in normoxia or hypoxia. Hypoxia increased TNFα secretion in naïve Mo, but inhibited it in IFNβ-primed cells. TNFα was determined in the supernatants using ELISA.

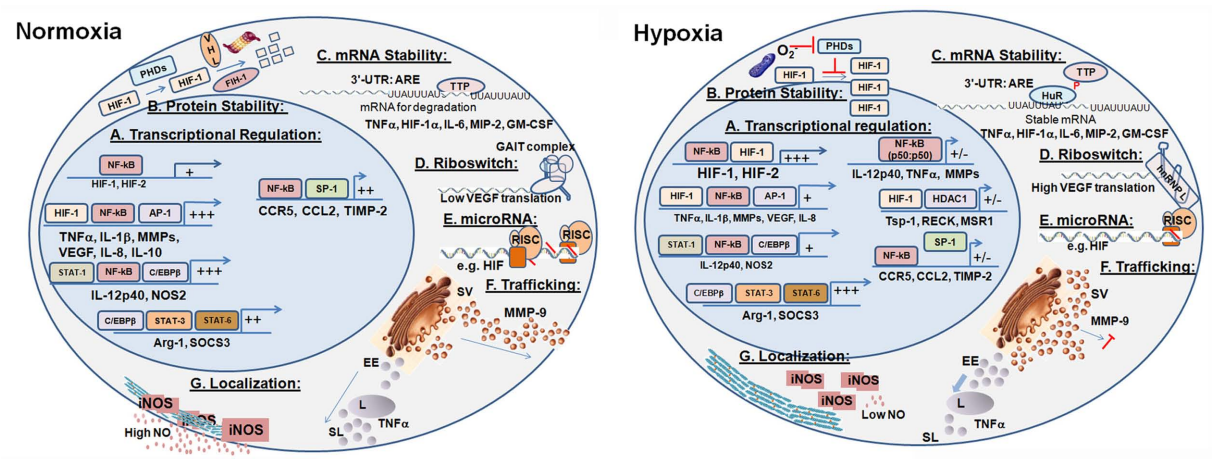

FIGURE 2 | Molecular mechanisms mediating the regulatory effects of hypoxia. Hypoxia affects the production, secretion, and activity of key Mϕ proteins in several regulatory checkpoints, generally keeping the pro-inflammatory Mϕ in check. **(A)** Transcriptional regulation in the M1-activated Mϕ in normoxia is based on the collaboration between NF-kB, low level of HIF-1, and additional transcription factors. In hypoxia, more HIFs are produced, and genes involved in angiogenesis, metabolism, and survival are triggered. **(B)** HIFs protein stability in hypoxia is increased, as the prolyl hydroxylases that target them for proteosomal degradation in normoxia are inhibited. **(C)** Stability of mRNA is regulated by RNA-binding proteins to the AU-rich elements (ARE) located in the 3′-UTR. **(D)** Post-transcriptional regulation – Riboswitch: The VEGF example illustrates how hypoxia changes secondary structures of 3′-UTR elements, and the binding of protein complexes to them, thus increasing their stability and translation. **(E)** Post-transcriptional regulation – microRNAs: Hypoxia modulates the expression of microRNAs that bind selected transcripts, thereby inhibiting or alleviating inhibition of their translation. **(F)** Post-translational regulation – Trafficking: Hypoxia retains secretory vesicles by inhibiting the actin cytoskeleton, thus attenuating the secretion of proteins such as MMP-9. Hypoxia also enhances the lysosomal degradation of other proteins, such as TNFα, which are secreted via the endosomal pathway. **(G)** Post-translational regulation – Localization: Hypoxia disrupts the interactions between iNOS and α-actinin-4, which anchors it to the cortical cytoskeleton, resulting in its inactivation. SV, secretory vesicles; EE, early endosomes; L, lysosome; SL, secretory lysosomes. +/− indicated reduced or inhibited transcription.

mRNA stability, regulation of translation via RBP and microRNA, protein trafficking and protein–protein interactions which may be regulated via reorganization of the cytoskeleton. We now begin to understand how some of these mechanisms allow Mϕ to integrate the different microenvironmental signals and to determine the Mϕ activation mode. Therefore, these mechanisms should be further studied in the context of Mϕ activation, to provide better insights and new approaches to manipulate Mϕ in the hypoxic microenvironment while taking advantage of their selective ability to home to and accumulate in hypoxic regions.

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New insights into the role of macrophages in adipose tissue inflammation and fatty liver disease: modulation by endogenous omega-3 fatty acid-derived lipid mediators

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Obesity is causally linked to a chronic state of "low-grade" inflammation in adipose tissue. Prolonged, unremitting inflammation in this tissue has a direct impact on insulin-sensitive tissues (i.e., liver) and its timely resolution is a critical step toward reducing the prevalence of related co-morbidities such as insulin resistance and non-alcoholic fatty liver disease. This article describes the current state-of-the-art knowledge and novel insights into the role of macrophages in adipose tissue inflammation, with special emphasis on the progressive changes in macrophage polarization observed over the course of obesity. In addition, this article extends the discussion to the contribution of Kupffer cells, the liver resident macrophages, to metabolic liver disease. Special attention is given to the modulation of macrophage responses by omega-3-PUFAs, and more importantly by resolvins, which are potent anti-inflammatory and pro-resolving autacoids generated from docosahexaenoic and eicosapentaenoic acids. In fact, resolvins have been shown to work as endogenous "stop signals" in inflamed adipose tissue and to return this tissue to homeostasis by inducing a phenotypic switch in macrophage polarization toward a pro-resolving phenotype. Collectively, this article offers new views on the role of macrophages in metabolic disease and their modulation by endogenously generated omega-3-PUFA-derived lipid mediators.

Keywords: obesity, adipocytes, M2 macrophages, resolvins, docosahexaenoic acid, Kupffer cells

OBESITY AND ADIPOSE TISSUE INFLAMMATION

White adipose tissue, once considered a mere storage depot of energy in the form of fat, is today recognized as an important endocrine organ. In fact, the adipocyte or fat cell is actively involved in the balance of our body homeostasis by releasing a number of factors, collectively known as adipokines (Ouchi et al., 2011). However, the expansion of adipose tissue mass seen in obesity inadvertently interrupt the interplay among these factors and other intracellular components yielding a chronic "low-grade" inflammatory scenario in this tissue (Ferrante Jr., 2007; Ouchi et al., 2011). This "low-grade" inflammation, also known as "metabolic-triggered inflammation" or "metainflammation," can be described as a long-term inflammatory response triggered by nutrients and metabolic surplus (Hotamisligil, 2006). It involves a similar set of molecules/signaling pathways to those involved in classical inflammation, but in this case these molecules/signaling pathways have a dual role as inflammatory mediators as well as regulators of energy metabolism. In fact, a rise in pro-inflammatory adipokines such as tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-1β, monocyte chemoattractant protein (MCP)-1, leptin, and resistin, accompanied by a reduction in the anti-inflammatory and insulin-sensitizing adipokine, adiponectin has been reported to signal the onset of metabolic dysfunction (Ouchi et al., 2011).

One of the most important sequela of adipose tissue inflammation is insulin resistance (**Figure 1**). In fact, stress sensors activate both the c-jun-N-terminal kinase (JNK) and inhibitor of κ kinase (IKK) pathways through classical receptor-mediated mechanisms (Shoelson et al., 2006). JNK and IKK activation induce insulin resistance by disrupting serine phosphorylation of IRS-1, a protein that connects the insulin receptor to the PI(3)K signaling cascade. In parallel to the activation of these kinases and their downstream signaling cascades, there is an increased production of pro-inflammatory adipokines (i.e., TNFα, IL-6, and MCP-1) in obese subjects, whose levels directly correlate with the degree of insulin resistance (Hotamisligil et al., 1996). Adipose tissue inflammation leading to insulin resistance also has negative consequences on the liver. In fact, adipose tissue and liver have immediate access to a vast network of blood vessels that facilitate a direct connection between these two organs. The exact mechanisms linking adipose tissue dysfunction and insulin resistance with metabolic liver disease are not completely understood, but several processes have been implicated. First, increased lipolysis from visceral fat resulting in increased free fatty acid efflux to the liver (Sanyal, 2005). Second, increased secretion of pro-inflammatory and insulin-resistant adipokines (TNFα and IL-6) by adipose tissue in parallel with a reduced release of adiponectin (Sanyal, 2005; **Figure 1**). Finally, a combined hepatic

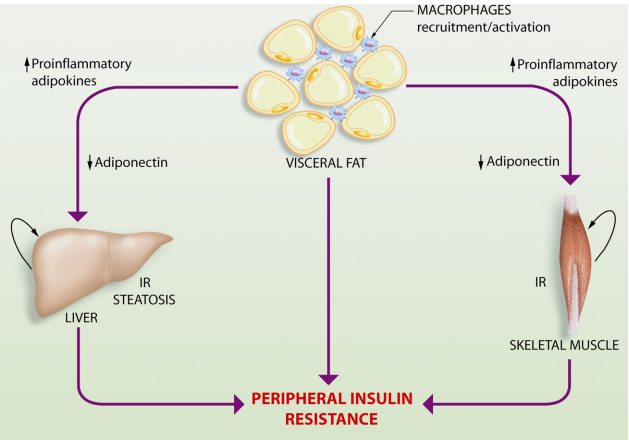

FIGURE 1 | Schematic representation of the interplay between adipose tissue, skeletal muscle, and the liver in the obesity-related perturbation of systemic metabolic control. Obesity results in expansion of adipose tissue mass that eventually leads to a characteristic inflammatory response driven by macrophage infiltration and aberrant production and release of pro-inflammatory adipokines, accompanied by a reduction in the anti-inflammatory and insulin-sensitizing adipokine, adiponectin. This altered profile of adipokine secretion leads to insulin resistance (IR) in the liver and skeletal muscle, which are the major organs contributing to the development of peripheral insulin resistance. Hepatic insulin resistance also triggers the progression of hepatic steatosis or fatty liver.

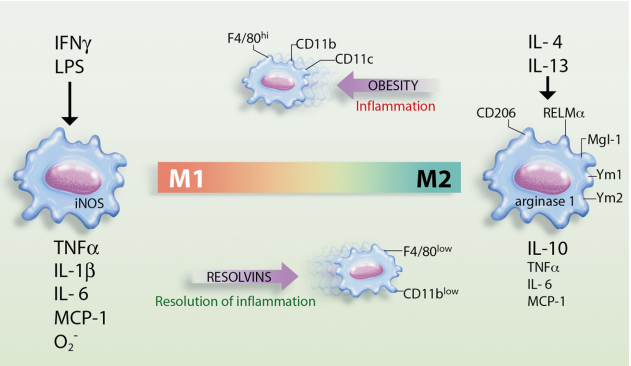

FIGURE 2 | Schematic representation of macrophage polarization in the adipose tissue and the actions of resolvins. Obesity promotes the polarization of macrophages into the M1 phenotype, which are highly inflammatory in nature and release pro-inflammatory cytokines/chemokines [e.g., tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, and monocyte chemotactic peptide (MCP)-1] and superoxide anion (O_2^-). These macrophages express inducible nitric oxide synthase (iNOs) and cell surface markers such as F4/80, CD11b, and CD11c and act as classically activated macrophages expressing interferon (IFN) γ and lipopolysaccharide (LPS)-responsive genes. Conversely, resolvins promote the resolution of inflammation by skewing macrophages toward the M2 phenotype, which release high levels of IL-10 in parallel with reduced levels of TNFα, IL-6, and MCP-1. M2 macrophages are alternatively activated macrophages, originally identified after IL-4 and IL-13 stimulation, that up-regulate scavenger, mannose (CD206), and galactose (Mgl-1) receptors, resistin-like molecule (RELM)-α, and chitinases Ym1 and Ym2 expression and arginase 1 activity.

dysregulation in free fatty acid oxidation and *de novo* lipogenesis secondary to altered hepatic insulin sensitivity (Tilg and Moschen, 2008).

MACROPHAGES AND ADIPOSE TISSUE INFLAMMATION

Obesity-induced adipose tissue inflammation is a unique process characterized by an inflammatory response driven by tissue macrophages (Lumeng and Saltiel, 2011). In fact, a pathological hallmark of obesity is the presence of an increased number of adipose tissue-infiltrating macrophages, which form the characteristic "crown-like structures" that surround necrotic adipocytes and perpetuate a vicious cycle of macrophage recruitment and exacerbated production of pro-inflammatory mediators (Weisberg et al., 2003; Wellen and Hotamisligil, 2003; Cancello et al., 2005; Lesniewski et al., 2007).

Tissue macrophages display an extensive receptor repertoire and a versatile biosynthetic capacity that confer them the plasticity to adapt to different tissue microenvironments (Gordon and Taylor, 2005). Accordingly, tissue macrophages are phenotypically heterogeneous and can exhibit either pro- or anti-inflammatory properties depending on the disease stage and the signals they are exposed. Although the classification based on the Th1/Th2 nomenclature needs to be revised, macrophages are broadly characterized by their activation (polarization) state according to the M1/M2 classification system (Mantovani et al., 2007; Martínez et al., 2009). According to this classification, the M1 designation is reserved for classically activated macrophages following stimulation with interferon (IFN) γ and LPS, whereas the M2 designation is applied to the alternatively activated macrophages after *in vitro* stimulation with IL-4 and IL-13 (**Figure 2**). M1 macrophages display enhanced microbicidal capacity and secrete high levels of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) and increased concentrations of superoxide anion (O_2^-) and oxygen and nitrogen radicals to increase their killing activity (Gordon and Taylor, 2005). Conversely, M2 macrophages dampen pro-inflammatory cytokine levels, secrete components of the extracellular matrix, and may be essential for the immune response to parasites, tissue repair, and resolution of inflammation (Gordon, 2003). In this classification system, M1 and M2 macrophages are merely regarded as two extremes of a continuum of functional stages (Mosser and Edwards, 2008). For instance, M2a designation defines those macrophages stimulated by IL-4/IL-13; M2b refers to macrophages activated by stimuli such as apoptotic cells in concert with LPS; and M2c relates to polarization in response to IL-10, transforming growth factor (TGF)-β, or glucocorticoids (Martínez et al., 2008). In mice, M1/M2 macrophage polarization can be monitored by assessing the expression of selected markers. M1-associated genes include inducible nitric oxide synthase (iNOs), the interferon responsive CXC chemokines, and classical pro-inflammatory mediators such as TNFα, IL-1β, IL-6, and MCP-1 as well as increased production of O_2^- (Gordon, 2003; Martínez et al., 2008; **Figure 2**). M2 macrophages display up-regulation of scavenger, mannose (CD206), and galactose (Mgl-1) receptors, arginase 1, which antagonizes iNOS activity, and IL-10, in parallel with down-regulation of IL-1β and other pro-inflammatory cytokines (Gordon, 2003; Scotton et al., 2005; Martínez et al., 2008). In addition, the panel of M2 markers comprises up-regulation of other genes with unknown function such

as chitinases Ym1 and Ym2, and resistin-like molecule (RELM)-α, also known as FIZZ (**Figure 2**).

In addition to the augmented infiltration of macrophages into the adipose tissue, obesity also induces a phenotypic switch in these cells toward the classically activated M1 phenotype (Olefsky and Glass, 2010). In fact, the majority of macrophages that accumulate in obese adipose tissue are M1-like and selectively express the cell surface markers F4/80, CD11b, and CD11c (Lumeng et al., 2007; Nguyen et al., 2007). In our laboratory, we have recently gathered data indicating the presence of a specific subset of macrophages with high expression of the surface glycoprotein F4/80 (F4/80*hi*) in adipose tissue from obese mice (Titos et al., 2011). This finding is consistent with that reported by Bassaganya-Riera et al. (2009) who identified two functionally distinct subsets of macrophages in adipose tissue based on their surface expression of F4/80 (F4/80*lo* macrophages predominate in adipose tissue of lean mice, obesity causes accumulation of both F4/80*lo* and F4/80*hi*). Importantly, lean adipose tissue macrophages are M2-like, display F4/80 and CD11b but are negative for CD11c and do not exhibit activation of the inflammatory pathways. In a series of elegant studies, Lumeng et al. (2007) and Nguyen et al. (2007) have demonstrated that adipose tissue macrophages undergo a phenotypic switch from the M2 polarization state to a more M1-like, $CD11c^+$ polarization state upon high-fat feeding. Moreover, Patsouris et al. (2008) have reported that selective depletion of $CD11c^+$ macrophages in adipose tissue reverses insulin resistance in high-fat diet-induced obese mice. Recently, Li et al. (2010) have reported that the M1-like, $CD11c^+$ macrophage subset can exhibit phenotypic plasticity between inflammatory and non-inflammatory states, depending on the presence or absence of insulin resistance.

MACROPHAGES AND LIVER DISEASE

Kupffer cells are specialized macrophages located in the liver lining the walls of the sinusoids (Ramadori et al., 2008). Kupffer cells are uniquely positioned within the liver and their location enables intimate contact with circulating blood and the clearance of pathogens and parasites by receptor-mediated phagocytosis or release of TNFα, reactive oxygen species, or proteinases. Kupffer cells are also professional antigen-presenting cells that trigger the adaptive immune system. Therefore, Kupffer cells act as true sentinels of the adaptive and immune system in the liver and protect our body from the extracorporeal environment. In cases of pathogenic infection or tissue damage, Kupffer cells act as the predominant inflammatory effector cell type to initiate the inflammatory cascade leading to liver injury (Ramadori et al., 2008). In fact, activation of Kupffer cells and the subsequent release of cytokines, reactive oxygen species, and inflammatory lipid mediators (i.e., eicosanoids) are considered an early step in the pathogenesis of liver damage and tissue remodeling, as they stimulate inflammatory and fibrogenic events in the liver (Titos et al., 2003, 2005; Ramadori et al., 2008; **Table 1**). Depletion of Kupffer cells by treatment with either gadolinium chloride, liposomal clodronate, or conditional ablation of the diphtheria toxin receptor appears to confer a protective role in the liver by reducing the production of inflammatory mediators and collagen content (Ramadori et al., 2008).

Recent studies have revealed a novel role for Kupffer cells in metabolic liver disease. In fatty livers, similar to that occurring

Table 1 | Kupffer cell-derived mediators and associated liver pathologies.

Mediators	Biological effects	Liver pathology	References
CYTOKINES/CHEMOKINES			
IL-1β, TNFα, IL-6	Hepatotoxicity, endothelial activation, steatogenic, hepatocyte proliferation	Alcoholic liver disease, acute liver injury, NAFLD, NASH, crucial for liver regeneration	Miura et al. (2010), Ramadori and Armbrust (2001), Taub (2004)
TGF-β, PDGF	Myofibroblast transformation and activation	Hepatic fibrosis and cirrhosis	Bataller and Brenner (2005), Pinzani (2002)
MCP-1, IL-8	Neutrophil, monocyte recruitment, angiogenesis, steatogenic	Acute liver injury, alcoholic liver disease, hepatic fibrosis	Devalaraja et al. (1999), Domínguez et al. (2009)
IL-12	Lymphocyte, natural killer activation	Alcoholic liver disease, viral hepatitis	Leifeld et al. (2002)
IL-10, IL-18, IFNα/β	Immunoregulatory, anti-inflammatory, anti-proliferative	Ischemia-reperfusion injury, viral hepatitis	Ellett et al. (2010), Takeuchi et al. (2004), Neuman et al. (2008)
EICOSANOIDS			
PGE_2, PGD_2	Cytoprotection/cytotoxicity	Ischemia-reperfusion injury	Quiroga and Prieto (1993), Planagumà et al. (2005)
LTB_4, cysteinyl-LTs	Vasoactive, hepatic stellate cell activation, chemotactic, steatogenic	Hepatic fibrosis and cirrhosis, NAFLD	Titos et al. (2000), Titos et al. (2003), Horrillo et al. (2010)
REACTIVE OXYGEN SPECIES			
O_2^-, H_2O_2, $ONOO^-$	Hepatotoxicity and necrosis, pro-inflammatory	Alcoholic liver disease, hepatic cirrhosis, ischemia-reperfusion injury, steatohepatitis	Lieber (1997), Muriel (2009)
OTHER			
Gelatinases	Extracellular matrix remodeling, collagen synthesis	Liver fibrosis	Wynn and Barron (2010)
Complement proteins	Pathogen destruction	Chronic liver disease	Bilzer et al. (2006)

in obese adipose tissue, macrophages are in close proximity to fat-laden parenchymal cells (the hepatocytes) and may establish a cross-talk by secreting insulin-resistant cytokines such as TNFα and IL-6, thus regulating hepatic fat and glucose homeostasis and the progression of fatty liver (Baffy, 2009). In fact, excessive exposure of Kupffer cells to fatty acids may induce the activation of these cells via Toll-like receptors thus connecting an important mechanism by which lipids regulate inflammation and immune response in the liver (Kim, 2006). In a mouse model of steatohepatitis, Miura et al. (2010) convincingly showed that TLR9 signaling induces production of IL-1β by Kupffer cells, leading to steatosis, inflammation, and fibrosis. These authors have also shown that JNK activation in Kupffer cells contribute to the development of chronic inflammation and fibrosis in an experimental model of diet-induced steatohepatitis (Kodama et al., 2009). Lanthier et al. (2010) have elegantly demonstrated that early hepatic insulin resistance and steatosis are concurrent with Kupffer cell activation, and that selective Kupffer cell depletion through intravenous clodronate injection is sufficient to improve hepatic insulin signaling. Interestingly, as earlier described for adipose tissue macrophages, alternative M2 activation of Kupffer cells appears to ameliorate insulin resistance and to retard the progression to steatohepatitis in mice (Odegaard et al., 2008).

CLINICAL IMPACT OF OMEGA-3-PUFAs IN DIABETES AND METABOLIC LIVER DISEASE

The first evidences of beneficial actions of omega-3-PUFAs in humans were provided by Endres et al. (1989). Since then, several *in vivo* and *in vitro* studies both in human and rodents have demonstrated the therapeutic potential of omega-3-PUFAs in pathologies with an important inflammatory component (Dinarello, 2010). A number of pre-clinical and clinical studies have demonstrated that regular consumption of modest amounts of omega-3-PUFAs (≤3 g/day) improves serum lipid profiles, exerts cardiovascular protective actions, and may reduce the risk of conversion from impaired glucose tolerance to type-2 diabetes (Nettleton and Katz, 2005). The use of enriched omega-3-PUFA diets in patients with non-alcoholic fatty liver disease could also represent an important nutritional strategy for their clinical management (Shapiro et al., 2011). However, there is a concern that most of studies addressing the effects of omega-3-PUFAs on glucose metabolism and insulin sensitivity did not have a control group and that dosages of fatty acids were sometimes higher than those sufficient to obtain beneficial end-points in these patients (De Caterina et al., 2007). This point out that new, more specific approaches are needed (i.e., compare potency and specificity of resolvins to their substrate precursors, see below).

EFFECTIVE RESOLUTION OF INFLAMMATION: ROLE OF MACROPHAGES

Since prolonged inflammation is detrimental to the host, higher organisms have evolved protective mechanisms to ensure resolution of the inflammatory response in a limited and specific time- and space-manner (Serhan et al., 2007). Once thought as a mere passive process of dilution of inflammation, resolution is today envisioned as a highly orchestrated process coordinated by a complex regulatory network of cells and mediators. Among the molecules that facilitate resolution, lipoxins generated from the omega-6-PUFA arachidonic acid, and resolvins and protectins generated from omega-3-PUFAs, are the lipid mediators that have attracted most attention. These endogenous anti-inflammatory and pro-resolving mediators counteract the effects of pro-inflammatory signaling systems and act as "braking signals" of the persistent vicious cycle leading to unremitting inflammation (Serhan et al., 2008). In fact, the same pro-inflammatory factors that initially trigger the inflammatory response also signal the termination of inflammation by stimulating the biosynthesis of pro-resolving lipid mediators (Serhan et al., 2008). For instance, both PGE_2 and PGD_2 transcriptionally activate the expression of 15-LO in human PMN, switching the mediator profile of these cells from the pro-inflammatory LTB_4 to the anti-inflammatory lipoxin A_4, which was the first identified omega-6-PUFA-derived anti-inflammatory lipid mediator (Serhan et al., 2007, 2008). Another example of this class switch is the displacement of pro-inflammatory lipid mediators derived from omega-6-PUFAs by anti-inflammatory mediators (i.e., resolvins and protectins) derived from omega-3-PUFAs (Serhan, 2011). These anti-inflammatory and pro-resolving mediators exert a strict control of the resolution process and pave the way for monocyte migration and their differentiation to phagocytosing macrophages, which remove dead cells and then terminate the inflammatory response (Tabas, 2010; Serhan, 2011).

RESOLVINS

Resolvins are a novel family of anti-inflammatory and pro-resolving mediators generated from the omega-3-PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). By using a lipidomics-based approach that combines liquid chromatography and tandem-mass spectrometry, Serhan et al. (2000, 2002) identified a library of omega-3-PUFA-derived lipid mediators present within exudates obtained from mice dorsal skin pouches during the "spontaneous resolution" phase of acute inflammation. These novel bioactive lipid autacoids were termed resolvins and were classified as either resolvin E-series, if the biosynthesis is initiated from EPA, or resolvin D-series, if they are generated from DHA. Schematically, the biosynthesis of resolvin E1 is initiated when EPA is converted to 18R-hydroperoxy-EPE by endothelial cells expressing COX-2 treated with aspirin (Serhan et al., 2000). Alternatively, 18R-hydroperoxy-EPE can be produced through cytochrome P450 activity (Haas-Stapleton et al., 2007). By transcellular biosynthesis, 18R-hydroperoxy-EPE generated by endothelial cells is transformed by 5-LO of neighboring leukocytes into resolvin E1 (5S,12R,18R-trihydroxy-EPA) via a 5(6)epoxide intermediate (Serhan et al., 2000, 2002). Resolvin D1 biosynthesis is also initiated in endothelial cells expressing COX-2 treated with aspirin, which transform DHA into 17R-hydroxy-DHA which is further transformed by leukocyte 5-LO into resolvin D1 (Serhan et al., 2000, 2002). More importantly from a physiological point of view, resolvin D1 can also be formed from endogenous sources of DHA without the requirement of aspirin. In this case, endogenous DHA is converted via 15-LO/5-LO interactions that give rise to a 17S alcohol-containing series of resolvins, including resolvin D1 and resolvin D2 (Hong et al., 2003). Finally, DHA is also transformed into a dihydroxy-containing DHA derivative,

17S-hydroxy-DHA via an intermediate epoxide that opens via hydrolysis and subsequent rearrangements to form protectin D1 (10R,17S-dihydroxy-docosa-DHA) (Serhan et al., 2000, 2002; Hong et al., 2003).

Unlike their precursors DHA and EPA, resolvins exert biological actions at the nanomolar range. Resolvin E1, decreases PMN infiltration and T cell migration, reduces TNFα and IFNγ secretion, inhibits chemokine formation and blocks IL-1-induced NF-κB activation (Gronert et al., 2005; Schwab et al., 2007; Bannenberg and Serhan, 2010). Resolvin E1 also stimulates macrophage phagocytosis of apoptotic PMN and is a potent modulator of pro-inflammatory leukocyte expression adhesion molecules (i.e., L-selectin) (Schwab et al., 2007; Dona et al., 2008). *In vivo* resolvin E1 exerts potent anti-inflammatory actions in experimental models of periodontitis, colitis, and peritonitis and protects mice against brain ischemia-reperfusion (Arita et al., 2005; Bannenberg and Serhan, 2010). Furthermore, Haworth et al. (2008) have identified a resolvin E1-initiated resolution program for allergic airway responses. Finally, a recent study has identified resolvin D2 as a potent endogenous regulator of excessive inflammatory responses in mice with microbial sepsis (Spite et al., 2009).

Our laboratory has recently provided evidence that adipose tissue expresses all the enzymes necessary for the formation of bioactive lipid mediators derived from both omega-6 and omega-3-PUFAs (i.e., 12/15-LO, 5-LO, FLAP, LTA4 hydrolase, and LTC4 synthase; Horrillo et al., 2010). Importantly, by means of liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis we have detected the presence of the omega-6 products PGE_2, $PGF_{2\alpha}$, TXB_2, 5-HETE, 12-HETE, and 15-HETE as well as the formation of the omega-3-derived mediators resolvin D1, protectin D1, and 17-hydroxy-DHA (González-Périz et al., 2009). Interestingly, the administration of a DHA-enriched diet to *ob/ob* mice, an experimental model of obesity-induced insulin resistance and fatty liver disease, resulted in the amplification of the formation of resolvin D1, protectin D1, and 17-hydroxy-DHA, accompanied by an inhibition of the formation of omega-6-derived inflammatory mediators (González-Périz et al., 2009). In these animals, DHA significantly increased adipose tissue levels of adiponectin which alleviated hepatic steatosis and insulin resistance (González-Périz et al., 2009). Of interest, intraperitoneal injection of resolvin E1 at the nanomolar levels elicited significant insulin-sensitizing effects by inducing adiponectin, GLUT-4, and IRS-1 expression in adipose tissue and conferred significant protection against hepatic steatosis (González-Périz et al., 2009). There is also evidence that omega-3-PUFAs may also signal independently of the 12/15-LO pathway by exerting potent anti-inflammatory and insulin-sensitizing actions through a G-protein-coupled 120 receptor (GPR120) (Oh et al., 2010).

Recent findings from our laboratory also indicate that DHA (at micromolar concentrations) and resolvin D1 (at nanomolar concentrations) consistently induce hallmarks of alternative macrophage activation in adipose tissue including stimulation of arginase 1 expression and non-phlogystic macrophage phagocytosis and attenuation of IFNγ/LPS-induced Th1 cytokine secretion (Titos et al., 2011). These results are in agreement with those reported by Schif-Zuck et al. (2011) who recently identified a novel phenotype of macrophages with pro-resolving properties emerging during the resolution of murine peritonitis. These macrophages had a low marker expression of CD11b ($CD11b^{low}$), engulfed significantly higher numbers of apoptotic PMN than $CD11b^{high}$ macrophages, responded poorly to activation by different TLR ligands in terms of cytokine and chemokine secretion, lost their phagocytic potential and were prone to migrate to lymphoid organs (Schif-Zuck et al., 2011). In addition, these $CD11b^{low}$ macrophages expressed low or moderate levels of COX-2, metalloproteinase-9, and 12/15-LO, but not detectable levels of iNOS and arginase 1. Importantly, *in vivo* administration of resolvin E1, resolvin D1, and glucocorticoids to peritonitis-affected mice clearly enhanced the appearance of $CD11b^{low}$ macrophages by reducing the number of engulfment-related events required for macrophage deactivation and by reducing the ability of peritoneal macrophages to produce pro-inflammatory cytokines upon LPS stimulation (Schif-Zuck et al., 2011). The ability of resolvins to modify tissue macrophage plasticity has also been demonstrated by Hellmann et al. (2011). These authors were able to improve insulin resistance by administering resolvin D1 to obese-diabetic mice, which reduced macrophage $F4/80^{+}CD11c^{+}$ cell accumulation and increased the percentage of positive F4/80 cells expressing Mgl-1, a marker of alternatively activated macrophages, in adipose tissue (Hellmann et al., 2011).

Studies on experimental models of liver injury have elucidated a protective role of DHA and DHA-derived lipid mediators against hepatic inflammation. In fact, feeding of a DHA-enriched diet ameliorated hepatotoxic-induced necroinflammatory liver injury in mice (González-Périz et al., 2006). The hepatoprotective actions of DHA were associated with a decrease in the hepatic formation of PGE_2 and a concomitant increase in the generation of protective DHA-derived lipid mediators (i.e., PD1 and 17*S*-HDHA). The beneficial role of these DHA-derived lipid signals was further supported by experiments *in vitro* demonstrating attenuated DNA damage and oxidative stress in hepatocytes. More important, DHA and DHA-derived autacoids reduced TNFα release in macrophages, recognized as the predominant effector cells involved in the inflammatory cascade leading to hepatocyte damage (Decker, 1990). A significant down-regulation of 5-LO protein expression was also noticed in macrophages treated with 17*S*-HDHA and in liver tissue from mice receiving DHA in the diet (González-Périz et al., 2006). This is relevant because the presence of an active 5-LO pathway in the liver is restricted to Kupffer cells and inhibition of the 5-LO pathway in these resident macrophages has been shown to attenuate necroinflammatory liver injury and fibrosis (Titos et al., 2000, 2003, 2005).

CONCLUSION

Obesity is not only a matter of appearance and beauty, but a serious health issue because the global obesity epidemic will result in increased incidence and risk of cardiovascular disease, type-2 diabetes, dyslipidemia, and fatty liver disease. The prevalence of obesity-related metabolic disorders is tightly associated with the appearance of a chronic "low-grade" inflammatory state in the adipose tissue, which severely disrupts the endocrine function of this organ. Indeed, a number of studies have appreciated

that expansion of adipose tissue during weight gain is associated with an inflammatory phenotype characterized by the recruitment of inflammatory cells, mainly macrophages, in this tissue. A very provocative strategy to manipulate this exacerbated inflammatory response is to replace the use of drugs that inhibit the formation of pro-inflammatory mediators by the use of endogenous-generated autacoids that boost the resolution of inflammation. Therefore, adipose tissue inflammation in obesity appears to be the perfect scenario for testing the novel anti-inflammatory and pro-resolving lipid mediators, designated resolvins. Notably, these inflammation-resolving factors can induce a proper skew of macrophages toward a unique pro-resolving phenotype, thus ameliorating the incidence of obesity-related metabolic disorders.

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Macrophages in synovial inflammation

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Synovial macrophages are one of the resident cell types in synovial tissue and while they remain relatively quiescent in the healthy joint, they become activated in the inflamed joint and, along with infiltrating monocytes/macrophages, regulate secretion of pro-inflammatory cytokines and enzymes involved in driving the inflammatory response and joint destruction. Synovial macrophages are positioned throughout the sub-lining layer and lining layer at the cartilage–pannus junction and mediate articular destruction. Sub-lining macrophages are now also considered as the most reliable biomarker for disease severity and response to therapy in rheumatoid arthritis (RA). There is a growing understanding of the molecular drivers of inflammation and an appreciation that the resolution of inflammation is an active process rather than a passive return to homeostasis, and this has implications for our understanding of the role of macrophages in inflammation. Macrophage phenotype determines the cytokine secretion profile and tissue destruction capabilities of these cells. Whereas inflammatory synovial macrophages have not yet been classified into one phenotype or another it is widely known that TNFα and IL-l, characteristically released by M1 macrophages, are abundant in RA while IL-10 activity, characteristic of M2 macrophages, is somewhat diminished. Here we will briefly review our current understanding of macrophages and macrophage polarization in RA as well as the elements implicated in controlling polarization, such as cytokines and transcription factors like NFκB, IRFs and NR4A, and pro-resolving factors, such as LXA4 and other lipid mediators which may promote a non-inflammatory, pro-resolving phenotype, and may represent a novel therapeutic paradigm.

Keywords: macrophage, arthritis, inflammation

INTRODUCTION

Macrophages (Mϕ) are one of the resident cell types in synovial tissue, along with fibroblasts. While quiescent in health, Mϕ become activated in the inflamed joint, where they make up around 30–40% of the cellular content, and regulate secretion of pro-inflammatory cytokines and enzymes involved in driving the inflammatory response and joint destruction (Firestein and Zvaifler, 1990). Their position throughout the sub-lining layer and lining layer at the cartilage–pannus junction facilitates their role mediating articular destruction. It is estimated that rheumatoid arthritis (RA) and psoriatic arthritis (PsA) each affects approximately 1% of the population (Firestein, 2003; Gladman, 2009), leading to patient pain and disability as well as contributing to a great economic burden in terms of lost working days and patient health services (Cooper, 2000) and therefore is an area of intense investigation.

As our understanding of inflammation progresses, including the recent concept that resolution of inflammation is an active process rather than a passive return to homeostasis, the role of Mϕ is increasingly appreciated. The inability to resolve acute inflammation may lead to a chronic inflammatory state. Depending on their phenotype, Mϕ can secrete either pro- or anti-inflammatory cytokines and mediate matrix destruction or deposition. Synovial Mϕ participate in many of the events driving inflammation including the stimulation of angiogenesis, leukocyte and lymphocyte recruitment, fibroblast proliferation, and protease secretion leading to eventual joint destruction (Burmester et al., 1997; Vallejo et al., 2003; Abeles and Pillinger, 2006). While RA and PsA are considered more inflammatory than osteoarthritis (OA), it can still contain an inflammatory component, of which Mϕ play a large part. In all of these conditions Mϕ derived mediators can drive inflammation and cartilage destruction. Depletion of Mϕ from OA synovial cell cultures significantly reduced TNFα and IL-1β levels. Depletion of Mϕ from both RA and OA synovial cell cultures leads to reduced synovial fibroblast responses such as cytokine and MMP production (Janusz and Hare, 1993; Bondeson et al., 2010). Both macrophages and fibroblasts display an activated cell phenotype with increased cell surface expression of HLA-DR and leukocyte adhesion molecules (Athanasou et al., 1988; Alvaro-Gracia et al., 1990) participating in T-cell activation. Interaction of Mϕ with T-cells potentiates the expression of several pro-inflammatory mediators such as IL-1α and β and MMPs (McInnes et al., 2000).

Important pro-inflammatory cytokines like TNFα and IL-l are abundant in the inflamed synovium and are characteristically released by classically activated (M1) Mϕ. These cytokines are

central to joint destruction. The importance of Mϕ in driving the inflammatory response has been highlighted by several quantitative microscopic studies, where they have shown that Mϕ number; correlates with disease activity (Tak et al., 1997), has potential use as a biomarker for disease (Kruithof et al., 2006; Bresnihan et al., 2009) and declines in response to therapy (Goedkoop et al., 2004; Canete et al., 2010). Mϕ can induce angiogenesis (Leibovich et al., 1987), and hypoxia, a prominent feature of the inflamed joint, promotes the survival of monocytes/macrophages and induces their anaerobic adaptations including glycolysis (Roiniotis et al., 2009).

It is long appreciated that Mϕ play an important role in the pathogenesis of arthritis and this observation was supported by studies showing that the number of Mϕ was increased in clinically affected joints compared to non-affected joints (Kraan et al., 1998). Several studies also linked the number of synovial Mϕ to inflammatory cytokine production joint destruction (Mulherin et al., 1996). As the search for a reliable biomarker in RA continued, the role of Mϕ was again highlighted. The culmination of this work has led to sub-lining CD68 positive synovial Mϕ currently being the only validated biomarker for disease severity (Tak et al., 1997) and response to therapy in arthritis (Haringman et al., 2005), further confirming their importance in the pathogenesis of this disease, a finding which is independent of treatment type (Haringman et al., 2005; Thurlings et al., 2008). Considering the similarities between synovial inflammation in RA and PsA, Mϕ have also been proposed as a biomarker for response to therapy in PsA. Several studies have concluded that Mϕ number is decreased in PsA synovial tissue following therapy (Goedkoop et al., 2004; Kruithof et al., 2006; Canete et al., 2010).

ACTIVATION OF SYNOVIAL MACROPHAGES

Besides the abundant pro-inflammatory cytokines and chemokines present in inflamed synovial tissue, activation, and survival of Mϕ can be achieved through acetylation or de-acetylation of histones. Downstream effects of TNFα and other molecules results in the induction of histone acetyltransferase (HAT) activity in Mϕ which causes acetylation of histones and subsequent modulation of transcriptional activity. HAT activity is counteracted by histone deacetylases (HDAC). Two recent studies have found evidence of depressed HDAC activity in RA, particularly in synovial macrophages and fibroblasts. The ratio of HDAC:HAT activity was significantly lower in RA synovial tissue compare to healthy controls. In combination with this, HDAC inhibition decreases IL-10 production from whole tissue synovial explants cultures, indicating a negative effect on anti-inflammatory pathways, which would lead us to believe that a lack of HDAC may contribute to perpetuation of inflammation (Huber et al., 2007; Grabiec et al., 2008, 2010). Despite this, HDAC inhibition is showing promise for inflammatory diseases. HDAC inhibitors reduced IL-6 production from TNFα stimulated Mϕ and induced apoptosis of RA synovial fluid (SF) Mϕ, even in the presence of a pro-inflammatory stimulus (Grabiec et al., 2010). This is of interest considering the ability of synovial cells and infiltrating cells to evade apoptosis during joint inflammation contributing to synovial hypercellularity (Salmon et al., 1997; Perlman et al., 2001). The potential use of HDAC inhibitors has been further promoted by their success in suppressing synovial inflammation and cartilage destruction in a CIA mouse model (Nasu et al., 2008).

Toll like receptors (TLR) are pattern recognition receptors that mediate response to infection. However, it is becoming apparent that some of these receptors may become activated by non-infectious agents from within the body and may therefore play a role in autoimmune conditions such as RA. Engagement of TLRs induces signaling through a well defined pathway involving MyD88 that leads to transcriptional activation (Joosten et al., 2003). TLR2 and TLR4 appear to be particularly associated with RA. TLR knockout and arthritis mouse models, or a combination of both, have highlighted the position of TLRs in the pathogenesis of arthritis. In a model of spontaneous arthritis due to IL-1 receptor antagonist knockout, simultaneous knockout of TLR4 attenuated inflammation while TLR2 knockout produced a more severe arthritis. Knockout of TLR9 had no effect (Abdollahi-Roodsaz et al., 2008). This clearly indicates a potential benefit for TLR4 antagonism in RA. However the role of TLR2 seems less defined as other studies have shown that knockdown of TLR2 produces beneficial effects in arthritis (Joosten et al., 2003). Further to this, many TLR ligands have been identified in synovial inflammation (Okamura et al., 2001; Park et al., 2004). Acute serum amyloid A (SAA), which is significantly upregulated in arthritis and propagates pro-inflammatory effects similar to TNFα (O'Hara et al., 2000; Mullan et al., 2006; Connolly et al., 2011), is a functional ligand for TLR2 and may contribute to the deleterious effects of SAA in arthritis (Cheng et al., 2008). RA Mϕ are more responsive to stimulation than Mϕ from other forms of inflammatory arthritis, despite no difference in Mϕ number (Huang et al., 2007). Therefore, engagement of TLR2 and 4 may contribute to Mϕ activation and a sustained Mϕ response in RA.

Rheumatoid factor (RF) is one of the diagnostic criteria for RA and can help to distinguish RA from similar arthropathies like PsA. Classification of RA as an autoimmune disease came initially from the discovery of IgG auto-antibodies in the blood of patients (Waaler, 1940; Franklin et al., 1957). RF is mostly IgM-RF, but IgG-RF and IgA-RF can also be detected in some patients. The cellular receptors for IgG are the Fcγ receptors, FCγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). All three receptors are expressed on synovial Mϕ (Laurent et al., 2011) as well as lymphocytes. FcγRIII has been demonstrated to play a role in the development of arthritis through animal models. Mice deficient in FcγRIII are protected from the development of collagen induced arthritis without alteration of their humoral response, and therefore the protection is not due to alterations in T-cell responses (Ståhl et al., 2002; Andrén et al., 2006). Polymorphisms in Fcγ receptors are associated with incidence of RA as well as response to therapy (Morgan et al., 2006; Canete et al., 2009; Thabet et al., 2009; Morales-Lara et al., 2010).

ARE SYNOVIAL MACROPHAGES POLARIZED?

In the immune system Mϕ are effective antigen presenting cells with phagocytic activity which respond to lymphocyte derived cytokines. However, the responses elicited by Mϕ are variable and depend entirely on the tissue environment. We now know these responses can be either pro- or anti-inflammatory. Dedicated reviews on this topic discuss in more detail the cytokines and chemokines involved in promoting one phenotype over another

(Mantovani et al., 2004; Murray and Wynn, 2011) but an overview of the main components are outlined in **Figure 1**. Classically activated M1 Mϕ have a pro-inflammatory phenotype, producing high levels of TNFα, IL-1, IL-6, IL-12, IL-23, reactive oxygen species, and low levels of IL-10. Alternatively activated Mϕ, of which there are three subsets (Mantovani et al., 2004; Martinez et al., 2008), display and anti-inflammatory phenotype, producing high levels of IL-10, IL-1 receptor antagonist, decoy IL-1RII, TGFβ, and low levels of IL-12. Both types are necessary for correct resolution of inflammation. An interesting, and potentially useful, property of these Mϕ is that they remain plastic and polarization into one phenotype does preclude re-polarization (Stout et al., 2005). Therefore, if we could elucidate the exact pathways and transcription factors involved in promoting one phenotype over the other *in vivo*, this system could be exploited for therapeutic gain.

There appears to be a lack of evidence for Mϕ polarization in either direction in the inflamed joint. It has been suggested that spondyloarthropathies such as PsA display a more M2 profile compared to RA patients and that M1 mediators correlate with joint inflammation in RA (Vandooren et al., 2009). However, in general, most studies of Mϕ in arthritis focus on important Mϕ functions and not polarization. The mediators that can control Mϕ polarization are indeed present in the synovium and some show potential as therapeutic targets. Synovial lining layer thickness is greater in RA, compared to PsA or healthy control subjects, which is associated with an increase in synovial Mϕ and fibroblasts. PsA patients tend to have less lining layer Mϕ than RA patients. This has been observed in many comparative studies. Danning et al. (2000) also found similar levels of IL-10 in RA and PsA synovium, despite the difference in synovial lining layer Mϕ numbers, however levels were described as being quite low. It is difficult to determine if this lack of IL-10 is a contributor to or consequence of the overwhelming inflammation in the joint. A study by Mottonen et al. (1998) found that 68% of Mϕ isolated from RA SF were CD86 positive and that SF Mϕ can take on a dendritic cell phenotype when exposed to a combination of IL-4 and GM-CSF and that these cells were more effective at activating T-cells than control or TNFα stimulated Mϕ. The effects of IL-4 + GM-CSF were mediated through CD86, a marker of classically activated Mϕ. IL-10 was able to inhibit the observed effects with IL-4 + GM-CSF as it downregulated the expression of CD86, as well as CD-40 and HLA-DR which also participate in Mϕ mediated T-cell activation. This is consistent with the classification that M2c Mϕ, which are driven by IL-10 are involved in suppression of the immune response (Mantovani et al., 2004). These results may appear confusing as IL-4 along with IL-13 drive the M2a or alternative Mϕ phenotype which should be involved in anti-inflammatory responses. However GM-CSF drives the M1 phenotype in monocyte derived macrophages so this may be the driving force for inflammatory responses in these experiments.

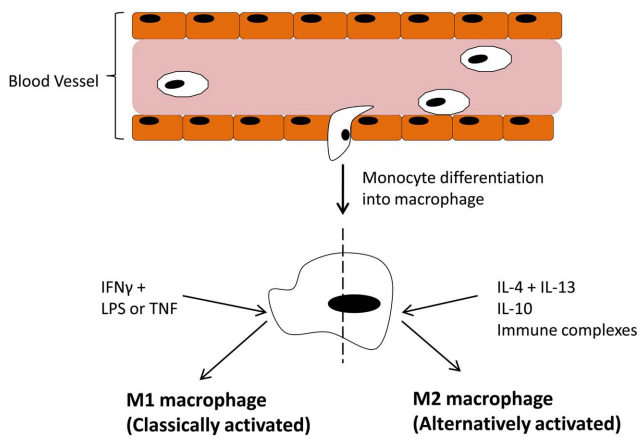

FIGURE 1 | Blood-borne monocytes exit the blood stream and differentiate into macrophages. The macrophage response depends on the stimulus provided by the microenvironment. IFNγ along with LPS or TNFα drive polarization of M1 (classically activated) macrophages which participate in pro-inflammatory activities. On the other hand, IL-4 + IL-13, IL-10, or immune complexes drive M2 (alternatively activated) macrophages, which participate in anti-inflammatory responses.

WHAT REGULATORS IN SYNOVIAL INFLAMMATION COULD POTENTIALLY INFLUENCE MACROPHAGE POLARIZATION?

Figure 1 has outlined the cytokines and regulators that promote M1 or M2 polarization. The extent of expression of these factors in the joint varies. The M1 Mϕ phenotype is induced by interferon-γ (IFNγ) in combination with either lipopolysaccharide (LPS) or TNFα. IFNγ is highly expressed in RA synovial tissue and its levels significantly correlate with disease severity (Milman et al., 2010). Exposure to INFγ increases the response of Mϕ exposed to other stimuli by either upregulating pro-inflammatory cytokines, like TNFα, or downregulating anti-inflammatory cytokines, like IL-10 (Erwig et al., 1998; Wallet et al., 2010). TNFα is a master cytokine in inflammation and as such is a potent inducer of other pro-inflammatory cytokines (Nawroth et al., 1986; Butler et al., 1995), is chemotactic for leukocytes, is a potent inducer of angiogenesis (Leibovich et al., 1987), stimulates adhesion molecule expression in SFC *in vitro* (Marlor et al., 1992), and lymphoid migration into inflamed synovial tissue *in vivo* (Wahid et al., 2000). Within the inflamed joint macrophages, fibroblasts, lymphocytes, and endothelial cells produce TNFα. An important role for TNFα in arthritis was confirmed by studies which showed its potential to degrade both cartilage (Dayer et al., 1985) and bone (Bertolini et al., 1986). Further rationale for the involvement of TNFα in the progression of inflammatory arthritis was provided when transgenic mice expressing a modified human TNFα gene spontaneously developed arthritis which exhibited increased human TNFα protein, joint inflammation, bone erosion, and cartilage destruction. In this study, antibodies specific for human, but not mouse TNFα reduced disease severity (Keffer et al., 1991). In subsequent studies administration of a monoclonal antibody to TNFα ameliorated inflammation and joint damage after disease onset in a CIA model of arthritis (Williams et al., 1992). TNFα cytokine targeted therapies have now been developed for inflammatory arthritis. The first clinical trial was undertaken in the UK in 1992 and demonstrated that targeted biologic therapy decreased serum IL-6 levels, swollen joint numbers and levels of the acute phase proteins CRP and A-SAA which are markers of inflammation (Elliott

et al., 1993). Alternatively, anti-inflammatory and M2 polarizing cytokines like IL-10 are lowly expressed in arthritis as its signaling is blocked during FCγ receptor ligation (Ji et al., 2003), and treatment with the pro-resolving mediator annexin A1 stimulates release of IL-10 (Ferlazzo et al., 2003). Treatment of PBMC with IL-10 caused a change in the ratio of T_h17:Treg cells in favor of Treg cells and decreased production of the pro-inflammatory cytokine IL-17 (Heo et al., 2010). Animal models of arthritis have also demonstrated how treatment with IL-10 can suppress the development and progression of joint inflammation, even in established disease (Walmsley et al., 1996; Whalen et al., 1999; Mauri et al., 2003).

The cytokines involved in promoting polarization are well defined, however less is known about which transcription factors are utilized to induce polarization. IRF5 (interferon regulatory factor 5) has been implicated in driving the M1 phenotype as well as actively suppressing M2 polarization and driving T_h1 and T_h17 responses (Krausgruber et al., 2011). While the study by Krausgruber et al. (2011) was not performed in synovial Mϕ, animal studies suggest that inflammation in RA is driven by T_h1 cytokines such as IFNγ, which is upregulated early in the disease process (Miltenburg et al., 1992; Schulze-Koops and Kalden, 2001) and a rapid growth in interest in the T_h17 pathway and indeed IL-17 itself in the last few years would suggest that this would warrant investigation in the inflamed joint. Recent reports confirm that alterations in the IRF5 gene confers susceptibility to RA (Dieguez-Gonzalez et al., 2008; Han et al., 2009; Dawidowicz et al., 2011) as well as many related illnesses such as inflammatory bowel disease, Sjogrens syndrome, and systemic lupus erythematosus (Dideberg et al., 2007; Graham et al., 2007; Miceli-Richard et al., 2007). Other transcription factors in the IRF family, like IRF3 (Biswas et al., 2006) and IRF4 (Satoh et al., 2010) have been implicated in promoting Mϕ polarization in other disease settings, and IRF family members contribute to determination of dendritic cell fate (Tamura et al., 2005). These findings make the IRF family attractive candidates to study in the context of Mϕ's in arthritis.

NR4A is part of the orphan nuclear receptor superfamily which have roles in lipid metabolism and inflammation (Desreumaux et al., 2001; Oosterveer et al., 2010; Hong et al., 2011). Receptors in the same superfamily as NR4A are downregulated in arthritic tissue and their activation appears to play a role in inhibiting disease progression (Bonnelye et al., 2008; Park et al., 2010). However members of the NR4A subfamily appear to have less clearly defined effects to the anti-inflammatory family members liver X receptor and peroxisome-proliferator-activator receptor and drive inflammation in human synovial tissue (Murphy et al., 2001). The role of NR4A receptors specifically in Mϕ polarization has not yet been elucidated, however, any role for NR4A in Mϕ polarization would be an interesting finding due to the modulation of NR4A by both dexamethasone and methotrexate, which are effective treatments for joint inflammation in some patients. NR4A receptors can also activate NFκB in murine Mϕ (Pei et al., 2006) where it promotes transcription of pro-inflammatory genes. In arthritis, and inflammation in general, NFκB can be considered a master transcription factor as it is utilized by many ligand–receptor complexes to modulate gene transcription. TNFα and IL-1β which are abundant in the inflamed joint employ this transcription factor and in turn can be regulated by it. NFκB activation has been detected prior to the clinical onset of arthritis in animal models (Tsao et al., 1997; Han et al., 1998) and the NFκB pathway has been directly targeted as a treatment method for RA confirming its essential role in the pathogenesis of this disease (Wakamatsu et al., 2005). Immunohistochemical staining has confirmed nuclear expression of NFκB subunits in synovial Mϕ (Handel et al., 1995). The NFκB family consists of five proteins; p50, p52, RelA (p65), RelB, and c-Rel. These proteins form homo or heterodimers to determine gene transcription. RelA, RelB, and c-Rel contain a transcriptional activation domain and therefore upregulate gene expression, however p50 and p52 do not contain the transcriptional activation domain and homodimers of these proteins can sometimes have a repressing function (Bohuslav et al., 1998). NFκB p50 activation has been linked to promoting M2 polarizing genes in Mϕ (Porta et al., 2009). This study found that knockout of the NFκB p50 subunit prevented the development of tolerance in LPS challenged Mϕ by restoring M1 mediators and inhibiting M2 cytokines. Similarly, Saccani et al. (2006) found that accumulation of the p50 homodimer occurred in the nuclei of tumor associated Mϕ and that these Mϕ expressed an M2 phenotype. Therefore due to the prominence of the NFκB pathway in RA it remains an interesting candidate for influencing Mϕ polarization. A summary of all factors discussed here are outlined in **Table 1**.

MACROPHAGES AND RESOLUTION OF INFLAMMATION

There is a growing understanding of the molecular drivers of inflammation and an appreciation that the resolution of inflammation is an active process rather than a passive return to homeostasis. Endogenously produced mediators that actively promote the resolution of inflammation are now under investigation

Table 1 | Description of the regulators in synovial inflammation that could potentially influence macrophage polarization.

Polarizing factors	Mϕ subset promotion	Reference
CYTOKINES		
IFNγ + (LPS/TNFα)	M1	Erwig et al. (1998), Wallet et al. (2010)
IL-4 + IL-13	M2	See review Mantovani et al. (2004)
IL-10	M2	See review Mantovani et al. (2004)
Immune complexes	M2	See review Mantovani et al. (2004)
TRANSCRIPTION FACTORS		
IRF3	M2	Biswas et al. (2006)
IRF4	M2	Satoh et al. (2010)
IRF5	Promotes M1, actively inhibits M2	Krausgruber et al. (2011)
NFκB p50	M2	Porta et al. (2009), Biswas et al. (2006)
NR4A	Not yet investigated in Mϕ polarization	

for their therapeutic use. These are molecules such as lipoxins, resolvins, protectins, and annexins. Lipoxin A4 (LXA4) is an eicosanoid produced by the transcellular metabolism of arachidonic acid by 15/5- or 5/12-lipoxinagese (Serhan et al., 1984). Its biosynthesis is co-incident with the resolution phase of inflammation and many of its bioactions are mediated through ligation of its receptor, ALX/FPR2 (Fiore et al., 1994). LXA4 is produced in inflamed synovial tissue (Thomas et al., 1995) where it can downregulate pro-inflammatory activities of activated fibroblasts and upregulate anti-inflammatory activities, even in the presence of a pro-inflammatory stimulus which acts through the same receptor (Sodin-Semrl et al., 2004; Kronke et al., 2009; Chan and Moore, 2010). In other disease models, LXA4 has been shown to induce anti-inflammatory/pro-resolving actions such as inhibition of neutrophil recruitment and activation (Filep et al., 1999), regulation of NFκB activation (Decker et al., 2009), and the clearance of apoptotic cells by Mϕ (Godson et al., 2000). Neutrophils are the first effector cells at the site of inflammation. Once these cells have carried out their functions in regard to host defense they are programmed to die by apoptosis. Resolution of inflammation and return to homeostasis involves phagocytosis of apoptotic neutrophils to prevent the persistence to necrosis and leakage of cellular contents, which may itself begin an inflammatory reaction. Despite the lack of apoptosis occurring in all cell types in the inflamed synovium, resident synovial Mϕ retain the capacity to phagocytose apoptotic cells, even at an early timepoint after arthritis induction (van Lent et al., 2001). If normal apoptosis and phagocytosis could be induced in the inflamed synovium, possibly by native LXA4 or its stable analogs, this process may trigger a normal resolution of inflammation.

In order to encourage phagocytosis, apoptotic cells release mediators that attract phagocytes toward them, essentially flagging themselves for engulfment. One such mediator is the anti-inflammatory compound annexin A1 (Arur et al., 2003; Scannell et al., 2007). Annexin A1 is a 37-kDa protein of the annexin superfamily where all family members contain a similar core region and a distinct N-terminal region which confers specificity of function. Generally annexin A1 is localized to the cytoplasm where, upon stimulation, it is mobilized to the cell membrane and secreted (for an extensive review on annexin A1 see Perretti and Dalli, 2009). Interestingly annexin A1 also signals through ALX/FPR2, the same receptor utilized by LXA4 and SAA. Annexin A1 is widely expressed in many cell types including Mϕ. Immunohistochemical analysis has demonstrated an increased expression of annexin A1 in the RA synovial lining layer macrophages and fibroblasts compared to OA and normal joints. This may, however, be due to the increased lining layer thickness in this condition (Goulding et al., 1995) as other studies have shown decreased binding of annexin A1 to several cell types in RA (Goulding et al., 1992; Sampey et al., 2000). Glucocorticoid stimulation causes annexin A1 mobilization to the cell surface and secretion where it mediates glucocorticoid induced anti-inflammatory effects. This is of particular interest in arthritis as glucocorticoid therapy is one of the current treatments for this condition (Flower, 1988; Podgorski et al., 1992; Yang et al., 1998, 1999; Maderna et al., 2005). However, as is increasingly the case for many mediators, the role of annexin A1 may not be as unambiguous as initially described and it may also potentiate pro-inflammatory actions in arthritis. An investigation by Tagoe et al. (2008) has revealed synergistic actions with TNFα and annexin A1 in terms of MMP production from synovial fibroblast cells. They saw firstly that TNFα can induce expression of endogenous annexin A1 and secondly that TNFα along with the annexin A1 mimetic peptide Ac2-26 enhanced secretion of MMP-1 which was dependent on FPR2/ALX, Erk, Jnk, and NFκB (Tagoe et al., 2008). As mentioned, this study was not performed in synovial Mϕ, but as they have similar actions to synovial fibroblasts, the same results may be produced by these cells once investigated. Further to this, it has also been shown that administration of human recombinant annexin A1 during the immunization phase of the collagen induced arthritis model perpetuated the development of the signs and symptoms of arthritis. This may have been due to the increased T-cell activation and skewing toward a T_h1 phenotype by annexin A1 acting through FPR2/ALX (D'Acquisto et al., 2007). T-cells from RA patients 48 h post steroid therapy demonstrated depressed expression of annexin A1 (D'Acquisto et al., 2008) further lending support to the possibility that annexin A1 may also mediate pro-inflammatory actions. However we must be careful to acknowledge the actions of specific cleavage products from full length annexin. One such cleavage product has been identified as causing neutrophil extravasation, an important event in inflammation, where other truncated forms of annexin cannot (Williams et al., 2010).

CONCLUSION

In the study of inflammation and our efforts to promote its normal resolution, Mϕ remain to the fore of our interest. In the inflamed joint, Mϕ will continue to be a focal point for therapeutic intervention which, currently, centers around cytokine blockade but now has the possibility of extending into Mϕ re-programming. This remains an interesting and a yet to be fully explored option in terms of treatment for synovial inflammation.

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Frustrated phagocytosis on micro-patterned immune complexes to characterize lysosome movements in live macrophages

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Lysosome mobilization is a key cellular process in phagocytes for bactericidal activities and trans-matrix migration. The molecular mechanisms that regulate lysosome mobilization are still poorly known. Lysosomes are hard to track as they move toward phagosomes throughout the cell volume. In order to anticipate cell regions where lysosomes are recruited to, human and RAW264.7 macrophages were seeded on surfaces that were micro-patterned with immune complexes (ICs) as 4 μm-side squares. Distances between IC patterns were adapted to optimize cell spreading in order to constrain lysosome movements mostly in two dimensions. Fcγ receptors triggered local frustrated phagocytosis, frustrated phagosomes appeared as rings of F-actin dots around the IC patterns as early as 5 min after cells made contact with the substratum. Frustrated phagosomes recruited actin-associated proteins (vinculin, paxillin, and gelsolin). The fusion of lysosomes with frustrated phagosomes was shown by the release of beta-hexosaminidase and the recruitment of Lamp1 to frustrated phagosomes. Lysosomes of RAW264.7 macrophages were labeled with cathepsin-D-mCherry to visualize their movements toward frustrated phagosomes. Lysosomes saltatory movements were markedly slowed down compared to cells layered on non-opsonized patterns. In addition, the linearity of the trajectories and the frequency and duration of contacts of lysosomes with frustrated phagosomes were measured. Our experimental set-up is the first step toward deciphering molecular mechanisms which are involved in lysosome movements in the cytoplasm (speed, directionality, and interaction with phagosomes), and opens the door to approaches such as RNA interference, pharmacological inhibition, or mutant expression.

Keywords: lysosome, frustrated phagocytosis, macrophages, micro-patterned immune complexes

INTRODUCTION

Secretion of lysosomes and related granules is a cellular process that is essential for the bactericidal functions of specialized immune cells such as phagocytes (see Luzio et al., 2007 for review). In macrophages, these secretory lysosomes constitute a population of vesicles which is different from the classical ubiquitous lysosomes dedicated to the digestion of nutriments (Rabinowitz et al., 1992; Claus et al., 1998; Astarie-Dequeker et al., 2002). Lysosomes are also at least in part associated with the biogenesis of podosomes that are F-actin rich structures responsible for adhering to and degrading the extracellular environment of phagocytes. Lysosome mobilization is therefore a crucial process for phagocyte migration and bactericidal function, but the molecular mechanisms that control this process remain unclear. It was shown that lysosomes and lysosome-related organelles travel over long distances along microtubules within the cell cytoplasm. This movement mainly entails kinesin motors, with a switch to actin rails when lysosomes reach the cell periphery (Barral and Seabra, 2004). However, detailed characterization of lysosome movements during phagocytosis has not yet been carried out, probably because of the technical challenge of tracking vesicles in three dimensions within the cytoplasm, where phagosomes themselves move around. Here, in order to (1) anticipate where lysosomes are recruited to, and (2) spread cells out as much as possible to neglect the third dimension during vesicle tracking, we set-up frustrated phagocytosis on micro-patterned immune complex surfaces. Frustrated phagocytosis has already been used in the past in immobilized phagocytosing macrophages (Wright and Silverstein, 1984; Takemura et al., 1986; Bainton et al., 1989). More recently, Eng et al. (2007) were able to demonstrate and measure the reorientation of the Golgi apparatus during frustrated phagocytosis in live RAW264.7 cells. In a more sophisticated experimental set-up, patterned antigen arrays were used to study exocytosis in mast cells in response to local activation of the IgE receptor (Wu et al., 2007). In that study, the authors used total internal reflection fluorescence microscopy (TIRF) to differentiate

the location of exocytosis of early endosomes and lysosomes in response to local activation of IgE receptors. TIRF and frustrated phagocytosis have been combined (Touret et al., 2005) to visualize whether the endoplasmic reticulum fuses with the plasma membrane in response to the activation of phagocytic receptors. None of the above studies, however, explored the regulation of the mobilization of lysosomes during their upstream movement through the cell cytoplasm, toward their fusion with the target membrane. Here, for the first time, we have been able to follow and characterize lysosome movements during phagocytosis in live macrophages.

MATERIALS AND METHODS

PRODUCTS AND ANTIBODIES

Ovalbumin, Nocodazole, Cytochalasin-D1, and Latrunculin-A, antibodies against gelsolin (1/100), paxillin (1/100) and vinculin (1/100) were purchased from Sigma-Aldrich (France). Lysotracker Red, phalloidin-Alexa488 and -Alexa350 were from Molecular Probes (Fisher Scientific, France) and used at 50 nM and a 1/500 dilution, respectively. Anti-CD16/CD32 monoclonal antibodies were from Immunostep (clone 2.4G2, Caltag medsystems, UK). Rabbit anti-ovalbumin serum was home-made by classical intradermal immunization and used at a 1/10 dilution to generate immune complexes (ICs). Bovine serum albumin (BSA, fraction V) was from Euromedex (France).

CELL CULTURE

RAW264.7 stably expressing actin-GFP (RAW–GFP) were kindly provided by S. Grinstein (Canada). They were cultured as described (Verollet et al., 2010). RAW–GFP cells were transfected with 2 μg of the cathepsin-D-mCherry construct kindly provided by F. Darchen (Paris) and with the Amaxa electroporator apparatus according to the manufacturer's indications. Human monocyte-derived macrophages (hMDM) were prepared from healthy donors and cultured as described (Van Goethem et al., 2010). Bone-marrow derived macrophages (BMDM) were prepared from wild-type C57/bl6 mice and cultured as described (Cougoule et al., 2005).

MICRO-CONTACT PRINTING AND OPSONIZATION OF THE SUBSTRATA

PDMS stamps patterned with squares of 4 μm edges spaced by 5 μm were prepared as described (Labernadie et al., 2010). Stamps were incubated for 20 min at room temperature (RT) in a phosphate-buffer saline (PBS) solution containing 50 μg/mL of either unlabeled ovalbumin or a 8:2 mixture of unlabeled ovalbumin and ovalbumin-TRITC (OVA-TRITC), or human IgG, as indicated in the text. Stamps were then dried under a nitrogen flow and brought into contact with glass cover slips for 1 min. These cover slips had been pre-cleaned in HCl/methanol solution 1/2 vol/vol for 2 h, then rinsed for 1 h in distilled water, for 30 min in ethanol (75% vol), and dried under airflow and sterilized. For studies of fixed samples, patterned cover slips of 12-mm diameter were placed at the bottom of 24-wells plates (VWR, France) and incubated with cell culture medium for 30 min at 37°C and 5% CO_2 before adding the cells. For live cell imaging, we patterned 24-mm cover slips that had been glued to the bottom of pre-drilled plastic Petri dishes (3-cm diameter). Alternatively, two-chamber Labtek-II (Nunc, Fisher Scientific, France) were micro-patterned. Ovalbumin-patterned cover slips were then opsonized with home-made anti-ovalbumin sera from rabbit at a 1/10 dilution for 30 min at RT, leading to patterned ICs. IgG-patterned cover slips were used directly. Cells were trypsinized at 37°C, washed in PBS and directly added to the prepared cover slips, either for later fixation and immunofluorescence studies or for live imaging.

FRUSTRATED PHAGOCYTOSIS

RAW cells or hMDM were trypsinized, washed in PBS and layered on micro-patterned cover slips at a density of 5×10^4 cells/mL or 10^4 cells/mL, respectively. For blocking experiments, RAW cells were treated with the anti-CD16/CD32 antibodies at a 1/50 dilution for 1 h at RT, before being washed in PBS and layered on the micro-patterned surfaces.

IMMUNOFLUORESCENCE

Cells were fixed for 45 min at RT with 3.7% paraformaldehyde in PBS containing 15 mM sucrose. Unreacted aldehyde functions were quenched with 50 mM NH_4Cl in PBS for 2 min at RT. Cells were then permeabilized with PBS containing 0.3% TX100 for 10 min at RT before being saturated with PBS containing 5% BSA for 1 h at RT. Cells were then stained with primary antibodies at indicated dilutions (see above) in the presence of 5% BSA (PBS/BSA) for 1 h at RT, washed three times in PBS/BSA and incubated with a mixture of the corresponding secondary antibodies and fluorescent phalloidin for 30 min at RT in the dark. Samples were then mounted on slides with the fluorescent mounting medium from DAKO (France). Immunofluorescent samples were observed with a DM-RB up-right microscope (Leica Microsystems, Paris, France) equipped with a CoolSnap HQ camera (Roper Scientific, France) and the appropriate FITC or TRITC filter cubes.

ATOMIC FORCE MICROSCOPY

Height measurements and profiles of micro-patterned ovalbumin alone or ICs were obtained by atomic force microscopy (AFM) in contact mode in liquid at RT. See (Labernadie et al., 2010) for technical details.

LIVE CELL IMAGING AND IMAGE PROCESSING

Cells performing frustrated phagocytosis on micro-patterned surfaces were imaged with a DM-IRB inverted microscope equipped with a 63× objective (n.a. = 1.3), a heated stage and a chamber to maintain temperature and CO_2 at constant values. Images of CathD-mCherry- or LysoTracker Red-positive lysosomes were acquired every 250 ms and images of actin-GFP were acquired every 10 s. Actin images (green) were used to delineate frustrated phagosome areas on the lysosomes images (red). Distances were calibrated using a graduated slide (1 pixel corresponds to 0.102 μm) and lysosome movements were tracked with the manual tracking plug in imbedded in the Image J software version 1.44F. Instant velocities were calculated between two consecutive frames. Linearity of trajectories was measured as the correlation coefficient (r^2) of the linear regression of sets of three consecutive positions, sliding along individual tracks.

STATISTICS

A t test or one-way ANOVA analysis with Tukey's multiple comparisons posttest was performed using GraphPad Prism. Statistical significance is indicated as follows: $^{***}P < 0.001$;$^{**}P < 0.01$; $^{*}P < 0.05$.

RESULTS

PREPARATION OF THE SUBSTRATA

Glass cover slips were micro-texturized with ovalbumin (OVA) or OVA-TRITC by micro-contact printing as described recently, and outlined briefly in the Section "Materials and Methods" (Labernadie et al., 2010). Patterns were then opsonized with anti-ovalbumin rabbit serum. We used AFM to characterize the OVA-TRITC patterns, and found out that they have an average height of 7 nm, whereas height was about 14 nm when anti-OVA IgGs were added (**Figures 1A,C**). Their shape was very regular with very sharp and vertical edges (**Figure 1B**). As anticipated from the AFM results, fluorescence microscopy confirmed that anti-ovalbumin IgGs did not bind outside the OVA patterns (**Figure 1D**).

FRUSTRATED PHAGOCYTOSIS ON OPSONIZED OVALBUMIN PATTERNS

When RAW264.7 cells were layered on IgG-ovalbumin patterns, actin rearranged rapidly as dots mainly present around the

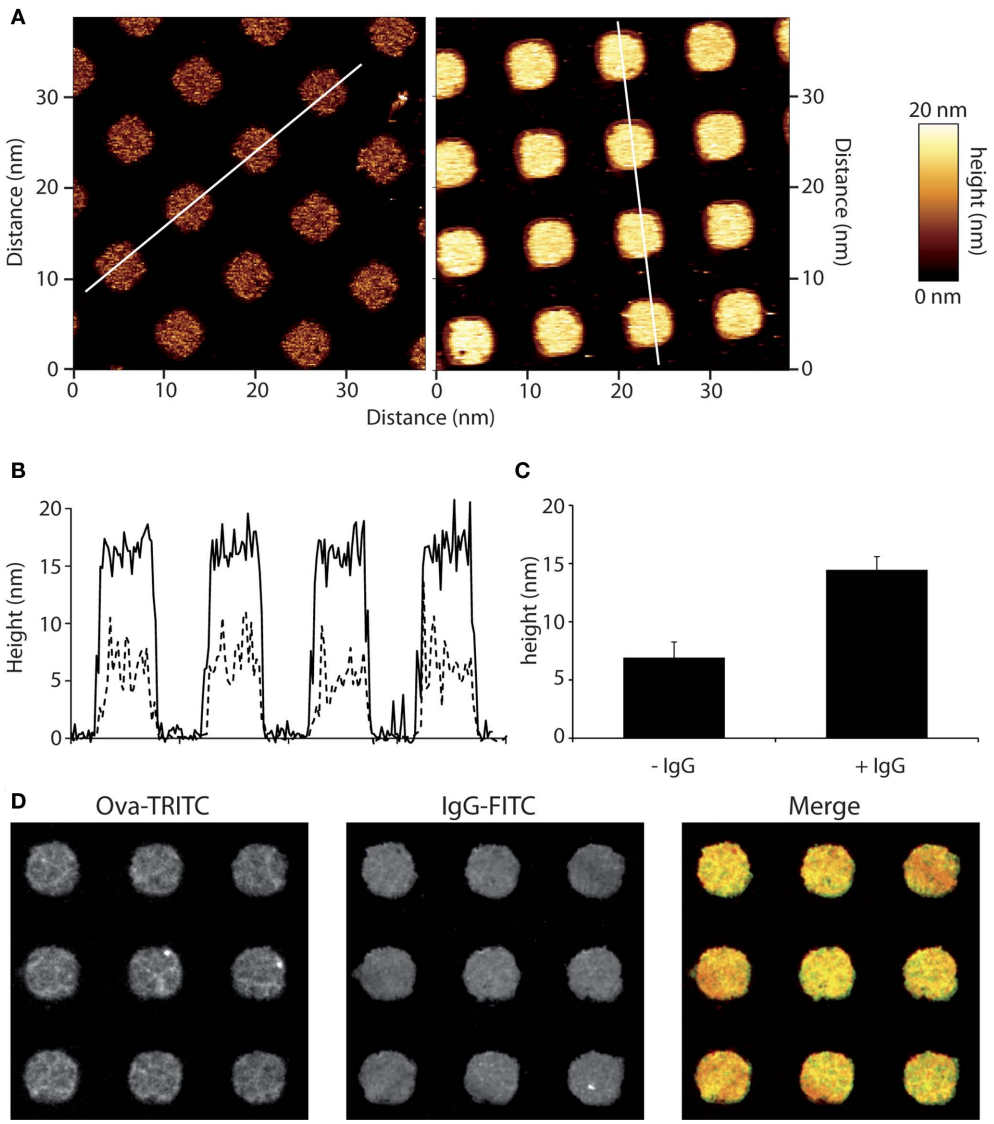

FIGURE 1 | Micro-contact printing of immune complexes. Micro-patterns of ovalbumin (OVA) were applied to glass cover slips and those were then incubated with anti-OVA antibodies. **(A)** Micro-patterned surfaces were scanned by AFM. Squares of 4 μm-sides are separated by 5 μm. Heights were measured as detailed in the Section "Materials and Methods" for OVA alone (left) and OVA-IgG (right) patterns." **(B)** Profiles of OVA patterns (dotted line) are compared to that of immune complexes patterns (solid line). **(C)** Heights are measured for more than 50 patterns and mean ± SD are plotted. **(D)** Immune-detection of micro-patterned ovalbumin-TRITC using an anti-OVA primary serum and Alexa488-coupled secondary antibodies revealed a homogenous repartition of immune complexes on micro-patterned surfaces.

patterns (**Figure 2A**). As early as 5 min after cell suspension was added to the micro-texturized cover slips, 50% of cells presented such actin rearrangements, and this number rose up to 83% after 30 min of adhesion. Formation of frustrated phagosomes was dependent on the Fcγ receptors since it was not observed in the absence of opsonization (**Figure 2B**) or when macrophages were pre-incubated withanti-CD16/CD32 blocking antibodies (**Figure A1** in Appendix). In our experimental conditions, about 85% of the patterns that were covered by a cell triggered a frustrated phagosome (not shown). Frustrated phagocytosis was also obtained with human macrophages derived from monocytes (**Figure 2C**) and mouse BMDM (see **Figure 5**). These larger cells could form up to 30 frustrated phagosomes with the actin rings sometimes surrounding several patterns (**Figure 2C**). As expected, biogenesis of frustrated phagosomes was dependent on actin dynamics since it was completely abolished when cells where pre-treated for 15 min with 0.5 μM Latrunculin A or 10 μM Cytochalasin-D1 (data not shown). Their stability was also dependent on actin polymerization since frustrated phagosomes disassembled after a 10-min treatment with either drug (not shown).

In order to observe frustrated phagocytosis in live cells, we used RAW264.7 cells that constitutively express actin-GFP, hereafter designated as RAW–GFP. The process could be divided into three phases. First, actin concentrated as dots at the point of contact of the cells with the substratum; second, actin dots moved rapidly to the cells' periphery like a growing belt as the cells spread on the cover-slip, and third, dots rearranged into rings around patterns when the cells were completely spread out (see Movie S1 in Supplementary Material). These actin rings could last as long as 10 min, but could also disassemble as cells moved to another position. Sometimes, one could observe a dynamic exchange of actin between two rings (see Movie S2 in Supplementary Material). Whatever the behavior of the ring was, each actin dot proved to be very dynamic by itself, with cycles of appearance/disappearance of about 2 min 40 s (not shown).

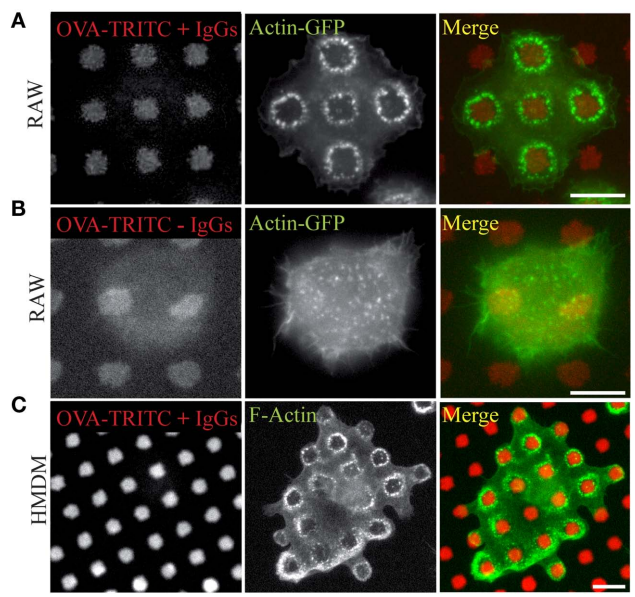

FIGURE 2 | Frustrated phagocytosis of macrophages on micro-texturized surfaces. Micro-patterns of OVA-TRITC were laid on glass cover slips as in **Figure 1**, and then incubated **(A,C)** or not **(B)** with anti-OVA antibodies. RAW–GFP **(A,B)** or hMDMs **(C)** were layered on the substrata for 15 min, then fixed and stained **(C)** with phalloidin-FITC and imaged. OVA-TRITC and actin-GFP **(A,B)**. Bars = 5 μm.

CHARACTERIZATION OF THE FRUSTRATED PHAGOSOMES

We further went on to characterize the rings of actin dots by staining cells undergoing frustrated phagocytosis for several actin-linked proteins that are classically found at phagosomes. **Figure 3** shows that vinculin, paxillin, and gelsolin were present at the actin rings. Paxillin described a clear outline of the rings, whereas vinculin was found around each dot of actin and gelsolin colocalized with actin.

Since we wanted to use this model to measure lysosome mobilization in live cells, we first looked for evidence that lysosomes fused with the frustrated phagosomes. As a first indirect approach, we measured the release of the lysosomal enzyme beta-hexosaminidase in the extracellular environment. We observed that RAW264.7 macrophages increased their secretion activity by 25.8 ± 4.4% (mean ± SD) when they were layered on IgG-opsonized patterns for 1 h, as compared to non-opsonized patterns (not shown, two independent experiments with measurements in triplicates). However, this protocol did not tell us whether secretion was occurring at the frustrated phagosomes or not. If lysosomes fuse with the frustrated phagosome, then we should detect the accumulation of lamp1 at the level of the patterns. **Figure 4** shows that Lamp1 was indeed recruited to the membrane of the frustrated phagosome as early as 10 min after cells spread on the micro-patterned ICs.

It has been described that the integrity of the microtubule network was dispensable for the biogenesis of FcγR-triggered phagosomes (Newman et al., 1991) and we wanted to check whether this holds true for frustrated phagosomes. Mouse bone-marrow derived macrophages were treated with nocodazole either pre- or post-adhesion on patterned ICs, and we observed that neither of these treatments had an effect on the formation or the stability of frustrated phagosomes (**Figure 5**).

TRACKING LYSOSOMES IN LIVE MACROPHAGES

The advantages of this experimental set-up are (1) to have all the phagocytosis-related signaling pathways initiated at the ventral side of the cell; (2) to anticipate where lysosomes get recruited to fuse with the phagosomal membrane; (3) to have the cells as spread out as possible in order to minimize the third dimension during characterization of the vesicle movements. RAW cells spread reasonably well on 5 μm-spaced patterns. However, they would form frustrated phagosomes that were too close to each other to unambiguously evaluate the direction of moving lysosomes. Thus, we looked for the appropriate distance between patterns to obtain cells that would be sufficiently spread out to form frustrated phagosomes which are sufficiently distant from each other. We compared our initial 5 μm-spaced patterns to patterns with spaces of 7, 9, 15, or 20 μm (not shown). We found out that a distance

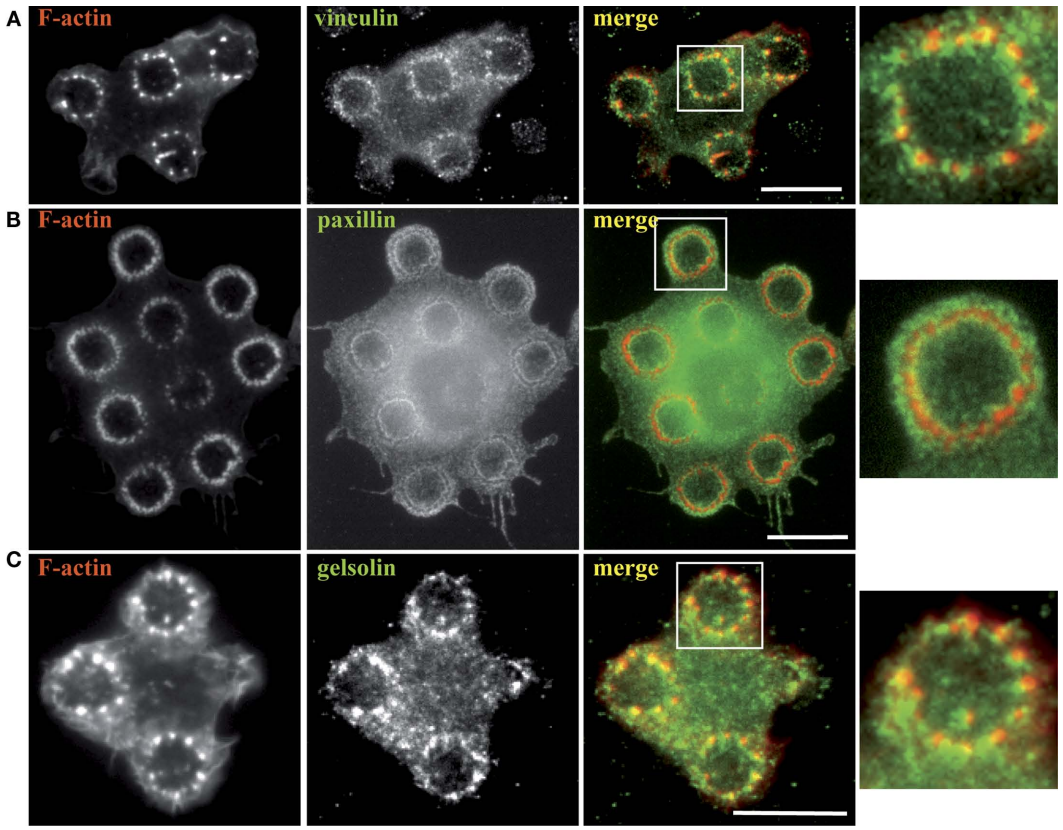

FIGURE 3 | Actin-associated proteins are recruited to the frustrated phagosomes. RAW–GFP **(A,C)** or hMDM **(B)** were plated on micro-patterned immune complexes for 15 min, fixed and stained for F-actin **(B)**, vinculin **(A)**, paxillin **(B)**, and gelsolin **(C)**. Actin in RAW cells was visualized thanks to GFP fluorescence **(A,C)**. Zoomed images correspond to the areas indicated by white boxes in the corresponding merged images. Bars = 5 μm.

of 7 μm between two frustrated phagosomes was not enough to track lysosome efficiently, and RAW cells layered on 15 μm-spaced patterns did not form more than one frustrated phagosome (not shown). When the distance between patterns was 9 μm, about 25% of the cells formed two to four frustrated phagosomes and lysosome tracking was feasible (not shown). Thus, 9 μm was a good compromise and we carried out all lysosome tracking with such a pattern.

For the following experiments, we used RAW–GFP macrophages either stably expressing the lysosomal protease Cathepsin-D (CathD)-mCherry or stained with LysoTracker Red®. Since the same results were obtained with both methods, only the results with CathD-mCherry are shown. Lysosomes are numerous in macrophages and even in cells that are extensively spread out on the substratum, we found that we could not use automated tracking software (see Movie S3 in Supplementary Material). Thus we decided to track vesicles manually, using the "manual tracking" PlugIn of the ImageJ software, after calibration of the images to have the pixel-to-μm correspondence. The actin/lysosome double staining allowed us to visualize frustrated phagosomes and lysosomes at the same time, hence to sort lysosomes moving "toward" a frustrated phagosome from those moving "outward" or "on" a frustrated phagosome, i.e., inside a ring of actin dots. Lysosomes with no clear destination over the time span of the experiment were called "Off" (**Figure 6A** and see also Movie S3 in Supplementary Material). Only lysosomes that could be individually tracked over a minimum of four frames, which corresponds to 1 s, were considered. Most of the time, tracking was stopped because lysosomes encountered an area of the cell over-crowded with lysosomes and could not be distinguished as an individual vesicle anymore. Very rarely, we had to stop tracking because the vesicle went out of

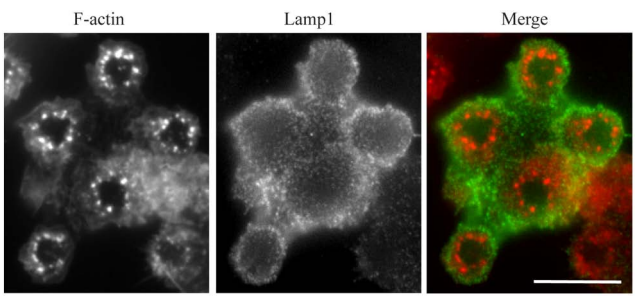

FIGURE 4 | Lamp1 is recruited to the frustrated phagosomes. RAW–GFP cells were added to micro-patterned immune complexes for 10 min before being fixed and stained for Lamp1 (red) without cell permeabilization. Actin was visualized thanks to GFP fluorescence (green). Bar = 5 μm.

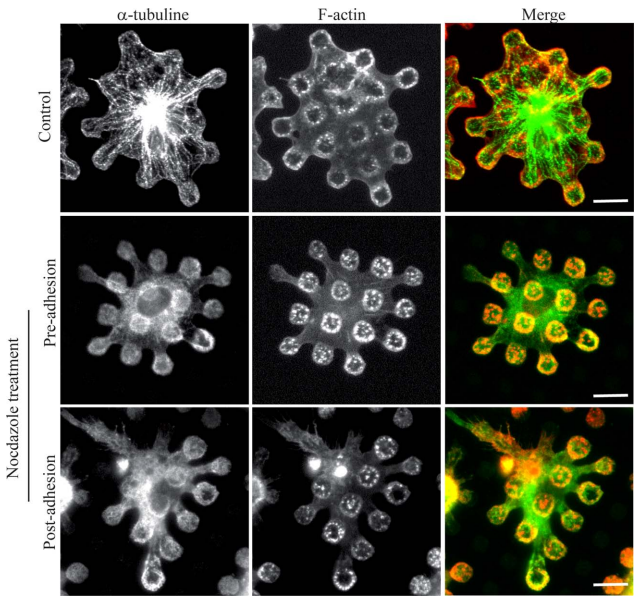

FIGURE 5 | Microtubules are dispensable for biogenesis of frustrated phagosomes and their stability. Bone-marrow derived macrophages from wild-type mice were treated or not (Control) with 10 μM nocodazole either before (pre-adhesion) or after (post-adhesion) being layered on micro-patterned immune complexes. Cells were then fixed, permeabilized, and stained for F-actin and α-tubulin. Bars = 5 μm.

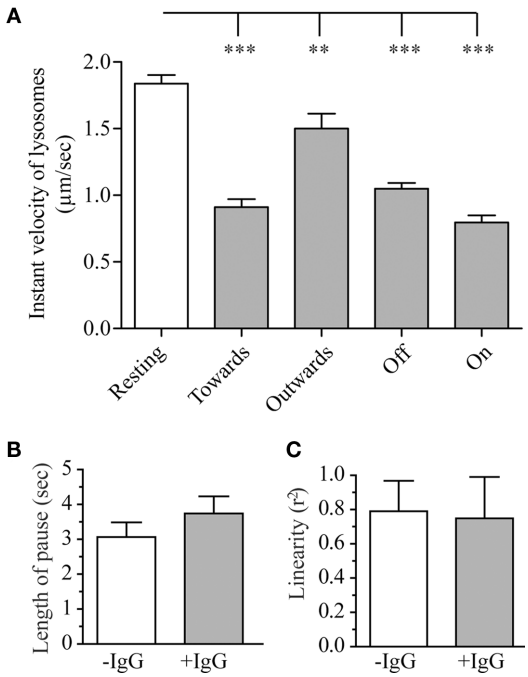

FIGURE 6 | Parameters of the lysosomal movement during frustrated phagocytosis and roles of Src kinases. RAW–GFP expressing cathepsin-D-mCherry were layered on glass cover slips that were micro-patterned with OVA alone (−IgG, "Resting") or OVA + anti-OVA antibodies (+IgG). Tracking of lysosomes was carried out as described in the Section "Materials and Methods." **(A)** Instant velocity of lysosomes as a function of their direction relative to the position of frustrated phagosomes (see text for details). **(B)** Length of the pauses of lysosomes during their saltatory movements. **(C)** Linearity of the trajectories for lysosomes moving toward a frustrated phagosome. For all graphs, 15–30 lysosomes were measured on three to six different cells per conditions; mean ± SEM.

focus. This observation comforted us into our decision to neglect the *Z* dimension in our measurements. As a control, we tracked lysosomes of resting macrophages that were layered on glass, in the absence of IgGs. We specifically chose "control" cells that were as spread as possible to be able to track lysosomes.

ACTIVATION OF PHAGOCYTOSIS RECEPTORS DECREASES LYSOSOME MOBILITY

As shown on **Figure 6A**, lysosomes in resting macrophages had an instant speed of about 1.8 μm/s. Surprisingly when macrophages were performing frustrated phagocytosis, lysosomes velocity was significantly lower than in resting macrophages (**Figure 6A**). This slowing down was observed for all lysosomes, but, lysosomes moving away from a frustrated phagosome were found to be less affected than lysosomes moving toward, on or off a frustrated phagosome. The duration of pauses during lysosomes saltatory movements (see Movie S3 in Supplementary Material) was slightly longer during frustrated phagocytosis but this difference remained below statistical significance (**Figure 6B**), and no difference was detected in the linearity of the trajectories (**Figure 6C**). This parameter is shown for lysosomes moving toward a frustrated phagosome but no difference was observed for lysosomes moving outward either (not shown). We showed that the integrity of the microtubule network was required for lysosome movement since no movement at all could be tracked when cells were treated with 10 μM nocodazole after they had formed frustrated phagosomes (not shown). We were also able to measure the frequency of lysosome arrival in the vicinity of a frustrated phagosome, and observed that, under our experimental conditions, 3.7 ± 0.5 lysosomes per minute (mean ± SEM) come into contact with the same frustrated phagosome. We did not look at the fusion process itself, but we could measure the length of time that a lysosome stays in contact with a frustrated phagosome. This parameter was called "interaction" and was measured to be 7.9 ± 0.8 s (mean ± SEM).

DISCUSSION

In this study, we describe a new experimental set-up to measure several parameters of lysosome movements in macrophages. When layered on micro-patterned ICs, macrophages spread, and formed frustrated phagosomes. As a consequence, we were able to anticipate where lysosomes were recruited to, to image them in live cells and measure their speed, saltatory movements, directionality, and interaction with frustrated phagosomes.

A frustrated phagosome was defined as a ring of actin dots surrounding an immune complex pattern. Previous experiments involving frustrated phagocytosis had been designed on glass supports uniformly coated with ICs. Under these conditions, macrophages adhere very strongly to the surface, forming a tight sealing zone that separates the area underneath the cell from the rest of the medium, where secreted material is confined (Wright

and Silverstein, 1984; Heiple et al., 1990), much like the sealing zone described for the macrophage-derived osteoclasts (Saltel et al., 2008). In our present study, we stained F-actin and noticed that frustrated phagosomes were delineated by F-actin dots triggered by activation of Fcγ receptors. The actin cores positioned just outside the IgG patterns and the space that was often visible between the dots and the pattern underneath may represent the scaffold of actin-linked proteins that are necessary to maintain the structure. Vinculin and paxillin did not co-localize with F-actin cores but displayed the same organization as in podosomes (Linder and Aepfelbacher, 2003; Van Goethem et al., 2011). The reason why actin rearranged as individual dots around the IgG patterns remains to be clarified. Under conditions of frustrated phagocytosis, actin polymerization is unable to form pseudopodia which normally appear in three dimensions around IgG-coated particles. Thus the actin dots could constitute "stable vestiges" of the protrusive machinery of frustrated pseudopodia.

We chose to work with the macrophage cell line RAW264.7 for its amenability to molecular manipulations. Our experimental procedure can also be applied to hMDMs and BMDMs. However, when we tried to label lysosomes of hMDMs with LysoTracker, most of the labeled compartments were tubular and interconnected, as previously described (Knapp and Swanson, 1990; not shown), and tracking lysosomes in such cells was not as easy as in RAW264.7 cells.

The optimization of the micro-pattern geometry for RAW264.7 cells led us to use squares that were spaced by 9 μm for optimal imaging of lysosomes. With this set-up, we could characterize for the first time the movement of lysosomes in phagocytic macrophages by anticipating where lysosomes are recruited to and by tracking lysosomes almost exclusively in two dimensions as cells spread strongly on patterned ICs and became very flat. The instant speed of lysosomes in macrophages adhering on glass was about 2 μm/s. We do not know of any previously published data documenting lysosome speed in macrophages. However, it appeared to be about five times greater than Hck-positive lysosomes in NIH-3T3 fibroblasts (Vincent et al., 2007) and twice as fast as the most rapid Lamp1-positive lysosomes in immortalized skin fibroblasts (Falcon-Perez et al., 2005). This, together with the observation that lysosomes are slowing down in macrophages that formed frustrated phagosomes thus suggests that lysosomes may be moving through some active, microtubule-dependent mechanism in the cytoplasm of non-phagocytic macrophages, and that these movements could then become hindered by the lysosomes becoming tethered to the cytoskeleton upon activation of phagocytosis. The decrease in the velocity of lysosomes that we observed during the frustrated phagocytosis process may be a feature of polarized secretion. We will modify our set-up to micro-texturize cover slips with proteins that induce the formation of podosomes (Labernadie et al., 2010), we will see whether lysosomes addressed to podosomes move also slowlier, when compared to lysosomes that are not targeted to podosomes.

Lysosome movements in macrophages were found to be dependent on intact microtubules, in agreement with previous studies (Astarie-Dequeker et al., 2002; Harrison et al., 2003; Huynh et al., 2007). The saltatory property of lysosome movement is clearly the same in resting and phagocytic macrophages. Pauses during movement have been described *in vitro* as time that vesicles spend at across-road of two microtubules or a microtubule and a microfilament before "choosing" to go on the same track or to switch track (Ross et al., 2008; Schroeder et al., 2010). However, such mechanisms have not been shown to exist in live cells, as yet.

Frustrated phagosomes being easily identified by the ring of actin dots, two additional parameters were measured: the frequency and the duration of lysosome/phagosome interactions. We called "interaction" the presence of a motionless lysosome in the vicinity of a frustrated phagosome, i.e., detected either inside the ring of actin dots or apparently touching the ring. Interaction of lysosomes with the frustrated phagosome lasted about 8 s, which is very close to the duration of the interaction of MT1-MMP-positive vesicles with podosomes actin rich structures where MT1-MMP is supposed to be delivered (Wiesner et al., 2010). Most of the time, interactions between lysosomes and phagosomes were transient and the same incoming lysosome was observed moving away from the phagosome. Although we could detect lysosomal enzymes in the extracellular medium, whether lysosomes delivered part of their content during the docking time in a kiss-and-run process (Desjardins, 1995) is not clear yet. Sometimes, cathepsin-D-positive lysosomes disappeared when the vesicles interacted with frustrated phagosomes, as if fusion occurred. In order to visualize the kiss-and-run and the fusion processes properly, we plan to use TIRF microscopy in future experiments, which should bring significant improvement to our current experimental set-up. This should also provide an answer to the questions raised by others as to whether lysosomes need to interact with actin structures before fusion with the phagosomes (Kjeken et al., 2004; Liebl and Griffiths, 2009).

The molecular mechanisms which control the movements and fusion of lysosomes in response to IgG receptor activation is of particular interest in the context of infections with pathogens which target these mechanisms to survive in the host (see Kumar and Valdivia, 2009 for review). Moreover, lysosomes are also secreted in a spatially controlled manner in different contexts than IgG-mediated phagocytosis. They fuse to the plasma membrane to repair injuries induced by migration on glass supports (Reddy et al., 2001); they fuse at podosomes to release their lytic content and degrade the extracellular matrix (Cougoule et al., 2005, 2010; Tu et al., 2008; Linder et al., 2010); and polarized exocytosis of secretory lysosomes takes place at the immunological synapse in cytotoxic lymphocytes and Natural Killers (see for Holt et al., 2006 review). Our set-up will be used to examine which parameter of lysosome movements actors of the lysosome trafficking such as Lyst [lysosomal trafficking regulator; (Stinchcombe et al., 2000)] or Hck (Cougoule et al., 2005; Vincent et al., 2007; Guiet et al., 2008) are involved in, using, for instance, sh- or siRNA-mediated knock-down approaches.

In conclusion, the experimental approach described here will facilitate studies dedicated to the identification of the actors which play a role in lysosome movement, directionality, speed, docking, and also fusion once TIRF microscopy is coupled to the present set-up.

Taking into account that lysosome secretion is a phenomenon that is crucial not only for bactericidal activities, but also for extracellular matrix degradation during trans-tissular migration, the molecular actors of this process could then be essential effectors of the inflammation response.

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SUPPLEMENTARY MATERIAL

The Movies 1–3 for this article can be found online at http://www.frontiersin.org/Inflammation/10.3389/fimmu.2011.00051/abstract

Movie S1 | RAW267.4 RAW267.4 macrophage stably expressing GFP-actin undergoes frustrated phagocytosis on micro-patterned immune complexes. The cell makes contact with the substratum at the top right corner of the field and form two successive frustrated phagosomes toward the left in the lower part of the field. Accelerated 2 times.

Movie S2 | RAW267.4 macrophage stably expressing GFP-actin undergoes frustrated phagocytosis on micro-patterned immune complexes. This cell rapidly forms two frustrated phagosomes, which seem to be linked by a dynamic actin structure. Accelerated 2 times.

Movie S3 | RAW267.4 macrophage stably co-expressing GFP-actin (left panel) and CathepsinD-mCherry (right panel). Images of GFP and mCherry fluorescence were taken every 10 sec to avoid bleaching, and 250 ms, respectively. Arrows show examples of tracked lysosomes. Accelerated 4 times.

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APPENDIX

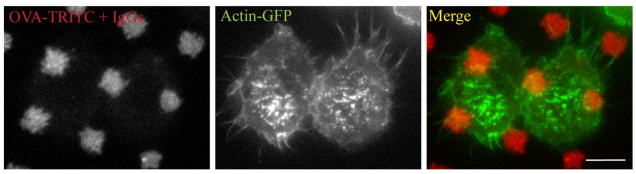

FIGURE A1 | Fc-receptors are necessary to trigger frustrated phagocytosis. RAW267.4 macrophage stably expressing GFP-actin (green) were pre-treated with anti-CD16/CD32 blocking antibodies, layered on micro-patterned immune complexes (red) for 15 minutes before being fixed.

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Modulation of macrophage efferocytosis in inflammation

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A critical function of macrophages within the inflammatory milieu is the removal of dying cells by a specialized phagocytic process called efferocytosis ("to carry to the grave"). Through specific receptor engagement and induction of downstream signaling, efferocytosing macrophages promote resolution of inflammation by (i) efficiently engulfing dying cells, thus avoiding cellular disruption and release of inflammatory contents, and (ii) producing anti-inflammatory mediators such as IL-10 and TGF-β that dampen pro-inflammatory responses. Evidence suggests that plasticity in macrophage programming, in response to changing environmental cues, modulates efferocytic capability. Essential to programming for enhanced efferocytosis is activation of the nuclear receptors PPARγ, PPARδ, LXR, and possibly RXRα. Additionally, a number of signals in the inflammatory milieu, including those from dying cells themselves, can influence efferocytic efficacy either by acting as immediate inhibitors/enhancers or by altering macrophage programming for longer-term effects. Importantly, sustained inflammatory programming of macrophages can lead to defective apoptotic cell clearance and is associated with development of autoimmunity and other chronic inflammatory disorders. This review summarizes the current knowledge of the multiple factors that modulate macrophage efferocytic ability and highlights emerging therapeutic targets with significant potential for limiting chronic inflammation.

Keywords: macrophage, efferocytosis, inflammation, alternative activation, classical activation, apoptotic cell

INTRODUCTION

Efferocytosis, or engulfment of apoptotic cells by macrophages is an essential process with roles in tissue homeostasis, embryologic development, immunity, and resolution of inflammation, the latter, the focus of this review. Apoptotic cells exhibit surface changes, especially exposure of the plasma membrane inner leaflet phospholipid phosphatidylserine (PS) or its oxidized forms (Fadok et al., 1992; Greenberg et al., 2006), that distinguish them from viable cells and allow recognition by a multiplicity of macrophage efferocytic receptors (**Table 1**). Other surface ligands on apoptotic cells, e.g., calreticulin and deposited complement, are reviewed elsewhere (Henson and Bratton, 2009). In many instances, factors in plasma (or serum), often produced by macrophages themselves, are utilized as bridge molecules to couple apoptotic cells to the macrophage receptors (**Table 1**). Engagement of efferocytic receptors initiates signaling events modulated by two main complexes, CrkII/ELMO/Dock180 (Gumienny et al., 2001) or ABCA1/GULP (Kinchen et al., 2005), both resulting in activation of Rac1, which initiates cytoskeletal rearrangement and subsequent engulfment. Rac1 and RhoA, two small Rho GTPases, have opposing roles in regulating efferocytosis; Rac1 enhances, while RhoA inhibits the process (Leverrier and Ridley, 2001; Nakaya et al., 2006). Thus, the relative balance between them plays a key role in determining macrophage efferocytic ability, and imbalance favoring active RhoA can lead to defective clearance.

Macrophages subserve key roles during inflammation and its resolution. As innate immune sentinels, resident tissue macrophages detect and interpret signals indicating tissue injury or pathogen infiltration and initiate responses through release of cytokines and other mediators. Consequently, leukocyte recruitment ensues, including blood monocytes that differentiate into macrophages. Changing cues in the inflammatory milieu alter macrophage programming and modulate various endocytic functions. Phagocytosis and macropinocytosis are essential for removal of infectious agents, antigen presentation, and activation of adaptive immune responses, while efferocytosis can help initiate restoration of tissue structure and function. Mechanistically, efferocytosis resembles stimulated macropinocytosis and is morphologically distinct from classic forms of phagocytosis (Ogden et al., 2001; Henson and Bratton, 2009). Due to differences in recognition mechanisms, engagement, and signaling pathways, it is not surprising that environmental factors have differential effects on the various forms of endocytosis (Gratchev et al., 2006; Feng et al., 2011).

Apoptotic cell recognition and efferocytosis by macrophages has a profound influence on resolution of inflammation, largely through secretion of anti-inflammatory cytokines, such as TGF-β and IL-10 that inhibit inflammatory mediator production (Voll et al., 1997; Fadok et al., 1998). Thus, macrophages responding to PS on apoptotic cells have been shown to accelerate resolution of LPS-induced lung inflammation in a TGF-β dependent manner (Huynh et al., 2002), and infusion of PS liposomes attenuated inflammation in murine skin edema and myocardial infarction models (Ramos et al., 2007; Harel-Adar et al., 2011). Additionally, local injection of PS liposomes decreased the amplitude of a $CD4^+$ T cell response (Hoffmann et al., 2005) indicating that apoptotic cell signaling can modulate adaptive immune responses. Finally, efferocytosis of dying cells prevents their deterioration and release

Table 1 | Phosphatidylserine, bridge molecules, and receptors for apoptotic cells including those known to be modulated by nuclear receptor signaling.

Ligand on apoptotic cell	Bridge molecules	Efferocytic receptor
PS	None	BAI1
PS	None	TIM1, TIM3, TIM4
PS	None	Stabilin-1, stabilin-2
PS	None	Receptor for advanced glycation end products (RAGE)
PS; oxidized PS	C1q*,**, MBL	Calreticulin/LRP (CD91)*
PS; oxidized PS	Gas6**, protein S	Mer*,**,^, Axl*,**, Tyro3
PS; oxidized PS	MFG-E8*,**	αVβ3/5 integrins
	Thrombospondin**	CD36*
PS; oxidized PS	Collectins (SP-A, SP-D)	LRP (CD91)*

Ligands, bridge molecules, and receptors are more comprehensively discussed in reference (Henson and Bratton, 2009) and references therein.

**Expression regulated by PPAR-γ (Berry et al., 2007; Majai et al., 2007; Roszer et al., 2011).*

***Expression regulated by PPAR-δ (Mukundan et al., 2009).*

^ Expression regulated by LXR (A-Gonzalez et al., 2009).

of phlogistic intracellular contents that can contribute to inflammation and autoimmunity. Given the impact of efferocytosis on suppression of inflammation, restoration of tissue homeostasis, and shaping the immune response, this review will focus on factors in the tissue environment that modulate the ability of macrophages to carry out these functions.

SECTION 1: MACROPHAGE PROGRAMMING AND MODULATION OF EFFEROCYTOSIS

The increasingly complex list of so-called macrophage "phenotypes" have been comprehensively reviewed elsewhere (Benoit et al., 2008; Mosser and Zhang, 2008; Martinez et al., 2009) The concept of phenotypes, while useful, is oversimplified, because macrophages exhibit substantial plasticity, with markers and functions readily altered by external signals (Gratchev et al., 2006; Fernandez-Boyanapalli et al., 2009). Accordingly, we argue that it is important to address the functional attributes of macrophages under study or discussion, leading us to focus, here, on those associated with differential capacity for efferocytosis. In general terms, "classically activated" or M1 macrophages demonstrate diminished efferocytosis, increased phagocytosis of foreign organisms, and enhanced production of pro-inflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO; Benoit et al., 2008; **Figure 1**). This M1 programming state results from stimulation with LPS and the innate cytokine IFNγ, or can be elicited by IFNα/β-inducing TLR agonists (Mosser and Zhang, 2008), leading to inhibition of efferocytosis in part through TNFα and/or oxidant-mediated mechanisms (McPhillips et al., 2007; Michlewska et al., 2009). Because M1 macrophages are also associated with Th1 T cell polarization, they exhibit protection against bacterial infection. Such macrophages are evident early in the course of infection or injury, e.g., peritonitis (Fernandez-Boyanapalli et al., 2010a) and are designated in this review as inflammatory, "efferocytic-low" macrophages.

On the opposite end of the spectrum, various "alternatively activated" or M2 macrophages can be elicited by IL-4 and IL-13 (M2a), a combination of TLR agonists and immune complexes (M2b), glucocorticoids and IL-10 (M2c), M-CSF, or TGF-β (Xu et al., 2006; Benoit et al., 2008; Mosser and Zhang, 2008; Martinez et al., 2009). Collectively, these macrophages exhibit increased expression and/or activity of the nuclear receptors, PPARγ and PPARδ, essential to their acquisition of "alternative activation" (Bouhlel et al., 2007; Odegaard et al., 2007). They often display increased levels of arginase, certain receptors (e.g., the macrophage mannose receptor) and anti-inflammatory cytokines, whereas production of pro-inflammatory cytokines, RNS, and ROS are downregulated (Benoit et al., 2008; Olefsky and Glass, 2010; **Figure 1**). The majority of these "alternative activation" states have been associated with enhanced efferocytosis of apoptotic cells (and likely also necrotic cells and cellular debris) supporting a role in resolution of inflammation. As such, these macrophages are seen later during the resolution phase of inflammation, and henceforth, are called pro-resolving, or "efferocytic-high" (Bystrom et al., 2008; Fernandez-Boyanapalli et al., 2010a; Schif-Zuck et al., 2011). Whether the *in vivo* shift from inflammatory to pro-resolving state is attributable to recruitment, or expansion of different macrophage populations within the milieu at differing stages of inflammation (Jenkins et al., 2011), or rather represents given macrophages responding to the changing milieu with a switch in programming (Bystrom et al., 2008), or a combination, remains an important, and largely unanswered question for most inflammatory processes. Importantly, two additional caveats deserve mention: (i) much of the literature is based on programming of murine macrophages which likely differs from human, and (ii) cultured macrophages (e.g., M-CSF-treated human monocyte-derived macrophages or murine bone marrow macrophages) are programmed during culture with substantial influences on subsequent responses (Fernandez-Boyanapalli et al., 2009).

MACROPHAGE PROGRAMMING FOR ENHANCED EFFEROCYTOSIS AND ANTI-INFLAMMATORY CONSEQUENCES

Of the pro-resolving programming states, those elicited by IL-4 and IL-13 are the most thoroughly studied in relation to enhanced efferocytic capacity. IL-4 and IL-13 increase expression and activity of the nuclear receptor PPARγ via STAT6 (Welch et al., 2003; Berry et al., 2007; Szanto et al., 2010). IL-4 also induces production of potential PPARγ-activating ligands, 13-HODE and15-HETE through 15-lipoxygenase activity (Huang et al., 1999). Macrophage PPARγ activation, in turn, has three consequences relevant to this review: (i) "alternative activation" with increased efferocytic surface receptors (**Table 1**) and secretion of the bridge molecule adiponectin; (ii) enhanced efferocytic capability; and (iii) suppression of inflammation. For some macrophage populations, IL-4/IL-13-induced PPARγ signaling enhances efferocytosis specifically (Fernandez-Boyanapalli et al., 2009), while in others, it non-specifically enhances other phagocytic functions: e.g., uptake of opsonized cells (Aronoff et al., 2004), parasitized RBCs (Serghides and Kain, 2001), and yeast (Gales et al., 2010). An overall increase in phagocytic ability, especially for fungal and parasitic pathogens,

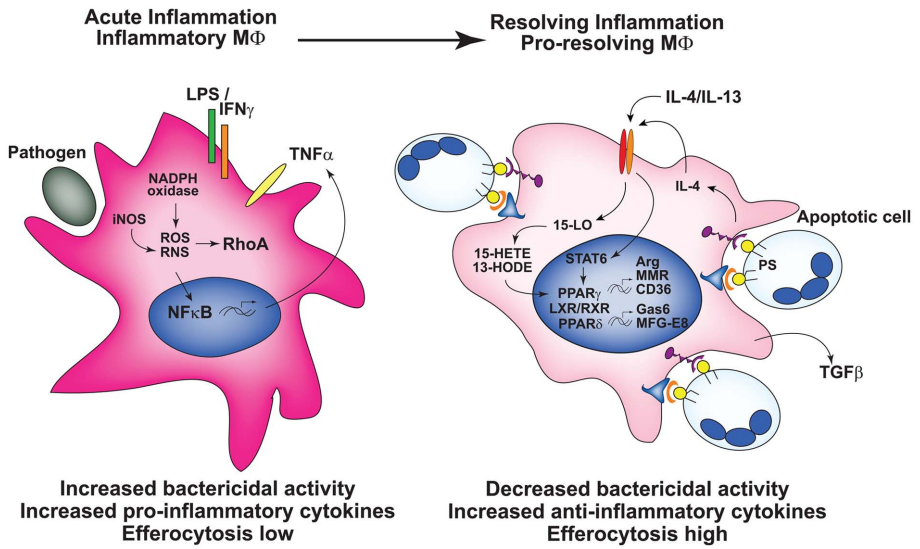

FIGURE 1 | Macrophage programming either *in vitro* or *in vivo* determines efferocytic capacity. *Left:* Inflammatory macrophages, e.g., generated by stimuli such as LPS + IFNγ have heightened bactericidal activity and production of pro-inflammatory mediators, and are programmed poorly for efferocytosis. *Right:* Following activation of the nuclear receptors, pro-resolving, or resolution-phase macrophages are programmed for heightened efferocytosis, with increased expression of receptors and bridge molecules required for the recognition of apoptotic cells, and the production of anti-inflammatory cytokines. *In vivo,* over the course of acute inflammation macrophage programming and function transitions from that of inflammatory macrophages to resolution-phase macrophages.

is likely associated with PPARγ-mediated upregulation of fungal and parasitic recognition receptors and related to the role of "alternatively activated" macrophages in immunity against Th2 response-inducing pathogens (Raes et al., 2005; Gales et al., 2010).

IL-4/IL-13 also enhanced macrophage PPARδ expression, and expression and release of bridge molecules (**Table 1**), and acquisition of anti-inflammatory functions (Kang et al., 2008). Both these PPARs are known to heterodimerize with other nuclear receptors to exert these actions, and accordingly, roles for LXR and RXRα in enhanced efferocytosis have been demonstrated (A-Gonzalez et al., 2009; Mukundan et al., 2009; Rebe et al., 2009; Roszer et al., 2011). Direct connections between IL-4/IL-13 and LXR and RXRα are still to be determined.

IL-4 also increases expression of the efferocytic receptors, stabilin-1 and stabilin-2, although connections with nuclear receptor signaling have not been made (Park et al., 2009). Efferocytic programming of macrophages by cytokines, such as M-CSF, IL-10, and TGF-β, are described but less understood. Likewise, pathways for the expression of other apoptotic cell receptors, and indeed the differences in the repertoire of receptors utilized by macrophages in different tissues/milieus is poorly defined (Henson and Bratton, 2009).

An important and emerging concept is that macrophage recognition of apoptotic cells themselves can reinforce signaling pathways that shift their programming toward enhanced efferocytic ability in a feedforward manner (**Figure 1**): e.g., apoptotic cell-induced PPARγ, PPARδ, and LXR activation results in enhanced CD36 and Mer expression and secretion of efferocytic bridge molecules (A-Gonzalez et al., 2009; Mukundan et al., 2009; Roszer et al., 2011). One mechanism by which apoptotic cells enhance efferocytic programming is through PS-dependent induction of IL-4 signaling to upregulate PPARγ (Fernandez-Boyanapalli et al., 2009). Autocrine stimulation by TGF-β produced in response to apoptotic cell recognition may similarly enhance PPARγ expression (Freire-de-Lima et al., 2006).

Suppression of inflammation also results from activation of these signaling pathways. Stimulation of PPARγ, PPARδ, and LXR, RXRα are associated with reduced production of pro-inflammatory mediators (Ghisletti et al., 2007; Mukundan et al., 2009; Roszer et al., 2011). For example, PPARγ and LXR associate with the co-repressor complex NCoR inhibiting its removal from transcriptional binding sites driven by inflammatory cytokines. Sustained NCoR occupation inhibits NF-κB down-regulating transcription of its target genes, e.g., TNFα and IL-1β (Pascual et al., 2005; Ghisletti et al., 2007; Jennewein et al., 2008). Recognition of apoptotic cells also down-regulates pro-inflammatory mediators through PS-induced stimulation of TGF-β production (Huynh et al., 2002; Freire-de-Lima et al., 2006). Observations such as these reinforce the concept of plasticity in macrophage programming during the course of an inflammatory response with early induction of protective properties and later development of restorative activities to promote resolution (**Figure 1**). Though the focus here is the role of efferocytic macrophages in the resolution of acute inflammation, it should also be noted that "pro-resolving" macrophages ameliorate chronic inflammation (Wang et al., 2007; Gordon and Martinez, 2010). Alternatively, depending on context, consequences of such programming may be detrimental. For instance, sustained production of TGFβ may lead to over-exuberant fibrotic responses, and there is increasing evidence that the cultivation of immunosuppressive

tumor-associated macrophages promote the growth, invasion, and immune evasion of tumors (Martinez et al., 2009; Sica, 2010). While important, these downstream consequences are beyond the scope of this review and the reader is directed to recent references on the subject.

MECHANISMS BY WHICH INFLAMMATORY MACROPHAGE PROGRAMMING DECREASES EFFEROCYTIC CAPACITY

In the other direction, suppression of efferocytosis, but not other forms of phagocytosis, is likely mediated both by early acting inhibitors (below) as well as inflammatory macrophage programming (Michlewska et al., 2009; Feng et al., 2011). This suggests effects on pathways that are unique to the recognition and uptake of apoptotic cells. Evidence for programming-related suppression of efferocytosis stems from studies of macrophages stimulated with LPS demonstrating decreased expression of PPARγ (Welch et al., 2003), and decreased transcription and serum levels of efferocytosis-associated bridge molecules Gas6 and MFG-E8 (Komura et al., 2009; Feng et al., 2011). Likewise, LPS induces the transcription factor IRF5, which suppresses expression of efferocytic receptors CD36 and CD14 along with "alternative activation" markers as it up-regulates pro-inflammatory cytokine production (Krausgruber et al., 2011). Thus, the balance between expression of IRF5 and IRF4, the latter driven by IL-4, governs "alternative activation" marker expression (El Chartouni et al., 2010) and likely plays a role in determining macrophage efferocytic capacity. Interestingly, stimulation of macrophages with the other "classical activation" stimulus IFNγ by itself enhances phagocytosis of both apoptotic cells and IgG opsonized targets in a NO-dependent manner involving Rac activation (Fernandez-Boyanapalli et al., 2010b) suggesting further complexity in the regulation of efferocytosis.

SECTION 2: EARLY ACTING ENHANCERS AND INHIBITORS IN THE INFLAMMATORY MILIEU THAT MODULATE EFFEROCYTOSIS

In addition to macrophage programming modulators, the inflammatory milieu contains numerous serum and cell-derived factors that have immediate (or near immediate) early effects on efferocytosis (**Figure 2**) separate from the slower and more prolonged consequences of macrophage programming. Many of these early acting effectors have been found to shift the balance between Rho A activation (inhibitory) and Rac 1 activation (enhancing; **Figure 2**).

EARLY ACTING INHIBITORS

Previous studies demonstrated that LPS-mediated suppression of efferocytosis was TNFα dependent (Michlewska et al., 2009; Feng et al., 2011) and that short-term exposure to TNFα inhibited macrophage efferocytosis in an oxidant-dependent manner involving RhoA activation (McPhillips et al., 2007; Moon et al., 2010). Similarly, lysophosphatidic acid (LPA) inhibits efferocytosis through activation of RhoA (Morimoto et al., 2006). Other inhibitors of efferocytosis in the inflammatory milieu act by blocking macrophage recognition of apoptotic cells. These include high mobility group box protein 1 (HMGB1), soluble receptor for advanced glycation end products (RAGE), and annexin A5. HMGB1 binds various macrophage receptors: its binding to RAGE blocks its recognition of PS, and binding to αV integrins blocks interactions with the bridge molecule, MFG-E8 (**Table 1**; Banerjee et al., 2010; Friggeri et al., 2010, 2011; He et al., 2011). HMGB1 binding to RAGE also reinforces an inflammatory macrophage state by stimulating NF-κB and enhancing pro-inflammatory cytokine production (Qin et al., 2009). Alternatively, masking of PS on apoptotic cells also impairs efferocytosis: soluble RAGE (He et al., 2011) and annexin A5 (unlike annexin A1, see below; Kenis et al., 2006) block macrophage recognition of apoptotic cells by this mechanism. Collectively, these signals downregulate efferocytosis and are likely to reinforce properties of inflammatory macrophages.

EARLY ACTING ENHANCERS

Early acting enhancers are defined operationally as agents that rapidly augment the capacity and/or the capability of macrophages to engulf apoptotic cells, often by increasing Rac 1 activity (**Figure 2**). Such factors are found on apoptotic cells and in the milieu, and many are made and released by macrophages themselves. Most enhancers identified to date appear to be modified lipids. A distinguishing feature is their inability to drive efferocytosis on their own (Frasch et al., 2011), i.e., they act by enhancing existing stimuli. By contrast, PS or oxidized PS (**Table 1**), termed inducers, drive macrophages to take up even viable cells when they are inserted into the target cell membrane (Fadok et al., 1992; Greenberg et al., 2006).

A modified phosphatidylserine species, lysophosphatidylserine (lyso-PS), is an early acting enhancer produced in activated and apoptosing neutrophils through an NADPH-oxidase dependent pathway. In sterile peritonitis, lyso-PS is localized to the neutrophil surface with its accumulation peaking at a time immediately preceding rapid neutrophil clearance by macrophages (Frasch et al., 2008). Lyso-PS acts through the macrophage G2A receptor stimulating the production of prostaglandin E_2 (PGE_2) leading to cAMP and PKA-dependent augmentation of Rac1 activity. While it has previously been reported that PGE_2 inhibits efferocytosis, this effect appears to be concentration dependent; concentrations less than or equal to 1 nM in fact enhance efferocytosis, whereas concentrations greater than 10 nM are inhibitory (Rossi et al., 1998; Frasch et al., 2011). Macrophage production of PGE_2 following efferocytosis (Voll et al., 1997; Fadok et al., 1998) has been demonstrated to impair anti-microbial functions of macrophages (Aronoff et al., 2004; Medeiros et al., 2009), and whether this PGE_2 ultimately enhances or inhibits subsequent efferocytosis is unknown and is likely to be context and concentration dependent.

Lipoxins are a class of pro-resolving eicosanoids derived from arachidonic acid that are produced and released by macrophages during the resolution phase of inflammation via lipoxygenase enzymes (Serhan et al., 2008). Lipoxin A_4 is reported to enhance efferocytosis *in vitro* (Godson et al., 2000) and *in vivo* (Mitchell et al., 2002) by acting through the macrophage ALX receptor (ALXR), which is also utilized by the PS-recognizing bridge molecule, annexin A1 (Scannell et al., 2007; Maderna et al., 2010). Several other pro-resolution lipids derived from essential omega-3 fatty acids, have also been identified, including resolvins, protectins (PD1), and maresins, all of which enhance macrophage phagocytosis of pathogens, particles, and apoptotic cells (Schwab et al., 2007; Serhan et al., 2008). How these pro-resolving lipids enhance

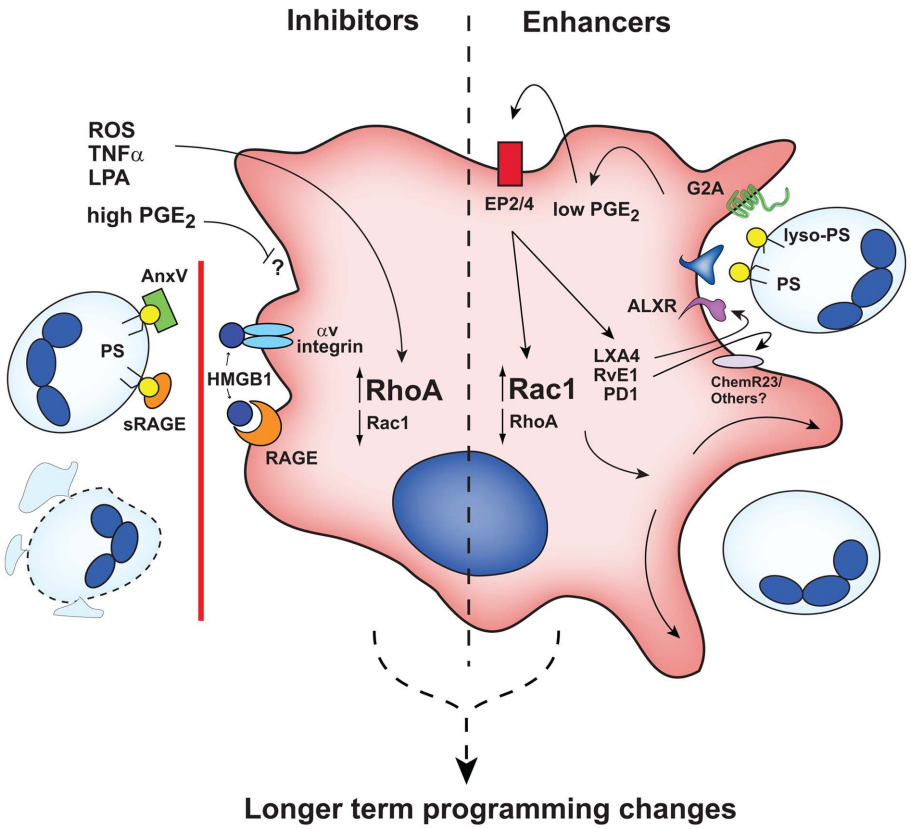

FIGURE 2 | Early acting inhibitors and enhancers influence efferocytic capacity of macrophages. Rho/Rac balance is altered by inhibitors (left) and enhancers (right). ROS, reactive oxygen species; AnxV, annexin A5; other abbreviations are defined in the text. It is likely that early acting agents also contribute to longer-term macrophage programming as illustrated in **Figure 1**.

efferocytosis, the mechanisms and precise receptors involved, are not well understood. For instance, resolvin E1 (RvE1), but not the related RvD1, exerts its effects in part via the chimerin receptor, ChemR23. Importantly, decreased production of pro-inflammatory cytokines, perhaps as a result of efferocytosis, have been observed with these pro-resolving lipids (Schwab et al., 2007). Interestingly, the transition toward production of these pro-resolving lipids (lipoxins, resolvins, and protectins) is mediated through PGE_2 (Serhan et al., 2008).

In addition to the short-term influences of enhancers or inhibitors, these factors are likely to have downstream contributions in shaping subsequent macrophage programming. For example, since apoptotic cell recognition and efferocytosis enhances the macrophage programming transition from inflammatory to pro-resolving, influencing this initial process may thwart this transition (inhibitors), or speed, reinforce, and/or sustain it (enhancers). For example, PGE_2 and subsequent cAMP production appear to be pivotal to programming inflammatory macrophages to a pro-resolving phenotype under some conditions (Bystrom et al., 2008). As such, early signals, e.g., lyso-PS, PGE_2, and cAMP, and possibly the other pro-resolving lipids, may contribute collaboratively to initiate the transition from inflammatory macrophage to resolving macrophage by carefully orchestrated signaling.

SECTION 3: CONSEQUENCES OF DECREASED EFFEROCYTOSIS AND PROLONGED INFLAMMATORY PROGRAMMING

Defective apoptotic cell clearance is associated with many autoimmune and chronic inflammatory disorders including SLE (Koh et al., 2000; Gaipl et al., 2007), type I diabetes (Haskins et al., 2003), chronic obstructive pulmonary disease (Hodge et al., 2008), and cardiovascular disease (Li et al., 2009). Mechanistically, disintegration of uncleared late apoptotic cells releases toxic intracellular contents that spur inflammation (e.g., HMGB1) and provide epitopes (e.g., nuclear DNA) for autoantibody production, a hallmark of autoimmune disorders such as SLE (Ma et al., 2010). As a consequence, the resulting increased production of pro-inflammatory cytokines (e.g., TNFα and IFNα; Koh et al., 2000; Haskins et al., 2003) prolongs inflammatory, efferocytic-low macrophage programming. Similarly, polymorphisms resulting in over-expression of the M1-associated transcription factor IRF5 are associated with many inflammatory diseases (Dideberg et al., 2007; Dieguez-Gonzalez et al., 2008; Kristjansdottir et al., 2008).

Ineffective apoptotic cell clearance, and enhanced inflammation are also demonstrated where there are defects in "alternative activation" mechanisms. Mice with PPARγ deficient macrophages have impaired efferocytosis, decreased expression of efferocytic

receptors including CD36 and Mer tyrosine kinase, increased kidney inflammation, and autoantibody production (Roszer et al., 2011). PPARδ and LXR-deficient mice also develop systemic autoimmune disease associated with defective clearance and decreased efferocytic receptor/bridge molecule expression (A-Gonzalez et al., 2009; Mukundan et al., 2009). In a murine model of chronic granulomatous disease (CGD), which exhibits chronic inflammation, and mild autoimmunity, macrophages maintain a "classically activated" phenotype, illustrated by enhanced pro-inflammatory cytokine production, impaired efferocytosis, and decreased expression of PPARγ, MMR, CD36, and CD14 (Fernandez-Boyanapalli et al., 2009, 2010a).

The above evidence suggests that defective efferocytosis in autoimmune and chronic inflammatory conditions may result from continual exaggerated inflammatory activation of macrophages and/or ineffective induction of signals associated with programming toward pro-resolving macrophages. As such, further investigation of the mechanisms leading to impaired macrophage efferocytosis in these conditions, and approaches for its reversal, may lead to the development of more effective therapeutic strategies.

SECTION 4: THERAPEUTIC STRATEGIES TO ENHANCE EFFEROCYTOSIS AND REDUCE INFLAMMATION

CURRENTLY AVAILABLE THERAPEUTICS

Based on the experimental evidence, enhancement of macrophage efferocytosis, either by early acting mediators or through programming changes, may have efficacy in treating disorders linked to chronic inflammation and aberrant macrophage function. Many anti-inflammatory drugs utilized clinically likely stimulate apoptotic cell uptake, though for the most part, mechanisms are poorly understood. For example, glucocorticoids with protean effects on inflammation also enhance efferocytosis (Rhen and Cidlowski, 2005). In this regard they appear to act through a number of mechanisms: (i) induced expression of the phospholipid binding protein annexin A1 and its receptor ALXR, which also binds lipoxin A_4 (Maderna et al., 2005), (ii) enhanced signaling via Mer and its ligand, protein S (McColl et al., 2009), (iii) altered Rac/Rho balance (Giles et al., 2001), and (iv) PPARγ-induced programming (Majai et al., 2007). Statins, cholesterol lowering drugs, are used with increasing frequency to treat inflammatory diseases also enhance phagocytosis of apoptotic cells. The mode of action is likely complex, but may work in part by reducing prenylation and membrane association of inhibitory RhoA (Morimoto et al., 2006), as well as by inducing programming-related changes through activation of PPARγ (Yano et al., 2007). Another class of anti-inflammatory agents known to enhance efferocytosis is the macrolide group of antibiotics. These likely alter macrophage programming as well as increase levels of bridge molecules *in vivo* to enhance uptake of apoptotic cells (Yamaryo et al., 2003; Hodge et al., 2008). Many of these current treatments have diverse targets, making it difficult to determine the precise role of enhanced efferocytosis in their anti-inflammatory effects. Furthermore, many have adverse side effects. Accordingly, novel therapies that more closely mimic normal resolution processes or target re-programming of macrophages to specifically enhance efferocytosis are needed.

NOVEL THERAPEUTIC TARGETS TO ENHANCE EFFEROCYTOSIS

Potential new therapeutic targets include modulation of signaling pathways and mediators involved in both early acting events and subsequent macrophage programming. Oxidant-mediated activation of RhoA and suppression of efferocytosis, may be amenable to antioxidant treatment. In a LPS-induced lung injury model, antioxidants reduced inflammation, and improved macrophage efferocytosis by inhibiting RhoA activation (Moon et al., 2010). Similarly, antioxidant treatment in autoimmune NOD mice dampened pro-inflammatory cytokine production and repaired the macrophage efferocytic defect (Haskins et al., 2003). Another potential therapy to enhance efferocytosis could be via lipoxins, including the related aspirin triggered 15-epi-lipoxins (Spite and Serhan, 2010) that signal through ALXR. Lipoxins as well as resolvins and protectins have been shown to reduce inflammation and tissue damage in a variety of rodent models (Serhan et al., 2008). Whether, and to what degree, any of these effects are a direct result of enhanced efferocytosis remains to be determined.

Given that nuclear receptor signaling is strongly associated with enhanced efferocytosis and suppression of inflammation, PPARγ, PPARδ, and LXR agonists are natural therapeutic targets for inflammatory diseases. LXR agonists were shown to reverse defective macrophage efferocytosis and reduce disease severity in a murine model of SLE (A-Gonzalez et al., 2009). Additionally, PPARγ agonists reduced neutrophil numbers in rodent models of acute inflammation, COPD, and asthma (Belvisi and Hele, 2008; Fernandez-Boyanapalli et al., 2010a). PPARγ and PPARδ activation were also shown to decrease inflammation and disease severity in experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis (Feinstein et al., 2002; Polak et al., 2005). Recently, a direct association between improved efferocytosis and treatment efficacy has been demonstrated: treatment with a PPARγ agonist resolved prolonged zymosan-induced inflammation in a mouse model of CGD, which corresponded with macrophage "alternative activation" enhanced efferocytosis, and increased TGFβ and IL-10 production (Fernandez-Boyanapalli et al., 2010a).

In vivo administration of apoptotic cells exploits their signaling to alter inflammatory programming in inflamed tissues; their stimulation of phagocytes shapes subsequent immune and inflammatory responses (Jonson et al., 2008). This has been especially successful in graft versus host disease where a shift in Th1 to Th2 T cell responses was driven by macrophage-dependent induction of tolerogenic dendritic cells (DC) and regulatory T cells (Gorgun et al., 2002; Kleinclauss et al., 2006). In EAE, infusion of autoantigen-expressing apoptotic cells induced tolerance to self antigens and reduced disease progression in a manner-dependent on macrophage-mediated regulation of DC efferocytosis (Miyake et al., 2007). The mechanisms involved in apoptotic cell infusion-induced regulation of inflammation and induction of tolerance are not well studied, however, it is likely mediated in part through apoptotic cell-induced alterations in macrophage programming. Treatment with PS liposomes, which simulate signaling by apoptotic cells, has also been effective. In sterile inflammatory models, administration of PS liposomes led to macrophage re-programming from pro-inflammatory to anti-inflammatory states. As consequences,

efferocytosis was enhanced (Fernandez-Boyanapalli et al., 2009), tissue swelling and pro-inflammatory cytokine production were reduced (Ramos et al., 2007), and repair of infarct tissue was enhanced (Harel-Adar et al., 2011). As a cautionary note, however, administration of both apoptotic cells and PS liposomes into inflamed tissues, and likely to inflammatory macrophages, can also contribute (at least transiently) to exacerbated inflammation (Medan et al., 2002; Borges et al., 2009), and disabled host defense against pathogens (Medeiros et al., 2009). These observations underscore the need to better understand the signaling pathways involved in therapeutic induction of macrophage anti-inflammatory programming and enhancement of efferocytic capacity.

CONCLUSION

Macrophages, through their phagocytic functions and production of cytokines and mediators, profoundly shape the course of inflammation. Interpreting cues from the environment, they orchestrate early pro-inflammatory responses to pathogens and tissue injury, and ultimately produce anti-inflammatory lipids and cytokines for the active suppression of inflammation and restoration of tissue homeostasis. Their recognition and removal of apoptotic cells are crucial to these latter events, and a considerable body of data supports that their dysregulation contributes to diverse autoimmune and chronic disease states. An increasing understanding of the normal resolution mechanisms, especially those that modulate apoptotic cell clearance through the stimulation and programming of macrophages will undoubtedly bring new therapeutic strategies to the forefront and will allow for the development of more effective targeted and/or combinatorial treatments.

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Macrophages in injured skeletal muscle: a *perpetuum mobile* causing and limiting fibrosis, prompting or restricting resolution and regeneration

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Macrophages are present in regenerating skeletal muscles and participate in the repair process. This is due to a unique feature of macrophages, i.e., their ability to perceive signals heralding ongoing tissue injury and to broadcast the news to cells suited at regenerating the tissue such as stem and progenitor cells. Macrophages play a complex role in the skeletal muscle, probably conveying information on the pattern of healing which is appropriate to ensure an effective healing of the tissue, yielding novel functional fibers. Conversely, they are likely to be involved in limiting the efficacy of regeneration, with formation of fibrotic scars and fat replacement of the tissue when the original insult persists. In this review we consider the beneficial versus the detrimental actions of macrophages during the response to muscle injury, with attention to the available information on the molecular code macrophages rely on to guide, throughout the various phases of muscle healing, the function of conventional and unconventional stem cells. Decrypting this code would represent a major step forward toward the establishment of novel targeted therapies for muscle diseases.

Keywords: macrophages, skeletal muscle, innate immunity, wound healing, alternative activation

MUSCLE INJURY AND INFLAMMATION

Resident leukocytes in the healthy skeletal muscle are exceedingly rare. Thereafter, the skeletal muscle represents a microenvironment in which immunologic reactions depend on the characteristics of the noxious event and on the nature and the function of newly recruited immune cells (Wiendl et al., 2005). Muscle inflammation is a common physiologic response to exercise and the hallmark of acute and chronic damages such as strain injury or muscular dystrophies. Muscle inflammation has been felt to run a rather stereotypical course; recent data however indicate that when persistent triggers cause muscle damage, differences exist in the recruitment of the humoral innate immunity at the site of tissue injury. For example, activation of the complement cascade occurs and contributes to the disease in dysferlin-deficient mice, a model for the muscle wasting diseases referred to as dysferlinopathies, but not in *mdx* mice, a mouse model of Duchenne Muscle Dystrophy (Han et al., 2010).

The inflammation in acutely damaged muscle is characterized by a rapid and sequential invasion of leukocyte populations that persist while muscle repair, regeneration, and growth occur (Paulsen et al., 2010). Neutrophils represent the first leukocyte population in the damaged tissue. They were found in muscle early after exercise completion (Fielding et al., 1993) and infiltrate the tissue for as long as 5 days. Neutrophils release molecules that may contribute to the muscle membrane lysis that follows injury (Nguyen et al., 2005). The actual final effect of neutrophil recruitment in damaged skeletal muscle is however not completely elucidated and recent results suggest that infiltration by neutrophils *per se* may not be harmful but additional inflammatory stimuli are required to reveal their detrimental potential (Dumont et al., 2008). Some such stimuli may be directly generated as a consequence of myofiber lysis and ensuing release of endogenous inflammatory molecules or may be contributed by other recruited cells, such as platelets (Maugeri et al., 2009; Manfredi et al., 2010).

Interestingly, genetic disruption of the chemokine pathway related to inflammatory leukocyte recruitment reveals an apparent balance between the extent and the duration of tissue infiltration by leukocytes: absence of CXCL16 not only resulted in defective homing of macrophages and severely jeopardized tissue regeneration, but was associated with a persistent and important infiltration of the tissue by neutrophils, with unrestrained inflammation and eventual fibrosis (Zhang et al., 2009b). Conversely, a significant increase in macrophage accumulation and cell proliferation was observed in mice in which a transient neutropenia was induced at early times after injury (Godbout et al., 2010). In general, macrophages represent the dominant leukocyte population in the late phases of the homeostatic response to injury or in conditions in which the original inflammatory *noxa* persists. Their ability to perceive environmental cues and orchestrate the functional activities of other cells populations, such as immune cells or myogenic precursors (**Figure 1**), makes them an intriguing field of study in muscle biology.

MACROPHAGES: WHAT'S IN A NAME?

Macrophages were originally identified as phagocytic cells responsible for pathogen elimination and housekeeping functions in

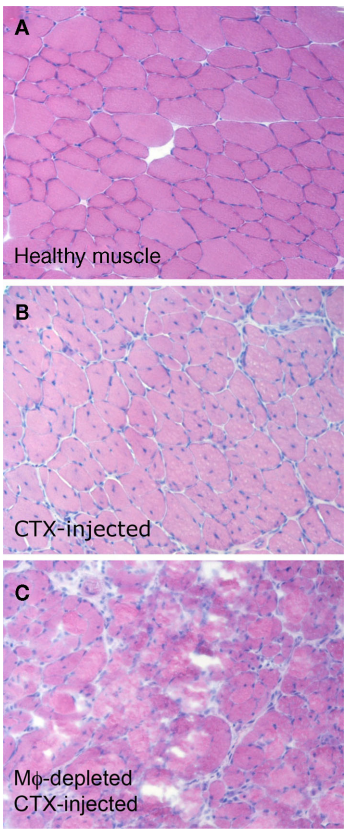

FIGURE 1 | Macrophages, recruited to the skeletal muscle after acute sterile injury, are necessary for effective tissue regeneration. Two months old C57BL/6 mice were treated i.m. with cardiotoxin (CTX). Mice were treated or not with clodronate-containing liposomes to deplete macrophages and sacrificed 15 days after CTX injection. *Tibialis anterior* and *quadriceps* muscles were collected. **(A)** Healthy muscles of untreated control mice. **(B)** The muscle of macrophage-competent mice undergoes effective and almost complete regeneration in 15 days: regenerating centronucleated fibers are evident throughout the section. **(C)** In the absence of macrophages, 15 days after acute sterile injury regenerating fibers are hardly evident. Degenerated fibers and cell remnants persist.

a wide range of organisms (Metchnikoff, 1905): they were thus included in the Mononuclear Phagocyte System, a population of cells, derived from bone marrow progenitors, that differentiate, enter the blood as monocytes and then the peripheral tissues to become resident macrophages or antigen presenting cells (Van Furth and Cohn, 1968).

Monocyte half-life in the blood is of about 1 day. This observation has fostered the concept that blood monocytes replenish macrophage or dendritic cell (DC) pools in peripheral tissues to maintain homeostasis (Ziegler-Heitbrock, 2000). This loop, by which tissues control the size and distribution of their macrophage populations, becomes evident when acute events, such as injury or infection occur.

Inflammation restricts the growth of invading microbes and guides, when the pathogen has been eliminated, its healing. Macrophages in particular represent an active link between innate and adaptive immunity, by regulating T lymphocyte activation and possibly shaping their polarization and function. The pioneering work on the role of T-cell-dependent protective autoimmunity in the healing of sterile spinal cord injuries makes this contention particularly cogent (Schwartz and Ziv, 2008; Shechter et al., 2009).

The role of macrophages is non-redundant. Depletion in the spleen of marginal zone macrophages, which interact with apoptotic material entering from the circulation, accelerated autoimmunity in mice genetically prone to systemic lupus erythematosus and caused significant mortality in wild-type mice repeatedly exposed to apoptotic cells (Mcgaha et al., 2011). Accumulation of apoptotic cell material *per se* triggers acceleration of systemic lupus erythematosus (Bondanza et al., 2003, 2004; Munoz et al., 2010). Macrophages recognize tags expressed by apoptotic cells: as a consequence they on one hand dispose of potentially reservoirs of autoantigens; on the other hand secrete regulatory cytokines that contribute to maintain self-tolerance (Manfredi et al., 2009; Elliott and Ravichandran, 2010). The clearance function of macrophages is crucial to limit the actual cross-presentation of apoptotic cell antigens and possibly to modify the cytokine environment in which the productive T-cell activation take place, thus favoring the establishment of protective, or at least not directly damaging, immune responses (Acharya et al., 2010; Elliott and Ravichandran, 2010; Brereton and Blander, 2011; Peng and Elkon, 2011).

Macrophages also support matrix remodeling and neoangiogenesis and have been implicated in conditions in which neoangiogenesis is potentially deleterious, including cancer (Qian and Pollard, 2010) but also non-neoplastic conditions, such as rheumatoid arthritis or endometriosis (Barrera et al., 2000; Bacci et al., 2009; Capobianco et al., 2010, 2011). All-together the data strongly support the ability of macrophages to perceive ongoing injury of various tissues, and to activate homeostatic programs that through the clearance of dying cells, the organization of neovessel generation, the regulation of the extracellular matrix remodeling and the activation of appropriate T lymphocyte responses leads to the effective healing.

Macrophage activation is clearly protective in the case of intense, short lasting injuries. In contrast, macrophage action sustaining regenerative and vascular responses can be deleterious in conditions such as persisting infectious diseases, chronic tissue damage, or event more notably tumors, in which the initial stimulus perceived by macrophages persists. Several excellent reviews have addressed the latter issue (e.g., see Biswas and Mantovani, 2010; Gordon and Martinez, 2010; Mantovani and Sica, 2010; Qian and Pollard, 2010; Squadrito and De Palma, 2011) and we will not discuss the issue further in this essay.

Dedicated pattern-recognition receptors (PRRs) are non-clonally expressed by most innate immune cells (Palm and Medzhitov, 2009). PRRs allow to indentify molecular structures shared by ample classes of microbes, referred to as pathogen-associated molecular patterns (PAMPs; Janeway, 1992). The activation of PRRs results in cascade of tightly coordinated events, including: (i) the production of cytokines and chemokines, which attract and activate leukocytes (Nathan, 2002) (ii) the activation of an acute phase response, with the production of conserved soluble PRRs, such as pentraxins (Manfredi et al., 2008) which tune leukocyte activation and limit their ability to damage the tissue; (iii) the migration of APC to draining lymph nodes, with

productive activation of naïve T lymphocytes. The expansion of antigen-specific T-cells is a key event in the establishment of an adaptive immunological response (Bevan, 2011).

Damage-associated molecular pattern (DAMP), an array of heterogeneous molecules that are released during cell and tissue necrosis, although non-microbial, share the ability to activate PRRs (**Table 1**). Ss a consequence DAMPs recognition elicits inflammation and prompts tissue regeneration and acquired T-cell-dependent immune responses even in the context of sterile injuries (Bianchi, 2007; Lotze et al., 2007; Rubartelli and Lotze, 2007; Urbonaviciute et al., 2008; Bianchi and Manfredi, 2009; Manfredi and Rovere-Querini, 2010; Maroso et al., 2010; Castiglioni et al., 2011; Liu et al., 2011b; Zhang et al., 2011). Macrophages undergo an extensive reprogramming of their functional properties in response to PAMPs (Nau et al., 2002; Martinon et al., 2010), but also to signals released directly from damaged tissues (London et al., 2011) and from lymphocytes (Tiemessen et al., 2007; Wong et al., 2010; Liu et al., 2011a).

Studies with various probes reveal a phenotype heterogeneity in macrophages that possibly reflects peculiar features and function of macrophages sub-populations within the microenvironment. In response to microenvironmental cues, macrophages in the tissue become potent effector cells integrated in a T helper (Th)-1 response, which kill microorganisms and tumor cells and produce copious amounts of cytokines. Microbial destruction is mediated at least partially by the production of reactive oxygen species (ROS) and nitric oxide (NO). The amount of NO produced is instrumental for the ability of macrophages to control the intracellular parasite *L. mexicana* (Mylonas et al., 2009). In humans, classically activated macrophages are important for resistance to mycobacteria and *Leishmania major* infection (Darrah et al., 2007). Macrophages may undergo an alternative activation pathway (referred to as "alternative activation") that endows them with the ability to tune inflammatory responses and adaptive immunity, scavenge debris, and promote angiogenesis, tissue remodeling, and repair (Mantovani et al., 2004).

Macrophages that infiltrate regenerating tissues in general belong to the second class of healing macrophages (Corna et al., 2010; Daley et al., 2010; O'brien et al., 2010; Schwartz, 2010; Brancato and Albina, 2011; Cairo et al., 2011; David and Kroner, 2011; Harel-Adar et al., 2011; Jaeschke, 2011; London et al., 2011; Wang and Harris, 2011), while unrestrained polarization toward a classically activated phenotype associates with defective healing and persistent inflammation (Sindrilaru et al., 2011). Healing (or alternatively activated) macrophages can be propagated *in vitro* by exposure to monocyte precursors to low concentrations of M-CSF in the presence of IL4, IL13, or IL10 (Mantovani et al., 2004). In contrast exposure to microbial components such as LPS in the presence of γIFN or to GM-CSF is an effective stimulus to elicit classically activated, inflammatory macrophages (**Figure 2**).

The dichotomy between inflammatory and tissue healing macrophages represents a "useful over-simplification" (Mantovani et al., 2009) of a sophisticated array of functions exerted by macrophage populations in injured tissues. The plasticity of macrophages in response to environmental cues has been characterized with particular attention in a model tissue, the skeletal muscle (Brunelli and Rovere-Querini, 2008; Chazaud et al., 2009; Tidball and Villalta, 2010).

Table 1 | Inflammatory sterile stimuli and associated diseases (Manfredi and Rovere-Querini, 2010; Rock et al., 2010, 2011; Zhang et al., 2010a; Castiglioni et al., 2011).

Molecule	Associated inflammatory conditions
Asbestos*	Asbestosis: lung inflammation and fibrosis. Mesothelioma and lung cancer
ATP, ADP, adenosine	Airway inflammation
Calcium pyrophosphate	Pseudogout: chronic inflammatory arthritis
Cholesterol crystal	Atherosclerosis: arterial inflammation and occlusion
DNA constituents	Systemic autoimmunity (SLE)
HMGB1	Sepsis, systemic autoimmunity (SLE), rheumatoid arthritis
Matrix constituents (hyaluronate, heparan sulfate, fibronectin, fibrinogen, elastin, and collagen derived peptide)	Idiopathic pulmonary fibrosis, COPD, nephritis, arthritis
Mitochondrial DNA	Trauma, systemic inflammatory response syndrome (SIRS)
Mitochondrial formyl peptides	Trauma, SIRS
Silica	Silicosis: lung Inflammation and fibrosis
Uric acid	Gout: chronic inflammatory arthritis

*via HMGB1 release? See (Yang et al., 2010).

A CASE FOR MACROPHAGES IN THE SKELETAL MUSCLE

Macrophages have been known for a long time to be associated with skeletal muscle injury (Robertson et al., 1993; Mclennan, 1996). Moreover they play a critical role in the pathogenesis and in the natural history of self sustaining muscle diseases, specifically including primary inflammatory myopathies (necrotizing autoimmune myositis, inclusion body myositis and polymyositis) and genetic diseases of the tissue (Duchenne and Becker muscular dystrophies; Dalakas, 2002; Villalta et al., 2009). *In vivo* studies have unequivocally shown that macrophages actually participate in the tissue repair process (St Pierre and Tidball, 1994; Mclennan, 1996; Chazaud et al., 2003; Warren et al., 2005; Summan et al., 2006; Arnold et al., 2007; Tidball and Wehling-Henricks, 2007; Segawa et al., 2008; Ruffell et al., 2009; Sun et al., 2009; Brigitte et al., 2010; Dumont and Frenette, 2010; Martinez et al., 2010; Vezzoli et al., 2010; Lu et al., 2011b). Macrophages, as discussed above, are professional scavengers of apoptotic cells and debris and produce a vast array of signals involved in matrix remodeling and neovessel formation. Data in various models of skeletal muscle injury, including hindlimb ischemia, freeze-injury, unloading/reloading sequences, and myotoxic agent injection indicate

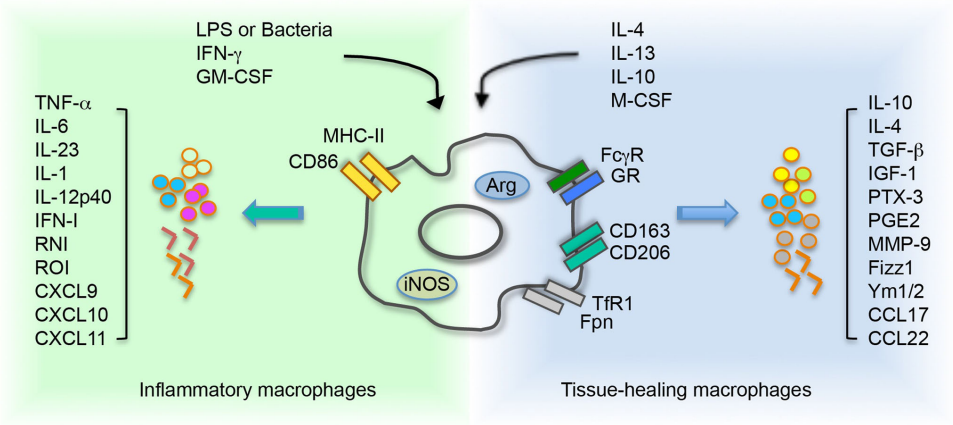

FIGURE 2 | Environment cues control and influence macrophage polarization. Inflammatory macrophage activation is driven by stimulation with LPS or bacteria, and/or IFN-γ or GM-CSF. These molecules induce iNOS activity on macrophages and consequent production of RNI. Treatment with IL4 and/or IL13, IL10 and/or M-CSF supports the activation of tissue healing macrophages. The cytokine network activates an arginase-dependent metabolism of arginine. In macrophages, acquisition of a specific polarization state determines a differential expression of receptors and secretion of molecules. LPS, lipopolysaccharide; IFN-γ, interferon-γ; GM-CSF, granulocyte macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; MHCII, major histocompatibility complex class II; GR, galactose receptor; CD163, haptoglobin–hemoglobin complex receptor; CD206, mannose receptor; TfR1, transferrin receptor 1; Fpn, ferroportin; TGF-β, transforming growth factor β; IGF1, insulin like growth factor 1; PTX-3, pentraxin 3; PGE2, prostaglandin E2; MMP-9, matrix metalloproteinase 9; Fizz, resistin like molecule; Ym1/2, chitinase like protein 1/2; TNF-α, tumor necrosis factor α; IFN, interferon type I; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate.

that the recruitment of macrophages in the tissue occurs regardless of the characteristics of the original *noxa* (Tidball, 2002).

Arnold et al. (2007) identified a population of circulating monocytes selectively recruited in damaged muscle where they acquire a anti-inflammatory phenotype, correlated to tissue healing. Which are the signals involved in the functional polarization of macrophages in the injured/regenerating muscles? The phagocytosis of muscle cells debris is most likely to favor this transition (Arnold et al., 2007).

A macrophage population associated to the epimysial and perimysial connective tissue plays a crucial non-redundant role in monocyte attraction and activation in acutely injured skeletal muscle, providing signals that control their switch to tissue healing macrophages (Brigitte et al., 2010). Resident macrophages also attract in the injured skeletal muscle cells that express the CD11c integrin, a *bona fid*e marker of myeloid DCs. These cells are endowed with antigen presenting capacity and with the ability to migrate from the muscle into draining lymph nodes (Brigitte et al., 2010). They represent attractive candidates to link the response to injury in the tissue to the local activation and recruitment of T lymphocytes, a hallmark of persistent skeletal muscle inflammation.

Activated myogenic precursors also generate chemoattractive signals for inflammatory cells, and their ability to recruit them at the site of muscle injury is further upregulated by the interaction with macrophages (Chazaud et al., 2003). After injury, myogenic precursors, injured fibers, resident macrophages, and recruited monocytes are a source of CCL2/MCP1 (Chazaud et al., 2003; Brigitte et al., 2010; Lu et al., 2011a). Indeed, severe impairments in skeletal muscle regeneration occur in mice defective of the chemokine-CC-motif receptor 2-deficient ($CCR2^{-/-}$), which is activated by CCL2/MCP1 (Warren et al., 2005). CCR2 expression on bone marrow derived cells is essential for robust macrophage recruitment after acute sterile injury and muscle regeneration. Surprisingly, injured muscle of lethally irradiated mice transplanted with CCR2-deficient bone marrow cells contain, despite impaired muscle regeneration, increased numbers of myogenic progenitor cells (Sun et al., 2009), suggesting that macrophages are required for precursor cells to fuse and yield effective myofiber formation. Drastic reduction of macrophage recruitment in injured muscle of $CCR2^{-/-}$ mice associates to a dramatically reduced expression of insulin like growth factor 1 (IGF1), a central regulator of muscle regeneration (Lu et al., 2011b). This observation suggests that macrophages regulate muscle healing through IGF1. Indeed, in $CCR2^{-/-}$ mice, local IGF1 injection at least partially makes up for the lack of recruited macrophages (Lu et al., 2011b).

In vitro, IGF1 elicits in muscle cells a biphasic response, first stimulating cell proliferation and subsequently enhancing myogenic differentiation (Rosenthal and Cheng, 1995), a sequence of events that could be teleologically suited to sustain the repair of damaged tissue. *In vivo*, expression of a muscle specific transgene encoding a locally acting isoform of IGF1 prompts hypertrophy and regeneration in senescent skeletal muscle (Musaro et al., 2001).

THE MUSCLE MICROENVIRONMENT AND THE INFLAMMATORY RESPONSE

The muscle environment and the mechanisms through which it modulates regeneration have attracted much attention in the recent years (Paylor et al., 2011). Several studies have investigated in particular whether similar events occur during the regeneration of adult skeletal muscle and during embryogenesis (Charge and Rudnicki, 2004). The microenvironment in which the two events occur is strikingly diverse: specifically muscle development occurs without any substantial contribution by infiltrating cells,

which are instead present in regenerating muscle at concentrations that exceed 100,000 inflammatory cells/mm^3 of tissue (Wehling et al., 2001; Paylor et al., 2011). Recent evidences suggest that a model in which myogenesis occurs independently of the activation of inflammatory pathway may be far too simplistic: for example, myoblasts of mouse embryos and regenerating myocytes in injured adult mouse skeletal muscle, but not mature myocytes, express the receptor for granulocyte colony stimulating factor (G-CSF). Moreover, the C-CSF/G-CSF receptor pathway is crucial for skeletal myocyte development and regeneration (Hara et al., 2011).

Although several molecules have been identified not to be dispensable for muscle regeneration, the overall array of signals macrophages deliver in the tissue and the hierarchy among them is far from being elucidated. At early stages after acute injury macrophages mostly secrete inflammatory molecules, including CCL2/MCP1 and TNFα, which may favor tissue wasting *via* activation of the FoxO transcription factor (Sandri et al., 2004; Zhao et al., 2007). Simultaneously, they dispose of apoptotic cells and fiber remnants: apoptotic cell clearance has been shown in other systems to trigger the release of cytokines involved in the termination of the inflammatory response and in immune regulation, such as TGFβ and IL10 (Huynh et al., 2002; Zhang et al., 2011; see also below).

At later stages macrophages actively sustain fiber reconstitution (Summan et al., 2006; Arnold et al., 2007; Shireman et al., 2007). At this stage they mainly secrete cytokines that may play a trophic function, such as IGF1 (Musaro et al., 2001; Summan et al., 2006; Pelosi et al., 2007) or IL10 (Strle et al., 2007, 2008; Tidball and Villalta, 2010).

In *mdx* mice, a well-accepted model for human Duchenne's muscular dystrophy, the expression of IL10 modulates macrophage activation and reduces the membrane damage: in this system, IL10 has been proposed to deactivate the inflammatory profile of macrophages infiltrating damaged muscle at the early, acute stage of muscle disease, promoting a switch toward an alternative activation profile (Villalta et al., 2010). At later phases however the persistence of alternatively activated macrophages in conditions in which the tissue can not heal may actually play a deleterious role: for example the sustained production in *mdx* mice of TGFβ may be involved in the fibrotic substitution of the myofibers, a hallmark of the advanced phases of muscular dystrophy: the cytokine indeed is associated to fibroblast activation and proliferation, leading to sustained collagen production and eventually to fibrosis (Vidal et al., 2008).

An exclusive population of progenitors of both fibroblasts and adipocytes has been recently clearly identified in skeletal muscle (Joe et al., 2010; Uezumi et al., 2010), referred to as fibro/adipogenic progenitors (FAP). These cells remain in a quiescent state in normal conditions, undergo a dramatic but transient proliferation in response to injury, contextually delivering trophic signals for proliferating myogenic precursors. Fibrotic scar substitution and accumulation of lipid filled adipocytes is a feature of conditions of the failed regeneration of the skeletal muscle, like it occurs dramatically in muscle dystrophies but at some extent also during physiological aging: when myogenic precursors fail to replace the damaged tissue, FAP would according to recent models (Rodeheffer, 2010; Paylor et al., 2011) take over, differentiating into adipocytes and possibly fibroblasts, thus ensuring the structural continuity of the tissue.

Various stem cell populations, including mesenchymal stem cells (MSC) and neural precursor cells (NPC), regulate the leukocyte fate, through mechanisms involving cell–cell contact and/or various soluble factor. MSC inhibit proliferation of various immune cells, including T, B, and NK lymphocytes (Groh et al., 2005; Krampera et al., 2006; Sotiropoulou et al., 2006; Spaggiari et al., 2006) and specifically influence affect DCs function through the release of IL6, (Djouad et al., 2007), of the Notch ligand Jagged-2, which induces the generation of regulatory DCs (Zhang et al., 2009a) and of prostaglandin E2 (Spaggiari et al., 2009), while NPC restrict the activation of DCs via a BMP-4-dependent-mechanism (Pluchino et al., 2009). MSC also reprogramming macrophages toward an alternatively activated profile (Ohtaki et al., 2008; Kim and Hematti, 2009), which is instrumental in a model of skin wound healing for effective wound repair (Zhang et al., 2010b). The possible cross-talk between FAPs and other precursors of mesenchymal origin and inflammatory cells infiltrating injured skeletal muscle, although demonstrated in other model tissues (Stappenbeck and Miyoshi, 2009; Zhang et al., 2010b; Ehninger and Trumpp, 2011), has not to the best of our knowledge been directly investigated so far.

MACROPHAGES AND MYOGENIC PRECURSORS

In the absence of macrophages injured muscle fail to regenerate (**Figure 1**; see also above), even if the number and function of the cell populations with myogenic potential in the tissue, the eventual effectors of muscle healing that repair or replace injured or dead fibers (Mauro, 1961), are not directly affected. The results indicate that macrophages actively "license" myogenic precursors to carry out their program, i.e., to proliferate, differentiate, and fuse and thus to regenerate the tissue. This is not an isolated feature of the skeletal muscle: macrophages for example sustain the stem cell survival in various tissues, including the skin and the bone marrow, a limiting step for their regeneration (Tothova and Gilliland, 2007; Blanpain and Fuchs, 2009; Discher et al., 2009; Gurumurthy et al., 2010).

It is still not clear at which level(s) macrophages actually specifically act. For example, myogenic progenitor cells were significantly increased in injured muscle of mice with CCR2-defective bone marrow cells even if muscle regeneration is severely affected (Sun et al., 2009). Satellite cells are considered the resident "stem-like" cells in skeletal muscle and are responsible for muscle growth and regeneration in postnatal life (Holterman and Rudnicki, 2005). In response to muscle injuries, quiescent satellite cells undergo activation, proliferate, and fuse with each other or with damaged fibers (Kuang et al., 2008); conversely, some precursors undergo self-renewal, and thus maintain the integrity of the quiescent satellite cell pool (Zammit, 2008; Kang and Krauss, 2010). A pathway strictly associated to the control of neoangiogenesis, which comprises the interaction between angiopoietin 1 (Ang1) and its receptor Tie-2 and the downstream activation of the ERK1/2 kinase, has been in elegant studies implicated in the ability of satellite cells to re-enter the stem cell niche (Abou-Khalil et al., 2009; Mounier et al., 2011), thus suggesting that endothelial cells and possibly other non-muscle cells, are involved in the maintenance of the

satellite cell niche. The observation that even single transplanted satellite cells both differentiate and self-renew after transplantation *in vivo* (Sacco et al., 2008) provides a formal demonstration that satellite cells are indeed endowed with stem cell properties. Telomeres length in muscle cells and the control of muscle stem cell regenerative capacity represents, in particular in the setting of muscular dystrophies, a particular attractive target for the action of inflammatory molecules (Sacco et al., 2010).

Despite the concentrated effort of several groups, our actual insight on the role of the microenvironment in determining the overall outcome of the tissue response to injury is still fragmentary. Recent studies specifically highlight the importance of mechanical factors, such as tissue rigidity/elasticity in regulating the fate of muscle stem cells (Gilbert et al., 2010), revealing important caveats that apply to the *in vitro* systems that are commonly used. Other environmental influences are possibly as relevant: for example, we have recently observed that regeneration after an acute sterile injury of the skeletal muscle is accompanied by the substantial generation of ROS production, which is counterbalanced and rapidly overcome by the generation of antioxidant moieties. Mitochondria are initially responsible for ROS formation while at later time points, non-mitochondrial sources are involved. Both regenerating fibers and macrophages express high levels of free thiols and antioxidant enzymes, such as superoxide dismutase 1 (SOD1) and thioredoxin (Vezzoli et al., 2011). The well-characterized role of a reduced environment in maintaining the extracellular function of DAMP molecules (Lotze et al., 2007; Rubartelli and Lotze, 2007; Carta et al., 2009; Rubartelli and Sitia, 2009), either directly released by damaged fibers of actively secreted by infiltrating macrophages suggests that the antioxidant response directly contributes in the acutely injured tissue to homeostasis.

Preliminary data from our laboratory indicate that macrophages also influence other cells with myogenic potential, such as mesoangioblasts. Mesoangioblasts are vessel-associated progenitors that ameliorate defective muscle structure and function in dystrophic mice and dogs (Sampaolesi et al., 2003, 2006; Tedesco et al., 2010). A clonal analysis of embryonic explanted organ rudiments led to the positive identification of a "mesoangioblast" cell population in the embryonic dorsal aorta (De Angelis et al., 1999) and later studies implicated pericytes associated with microvascular walls in the human skeletal muscle as their human counterpart (Dellavalle et al., 2007). Mesoangioblasts are endowed with the ability to cross the vessel wall, a feature missing in satellite cell-derived myogenic precursors (Dellavalle et al., 2007): when injected into the blood, mesoangioblasts are indeed able to migrate outside the vessel toward injured and inflamed tissues, including the dystrophic muscle (Sampaolesi et al., 2003), possibly following chemotactic signals generated by activated innate cells, macrophages in particular (Lolmede et al., 2009). These features allow a systemic delivery of mesoangioblasts, thus overcoming migration-related problems described for other stem cell populations. In the recent years various tools were investigated to improve mesoangioblast migration toward damaged muscle and their local differentiation to optimize future cell therapy protocols for muscular dystrophies (Tedesco et al., 2010).

Macrophages are important to recruit and locally activate mesoangioblasts, committing them to myogenic differentiation. Conversely, we have observed that mesoangioblasts regulate gene expression of *in vitro* bone marrow derived macrophages (Bosurgi et al., unpublished results). Genes associated to macrophage scavenger function, phagocytic activity, chemokines release, and response to cytokines, which are regulated at the expression level, are all targets of mesoangioblast action. Their regulation is selective, since several other genes associated to macrophage housekeeping functions, to the iron metabolism and to the redox control are unaffected. We are actively verifying the possibility that mesoangioblasts prime the macrophages function toward a regulatory activity and this finely tuned cross-talk regulates the outcome of tissue remodeling.

CONCLUSION

In summary, myogenic precursors derived from satellite cells and other muscle stem cells are the final effectors of muscle regeneration. Substantial evidence indicates that they need licensing by accessory cells, in particular by inflammatory cells such as macrophages. Other mesenchymal precursors expand when muscle regeneration fails, leading to the eventual fibrotic scar and fat replacement of the tissue. The latter event is again possibly dependent on the inflammatory environment of the tissue. The efforts of the next years are likely to break the code by which the immune response controls the regeneration of the skeletal muscle, thus leading to the development of effective targeted therapies for genetic defects of the tissue and for the most common causes of physiological muscle wasting and sarcopenia.

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Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair

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Lung macrophages are long living cells with broad differentiation potential, which reside in the lung interstitium and alveoli or are organ-recruited upon inflammatory stimuli. A role of resident and recruited macrophages in initiating and maintaining pulmonary inflammation in lung infection or injury has been convincingly demonstrated. More recent reports suggest that lung macrophages are main orchestrators of termination and resolution of inflammation. They are also initiators of parenchymal repair processes that are essential for return to homeostasis with normal gas exchange. In this review we will discuss cellular cross-talk mechanisms and molecular pathways of macrophage plasticity which define their role in inflammation resolution and in initiation of lung barrier repair following lung injury.

Keywords: macrophage, lung, inflammation, resolution, repair

INTRODUCTION

Alveolar macrophages are tissue-resident or recruited cells with key functions in recognition of pathogens, initiation of host defense via protective inflammation, and in clearance of pathogens from the airways. Forming the first line of defense toward foreign invaders, alveolar macrophages scavenge and phagocytose pathogens and sense microbial patterns via toll-like receptors (TLRs), NOD-like receptors (NODs), and intracellular helicases like retinoic acid inducible gene I (RIG-I) and other pattern recognition receptors. Upon activation they release early response cytokines such as type I IFN, TNF-α, and IL-1β in an IRF- or NF-κB-dependent way. These cytokines stimulate neighboring alveolar epithelial cells and tissue-resident macrophages in an auto- and paracrine manner to produce a variety of chemokines which in turn mediate the recruitment of neutrophils, and later on, exudate macrophages and lymphocytes to the site of infection, ultimately resulting in clearance of pathogens.

Lung inflammation is not merely terminated when the pathogen is cleared and pro-inflammatory signaling events, previously initiated by recognition of foreign antigen or host-derived alarmins, decline. In fact, resolution of lung inflammation and return to tissue homeostasis is an active, tightly coordinated process which reverses all of the steps involved in initiation of the inflammatory response and induces counter-regulatory mechanisms which terminate these. This process includes cessation of granulocyte emigration from blood vessels, restoration of normal vascular permeability and removal of extravasated fluids, termination of monocyte emigration and induction of their maturation into resident alveolar macrophages, removal of apoptotic neutrophils, and finally, repair of "bystander" injury to restore functional endothelial and epithelial monolayers. Apart from their well-known role in phagocytosis and recognition of foreign antigens it is increasingly recognized that alveolar macrophages are endowed with high functional plasticity allowing them to acquire different pro- or anti-inflammatory as well as tissue-reparative phenotypes during the course of inflammation, dependent on the signals they receive from surrounding cells or from the pathogen itself. The ability to integrate these various signals in the course of inflammation and to mount a differential response empowers the mononuclear phagocyte, either lung resident or recruited, to terminate and resolve alveolar inflammation in the later phases of acute lung injury and to tightly coordinate parenchymal repair processes that are essential for return to homeostasis (**Figure 1**).

CHANGE IN LOCAL LIPID AND MEDIATOR PROFILE INITIATES MACROPHAGE-MEDIATED RESOLUTION OF INFLAMMATION

LIPOXINS

Lipid mediators are key players in termination of pulmonary inflammation and initiation of resolution (Serhan et al., 2008), characterized by an active switch of the lipid mediator profile found at the inflamed site (Levy et al., 2001). During the initial inflammatory response, prostaglandins and leukotrienes, generated from arachidonic acid, an omega-6 polyunsaturated fatty acid (PUFA) by endothelial cells, neutrophils, and tissue-recruited and resident macrophages, amplify inflammation (Funk, 2001). Later on, the prostaglandins PGE_2 and PGD_2, generated in a cyclooxygenase-dependent way, gradually promote the synthesis of lipid mediators with anti-inflammatory and pro-resolving activity, such as the lipoxins. Lipoxins are lipoxygenase-derived double oxygenated eicosanoids which were shown to inhibit neutrophil recruitment to inflamed sites and suppress their pro-inflammatory actions, but promote recruitment of macrophage precursors (Maddox et al., 1997; Chiang et al., 2006). Lipoxin A_4 rapidly stimulates macrophages to phagocytose apoptotic neutrophils (Godson et al., 2000), induces RhoA- and Rac-dependent cytoskeleton re-organization of macrophages (Maderna et al.,

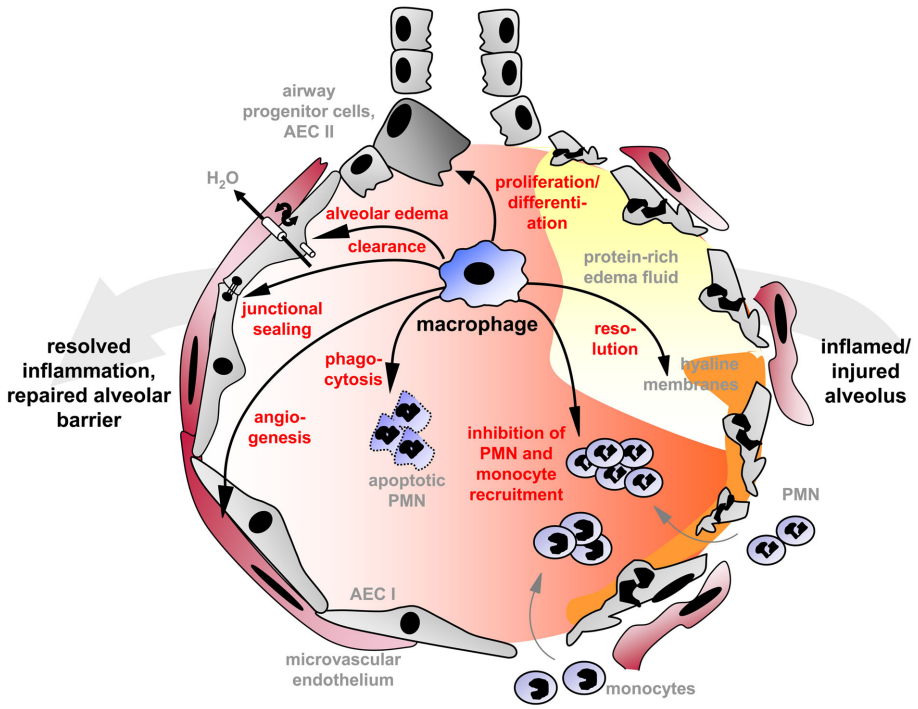

FIGURE 1 | Macrophages terminate and resolve alveolar inflammation after acute inflammatory lung injury and coordinate structural and functional parenchymal repair processes that are essential for return to homeostasis. Inflammation resolution and tissue repair after injury involve a variety of timely coordinated, active processes in which lung macrophages are directly or indirectly involved: Inhibition of granulocyte (PMN) and monocyte recruitment from the circulation, phagocytosis of apoptotic neutrophils or parenchymal cells, removal of fibrin, clearance of alveolar edema fluid, and repair of the endo- and epithelial barrier by junctional sealing and induction of angiogenesis and proliferation/ differentiation of epithelial progenitor cells including type II alveolar epithelial cells (AEC).

2002), and inhibits macrophage CXCL8 release (Jozsef et al., 2002), supporting macrophage-mediated resolution of inflammation. In turn, as a result of engulfment of apoptotic neutrophils, macrophages themselves become a primary source of lipoxins (Freire-de-Lima et al., 2006). With regard to acute lung injury, it was recently demonstrated that Lipoxin A_4 acts as a potent pro-apoptotic signal for alveolar neutrophils, thereby increasing their engulfment by macrophages (El Kebir et al., 2009) and triggering further release of anti-inflammatory agents.

RESOLVINS AND PROTECTINS

Resolvins and protectins represent another class of pro-resolving lipid mediators derived from omega-3 PUFA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA; Serhan et al., 2002, 2008; Ariel and Serhan, 2007). Resolvin (Rv)E_1 binds to the receptor ChemR23 expressed on macrophages and their precursors and attenuate TNF-mediated NF-κB activation, thus activating an anti-inflammatory signaling pathway (Arita et al., 2007). Ligation of the pro-inflammatory leukotriene B_4 receptor BLT1 on leukocytes by RvE_1 has antagonizing, anti-inflammatory effects (Arita et al., 2005, 2007). Similar to the lipoxins, RvD_1 and the related DHA-derived lipid mediator protectin D_1 stimulate clearance of inflammatory infiltrates by macrophage phagocytosis (Schwab et al., 2007). Recently, another anti-inflammatory lipid mediator termed macrophage mediator in resolving inflammation 1 (maresin 1) was identified (Serhan et al., 2009) which is synthesized by conversion of DHA by resident tissue macrophages involving 12/15-lipoxygenase. Similarly to resolvins, maresin 1 was found to decrease neutrophil accumulation while enhancing the recruitment of macrophage precursors to sites of inflammation in a murine peritonitis model. Furthermore, maresin 1 induces the uptake of zymosan particles by macrophages and might therefore promote macrophage uptake of apoptotic neutrophils (Serhan et al., 2009). Recent evidence highlights a crucial role of resolvins in mediating the emergence of a "pro-resolution" $CD11b^{low}$ tissue macrophage subset, which was characterized by a distinct protein expression profile, enhanced apoptotic leukocyte engulfment, unresponsiveness to TLR ligands, and increased emigration to draining lymph nodes (Schif-Zuck et al., 2011). Resolvins were demonstrated to be important players in resolution of chronic (Uddin and Levy, 2011) and acute lung injury, as demonstrated in mouse models of aspiration and intratracheal LPS challenge, by decreasing the pro-inflammatory potential of macrophages via cross-talk with the lipoxin A_4 pathway (Seki et al., 2009; Wang et al., 2011). Increasing the Rv precursor omega-3 PUFA in the transgenic *fat*-1 mouse model likewise attenuated LPS-induced lung injury (Mayer et al., 2009). Acute administration of these PUFA seems to exert beneficial effects on alveolar macrophages and monocytes by decreasing the adhesion and release of pro-inflammatory cytokines like TNF-α. These effects were mediated

in part by platelet activating factor (PAF), another important lipid mediator (Mayer et al., 2002; Schaefer et al., 2007).However, other pathways seem to be operative under chronic exposure as in *fat*-1 mice (Mayer et al., 2009) the TNF-α generation remained unchanged.

CHEMERIN

A similar role was recently ascribed to chemerin-derived peptides. Chemerin is a chemoattractant present in diverse inflammatory exudates. It was identified as a natural ligand for the G protein-coupled receptor ChemR23 expressed by epithelial cells (Campbell et al., 2007), dendritic cells (Vermi et al., 2005), and macrophages (Luangsay et al., 2009). ChemR23 binds RvE_1 and shares phylogenetic homology with other chemoattractant receptors, including those for lipoxin A_4 and the neutrophil chemotaxins C5a and C3a. Recently, another chemerin receptor, GPR1, was identified (Cash et al., 2008, 2010). In a murine model of LPS-induced acute lung injury, chemerin binding to ChemR23 decreased both neutrophil invasion into the lung and pro-inflammatory cytokine generation while increasing recruitment of macrophages (Luangsay et al., 2009).

MACROPHAGES ACTIVELY TERMINATE AND RESOLVE NEUTROPHIL INFILTRATES

TERMINATION OF NEUTROPHIL INFLUX

Apart from initiating neutrophil influx after recognition of pathogens or intrinsic danger signals, macrophages acquire functional profiles which actively terminate neutrophil recruitment. As recently outlined by our group, GR-1^{high}CCR2^{high} exudate macrophages express IL-1ra upon recruitment into the lung parenchyma in LPS- and *Klebsiella pneumoniae*-induced lung injury. Upon blockade of IL-1β actions at the receptor IL-1R1 expressed on alveolar epithelium, macrophage-derived IL-1ra downregulates alveolar release of the neutrophil chemokine MIP-2 and of the epithelial adhesion molecule ICAM-1, attenuating alveolar neutrophil recruitment (Herold et al., 2011). In a model of LPS-induced lung inflammation (Dean et al., 2008), MMP12 that is mainly macrophage-derived cleaves CXC-chemokines within the ELR motif, which is crucial for receptor binding resulting in loss of neutrophil-recruiting activity. MMP-dependent chemokine cleavage also affects CC-chemokines such as CCL7, which may result in dampened inflammation. Similar findings were reported for CCL2, CCL8, and CCL13 following cleavage by MMP1 and MMP3 (McQuibban et al., 2000, 2002).

INDUCTION OF NEUTROPHIL APOPTOSIS

Neutrophils are rather short-lived cells, but once they have reached inflammatory sites they might initially be exposed to survival signals such as G-CSF or IL-1β (Kantari et al., 2008) to prolong their anti-bacterial actions. By providing IL-1β antagonism at the receptor level, it is likely that exudate macrophage-derived IL-1ra might force neutrophil apoptosis (Herold et al., 2011) as a first step to clear the inflammatory infiltrate in the lung. Furthermore, alveolar macrophages are a primary source of TNF-α in different models of pulmonary inflammation (Herold et al., 2006; Cabanski et al., 2008; Cakarova et al., 2009) which, at higher concentrations such as found during human ARDS (Maus et al., 1998; Park et al., 2001), promotes apoptosis of neutrophils (van den Berg et al., 2001). Similarly, resident and GR-1^{high}CCR2^{high} exudate alveolar macrophages were found to highly express the death ligand TRAIL in murine and human influenza and RSV infection (Zhou et al., 2006; Herold et al., 2008; Bem et al., 2010) and TRAIL significantly contributed to neutrophil apoptosis in LPS-induced lung injury (McGrath et al., 2011).

PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS – "FIND ME" AND "EAT ME"

Coordinated removal of apoptotic cells by alveolar macrophages prevents the release of their toxic, tissue-damaging intracellular contents. In contrast to necrosis, apoptosis of neutrophils provides signals to alveolar macrophages to initiate clearance to limit tissue injury and to promote resolution, rather than persistence, of inflammation. First, apoptotic neutrophils advertise their own presence at the earliest stages of death and attract their scavengers via specific "*find me*" signals. Apart from the well-described lysophosphatidylcholine, recognized by the G-protein-coupled macrophage chemotaxis receptor G2A (Peter et al., 2008), these include fractalkine (CX_3CL1), the nucleotides ATP and uridine 5′ triphosphate (UTP), S19 ribosomal protein dimer, split tyrosyl-tRNA synthetase, thrombospondin 1, and sphingosine-1-phosphate (S1P; Savill and Fadok, 2000; Ravichandran, 2010; Soehnlein and Lindbom, 2010). Just recently, Pannexin 1 channels were identified as mediators to release nucleotides as "*find me*" signals (Elliott et al., 2009). All of these are capable of attracting macrophages or their precursors, although only fractalkine and nucleotides have been shown to act as "find me" signals *in vivo* (Truman et al., 2008; Elliott et al., 2009). Whereas ATP and UTP are recognized by the G-protein-coupled macrophage receptor P2Y2, the receptor for CX_3CL1, CX_3CR1, defines a GR-1^{low}CCR2^{low} circulating lung macrophage precursor (Landsman et al., 2007) which has been attributed a wound healing and tissue-reparative phenotype similar to the one ascribed to "alternatively activated" macrophages (Geissmann et al., 2010).

Surfaces of dying cells express or allow the access to a number of "*eat me*" signals that replace the native "*don't eat me*" signals such as CD31 or CD47/SIRP-α present on living cells (Janssen et al., 2008). These signals may be membrane-associated (e.g., phosphatidylserine) or are released from intracellular compartments at later stages of programmed cell death (Savill and Fadok, 2000). Macrophages express a variety of receptors that bind either directly to the exposed "eat me" flags or indirectly through bridging molecules. These receptors include a phosphatidylserine receptor, the tyrosine kinase receptor MeR, integrins, scavenger receptors, and complement receptors (Mevorach et al., 1998; Grimsley and Ravichandran, 2003; Li et al., 2003; Greenberg et al., 2006; Miyanishi et al., 2007; Kennedy and DeLeo, 2009). Soluble innate immune pattern recognition proteins identifying non-self or altered-self molecular patterns are found in the immune-privileged surfaces of the lung and serve as bridging molecules. These include ficolins, pentraxins, thrombospondin, sCD14, MFG-E8, natural IgM, collections, C1q, and annexin A1 (Janssen et al., 2008; Kennedy and DeLeo, 2009; Litvack and Palaniyar, 2010). Annexin A1, released from neutrophil granules upon activation, inhibits the recruitment of leukocytes including

inflammatory macrophage precursors, promotes neutrophil apoptosis, and acts on macrophages to enhance removal of dead neutrophils (Perretti and D'acquisto, 2009). In a mouse model of LPS-induced acute lung injury alveolar recruited, exudate rather than resident macrophages were shown to clear apoptotic granulocytes from the airways (Janssen et al., 2008).

INGESTION OF APOPTOTIC NEUTROPHILS CHANGES THE MACROPHAGE PHENOTYPE

Following ingestion of apoptotic neutrophils, macrophages are stimulated to release anti-inflammatory and pro-repair mediators. One of the first studies in this field showed that co-culture of LPS-activated monocytes with apoptotic lymphocytes inhibited monocyte expression of the pro-inflammatory TNF-α and increased the release of the immunosuppressive cytokines TGF-β and IL-10 (Voll et al., 1997). In following studies, ingestion of apoptotic neutrophils by macrophages, more recently termed "*efferocytosis*," had a similar effect on human monocyte-derived and murine alveolar macrophages, inducing the anti-inflammatory mediators TGF-β, PGE_2, and PAF (Fadok et al., 1998; Medeiros et al., 2009). Phagocytosis of apoptotic – but not of necrotic – cells not only prevented these macrophages from killing tissue-resident cells but also triggered the release of growth factors such as vascular endothelial growth factor (VEGF; Golpon et al., 2004) or hepatocyte growth factor (HGF; Amano et al., 2004) being crucial for tissue repair after injury. Impairment of efficient phagocytosis of apoptotic airway cells may therefore contribute to the pathogenesis of chronic airways diseases like COPD, asthma, and cystic fibrosis (Krysko et al., 2010; Mukaro and Hodge, 2011).

The signaling pathways activated during the phagocytosis-dependent induction of an anti-inflammatory macrophage program in the resolution phase of tissue injury were studied in detail (Patel et al., 2007). The anti-inflammatory activity of apoptotic cells lead to an inhibition of the release of pro-inflammatory mediators from phagocytosing macrophages (Voll et al., 1997; Fadok et al., 1998). In contrast, necrotic cells, which are recognized by another distinct mechanism, rather enhance a pro-inflammatory macrophage program (Cocco and Ucker, 2001). Acquisition of anti-inflammatory activity consists in the loss of the pro-inflammatory response to inflammatory stimuli and a shift to an anti-inflammatory profile that is induced by the apoptotic neutrophil. This anti-inflammatory potential is maintained at all stages of neutrophil apoptotic cell death, irrespective of cell membrane integrity (Cocco and Ucker, 2001; Cvetanovic and Ucker, 2004; Patel et al., 2006). Recognition of apoptotic cells targets the pro-inflammatory transcriptional machinery of interacting macrophages, without apparent effect on proximal steps of TLR signaling. This modulatory activity is exerted directly upon binding to the macrophage and decreases IL-6, IL-8, and TNF-α expression in an NF-κB-dependent way. These effects were dependent on apoptotic cell recognition and independent of engulfment (Cocco and Ucker, 2001; Cvetanovic and Ucker, 2004). Apart from the counter-inflammatory response, phagocytosis (but not mere recognition of apoptotic cells) provides a PI3K/AKT-dependent survival signal to prolong the macrophage life-span to facilitate clearance of neutrophil corpses (Reddy et al., 2002). In contrast, the effects of apoptotic versus necrotic targets on the MAPK pathway depended on recognition. Exposure to apoptotic cells strongly inhibited phosphorylation of ERK1/2 but induced activation of JNK1/2 and p38, a process which did not require phagocytosis. Exposure to necrotic cells stimulated proliferation and activated ERK1/2 (Reddy et al., 2002; Patel et al., 2006).

MECHANISMS AND EFFECTS OF ALTERNATIVE MACROPHAGE PROGRAMMING

MACROPHAGE SUBSETS AND POLARIZATION

Pathogen elimination and restoration of homeostasis following infection and tissue damage requires resident tissue macrophages and a coordinated mobilization of two circulating precursor subsets defined according to lineage marker and chemokine receptor expression in mice, namely the GR-$1^{low}CCR2^{low}CX_3CR1^{high}$ and the GR-$1^{high}CCR2^{high}CX_3CR1^{low}$ peripheral blood monocytes. GR-$1^{low}CCR2^{low}CX_3CR1^{high}$ monocytes patrol the resting vasculature, populate normal or inflammatory sites CX_3CR1-dependently, and participate in resolution of inflammation and tissue repair (Auffray et al., 2007; Geissmann et al., 2010). GR-$1^{high}CCR2^{high}CX_3CR1^{low}$ monocytes are predominantly inflammatory and migrate to injured and infected sites. In humans, most monocytes are $CD14^{hi}CD16^{-}$ and are referred to as "classical" monocytes, whereas $CD14^{+}CD16^{+}$ monocytes are referred to as "non-classical" monocytes. CCR2 and its major ligand, CCL2, are evidently important in both emigration of these cells from the bone marrow into the blood stream and their immigration into inflamed tissues, where they undergo differentiation into macrophages that are categorized as either classically activated (CAM, M1) or alternatively activated (AAM, M2; Benoit et al., 2008; Martinez et al., 2008; Gordon and Martinez, 2010). Several genes define CAM and AAM, e.g., *inos*, *tnf*, *il*-12, and *arg*1, *ym*1, *ym*2, *fizz*1, *mrc*1, *ccl*22, respectively, although a clear-cut association of those genes with the functional profile of the respective subset is lacking, except for Fizz1, also known as RELM-α (Nair et al., 2009). The M1 program is associated with release of pro-inflammatory mediators such as iNOS-derived NO, TNF-α, IFN-γ, and IL-12 and critically contributes to pathogen elimination (Benoit et al., 2008; Serbina et al., 2008). In contrast, AAM, which secrete anti-inflammatory cytokines like IL-1ra, IL-10, and TGF-β, downregulate IL-12, upregulate scavenger receptors, promote angiogenesis, and support wound healing and tissue remodeling (Mosser and Edwards, 2008). They are renowned for their heterogeneity and plasticity, which is reflected by their further subdivision into M2a, M2b, and M2c subsets (Mosser and Edwards, 2008; Ricardo et al., 2008; Gordon and Martinez, 2010).

SIGNAL INTEGRATION IN THE SHAPING OF PULMONARY MACROPHAGE PHENOTYPES

As a key component of the inflammatory response that determines lung tissue destruction or recovery, increasing evidence suggests that pulmonary macrophages do not remain committed to a single activation profile. They may regress to a resting state and can subsequently be reactivated with a different polarization. Functionally distinct subsets of macrophages may exist in the same tissue and play critical roles in both initiation and recovery of inflammation. Therefore, the origin and activation state of the macrophages and the microenvironment, in which they reside, are

critical determinants of their response to lung injury. The heterogeneity of macrophages, their diverse role in lung inflammation and tissue remodeling, and the coordinated activation and programming by other inflammatory and parenchymal cells are not fully understood. However, it becomes increasingly evident that cross-talk of various signals at different levels impinges on the generation of functional macrophage programs, with a variety of signals being integrated to shape a distinct phenotype at a defined stage of inflammation.

With respect to lung inflammation and injury, several of those signal steps have been defined. First, growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) or M-CSF drive differentiation and activation of macrophage progenitors or lineage precursors but also of well-differentiated alveolar macrophages (Berclaz et al., 2002, 2007; Baleeiro et al., 2006; Ballinger et al., 2006). In addition, GM-CSF was shown to induce an M1 phenotype (Krausgruber et al., 2011). Second, at the stage of transendo/epithelial recruitment to the airspace, macrophages obtain signals from chemokines or CAMs (Srivastava et al., 2005). Then, at the site of inflammation, macrophages are primed by cytokines like IFN-γ (M1) or IL-4 and IL-13 (M2), or via Th2 cell-expressed IL-25 and IL-33 (M2; Gordon and Martinez, 2010). Next, PAMPs or DAMPs deliver signals via TLR, NLR, or other pattern recognition receptors. Exposure to LPS promotes the differentiation toward M1-like cells, whereas addition of further cytokines differentiates them toward M2-like macrophages (Martinez et al., 2008; Cabanski et al., 2009; Arora et al., 2011). Recently, a critical role for type I IFN/IFNAR signaling in differentiation of peripheral blood monocytes toward defined lung macrophage phenotypes, either classical or alternative, with different functions in control of alveolar inflammation, was demonstrated in influenza virus-induced lung injury (Seo et al., 2011). Later on, phagocytosis of apoptotic neutrophils by macrophages may add on these signals and support an anti-inflammatory, resolving and tissue-reparative phenotype with release of IL-10, TGF-β, VEGF, and HGF, as outlined above. IL-10 and TGF-β, for example, were shown to be protective in *P. aeruginosa*- (Buff et al., 2010) or LPS-induced lung injury (D'alessio et al., 2009) by abrogating alveolar neutrophil recruitment and by mediating counter-inflammatory effects of $CD4^+CD25^+FoxP3^+$ regulatory T cells, respectively (**Figure 2**).

With respect to the role of macrophage phenotypes in acute lung injury, the M1 program clearly correlates with pathogen

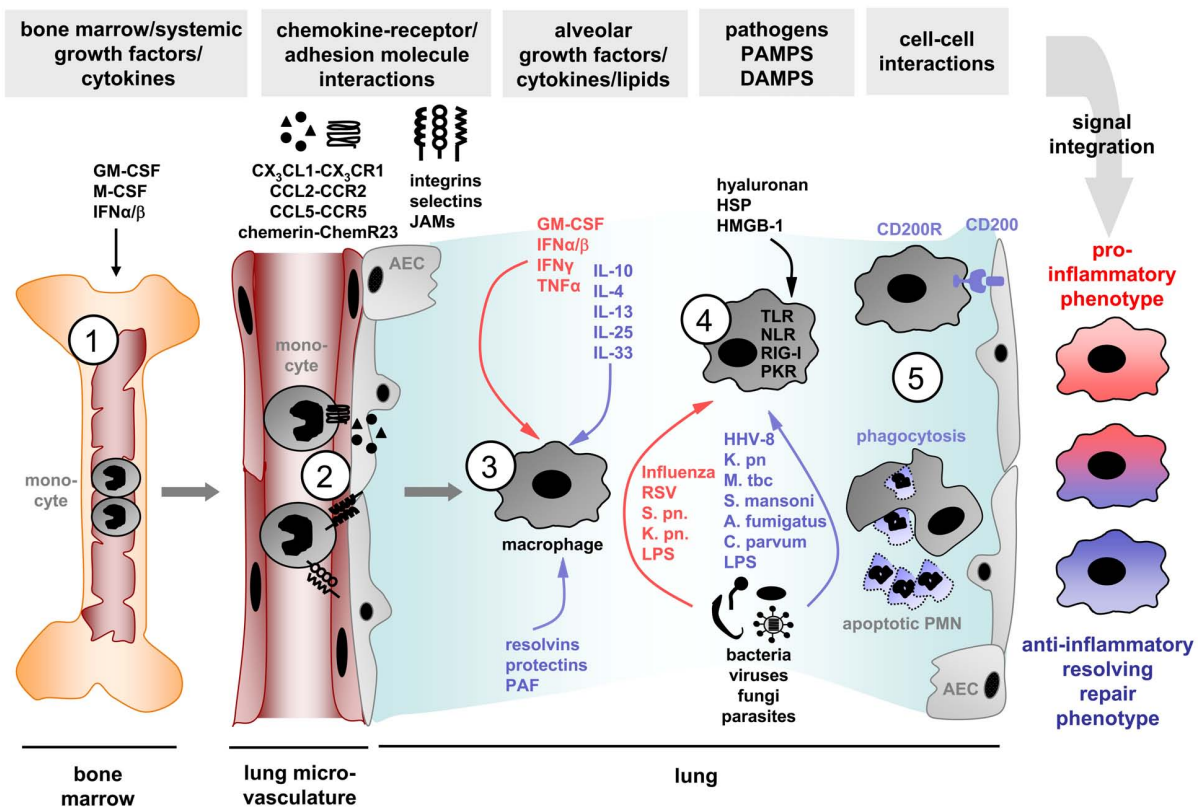

FIGURE 2 | Different extracellular signals are integrated to shape pulmonary macrophage phenotypes during lung inflammation. First, growth factors such as GM-CSF, M-CSF, or type I interferons (IFN) drive differentiation and activation of macrophage progenitors or lineage precursors (1), second, macrophages obtain signals from chemokines or cellular adhesion molecules upon transendo/epithelial recruitment to the alveoli (2), third, macrophages receive signals from cytokines like GM-CSF and interferons (M1) or IL-4, IL-13, IL-25, or IL-33 (M2) (3); fourth, pathogens, PAMPs, or DAMPs deliver signals via TLR, NLR, or other pattern recognition receptors (4). Later on, cell–cell communications during phagocytosis of apoptotic neutrophils (PMN) or via CD200–CD200R interaction with AEC add on these signals and may support an anti-inflammatory macrophage phenotype (5). JAMs, junctional adhesion molecules; HSP, heat shock proteins; HMGB-1, high mobility group box-1; S. pn., *Streptococcus pneumoniae*; K. pn., *Klebsiella pneumoniae*; AEC, alveolar epithelial cells.

clearance, but as well with inflammation and mortality, e.g., after *S. pneumoniae* infection (Smith et al., 2007). A mouse model of Sendai virus infection demonstrated that NKT cells produce IL-13 through a CD1d-glycolipid-dependent mechanism, initiating pulmonary M2 amplification in late stages of infection, when virus had already been cleared from the lungs (Kim et al., 2008). Similarly, macrophage-derived IL-13 induced an M2 phenotype via STAT6 in an autocrine way upon RSV-induced lung injury (Shirey et al., 2010). GR-1^{high}CCR2^{high} exudate macrophages, which are tissue-recruited in LPS- or *K. pneumoniae*-induced lung injury, express high levels of IL-1ra, a classical M2 marker (Benoit et al., 2008) which directly antagonizes IL-1β derived from (M1 activated) resident alveolar macrophages, thereby exerting anti-inflammatory and tissue-protective effects. Adoptive transfer studies using IL-1ra$^{-/-}$ monocytes revealed that IL-1ra-expressing, M2 polarized exudate macrophages reduced alveolar epithelial cell damage and increased pulmonary barrier function (Herold et al., 2011).

These data suggest that, in the inflamed lung, different macrophage phenotypes are induced by distinct signals at defined time points to fulfill discriminative tasks during infection, resolution, and repair. Moreover, these differentially programmed macrophage populations cross-talk during the time course of acute pulmonary inflammation. While resident versus recruited macrophages were found to be differentially polarized (Herold et al., 2011), the question arises to what extent a functional macrophage program might be lineage-confined in monocyte/macrophage subsets (Nahrendorf et al., 2007).

TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF MACROPHAGE POLARIZATION

The signal transduction pathways and transcription factors involved in macrophage polarization, especially with respect to lung inflammation, are still incompletely understood. Activation of the transcription factor NF-κB subunit p50 has been associated with the inhibition of M1-polarizing genes including IFN-β *in vivo* (Porta et al., 2009), whereas induction of the basic region-leucine zipper transcription factors CREB and C/EBPβ has been shown to upregulate M2 genes in macrophages, which promoted tissue repair after injury (Ruffell et al., 2009). Subsequent data suggested that in mice, an IRF4-dependent pathway initiates an M2 program by stimulating the expression of M2-specific markers (Satoh et al., 2010). In contrast, M1 macrophages were characterized by increased expression of IRF5, which was induced by GM-CSF during their differentiation. Forced expression of IRF5 in M2 macrophages drove M1-specific cytokines, chemokines, and costimulatory molecules and led to a potent Th1–Th17 response, whereas induction of M1-markers was impaired in *irf5*$^{-/-}$ macrophages (Krausgruber et al., 2011). Together with the data of Satoh et al. (2010) these findings establish a new paradigm of IRF5–IRF4 balance mediating M1–M2 polarization. Liao et al. (2011) identified Krüppel-like factor 4 (KLF4) as a critical regulator of macrophage polarization. KLFs represent a large family of transcription factors involved in development, differentiation, and activation of leukocytes. Macrophage KLF4 expression was robustly induced in M2 macrophages and strongly reduced in M1 macrophages, and was found to cooperate with Stat6 to induce an M2 genetic program and inhibit M1 targets via sequestration of coactivators required for NF-κB activation. KLF4-deficient macrophages demonstrated enhanced pro-inflammatory gene expression and increased bactericidal activity. Whether these transcriptional programs are operative during processes of lung macrophage polarization in lung infection, injury, and repair, however, remains to be established.

With respect to epigenetic control of macrophage polarization, reports showed that induced M2 signature genes of IL-4-treated mouse macrophages like *arg*1, *ym*1, *fizz*1, *and mrc*1, revealed reciprocal changes in histone H3K4 and H3K27 methylation (Ishii et al., 2009). These modifications depended on STAT6 activation, which bound to the demethylase Jmjd3 promoter, contributing to decreased H3K27 methylation, as well as to transcriptional activation of M2 marker genes. Moreover, the kinase AKT regulated LPS-induced microRNA in macrophages and was implicated in LPS tolerance. AKT1 and AKT2 isoforms thereby had differential effects on TLR4 and SOCS1 signaling in macrophages, depending on the microRNAs let7e and miR155 (Androulidaki et al., 2009). A possible effect of IL-4 and IL-13 on AKT isoforms has not been reported. Other studies have linked microRNA-dependent regulation with macrophage activation programs (Taganov et al., 2006; Tili et al., 2007).

AIRWAY EPITHELIAL–MACROPHAGE CROSS-TALK CONTROLS MACROPHAGE RESPONSES

Recently, a new concept of the pulmonary "*innate immune rheostat*" arose from findings by Snelgrove et al. (2008) demonstrating that the phenotype of airway macrophages depends on the fine-tuned balance between negative regulatory pathways and those that amplify immunity (Snelgrove et al., 2008; Wissinger et al., 2009). As innate immunity at lung surfaces requires restraint to prevent inflammation to innocuous antigens or commensals to guarantee gas exchange, the threshold above which airway macrophages become activated must be increased by local factors. Furthermore, excessive and prolonged pathogen-induced inflammation has to be controlled to resolve infiltrates after pathogen clearance and to prevent collateral lung tissue damage. Data from an influenza virus pneumonia model demonstrated that one such key regulator is CD200R, transmitting a suppressive signal and critically regulating activation of airway macrophages on which it is expressed at high levels. CD200R levels are maintained by epithelial expression of IL-10 and TGF-β. Its ligand, CD200, is exposed on the apical side of the airway epithelium and limits alveolar macrophage-mediated inflammation. *Cd*200$^{-/-}$ mice displayed increased pro-inflammatory macrophage activity and enhanced sensitivity to influenza infection, with delayed resolution of inflammation and increased mortality. These data suggest that macrophage pro- versus anti-inflammatory phenotypes are under tight control of nearby airway epithelial cells during the course of infection, which on one side represent primary targets for infection (that has to be effectively cleared by a mounted immune response) but on the other side have to maintain lung barrier integrity and organ function. Epithelial–macrophage cross-talk by soluble and surface-expressed factors therefore seems to be an important mechanism to keep the balance between efficient host defense and excessive inflammation and injury during pneumonia.

LUNG MACROPHAGES IN TISSUE REPAIR AND REMODELING

REPAIR OF THE AIRWAY EPITHELIUM

Acute lung inflammatory diseases or infection and the following innate and adaptive host responses leave a damaged alveolar endo/epithelial barrier. Alveolar epithelial cell apoptosis was found as major underlying cause of severe lung parenchymal damage in sterile or infectious lung injury (Albertine et al., 2002; Herold et al., 2008; Ma et al., 2010; Budinger et al., 2011). Re-epithelialization (given an intact basement membrane) and endothelial re-sealing following bronchoalveolar injury is considered as critical step to re-establish normal gas exchange conditions in the lung. Alveolar epithelium is comprised of two morphologically and functionally distinct cell types, alveolar epithelial cells type I (AEC I) and type II (AEC II). The flattened AEC I, covering a high percentage of the alveolar surface, are presumed to be terminally differentiated and exhibit a very limited potential to divide, features that make them particularly susceptible to irreparable damage (Fehrenbach, 2001; Tesfaigzi, 2003). The smaller, cuboidal AEC II retain progenitor cell properties and therefore, together with the $CCSP^+$ Clara cells, represent a distal transit-amplifying cell pool (Stripp, 2008; Rock and Hogan, 2011). AEC II are assumed to play a central role in alveolar repair processes after injury by trans-differentiation into AEC I. This involves tightly regulated alveolar epithelial cell proliferation, migration, and polar differentiation with restoration of junctional structures (Fehrenbach, 2001). More recently, studies in mice revealed that endogenous airway epithelial progenitor cells are located within the adult lung in the basal layer of the upper airways, or within bronchoalveolar junctions.. These cells, termed bronchoalveolar stem cells (BASCs) and expressing both AEC II and Clara cell properties, are defined as $EpCam^{high}$ $CD104^+$ integrin $\alpha6\beta4^+$, are resistant to damage, proliferate after injury *in vivo*, are multipotent in clonal assays *in vitro* and give rise to different ciliated and non-ciliated epithelial cell populations of the distal lung (McQualter et al., 2010; Chapman et al., 2011). In humans, recent data define lung stem cells as positive for c-kit (Kajstura et al., 2011), whereas others found them included in the $p63^+ck5^+$ basal cell pool (Whitsett and Kalinichenko, 2011). Following acute lung injury, in accordance with data obtained from a rat model (Berthiaume et al., 2006), we demonstrated that alveolar repair processes in terms of AEC II proliferation were initiated 4 days after LPS instillation, when alveolar inflammation decreased virtually to baseline levels. However as a first step, trans-differentiation of existing AEC II into AEC I might occur fast and precedes AEC II proliferation peaking at 48–96 h post injury. We were able to delineate this feature from the notion that alveolar leakage was associated with AEC I apoptosis and declined upon recovery of the AEC I pool (Cakarova et al., 2009).

Since the earliest reports on alternatively activated M2 macrophages, it has been assumed that these cells promote repair of host tissues after inflammation, e.g., by expression of fibronectin 1 (FN-1), the TGF-β-induced matrix associated proteins BIG-H3, and IGF-1, which provide signals for tissue repair and proliferation (Gordon, 2003). However, although involvement of resident or tissue-recruited macrophages in these processes has been demonstrated for several organ systems like liver, skin, heart, kidney, and gut mucosa (Duffield, 2003; Takaba et al., 2010; Harel-Adar et al., 2011; Lee et al., 2011; Lu et al., 2011; Mahdavian Delavary et al., 2011), studies demonstrating a direct contribution of macrophages in lung epithelial regeneration after injury, e.g., by using macrophage depletion strategies, are lacking.

Several studies at least indirectly suggest that these cells are similarly involved in repair of the injured lung. In this regard, the cytokines keratinocyte growth factor (KGF, FGF7), VEGF, epidermal growth factor (EGF), heparin-binding EGF-like growth factor, platelet-derived growth factor (PDGF), GM-CSF, fibroblast growth factors 2 and 10 (FGF2, FGF10) were shown to act as potent lung epithelial mitogens (Panos et al., 1993; Melloni et al., 1996; Huffman Reed et al., 1997; Van Winkle et al., 1997; Li et al., 2001; Ray, 2005; Mura et al., 2006; Pogach et al., 2007; Gupte et al., 2009; Crosby and Waters, 2010). Anti-inflammatory or regenerative alveolar macrophages were noted to directly release the epithelial growth factors PDGF, FGFs, HGF, TGF-β, and VEGF following inflammation or lung injury (Melloni et al., 1996; Leslie et al., 1997; Morimoto et al., 2001; Miyake et al., 2007; Medeiros et al., 2009; Granata et al., 2010). Notably, our own studies demonstrate that epithelial repair processes were primed already in the pro-inflammatory phase of acute lung injury and elucidate a key role of alveolar macrophage TNF-α inducing AEC repair via induction of autocrine epithelial GM-CSF signaling (Cakarova et al., 2009). In support of these findings, GM-CSF has been recognized as potent growth factor for AEC *in vitro* and in lung injury models *in vivo* (Huffman Reed et al., 1997; Paine et al., 2003). Furthermore, we demonstrated proliferative effects of the macrophage cytokine MIF (macrophage migration inhibitory factor) which were mediated by the MIF receptor CD74 expressed on AEC II (Marsh et al., 2009). The M2 phenotype-associated cytokines IL-4 and IL-13 stimulated proliferation and migration of both murine and human bronchial epithelial cells (Booth et al., 2001; White et al., 2009). As opposed to data derived from our group, demonstrating a detrimental, tissue-damaging role of pro-apoptotic, highly inflammatory $GR\text{-}1^{high}CCR2^+$ exudate macrophages in murine influenza virus pneumonia (Herold et al., 2008), Narasaraju et al. (2010) argued that HGF produced by this macrophage population may contribute to the resolution of inflammation and regeneration of alveolar epithelium.

RESTORATION OF STRUCTURAL AND FUNCTIONAL LUNG BARRIER INTEGRITY

Successful lung barrier repair after injury is critically linked to the survival of the patient (Ware and Matthay, 2001). Epithelial junction formation during alveolar repair represents a crucial event for restoration of alveolar barrier function. Tight junctions are important to maintain discrete compartments in the lung and tightly regulate the flow of molecules between apical and basolateral compartments, whereas gap junctions permit direct transmission of small signaling molecules between neighboring cells. Transmembrane proteins of the occludin and claudin families are the major transmembrane structural elements of tight junctions. It has previously been shown that alveolar epithelial cells express occludins and zona occludens 1 protein (ZO-1) as part of the tight junctional complex. Tight junctions are highly dynamic structures, whose degree of sealing varies in response to external stimuli (e.g., cytokines) via MAPK, PI3K, and PKC-mediated re-organization of their sub-structures (Gonzalez-Mariscal et al.,

2008). Ganter et al. (2008) previously demonstrated that IL-1β causes alveolar endothelial and epithelial permeability increase via integrin-mediated epithelial TGF-β release which induced phosphorylation of endothelial VE-cadherin and stress fiber formation. Although reports on the role of macrophages herein are limited, there is evidence that IL-1ra-expressing exudate macrophages prevent disruption and disassembly of the tight junctional protein ZO-1 in alveolar epithelial cells by IL-1β antagonism (Ganter et al., 2008; Herold et al., 2011). Macrophage-released growth factors might in turn increase tightness of junctions in airway epithelial cells (Terakado et al., 2011).

To restore normal gas exchange in the alveoli, edema fluid accumulating in the airspaces during lung injury is cleared by active sodium transport via apical membrane epithelial Na+ channels (ENaC). The electrochemical gradient for Na+ influx is maintained by the basolateral Na, K-ATPase. Transport of sodium promotes a transepithelial osmotic gradient, causing water to move passively from the airspaces to the interstitium thereby removing excess alveolar fluid (Morty et al., 2007; Eaton et al., 2009). Infection of the lung epithelium and release of pro-inflammatory mediators such as IL-1β and TNF-α, but as well TGF-β, were shown to inhibit ENaC function (Dickie et al., 2000; Kunzelmann et al., 2000; Chen et al., 2004; Roux et al., 2005; Hickman-Davis et al., 2006; Wolk et al., 2008). Similarly, LPS-stimulated, pro-inflammatory alveolar macrophages were found to decrease ENaC expression and activity (Dickie et al., 2000). In contrast, the epithelial growth factors KGF and EGF upregulated transepithelial sodium transport and increased alveolar fluid clearance in animal models of acute lung injury by affecting the Na, K-ATPase (Morty et al., 2007), and at least EGF was shown to be expressed by lung tissue macrophages *in vivo* (Temelkovski et al., 1997). Moreover, recombinant IL-1ra increased ENaC α and β subunit expression in primary murine and human AEC by antagonizing IL-1β *in vitro*, and IL-1ra-expressing M2-programmed GR-$1^{high}CCR2^{+}$ exudate macrophages similarly reverted IL-1β-induced downregulation of ENaC expression in lung parenchyma in an LPS injury mouse model *in vivo* (Herold et al., 2011).

Macrophages or subsets thereof are involved in neoangiogenesis either by secreting cytokines/growth factors or by providing a physical scaffold fostering endothelial cell fusion. However, signaling pathways activating an angiogenic program in macrophages, especially in the lung, are still poorly defined, and most data derive from *in vitro* studies in the field of tumor angiogenesis. Hence, it was shown that apoptotic cells release the lipid mediator sphingosine-1-phosphate (S1P), which activates S1P1/3 on macrophages to upregulate cyclooxygenase-2. The liberation of PGE_2 then stimulates migration of endothelial cells *in vitro* (Brecht et al., 2011). Other findings demonstrated a role for M2 macrophages in angiogenesis which was linked to release of IL-8 (Medina et al., 2011). In the lung microvasculature, the angiopoietin (Ang)-Tie ligand-receptor system has a key regulatory role in endothelial integrity and quiescence. Whereas Ang-1-mediated Tie2 activation is required to maintain the quiescent state of the resting endothelium, Ang-2 destabilizes the quiescent endothelium and primes it to respond to exogenous stimuli, thereby facilitating the activities of inflammatory mediators, but as well of the angiogenic cytokine VEGF to promote endothelial barrier repair (Fiedler and Augustin, 2006). As outlined above, the ingestion of apoptotic cells results in release of VEFG from macrophages, one of the most important growth factors for endothelial cells (Golpon et al., 2004; Granata et al., 2010). However, although recent data suggest a link between decreased pulmonary VEGF and impaired endothelial barrier function and angiogenesis (Jesmin et al., 2011), a clear role of macrophage-derived VEGF and pulmonary microvascular angiogenesis after injury has not been defined.

LUNG TISSUE REMODELING AND FIBROSIS

Tightly controlled remodeling processes are important to restore tissue homeostasis after injury and involve transient fibroblast proliferation and production and degradation of matrix components. Excessive scarring and tissue fibrosis may result from an imbalanced action of M1 and M2 polarized macrophages after prolonged lung inflammation. Whereas M1 macrophages play a role in resolution of scarring and matrix degradation by release of a variety of anti-fibrotic cytokines such as CXCL10 (Tighe et al., 2011) or of matrix metalloproteinases (Strieter, 2008), M2 macrophages were found to support fibroproliferative tissue remodeling (Meneghin and Hogaboam, 2007; Strieter, 2008) by increased expression of TGF-β, fibronectin, proline, arginase, and tissue inhibitors of metalloproteinase (TIMPs). Prolonged IL-13 effects on alveolar macrophages, as found in several lung infectious diseases (Meneghin and Hogaboam, 2007), promotes the presence of M2-programmed macrophages and, ultimately, excessive fibrogenesis. They also express the pro-fibrotic cytokines IL-10 (Sun et al., 2011), and CCL17 which binds CCR4, and the interaction between these two drives fibrogenesis in several mouse models of lung disease (Meneghin and Hogaboam, 2007). Arginase-1-mediated metabolism of L-arginine in M2 macrophages may result in the formation of L-proline, which is used by myofibroblasts to produce collagen (Strieter, 2008). M2 macrophage numbers were increased in the lungs of patients with idiopathic pulmonary fibrosis (IPF; Prasse et al., 2006; Pechkovsky et al., 2010). Analyses of cellular cross-talk within the lung mononuclear phagocyte system revealed that Gr-1^{+} circulating macrophage precursors directed M2-programmed, pro-fibrotic, tissue-resident macrophages to enhance lung fibrosis in a mouse model of bleomycin injury (Gibbons et al., 2011). Apart from macrophage-mediated fibrogenic tissue remodeling, as often observed after acute or chronic infectious lung disease (Meneghin and Hogaboam, 2007), a role for M2 polarized macrophages was described in similar processes resulting in COPD or pulmonary hypertension (Benoit and Holtzman, 2010; Vergadi et al., 2011). However, although there is increasing interest in the field of macrophage polarization in tissue remodeling, the definite role of the M1/M2 balance in the process of (scarless) alveolar regeneration after acute lung injury/pneumonia is still poorly defined.

CONCLUSION

Although numerous studies clearly demonstrate a crucial role of resident and recruited lung macrophages with an anti-inflammatory, regenerative potential in resolution of pulmonary inflammation and in initiation of tissue repair processes, much remains to be learned about the underlying signaling pathways and

the mechanisms of cell–cell communications herein. Important questions to be answered include the putative cross-talk between these macrophages and other anti-inflammatory, injury-resolving immune cells such as regulatory T cells, or their interaction with local endothelial or airway epithelial progenitors during the process of alveolar regeneration. Further issues to be addressed in future studies concern organ-related plasticity of macrophages *in vivo*, the question whether or to what extent macrophage phenotypes are lineage-confined or induced by an organ-specific inflammatory milieu, and the definition of robust markers for ´beneficial´ macrophage phenotypes in the context of acute lung disease, to ultimately decipher how their polarization can be manipulated to improve the outcome of acute lung disease.

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CRP/anti-CRP antibodies assembly on the surfaces of cell remnants switches their phagocytic clearance toward inflammation

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Systemic lupus erythematosus (SLE) is a chronic inflammatory disease characterized by the production of autoantibodies, formation of immune complexes (IC), and activation of complement that ultimately fuel acute and/or chronic inflammation. Accumulation in blood and tissues of post-apoptotic remnants is considered of etiological and pathological importance for patients with SLE. Besides receptors directly recognizing apoptotic cells, soluble opsonins of the innate immune system bind apoptotic material dependent on the stage of apoptosis. We describe the binding to the surface of secondary necrotic cells (SNEC) of the serum opsonin CRP and further opsonins. We show that anti-dsDNA and anti-CRP autoantibodies bind and sensitize SNEC. Autoantibody-sensitized SNEC were cleared by macrophages *in vitro* and induced a pro-inflammatory cytokine response. In conclusion, anti-CRP, CRP, and SNEC form a ternary pyrogen endowed with strong pro-inflammatory capabilities which is able to maintain and perpetuate chronic inflammation.

Keywords: immune complexes, opsonins, CRP, anti-dsDNA, inflammation, SLE

INTRODUCTION

Accumulation in blood and tissues of post-apoptotic remnants is discussed to be of etiological and pathological importance for patients with Systemic lupus erythematosus (SLE) since the pathognomonic autoantigens are sequestered inside viable cells. They are, therefore, not accessible to the immune system in healthy persons. These autoantigens are often exposed during cell death. The formation of major complexes containing cellular debris and autoantibodies has been observed in patients with SLE and proposed to be an important pathogen (Munoz et al., 2009). Sensibilization of cellular remnants with autoantibodies shifts the phagocytosis of dead cells from liver and spleen to blood–borne phagocytes, which do not take up unsensitized material. This antibody-dependent pro-inflammatory pathway initiates an amplification loop of inflammation and contributes to the chronification of the autoimmune response (Munoz et al., 2010c).

The recognition by macrophages, of dead cell remnants is based on phosphatidylserine (PS) or on sugar neo-epitopes exposed on the plasma membranes (Schlegel and Williamson, 2001; Bottcher et al., 2006; Franz et al., 2006; Ravichandran and Lorenz, 2007). PS is recognized either directly by receptors as a "nude" lipid, or in combination with soluble proteins working as "adaptor molecules" or "opsonins" that bridge phagocytes and dead cells. These opsonins include milk fat globule protein MFGE8 (Hanayama et al., 2002), growth arrest specific gene product GAS-6 (ligand for the receptor tyrosine kinase MerTK; Scott et al., 2001), β-2-glycoprotein-1 (Balasubramanian and Schroit, 1998), serum-derived protein S (Anderson et al., 2003), annexin I (Arur et al., 2003), annexin A5 (Bondanza et al., 2004), C-reactive protein (CRP; Janko et al., 2009), C1q (Taylor et al., 2000), mannose-binding lectin (Nauta et al., 2004), serum amyloid P-component (Manfredi et al., 2008), the long Pentraxin 3 (Rovere et al., 2000), and further phospholipid or carbohydrate recognizing proteins (Franz et al., 2006; Beer et al., 2008; Sarter et al., 2009). The binding of these opsonins is often dependent on the stage of apoptosis, with PS recognition preceding that of carboneoepitopes (Franz et al., 2007). All these molecules mediate recognition and uptake of dying cells by macrophages and act as bridging opsonins that modulate the inflammatory and immunogenic potential of apoptotic material. Some of them additionally participate in complement activation (Ogden and Elkon, 2006).

C-reactive protein binds to damaged cell membranes *via* (lyso)phosphatidylcholine and efficiently activates the classical complement pathway avoiding the assembly of the membrane attack complex (MAC). CRP acts as an opsonin supporting ingestion of apoptotic cells by human macrophages and plays a role in the clearance of apoptotic cells, especially during acute phase reactions (Gershov et al., 2000; Vogt et al., 2007). In the case of primary necrosis, the action of nucleases causes an increase in the binding of CRP to necrotic cells that may foster their silent elimination (Janko et al., 2009).

Generally opsonins for dead cells tend to be anti-inflammatory and ameliorate clearance. Their interaction with its cognate receptor of the phagocytic cell often attenuates inflammatory responses (Voll et al., 1997; Aderem and Underhill, 1999). In patients with chronic inflammatory autoimmune diseases these opsonins for dying or dead cells are often targets of autoantibodies (**Table 1**). Intriguingly, in almost all cases the autoantibodies target only the surface bound opsonins and ignore their circulating counterparts (Shoenfeld et al., 2007; Sjowall et al., 2007; Bigler et al., 2009). The autoimmune response is, therefore, directed against opsonized material and not against the fluid phase proteins (Bell et al., 1998; Shoenfeld et al., 2007; Schaller et al., 2009). The autoantibody-dependent engagement of Fcγ receptors may have devastating consequences since it is prone to shift the process of waste disposal toward inflammation.

Here we describe the exposure of binding sites of anti-dsDNA autoantibodies and of innate opsonins on the surfaces of post-apoptotic remnants and the recognition of these targets by autoantibodies against dsDNA and against surface bound CRP, respectively. The autoantibodies promote the release of inflammatory cytokines by macrophages in both conditions. We postulate that anti-opsonin autoantibodies, opsonins, and (post)apoptotic material form a ternary pyrogen that fuels chronic inflammation in patients with SLE.

MATERIALS AND METHODS

PATIENTS

Thirty nine patients from our outpatient clinic met the classification criteria of the American College of Rheumatology for SLE were included in this study (Hochberg, 1997). Thirty five NHD served as a control population. An informed consent was obtained from all blood donors and the study received the final approval from the ethics committee of the University Hospital Erlangen. Serum samples were obtained by centrifugation at 1000 *g* for 15 min of clotted blood, stored at −20°C, thawed once for ELISA, and stored again until phagocytosis assays.

Table 1 | Opsonins of dead and dying cells as targets of humoral autoimmunity.

Opsonin	IgG autoantibodies reported in
C1q	Siegert et al. (1991)
Protein S	Oosting et al. (1993)
Annexin A5	Matsuda et al. (1994)
β2-GP1	Tincani et al. (1996)
(PS)	Manfredi et al. (1998)
CRP	Sjowall et al. (2002)
MBL	Seelen et al. (2003)
SAP	Zandman-Goddard et al. (2005)
PTX3	Bassi et al. (2010)
Annexin A1	Kretz et al. (2010)
Galectins	Own data

ISOLATION OF CRP

Human CRP was purified from human serum by calcium-dependent affinity chromatography with immobilized phosphorylcholine (Thermo Scientific, Rockford, IL, USA). The monomeric CPR was removed by filtration through Amicon Ultra centrifugation columns (Millipore, MA, USA). The purity of isolated CRP was assured by SDS PAGE and analytical HPLC.

SEROLOGICAL PARAMETERS

Anti-dsDNA autoantibodies were detected by the ability of specific serum antibodies to bind radiolabeled dsDNA employing the Farr method (Wold et al., 1968) adapted by Kredich et al. (1973).

Anti-CRP autoantibodies were detected by ELISA, briefly, NUNC maxisorp 96-well microtiter plates (Nalgene Nunc, New York, NY, USA) were coated overnight at 4°C with 2 μg/well native human CRP in Coating buffer (0.1 M Na2CO3, 0.1 M Na HCo3, pH 9,6) and blocked for 2 h with 1% BSA in PBS. Patient sera were diluted 1:100 in 1% BSA/PBS-0.05% Tween, added in duplicates and incubated for 60 min. After washing with PBS-0.05% Tween, HRP-conjugated rabbit F(ab′)2 anti-human IgG (Southern Biotech) was diluted 1:10000 in PBS-Tween, added to each well and incubated for 60 min. After washing with PBS-Tween, the substrate (1 mg/ml TMB, 0.1 M Na_2HPO_4, 0.05 M Citric Acid, 0.006% H2O2, pH 5) was added to each well and incubated at room temperature. After 15 min the reaction was stopped with 25% sulfuric acid. Optical densities (OD) were measured at 450 nm. To discriminate positive from negative sera a cutoff was set at the mean value of NHD population plus 2 SD.

SECONDARY NECROTIC CELL-DERIVED MATERIAL

Peripheral blood mononuclear cells (PBMC) served as source for secondary necrotic cell-derived material (SNEC). PBMC were isolated from whole blood by Ficoll density gradient centrifugation using Lymphoflot (Bio-Rad, Dreieich, Germany). Remaining platelets were removed by centrifugation through heat inactivated fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany) and PBMC were subsequently reconstituted in PBS (Invitrogen, Karlsruhe, Germany). Finally SNEC was produced by UVB irradiation (180 mJ/cm^2) of PBMC. In order to achieve uniform staining with Trypan blue (0.02%) or propidium iodide (1 μg/ml) in late stages of apoptosis, the lymphocytes were treated with heat (56°C) for 30 min and stored at 4°C in PBS until use. All particles generated by this method showed homogeneous fluorescence staining, scatter, and ligand-binding characteristics detected by flow-cytometry. We further characterized SNEC by analyzing the binding of the following fluorescent labeled dying and dead cell ligands as well as control proteins: BSA, Narcissus pseudonarcissus lectin (Npn), acetylated low density lipoprotein (acLDL), chicken

annexin A5 (AxA5), human CRP. Human complement components C1q and C3c, and human IgG were detected employing fluorescent labeled specific antibodies, as control served heat inactivated human serum and NHD serum, respectively. Fluorescence microscopy pictures were done by staining SNEC with propidium iodide and monoclonal antibodies recognizing dsDNA and apoptotic nucleosomes. In the phagocytosis assays SNEC stained with trypan blue was incubated with patients' sera, respectively for 30 min. Not bound serum proteins were removed by a washing step.

For the evaluation of the role of the opsonin CRP in the formation of ternary complexes, selected anti-dsDNA negative sera from patients with SLE were previously adsorbed 2× against immobilized Phosphorylcholine (Thermo Scientific, Rockford, IL, USA) and SNEC to remove CRP and anti-SNEC antibodies, respectively.

LIGAND SPECIFIC INTERACTION EMPLOYING INERT PARTICLES

We employed Sephadex beads coated with protein G (Pharmacia) to immobilize the anti-dsDNA monoclonal antibody 33.C9 or normal human IgG. Sephadex beads coated with phosphorylcholine were used to immobilize CRP in the presence or absence of calcium. Coated beads were incubated with fluorescent SNEC for 30 min at room temperature and immediately analyzed by fluorescence microscopy.

MACROPHAGE PREPARATION

Leukocytes were isolated from heparinized human peripheral blood by density-gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). Monocytes were enriched by anti-CD14 microbeads (Miltenyi Biotec). Macrophages were generated by culturing $CD14^+$ monocytes for 6 days in RPMI (Biochrome) supplemented with 1% penicillin/streptomycin and 1% glutamine (both Gibco) and 10% heat inactivated FCS (Biochrome), in the presence of 50 U/ml GM-CSF (Behringwerke, Marburg, Germany).

PHAGOCYTOSIS ASSAYS

Phagocytosis assays were performed in two independent experiments with sera from two patients with SLE of each group (anti-dsDNA−/anti-CRP−, anti-dsDNA+/anti-CRP−, anti-dsDNA+/anti-CRP+, anti-dsDNA−/anti-CRP+) or NHD. Phagocytosis assays were performed as follows; macrophages were rinsed with PBS to wash off serum components of the macrophage differentiation medium. Macrophages were co-cultured with trypan blue-labeled SNEC (non-opsonized or opsonized with different patients sera) in a ratio of 1:10 in serum free medium containing 100 ng/ml LPS at 37°C. After 1 h non-internalized SNEC were removed and macrophages detached with trypsin/EDTA. Phagocytosing macrophages were quantified by flow-cytometry.

CYTOKINE QUANTIFICATION

Supernatants from phagocytosis cultures were collected after 18 or 24 h in co-culture with SNEC. Cytokines IL-8, TNF, and IL-10 were quantified by either ELISA (Peprotech) or multiplex bead array technology (Bender Medsystems, Vienna, Austria).

STATISTICS

Association between parameters were evaluated by a bivariate correlation test. Data are presented as mean ± SD, with $n =$ number of independent experiments. Statistical significance was evaluated using a Student's t test. All statistic calculations were done with the software SPSS-Statistics 18.0.

RESULTS

SECONDARY NECROTIC CELLS-DERIVED MATERIAL EXPOSES BINDING SITES FOR SEVERAL OPSONINS

Considering the importance of dsDNA as autoantigen in SLE and the tight interaction between degraded nuclear DNA and CRP, we analyzed its localization in SNEC by flow-cytometry, indirect immunofluorescence, and ligand specific interaction with inert particles. We observed that after execution of apoptosis, peripheral blood lymphocytes contain high amounts of degraded DNA (**Figure 1A**) and show low binding of C3c, high binding of acLDL, NPn lectin, AxA5, C1q, CRP, and IgG from patients with SLE (**Figure 1B**, gray curves). Black histograms correspond to BSA–FITC binding as control for AxA5, CRP, acLDL, NPn lectin; to anti-C3c and anti-C1q staining in the presence of decomplemented serum; and to anti-IgG in the presence of NHD serum, respectively. The binding to SNEC of C1q and of autoantibodies from patients with SLE but not that of CRP was abolished by treatment of SNEC with DNase. SNEC show a random distribution of autoantibody (anti-dsDNA and anti-apoptotic nucleosomes) targets on their surfaces that do not overlap with the nuclear chromatin (**Figure 1C**). To test whether the binding sites for anti-dsDNA and for CRP are not sequestered inside the SNEC but are accessible on their surfaces we employed anti-dsDNA antibodies and CRP immobilized on beads mimicking the curvature of effector phagocytes, respectively. Co-incubation of fluorescent SNEC demonstrates its specific interaction with both immobilized anti-dsDNA and immobilized CRP, respectively. SNEC was captured by beads coated with anti-dsDNA or with CRP but not by those coated with normal human IgG or with CRP-beads in absence of calcium (**Figures 1D,E**). These experiments reveal that (1) CRP binding sites are accessible for CRP and (2) that both dsDNA and CRP bound to CRP binding sites are accessible for autoantibodies and for professional phagocytes on the surfaces of SNEC.

AUTOANTIBODIES AGAINST THE DEAD CELLS OPSONIN CRP ARE FREQUENTLY FOUND IN PATIENTS WITH SLE AND DO NOT CORRELATE WITH ANTI-dsDNA

C-reactive protein interacts with SNEC and may facilitate their phagocytic clearance. SNEC–CRP complexes characterize SLE patients and are seldom found in healthy persons, since apoptotic cells rarely get secondary necrotic in the latter. In the absence of proper clearance bound CRP may itself become a target for the autoimmune response. Therefore, we measured the anti-CRP and anti-dsDNA reactivity in sera from 39 patients with SLE and 35 NHD.

Autoantibodies of the IgG isotype were detected in 61.5 and 2.7% ($p < 0.001$) of the patients with SLE and of healthy donors, respectively (**Figure 2A**). Anti-CRP did not correlate with anti-dsDNA (Farr assay; **Figure 2B**). This allowed us to study the

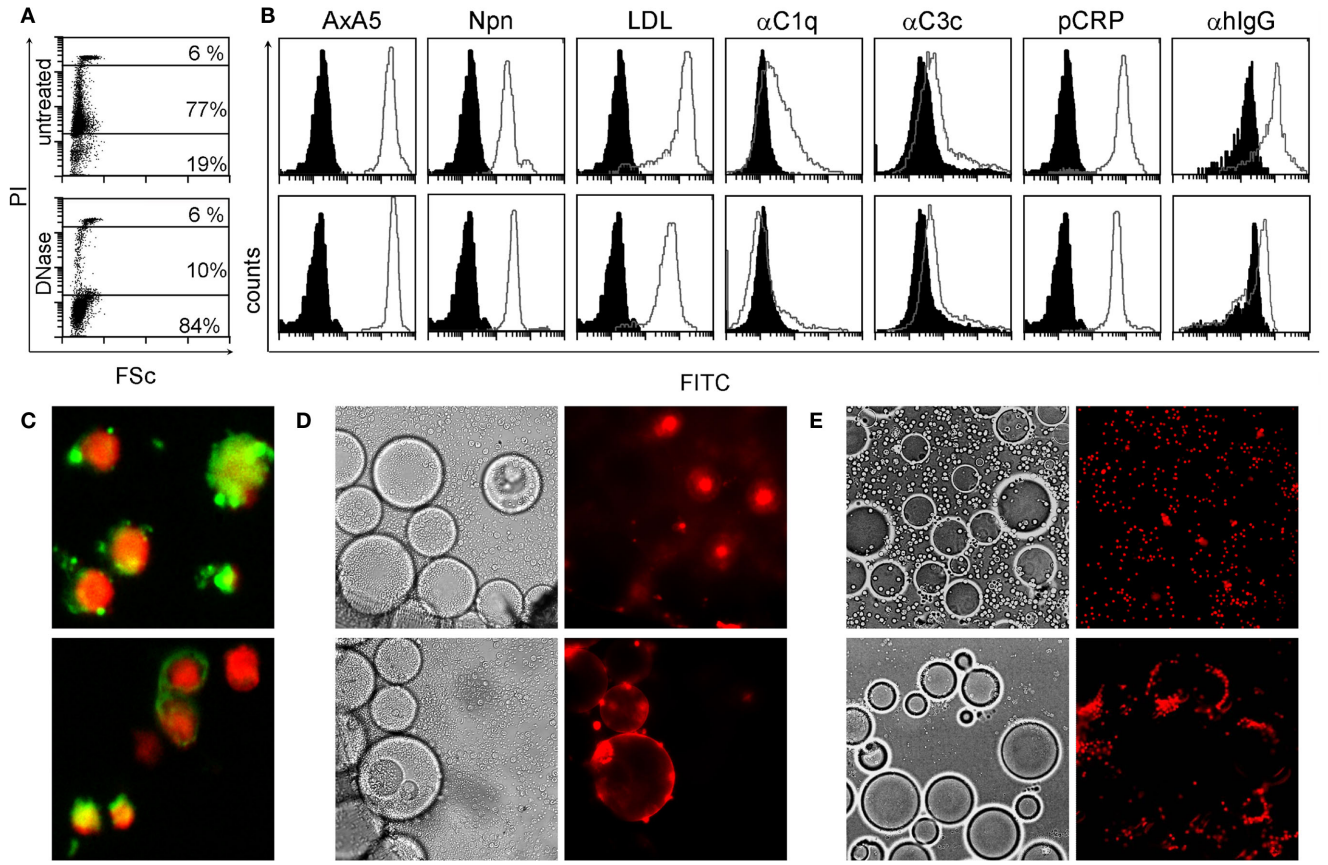

FIGURE 1 | SNEC contain DNA, detected by staining with propidium iodide (PI) (A). SNEC bind AxA5, NPn lectin, acLDL, CRP, serum C1q, and C3c depicted as gray curves as detected by flow-cytometry **(B)**. IgG from patients with SLE sensitize SNEC [**(B)**, right histograms]. BSA–FITC for labeled opsonins, heat inactivated human serum for complement, and NHD serum for SLE serum served as controls, respectively and are shown as black histograms. After treatment with DNAse-1 [**(B)**, lower row], the binding of complement proteins and of IgG from SLE patients is reduced. SNEC expose binding sites for monoclonal antibodies recognizing dsDNA [**(C)**, upper slide] and apoptotic nucleosomes [**(C)**, lower slide]. Binding sites for anti-dsDNA [**(D)** lower row] and for CRP [**(E)**, lower row] are accessible on the surfaces of SNEC and may, therefore, be employed by phagocytes for uptake. Negative controls (see Materials and Methods) are displayed in the upper rows of D/E.

individual contribution of these autoantibodies in the uptake by macrophages of sensitized SNEC and their subsequent cytokine responses.

SENSIBILIZATION OF SNEC WITH AUTOANTIBODIES PROMOTES THEIR UPTAKE BY MACROPHAGES AND FUELS AN INFLAMMATORY CYTOKINE RESPONSE

Macrophages were co-cultured with (I) pure SNEC or (II) SNEC pre-incubated with serum from healthy donors, or (III) sera from patients with SLE. The latter contained (IIIa) neither anti-dsDNA nor anti-CRP, (IIIb) anti-dsDNA only, (IIIc) anti-CRP only; (IIId) anti-dsDNA; and anti-CRP (**Figure 3A**). Treatment of SNEC with autoantibody positive sera results in an elevated uptake by macrophages, whereas phagocytosis of SNEC incubated with autoantibody negative serum does not. Sensibilization of SNEC with anti-dsDNA and anti-CRP antibodies promoted the highest phagocytosis index ($p < 0.05$; **Figure 3B**).

Phagocytosis of apoptotic cells is typically anti-inflammatory. We, therefore, analyzed the cytokine profile of LPS triggered macrophages after the uptake of SNEC in the presence and absence of opsonins. SNEC sensitized with serum of patients with SLE induces an increased production of IL-8 and TNF and a reduced

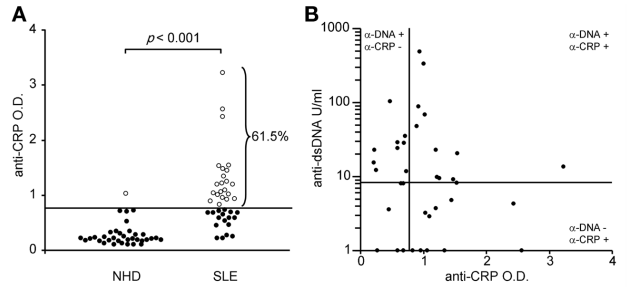

FIGURE 2 | Autoantibodies against CRP are frequently found in sera of patients with SLE (A). Anti-CRP levels do not correlate with anti-dsDNA levels **(B)**.

release of IL-10 when compared with SNEC treated with the serum of healthy donors. **Figures 3C,D** shows the ratios of IL-8 and TNF in relation to IL-10. However, phagocytosis of SNEC targeted with anti-dsDNA and anti-CRP antibodies promoted the strongest inflammatory cytokine response as seen by the highest IL-8 and TNF ratios (**Figures 3C,D**).

SNEC, THE OPSONIN CRP, AND ANTI-CRP AUTOANTIBODIES FORM TERNARY COMPLEXES THAT SHIFT CLEARANCE OF APOPTOTIC CELLS TOWARD INFLAMMATION

To evaluate the role of CRP, of anti-CRP autoantibodies during the clearance process of SNEC, we depleted CRP and anti-SNEC from anti-dsDNA negative/anti-CRP positive sera. These procedures remove the target structure for anti-CRP antibodies as well as other possible anti-SNEC antibodies. Depleted and reconstituted anti-CRP containing sera were used to sensitize SNEC for phagocytosis. Opsonisation with CRP or sensibilization with anti-CRP antibodies or both does not influence the phagocytosis by macrophages of SNEC (**Figure 4A**). However, the cytokine profile measured in the culture supernatants showed a significant higher TNF/IL-10 ratio if SNEC was opsonized with CRP and sensitized with anti-CRP autoantibodies, respectively (**Figure 4B**). The ternary complex of SNEC, CRP, and anti-CRP fosters the pro-inflammatory response of the macrophages.

DISCUSSION

In previous work, we have definitely demonstrated that autoantibodies promote phagocytosis of SNEC by blood–borne phagocytes and suggested that shuttling autoantigens into the intracellular milieu of phagocytes is an important trigger of inflammatory cytokine responses in patients with SLE (Munoz et al., 2009). SNEC–IC containing nucleic acids can be considered as a binary pyrogen able to induce much more damage than its single components apart (Munoz et al., 2010b). Since patients with SLE continuously produce SNEC, we proposed SNEC–IC as a lupus "pathogen" playing a role in the chronification of inflammation in patients with SLE (Munoz et al., 2010c).

In the actual work we have demonstrated that not properly cleared SNEC displays not only dsDNA on its surface but binding sites for several further opsonins. Employing inert particles we showed the presence of binding sites for anti-dsDNA and for CRP on the surfaces of SNEC. These are accessible to be recognized by opsonins, autoantibodies, and finally by phagocytes. CRP reportedly binds to SNEC *via* (lyso)phosphatidylcholine of disturbed membranes (Volanakis and Wirtz, 1979), to nuclear components like histones (Du Clos et al., 1988), and to snRNPs (Jewell et al., 1993). Such DNA-associated proteins become more accessible after DNA degradation during apoptosis and in post-apoptotic cells (Janko et al., 2009).

Anti-CRP autoantibodies have a high prevalence in patients with SLE. This finding has also been reported by several other authors (Bell et al., 1998; Sjowall et al., 2002). The titer of anti-CRP is associated with clinical activity but not with the levels of circulating CRP (Sjowall et al., 2005). It has also been shown that anti-CRP recognizes neo-epitopes of surface bound CRP which displays a monomeric conformation (Bell et al., 1998; Sjowall

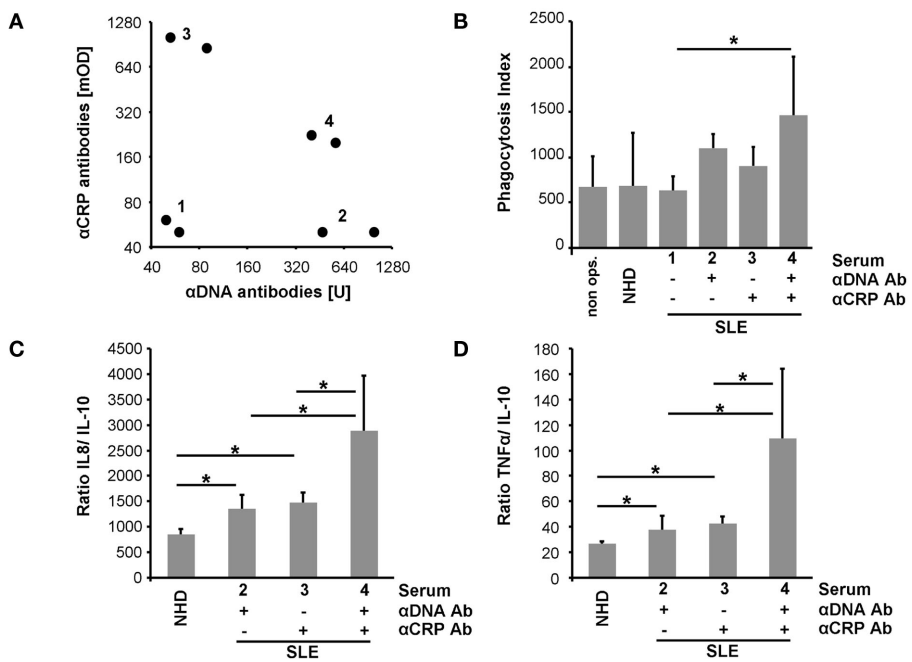

FIGURE 3 | Sensibilization with autoantibodies enhances uptake by macrophages of SNEC and drives an inflammatory cytokine response. Sera selected for phagocytosis assays are shown in **(A)**. SNEC treated with autoantibody positive sera are more efficiently engulfed by macrophages **(B)**. Sensibilization with serum of SNEC induces an elevated macrophage cytokine response upon uptake. In the presence of both anti-dsDNA and anti-CRP phagocytosis of SNEC induces the highest levels of IL-8 and TNF **(C,D)**. This indicates an autoantibody-dependent shift toward an inflammatory cytokine response.

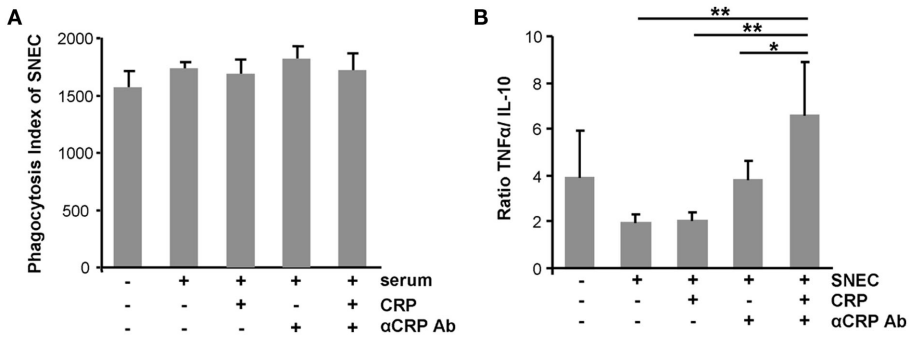

FIGURE 4 | Ternary complexes of autoantibodies and opsonized apoptotic cell remnants provoke inflammation upon uptake by macrophages. The phagocytosis by macrophages of SNEC was not influenced by the opsonisation with CRP or sensibilization with anti-CRP or both when compared to non-opsonized SNEC **(A)**. However, the cytokine profile measured in the culture supernatants showed significantly higher TNF/IL-10 ratio if SNEC was opsonized with CRP and sensitized with anti-CRP **(B)**.

et al., 2002). The levels of anti-CRP and of anti-dsDNA do not correlate as already been shown by Sjowall et al. (2002). The independency of anti-CRP from anti-dsDNA confers target-bound CRP a role as further autoantigen in autoimmune responses of patients with SLE.

In this work we report the ability of sera to sensitize SNEC for phagocytic clearance by macrophages. Enhanced phagocytosis of SNEC by macrophages is observed in all conditions where anti-dsDNA or anti-CRP, or both are present. We previously reported that anti-dsDNA sensitize SNEC and enhance their uptake by blood–borne phagocytes (Munoz et al., 2009, 2010a). Macrophages play a very important role in the swift engulfment of dying cells (Voll et al., 1997). The involvement of these professional phagocytes in the aberrant clearance process may have additional deleterious consequences for patients with SLE, which often show an impaired clearance capability.

During an acute phase response CRP usually increases dramatically. In patients with SLE CRP is not considered as a marker for inflammation – often CRP levels increase only moderately during flares. This is in striking contrast to other rheumatic diseases with comparable amounts of tissue inflammation such as RA and gout (Becker et al., 1980). High IFNα levels, prototypic for SLE flares, have been shown to suppress IL-6 induced CRP levels by human hepatocytes *in vitro* (Enocsson et al., 2009). CRP has early been identified as a component of immune complexes (IC) circulating in the plasma of patients with SLE (Maire et al., 1983). CRP bound to circulating or sessile targets may escape detection, resulting in artificially low values of measurable CRP. In a clearance deficiency scenario where post-apoptotic remnants accumulate, CRP can be massively sequestered by this mechanism. We propose that in patients with SLE dead cell bound CRP is an important target for anti-CRP antibodies compromising the normal clearance process.

The complement system and CRP act together as an important backup mechanism for cells that have escaped the early PS-dependent clearance process (Gaipl et al., 2001). After opsonization, CRP activates complement and facilitates silent clearance by macrophages (Gershov et al., 2000). If complement is low, CRP does not enhance the phagocytosis of late apoptotic neutrophils (Hart et al., 2005). To exclude influences of the serum levels of complement of individual sera we performed the phagocytosis assays in the absence of complement. This argues for an Fcγ-receptor involvement during recognition and engulfment by macrophages of sensitized SNEC. Sensibilization of SNEC with either anti-dsDNA or with anti-CRP present in sera from patients with SLE significantly increased the production by macrophages of inflammatory cytokines. We have shown that SNEC exposes binding sites for anti-dsDNA and for CRP on their surfaces and suggest that autoantibodies recognize these targets and thereby form IC that can be taken up by macrophages in an inflammatory fashion. The maximal enhancement of uptake and inflammatory cytokine production was observed employing sera containing both anti-dsDNA and anti-CRP. This observation suggests that concomitant sensibilization with both autoantibody specificities results in a higher density of antibodies bound to the surfaces of the target particles. The spatial vicinity of the Fc portions is critical to trigger Fcγ-receptor mediated phagocytosis (Allen and Aderem, 1996).

Kenyon et al. (2011) recently showed that anti-C3 antibodies in sera from autoimmune mice inhibited uptake of apoptotic cells by blocking C3 recognition by mouse macrophages suggesting that autoantibodies against the dead cell opsonin C3 may contribute to a further suppression of apoptotic cell disposal increasing severity and/or exacerbations in SLE (Kenyon et al., 2011). On the first view this contradicts our results. However, phagocytosis assays crucially depend on experimental details. In contrast to the settings of Kenyon et al. (2011) our phagocytosis assays were performed under serum free conditions. After opsonisation, SNEC was washed twice and resuspended in serum free medium. Therefore, only molecules bound to the target-cells influenced the outcome of phagocytosis.

We employed anti-dsDNA negative sera from patients with SLE and depleted CRP and anti-SNEC, to analyze if circulating CRP is part of a major IC containing SNEC and anti-CRP. Although the indices of SNEC phagocytosis by macrophages were not increased by opsonisation with CRP and/or sensibilization with anti-CRP, the presence of anti-CRP and SNEC-bound CRP induced a strong TNF response of macrophages. The presence of anti-CRP at the phagocytic synapse formed by phagocytes and SNEC might be

decisive to start the signaling process that results in cytokine production. Our findings that anti-opsonin antibodies shift the clearance toward inflammation are in agreement with previous findings by Rovere et al. (1999) who reported that anti-beta 2-GP1 antibodies bound to apoptotic cells skew their immunogenicity, enabling DCs to present their antigen with higher efficiency and secrete pro-inflammatory cytokines.

The fact that many opsonins for dead cells are also targeted by IgG autoimmune responses (**Table 1**) place them in the "crime scene" during the challenge of the tolerance in germinal centers of patients with SLE (Baumann et al., 2002). Anti-CRP in a patient with persistent deficiency for the early anti-inflammatory clearance of apoptotic cells provides the optimal condition to form ternary pro-inflammatory IC composed of anti-CRP, CRP, and SNEC. The **Figure 5** shows a schematic representation of the usual and the alternative interactions among SNEC, opsonins, and phagocytes. We conclude that sensibilization by autoantibodies of SNEC, either directly or indirectly, shifts the clearance process by macrophages toward inflammation. The role of the individual receptors involved in this alternative and pathological phagocytosis pathway may differ amongst individual patients.

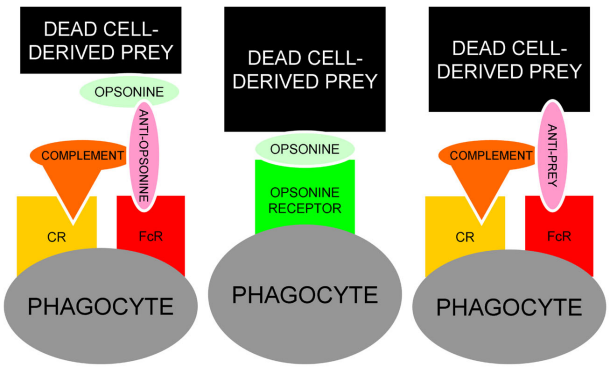

FIGURE 5 | Schematic representation of the usual interaction between SNEC, opsonins and phagocytes (central). In the presence of autoantibodies, alternative models for the recognition by phagocytes of antibody-sensitized SNEC are represented. Anti-opsonin antibodies, e.g., anti-CRP (left) or directly binding anti-SNEC antibodies, e.g., anti-dsDNA (right) may shift the clearance of SNEC toward inflammation engaging the receptors for Fcγ and complement. In addition to autoantibodies against dsDNA and/or opsonins, low complement levels are prone to influence the clearance of post-apoptotic remnants.

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New lives given by cell death: macrophage differentiation following their encounter with apoptotic leukocytes during the resolution of inflammation

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Monocytes that migrate into tissues during inflammatory episodes and differentiate to macrophages were previously classified as classically (M1) or alternatively (M2) activated macrophages, based on their exposure to different fate-determining mediators. These macrophage subsets display distinct molecular markers and differential functions. At the same time, studies from recent years found that the encounter of apoptotic leukocytes with macrophages leads to the clearance of this cellular "debris" by the macrophages, while concomitantly reprogramming/immune-silencing the macrophages. While some of the features of M2 differentiation, such as arginase-1 (murine) and 15-lipoxygenases (human and murine) expression, were also displayed by macrophages following the engulfment of apoptotic cells, it was not clear whether apoptotic cells can be regarded as an M2-like differentiating signal. In this manuscript we review the recent information regarding the impact of apoptotic cells on macrophage phenotype changes in molecular terms. We will focus on recent evidence for the *in vivo* existence of distinct pro-resolving macrophages and the role of apoptotic cells, specialized lipid mediators, and glucocorticoids in their generation. Consequently, we will suggest that these pro-resolving CD11b^{low} macrophages have metamorphed from M2-like macrophages, and modulated their protein profile to accommodate the changes in their function.

Keywords: resolution of inflammation, macrophage differentiation, efferocytosis, pro-resolving lipid mediators

INTRODUCTION

Macrophages are highly plastic monocyte-derived cells that acquire different molecular and functional phenotypes following exposure to different bioactive molecules and environments. The early studies on the interactions of macrophages and lymphocytes in battling bacterial infections revealed the T helper type 1 (Th1) secreted cytokine IFNγ to be involved in the classical activation of macrophages (Nathan et al., 1983). However, seminal studies by the groups of Gordon and Mantovani have extensively characterized additional macrophage subtypes activated in alternative manners (reviewed in Mantovani et al., 2004; Martinez et al., 2009). Since the major polarizing cytokines initially found to be involved in classical and alternative activation were derived from Th1 (IFNγ) and Th2 (IL-4 and IL-13) lymphocytes these activated macrophages were named M1 and M2, respectively. Later studies revealed that in addition to IL-4, alternative activation can also be induced by immune complexes and glucocorticoids (Martinez et al., 2008), and accordingly the subdivision of alternatively activated macrophages to M2a–c was instilled. M1 macrophages are important inducers and effectors in the Th1 response. They are instrumental in immune responses against intracellular microbes and tumors (Mantovani et al., 2005). M2 macrophages are more heterogeneous, but generally play a role in Th2 responses, such as killing and encapsulation of extracellular parasites, resolving type 1 inflammation, and promoting tissue repair and remodeling. M2 macrophages are also playing a role in immune regulation and promote tumor progression (Mantovani et al., 2005; Martinez et al., 2009). M1 and M2 macrophages are not only distinct in function, but also express different receptors and enzymes required for their activities. M1 macrophages express high levels of inflammatory cytokines (IL-12, IL-23, TNFα, IL-1β, and IL-6) and chemokines (CXCL9, 10, and 11, CCL2, 3, 4, and 5, and CXCL2), as well as enzymes involved in the generation of reactive oxygen species (ROS) and nitric oxide (NO; Mantovani et al., 2005). M2 macrophages express lower levels of inflammatory mediators, but high levels of IL-10, scavenger, mannose, and galactose receptors. Importantly, in mice, M2 express the enzyme arginase-1 that intercepts the NO generation pathway [though inducible NO synthase (iNOS)] to generate ornithine and polyamines that are instrumental in tissue repair and fibrosis (Hesse et al., 2001). Hence, the expression of iNOS and arginase-1 are major markers deciphering M1 and M2 macrophages. Additional markers of M2, such as YM1 and FIZZ1, were later identified in mouse macrophages (Raes et al., 2002, 2005).

Macrophages also undergo dramatic molecular and functional changes upon encounter, interaction with, and uptake of apoptotic cells (efferocytosis) during the resolution of inflammation. In this article we will highlight some of the similarities between M2

differentiation and transcriptional events activated by early efferocytosis. In addition, we will discuss recent results that support the notion that efferocytosis can eventually transform macrophages to another phenotype that is postulated to limit tissue repair/fibrosis and promote macrophage regulatory properties at remote sites. In this regard, it is important to note the early studies that indicated "non-phlogistic" activation of monocytes by the pro-resolving lipid mediators lipoxins. This bioactivity of lipoxins resulted in increased adhesion and migration of human monocytes (Maddox and Serhan, 1996; Maddox et al., 1997, 1998) hence prompting the notion that resolution-driven monocyte/macrophage activation promotes tissue repair and wound healing.

EFFEROCYTOSIS AS AN ALTERNATIVE MODE OF MACROPHAGE ACTIVATION

The recognition, engulfment, and responsiveness to apoptotic cells are cardinal properties of resident and inflammatory macrophages and play a role in processes, such as tissue morphogenesis and homeostasis, embryonic development, hematopoiesis, immunity, and the resolution of inflammation (Savill et al., 2002; Erwig and Henson, 2007; Ravichandran and Lorenz, 2007). The recognition and uptake of apoptotic cells by macrophages through "eat me" signals (and the absence of "do not eat me" signals) expressed on their surface and their cognate receptors have been extensively studied and reviewed (Ravichandran, 2011). However, apoptotic cells also transduce signals to the engulfing macrophages that result in significant molecular and functional adjustments that address physiological needs consequent to the identified cell death. During the resolution of inflammation, macrophages engulf apoptotic cells and consequently, apoptotic cell recognition evokes distinct signaling events (Patel et al., 2006) that block the release of pro-inflammatory mediators from macrophages. This release is activated by bacterial moieties, and its blockage, which is termed immune-silencing (Voll et al., 1997; Fadok et al., 1998; Kim et al., 2004), is accompanied by the production of TGFβ and IL-10 (Byrne and Reen, 2002; Huynh et al., 2002; Mitchell et al., 2002), cytokines that can promote resolution and wound repair. The engulfment of apoptotic leukocytes by macrophages also leads to inhibition of iNOS expression and stimulates the expression of arginase-1 in the RAW 264 macrophage cell line (Freire-De-Lima et al., 2006) thereby preventing reactive NO production. In addition, the production of angiogenic growth factors (Golpon et al., 2004) by macrophages is consequent to the uptake of apoptotic cells. Elucidation of the signaling pathways activated by efferocytosis revealed significant roles for nuclear transcriptional regulators, such as peroxisome proliferator activated receptor (PPAR)-γ (Freire-De-Lima et al., 2006; Johann et al., 2006) and -δ (Mukundan et al., 2009) as well as the liver X receptor (LXR; A-Gonzalez et al., 2009) in promoting anti-inflammatory properties.

It is important to note that while macrophages engulf tissue-infiltrating apoptotic PMN during the resolution of inflammation, different experimental models used different sources of apoptotic cells, including Jurkat T cells, mouse thymocytes, or human peripheral blood neutrophils. All types of apoptotic cells express phosphatidylserine on the outer leaflet of their cytoplasmic membrane, and this is apparently the major signaling module used by these cells to communicate their mortal status with phagocytic cells (Ravichandran, 2011). Nevertheless, it is conceivable that other molecules ("eat me signals") are expressed on apoptotic cells of different sources to give a more detailed "report" as to the consequences of their demise. Thus, the interpretation of the results obtained following incubations of macrophages with apoptotic cells of different sources should be evaluated carefully depending on the source of apoptotic cells used.

The prototypic Th2 cytokines IL-4, IL-13, and IL-10, as well as immune responses to parasites were found to promote many of the outcomes of efferocytosis in macrophages. These cytokines are well appreciated antagonists of the M1 response and macrophage pro-inflammatory properties (Martinez et al., 2009) while IL-4 and IL-13 can also promote fibrosis through TGFβ production (Fichtner-Feigl et al., 2006; Wynn, 2008). IL-13 was also found to promote vascular endothelial growth factor production during lung injury (Corne et al., 2000). Importantly, IL-4 and IL-13 also activate PPAR-γ (Huang et al., 1999; Berry et al., 2007) and PPAR-δ (Kang et al., 2008) to promote monocyte/macrophage alternative activation. LXR was recently found to synergize with IL-4 in the induction of arginase-1 expression and promotion of an M2 phenotype in regressive atherosclerotic lesions (Pourcet et al., 2011). Thus, efferocytosis induces phenotypic and molecular switches and activates signaling pathways in macrophages that resemble M2 polarization. Moreover, M2 polarization promotes efferocytosis through induction of different molecular modules, whereas M1 macrophages exert reduced uptake of apoptotic cells. Along these lines, recent studies also found that efferocytosis is a self-promoting process, and that M2 pathways play key roles in mediating this feature of macrophage function. These aspects of efferocytosis are covered by Korns et al. (2011) in this research topic and will not be elaborated on here. Nevertheless, while macrophages are paradoxically involved in both the generation of fibrosis and its resolution (Wynn and Barron, 2010) and efferocytosis and M2 polarization generate a positive feedback loop during resolution of inflammation, it is much less clear what are the events and mediators that stop M2 differentiation and tissue repair/remodeling short of excessive, fibrotic outcomes. Such events and mediators are inevitably required to complete the resolution of inflammation and restore homeostasis rather than end every infection with a debilitating scar.

15-LIPOXYGENASE AND ITS PRODUCTS

A major enzymatic pathway that mediates key events in the resolution of inflammation involves the expression and activation of 12/15-lipoxygenase (LO) in mice and 15-LO-1 in humans. 15-LO expression and activity are upregulated by IL-4 and IL-13 in murine and human monocytes, macrophages, and peripheral blood mononuclear cells (Levy et al., 1993; Nassar et al., 1994; Heydeck et al., 1998; Huang et al., 1999; Ariel et al., 2005). This upregulation leads to the production of 15-LO products from eicosatetraenoic and docosahexaenoic acids (ETA and DHA, respectively), such as 15-hydroxyeicosatetraenoic acid (15-HETE), lipoxin (LX) A_4 and B_4 (5*S*,6*S*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-EPA, and 5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12E-EPA, respectively), 17*S*-hydroxy-DHA (17*S*-hydroxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-DHA), and protectin D1

(10*R*,17*S*-dihydroxy-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-DHA). Macrophage expression of 12/15-LO was found to promote the production of resolvin (Rv) D1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA) and maresin 1 (7,14-dihydroxy-4*Z*,8,10,12,16*Z*,19*Z*-DHA), in addition to LXA_4 and PD1 (Merched et al., 2008; Serhan et al., 2009). The expression of 12/15-LO was also found to be upregulated in mouse macrophages following their incubation with apoptotic cells (Freire-De-Lima et al., 2006; Schif-Zuck et al., 2011) and resulted in the production of 15-HETE and LXA_4 (Freire-De-Lima et al., 2006). Macrophages from chronic granulomatous disease (CGD) mice display impaired efferocytosis that could be repaired by IL-4 through the expression of 12/15-LO and activation of PPAR-γ (Fernandez-Boyanapalli et al., 2009). Hence, 15-LO-mediated signaling seems to be a major convergence point for efferocytosis and M2 polarization, and its down-stream signaling pathways could play a paramount role in deciphering whether macrophages will become pro-fibrotic or will finalize the resolution sequel to restore tissue homeostasis.

Along these reasoning, 12/15-LO products have been shown to be anti-inflammatory and to promote tissue repair, while playing an anti-fibrotic and immune-regulatory role (Serhan, 2010). The major bioactive 12/15-LO products could be produced from arachidonic acid to yield 15-HETE or lipoxins, or from DHA to generate protectin D (PD)1, resolvins of the D series, and the recently identified macrophage product maresin 1 (Serhan, 2010). While 15-HETE binds PPARγ to mediate its anti-inflammatory actions (Huang et al., 1999), LXA_4, PD1, and resolvin D1 seem to act through binding to cell surface GPCRs (Serhan et al., 2011), as well as the aryl hydrocarbon receptor (that binds LXA_4; Machado et al., 2006). All these 12/15-LO products induce a broad spectrum of anti-inflammatory actions on neutrophils and macrophages, as well as other cell types (Wittwer and Hersberger, 2007; Serhan et al., 2011). Lipoxins and PD1 are produced during epithelial injury in the cornea and mediate wound repair in addition to counteracting inflammation (Gronert et al., 2005). On the other hand, 12/15-LO products also induce unique pro-resolving properties of macrophages and promote regulatory pathways in lymphocytes. LXA_4, PD1, RvD1, and PPARγ agonists were all found to promote efferocytosis and enhance PMN clearance during resolution (Godson et al., 2000; Schwab et al., 2007; Fernandez-Boyanapalli et al., 2009; Krishnamoorthy et al., 2010). In addition, PD1 and RvD1 were found to promote macrophage departure of resolving inflammation sites (Schwab et al., 2007; Schif-Zuck et al., 2011). LXA_4 and PD1 inhibited inflammatory cytokine secretion from T lymphocytes (Ariel et al., 2003, 2005) and enhanced CCR5 expression on apoptotic PMN to promote clearance of its pro-inflammatory ligands (Ariel et al., 2006). Moreover, LXA_4 was recently found to play a role in the generation of myeloid-derived suppressor cells (Zhang et al., 2010). Of note, LXA_4, PD1, and RvD1 are potent inhibitors of fibrosis in the lung and kidney (Duffield et al., 2006; Martins et al., 2009; Borgeson et al., 2011). Therefore, 15-LO products can be generated by macrophages following their interaction with apoptotic cells and/or polarization to the M2 phenotype. In turn, these products not only block inflammation but can also shift the macrophage healing balance from tissue repair/fibrosis to pro-resolution, anti-fibrotic, and regulatory functions. The exact mode of production and action for the different 15-LO products is probably dependent on substrate availability, concentration formed in the healing tissue and additional cues from the resolving environment. Nevertheless, they seem to act in concert to promote post-inflammation tissue healing and return to homeostasis.

CD11B^{LOW} MACROPHAGES – A NEW PHENOTYPE GENERATED FOLLOWING SATIATED-EFFEROCYTOSIS

Recent reports have indicated the co-existence of various macrophage phenotypes in resolving peritoneal cavities (Bystrom et al., 2008; Schif-Zuck et al., 2011). Macrophages from resolving murine peritonitis expressed an alternatively activated phenotype albeit with increase expression of M1 markers, such as cyclooxygenase 2 (COX 2) and iNOS (Bystrom et al., 2008). Thus, these macrophages were termed resolution-phase macrophages (rMs) and were postulated to have a hybrid phenotype of classically and alternatively activated macrophages (Bystrom et al., 2008). A recent report from the same group has indicated that rMs could be divided to at least three distinct populations based on F4/80 and Ly-6C expression, with varying expression of additional pro-inflammatory and anti-inflammatory markers as well as CD11b (Stables et al., 2011). Along these lines, we have recently characterized F4/80$^+$ macrophages from resolving peritoneal exudates into two distinct macrophage subtypes: CD11b^{high} and CD11b^{low} (Schif-Zuck et al., 2011). CD11b^{high} macrophages were found to express low to intermediate levels of the M1 markers iNOS, COX 2, and matrix metalloproteinase (MMP)-9 and high levels of the M2 marker arginase-1. These cells also expressed very low levels of 12/15-LO. In addition, these macrophages secret medium levels of inflammatory cytokines and chemokines, as well as IL-10, in response to TLR ligands, are highly phagocytic, and do not migrate to lymphoid tissues. CD11b^{low} macrophages express even lower levels of iNOS, COX 2, and MMP-9 than CD11b^{high} ones, but they also do not express arginase-1. In addition, these macrophages secrete very low levels of inflammatory cytokines and chemokines, and IL-10, but higher amounts of TGFβ. Moreover, CD11b^{low} macrophages, despite containing higher numbers of apoptotic PMN, are no longer phagocytic and are prone to emigrate to remote sites. Hence, CD11b^{low} macrophages were termed "satiated" (Schif-Zuck et al., 2011). A seminal report from Ravichandran and colleagues (Park et al., 2011) has recently revealed that the mitochondrial membrane protein UCP2 controls satiation vs. continued clearance of apoptotic cells, and it would be interesting to examine its role in the generation of CD11b^{low} macrophages. The integration of the results from Schif-Zuck et al., Bystrom et al., and Stables et al. suggests rM/CD11b^{high} macrophages are a mixed macrophage population with dominant M2-like characteristics, and some low-grade M1 activity and that early efferocytosis promotes the conversion of the M1-like population to an M2-like phenotype (Fadok et al., 1998; Freire-De-Lima et al., 2006; Korns et al., 2011) as well as enhanced phagocytosis/efferocytosis. However, the CD11b^{low} subset of macrophages, although converting from the CD11b^{high} subset *ex vivo* and *in vivo* (following late, threshold-meeting, efferocytosis; Schif-Zuck et al., 2011), are not M2-like, but rather display a distinct phenotype with its own molecular and functional characteristic (**Figure 1**). Of interest, a similar series of macrophage phenotype switches was found to take place

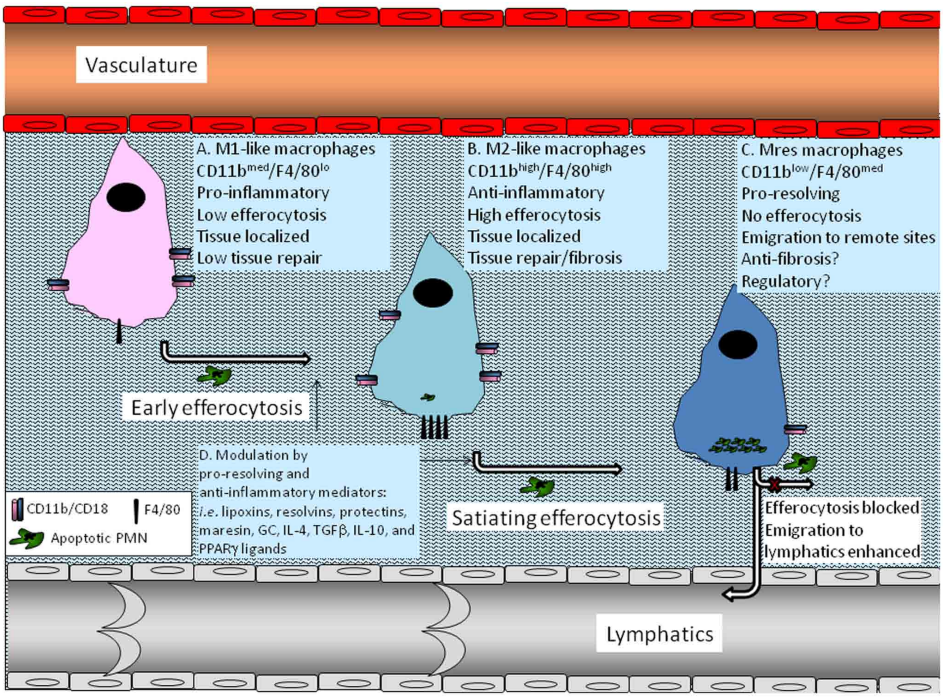

FIGURE 1 | Macrophage phenotype conversions induced by efferocytosis. A monocyte that infiltrated an inflamed tissue differentiates to a macrophage and adopts an M1-like phenotype previous to encounter with apoptotic PMNs **(A)**. Once it encounters apoptotic PMN and starts to engulf them (early efferocytosis), the macrophage switches to an M2-like phenotype that is anti-inflammatory, highly efferocytic, and involved in tissue repair and return to homeostasis, but can also promote fibrosis and scar formation **(B)**. As the engulfment of apoptotic PMN by the macrophage continues and reaches a threshold level determined by the resolving milieu (satiating-efferocytosis) the macrophage undergoes another switch to the Mres phenotype **(C)**. These macrophages reduce the expression of pro-fibrotic arginase-1 and display reduced phagocytosis of extracellular particle including apoptotic cells. Consequently, rapid Mres departure of the resolving tissue and emigration to remote sites takes place. At these target organs Mres macrophages presumably produce 12/15-LO-derived pro-resolving lipid mediators, and deliver homeostatic signals to antigen presenting cells and lymphocytes. Moreover, Mres that stay in the resolving tissue might express higher levels of anti-inflammatory, anti-fibrotic, and anti-oxidant proteins to limit tissue damage and fibrosis. 12/15-LO-derived lipid mediators probably also contribute to the anti-inflammatory and anti-fibrotic properties of Mres in the resolving tissue. Early and satiating-efferocytosis can be modulated by pro-resolving and anti-inflammatory mediators, such as lipoxins, resolvins, protectins, maresin, GC, IL-4, TGFβ, IL-10, and PPARγ ligands **(D)**. This modulation can enhance the immune-silencing and departure of Mres to the lymphatics, where they can contribute to the termination of acquired immune responses.

during muscle injury and repair. These switches were induced by the engulfment of muscle debris that promoted TGFβ production and muscle regeneration (Arnold et al., 2007; Perdiguero et al., 2011). Importantly, the macrophage phenotype switch was mediated by a signaling cascade involving MAPK (Perdiguero et al., 2011) an essential module in macrophage inflammatory signaling (Kim et al., 2008).

Macrophages are important in limiting inflammation, excessive tissue repair, and fibrosis (Wynn and Barron, 2010). They also act at remote sites, such as lymphoid organs and adipose tissue (Schwab et al., 2007; Mukundan et al., 2009; Odegaard et al., 2007; Titos et al., 2011) to regulate acquired immune responses and metabolism. Since CD11b^{low} macrophages are distinct from either M1 or M2, do not express the pro-fibrotic enzyme arginase-1, stop phagocytosing foreign particles and can be found at lymphoid organs and adipose tissue (Schif-Zuck et al., 2011; Titos et al., 2011), we suggest these macrophages display a new phenotype, now termed resolution-promoting macrophages (Mres), which might be involved in anti-fibrotic, immune-regulatory, and metabolic processes, and hence is critical for the local and systemic termination of inflammatory episodes. The "decision-making" of macrophages on which phenotype will be expressed at a given time and setting is probably controlled by multiple variants in their milieu, including the number of apoptotic PMN they acquired and local concentrations of pro-resolving lipid mediators (from 15-LO and other pathways) and glucocorticoids (Schif-Zuck et al., 2011; Titos et al., 2011). Other macrophage-inactivating and resolution-promoting cytokines, growth factors and lipid mediators, such as IL-10, TGFβ, and PPARγ ligands are likely to also be important in regulating the fate of macrophages during the resolution of inflammation and the return of tissues to homeostasis.

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Contrasting inflammation resolution during atherosclerosis and post myocardial infarction at the level of monocyte/macrophage phagocytic clearance

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In cardiovascular disorders including advanced atherosclerosis and myocardial infarction (MI), increased cell death and tissue destabilization is associated with recruitment of inflammatory monocyte subsets that give rise to differentiated macrophages. These phagocytic cells clear necrotic and apoptotic bodies and promote inflammation resolution and tissue remodeling. The capacity of macrophages for phagocytosis of apoptotic cells (efferocytosis), clearance of necrotic cell debris, and repair of damaged tissue are challenged and modulated by local cell stressors that include increased protease activity, oxidative stress, and hypoxia. The effectiveness, or lack thereof, of phagocyte-mediated clearance, in turn is linked to active inflammation resolution signaling pathways, susceptibility to atherothrombosis and potentially, adverse post MI cardiac remodeling leading to heart failure. Previous reports indicate that in advanced atherosclerosis, defective efferocytosis is associated with atherosclerotic plaque destabilization. Post MI, the role of phagocytes and clearance in the heart is less appreciated. Herein we contrast the roles of efferocytosis in atherosclerosis and post MI and focus on how targeted modulation of clearance and accompanying resolution and reparative signaling may be a strategy to prevent heart failure post MI.

Keywords: macrophage, phagocytosis, cardiovascular, myocardial infarction, clearance, hypoxia

INTRODUCTION

The sequence of events that are atherothrombosis, myocardial infarction (MI), and heart failure, combine to serve as a leading cause of morbidity and mortality in the industrialized world (Lloyd-Jones et al., 2010). Advanced atherosclerosis and MI are mutually characterized by accelerated cell death followed by inflammatory cell recruitment. Though intimately linked, each disorder individually is distinguished by unique cell populations and cell stressors (Libby et al., 2008). In the intimal vascular wall of the atherosclerotic plaque, lipid-laden macrophage foam cells predominate after responding to retained lipoproteins that are embedded in the sub-endothelium (Williams and Tabas, 1995). As atherosclerotic lesions mature, a combinatorial array of stressors, including excess free cholesterol, pattern recognition receptor ligands, and oxidative stress, additively signal to activate cellular stress pathways, secretion of inflammatory cytokines, and accelerate apoptosis (Lloyd-Jones et al., 2010; Moore and Tabas, 2011). When combined with reduced apoptotic cell clearance efficiency (i.e., defective "efferocytosis"), this leads to secondary necrosis and plaque destabilization, the precursor to atherothrombosis (Tabas, 2005; Schrijvers et al., 2007). In turn, plaque rupture and MI lead to the release of chemotactic factors into the bloodstream and subsequent influx of neutrophils and monocytes into the heart (Kumar et al., 1997). In contrast to advanced atherosclerosis leading to MI, inflammation after a heart attack is often acute and resolving. This response is necessary to heal the heart and promote scar formation. Interestingly, recent and not-so-recent reports, suggest that modulation of the inflammatory response post MI contributes to the quality of heart repair (Roberts et al., 1976; Frangogiannis et al., 2002; Nahrendorf et al., 2007). Marginated leukocytes clear dying and necrotic cardiomyocytes and promote fibrogenic and angiogenic responses. In some cases, especially in the elderly, sub-optimal clearance efficiency may lead to maladaptive vascular remodeling and tissue repair in the healing heart and therefore accelerate transition into heart failure (Chen and Frangogiannis, 2010). Herein, we compare basic mechanisms of inflammation resolution by phagocytes in the vascular wall during atherosclerosis and in the myocardium post infarction, with a focus on monocyte/macrophage-mediated phagocytic clearance of dying tissue, particularly post MI. These concepts form a working model (**Figure 1**) of how clearance may modulate downstream inflammation and tissue repair in cardiovascular disease.

DEFECTIVE INFLAMMATION RESOLUTION IN ATHEROSCLEROSIS

Though initially protective, inflammation must eventually subside in order to prevent further tissue damage. Many diseases of inflammatory cell recruitment, including advanced atherosclerosis leading to MI, are failures of inflammation to resolve that subsequently lead to tissue destabilization and injury. A key component of defective inflammation resolution in advanced atherosclerosis is defective efferocytosis (Schrijvers et al., 2007; Tabas, 2010). In non-diseased settings, apoptosis is typically followed by rapid and non-phlogistic uptake into neighboring phagocytic cells. During inflammation, active production of omega-3

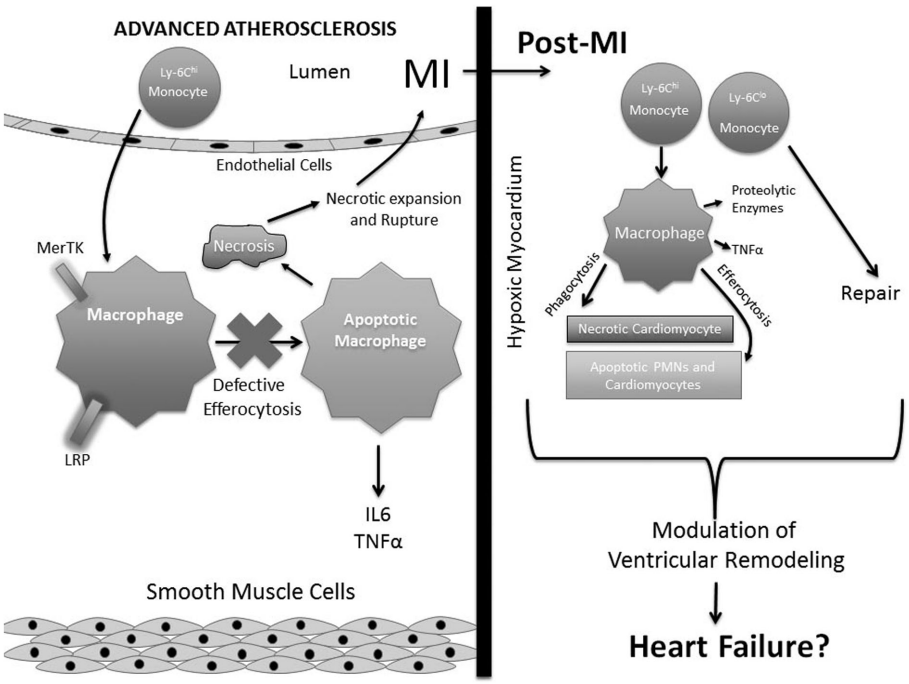

FIGURE 1 | Contrasting phagocytic clearance in advanced atherosclerosis and post myocardial infarction. Advanced atherosclerosis is characterized by recruitment of Ly-6C-HI monocytes that differentiate into macrophages. Macrophage apoptotic cell receptors, such as MerTK and LRP promote efferocytosis. However, in the inflammatory setting of mature atheromata, efferocytosis becomes defective (see Tabas, 2010) leading to secondary necrosis, necrotic core expansion, and susceptibility to myocardial infarction (MI). Post MI, both Ly-6C-HI and LO monocytes marginate into myocardial tissue to differentiate into macrophages and promote clearance of apoptotic and necrotic cells (see Nahrendorf et al., 2007). These acute events can affect later cardiac remodeling and inflammation that may lead to heart failure.

poly-unsaturated fatty-acid-derived mediators promotes further phagocytic removal of dying cells (Schwab et al., 2007). The act of efferocytosis also triggers anti-inflammatory, or pro-resolving signaling that assists in dampening the immune response and restoring tissue equilibrium (Serhan and Savill, 2005). Macrophages that have ingested apoptotic cells inhibit pro-inflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, prostaglandin E2, and platelet-activating factor (Fadok et al., 1998). An important anti-inflammatory cytokine and pro-resolving factor that is linked to efficient efferocytosis IL-10 (Lingnau et al., 2007). Both *in vitro* and *in vivo*, IL-10 has been reported to enhance efferocytosis and transgenic over-expression of IL-10 has been shown to reduce atherogenesis in experimental rodents (Pinderski et al., 2002). In humans, IL-10 levels are reduced in patients with cardiovascular disease, consistent with the notion that reduced levels of this cytokine may accelerate atherosclerotic progression (Seljeflot et al., 2004). *In vitro*, "alternatively" activated M2 macrophages preferentially clear apoptotic cells and are often characterized by secretion of anti-inflammatory cytokines such as IL-10 and TGFβ (Xu et al., 2006). In the case of early atherosclerosis, cell turnover within the developing atherosclerotic lesion is rapidly countered by neighboring macrophage phagocytes that promote efficient efferocytosis (Tabas, 2005). Consistent with this, early atherosclerotic lesions rarely exhibit TUNEL-positive apoptotic nuclei (Kockx et al., 1999). Efficient clearance in early atheromata limits the cellular density of the lesion and may also reduce further recruitment of blood-borne monocytes. In human advanced atherosclerosis, there is an accumulation of free, non-phagocytosed apoptotic cells (Schrijvers et al., 2005). The failure of these dying cells to be removed leads to the loss of cell membrane integrity, secondary post-apoptotic necrosis, liberation of potentially immunogenic epitopes, and release of damage associated molecular patterns (DAMPs) that stimulate cell activation. Failed clearance may also be responsible for the aforementioned reductions in anti-inflammatory/pro-resolving mediators such as IL-10. Necrotic plaques are strongly associated with clinical acute atherothrombotic events and are a source of procoagulant materials (Kolodgie et al., 2003). It is not entirely clear why early stable atherosclerotic lesions mature into non-resolving and necrotic inflammatory advanced lesions, however recent reports in experimental mice, shed some light on key clearance pathways that may be involved, as described below.

MOLECULAR MECHANISMS OF EFFEROCYTOSIS IN ATHEROSCLEROSIS

Recognition of apoptotic cells in advanced atheromata requires bridging of apoptotic cell ligands such as phosphatidylserine, with phagocyte receptors, that trigger downstream activation of the phagocyte actin cytoskeleton. Bridging molecules, such as complement factor C1q, link apoptotic cell receptors to their apoptotic ligands (Ogden et al., 2001). During atherosclerosis for example,

C1qa−/− mice on a fat–fed low density lipoprotein receptor (Ldlr) deficient background had larger atherosclerotic lesions and an increase in the number of lesional apoptotic cells, consistent with defective clearance (Bhatia et al., 2007). Another bridging molecule, milk fat globule-EGF-factor 8 (MFG-E8), a secreted glycoprotein, also links apoptotic cell receptors to their apoptotic ligands (Hanayama et al., 2002). MFGE-E8 (lactadherin) is expressed in atherosclerotic lesions and it promotes efferocytosis *in vitro* and *in vivo*. Mice lacking MFG-E8 in bone marrow precursors exhibit more necrosis and apoptotic cellular debris (Ait-Oufella et al., 2007). MFG-E8 is recognized by the macrophage cell-surface and protein cross-linking transglutaminase-2 (TG2). TG2, in cooperation with αvβ3 integrin, bind to MFG-E8 to promote efferocytosis (Toth et al., 2009). During atherosclerosis, mice reconstituted with Tg2−/− bone marrow cells exhibited larger necrotic cores relative to control (Boisvert et al., 2006). In addition, clearance of apoptotic cells also been reported to be significantly reduced in Ldlr related protein (Lrp) 1−/− lesions relative to control. By immunohistochemistry and relative to wild-type lesions, Lrp1−/− lesions exhibited more necrotic cores with more apoptotic cells not associated with macrophages (Yancey et al., 2010). LRP is activated to promote engulfment after binding calreticulin on apoptotic cells (Gardai et al., 2005). Another important efferocytosis receptor in atherosclerosis in MERTK. Mice deficient in the tyrosine kinase MER (MERTK), have a defect in macrophage efferocytosis and this correlated with an increase in plaque inflammation and plaque necrosis (Ait-Oufella et al., 2008; Thorp et al., 2008). MERTK is involved in both efferocytosis and in anti-inflammatory responses (Camenisch et al., 1999). It promotes clearance by binding to one of two bridging molecules, either GAS6 or protein S (Lemke and Rothlin, 2008). Interestingly, MERTK is proteolytically cleaved as a result of inflammatory stimuli such as LPS and this leads to the generation of a solubilized MER that can act as a competitive inhibitor of uptake (Sather et al., 2007). With the recent identification of the MERTK cleavage site, future tests will examine whether MERTK sheddase-mediated proteolysis contributes to defective efferocytosis in atherosclerosis (Thorp et al., 2011). The identification of the aforementioned key clearance players in atherosclerotic progression provides targets for testing relevance in humans with coronary artery disease. For example, in addition to soluble MER being linked to defective efferocytosis, it has also been identified in human inflammatory cardiovascular lesions (Hurtado et al., 2011). The fact that MERTK is rendered inactive through sheddase-mediated cleavage may provide a therapeutic opportunity. That is, if excess MERTK cleavage were a culprit of defective inflammation resolution through its anti-efferocytic properties in human advanced plaques, targeted inhibition of cleavage might suppress plaque necrosis and increase pro-resolving mediators as described above (Tabas, 2010). Thus, by defining the mechanisms of defective efferocytosis *in vitro* and establishing relevance in humans, specific hypotheses can be formulated toward designing clearance based therapeutic strategies that promote inflammation resolution. In the case of post MI inflammation and clearance, a more acute and resolving inflammation and dissimilar apoptotic and necrotic targets distinguish clearance in the heart from clearance in the vasculature, as described below.

CARDIOMYOCYTE CLEARANCE POST MI AND ITS ASSOCIATION WITH MYOCARDIAL INFLAMMATION RESOLUTION AND REPAIR

Healing of the heart after interruption of blood supply and generation of an infarct requires scavenging of necrotic cellular debris and preservation of the remaining and irreplaceable cardiomyocytes. This wound repair is accomplished in part through acute mobilization of innate inflammatory cells that assist in degrading released macromolecules. The recruited phagocytes, which initially include neutrophils and monocytes, act in turn to directly remove necrotic and apoptotic cells. This is followed by formation of granulation tissue and extracellular matrix deposition. Neutrophils likely contribute to the clearance of necrotic debris from the infarct; however they also potentially damage neighboring myocytes through release of their proteolytic enzymes. Neutrophil depletion in animals post MI and reperfusion have been shown to reduce infarct size and myocardial injury (Romson et al., 1983). Neutrophils may also contribute to inflammation resolution through programmed cell death leading to efferocytosis by macrophages. Efferocytosis, as described above, induces signaling pathways that promote pro-resolving factors such as TGF-B and IL-10. Importantly, the effects of neutrophils and other myeloid cells post MI may be exacerbated during reperfusion of the infarct. For example, hallmarks of reperfusion injury post MI include the production of reactive oxygen species, mitochondrial dysfunction, and recruitment of elevated neutrophils and monocytes. These events can lead to increased myocardial injury and cardiomyocyte apoptosis that would increase the burden for dead cardiomyocyte clearance (Foo et al., 2005). Clearance *per se* may also be affected after reperfusion. For example, in a non-MI mouse intestinal arterial occlusion and reperfusion model, investigators found decreased levels of the "come-eat-me" mediator MFG-E8 mRNA in remote organs. Administration of rmMFG-E8 suppressed intestinal I/R injury-induced organ injury and apoptotic cell accumulation (Matsuda et al., 2011). Thus, it will be important to compare and contrast clearance roles post MI versus post MI followed by reperfusion.

Post MI, most of the initial cell death is necrotic, and therefore, this promotes the release of pro-inflammatory intracellular contents such as heat shock proteins and DAMPs. These DAMPS, or "alarmins," activate innate phagocytes and may or may not exacerbate the repair response (Matzinger, 2002). For example, endogenous DAMPs such as HSP-60 and EDA can activate signaling pathways downstream of pattern recognition receptors. Pattern recognition receptors such as Toll-like receptor 4 (TLR4) activate post MI inflammation and are required for adverse myocardial left ventricular remodeling following infarction, indicating that part of the inflammatory response post MI is maladaptive (Timmers et al., 2008). This deficiency of TRL-4 is associated with reduced intercellular adhesion molecule expression and reduced monocyte homing to the infarct, in turn leading to markedly reduced myocardial inflammatory cytokine production and preservation of heart function. Importantly, clearance of dying cells is linked to phagocyte-mediated suppression of inflammation. For example, apoptotic cells promote their own clearance and activate of the nuclear receptor LXR to suppress inflammation (A-Gonzalez et al., 2009). Thus, clearance may play a role in dampening TLR-mediated inflammation post MI. Cardiomyocyte necrosis also

leads to the release of mitochondrial DAMPs (MTDs). MTDs include formyl peptides and mitochondrial DNA and can activate neutrophils through formyl peptide receptor-1 and TLR signaling (Zhang et al., 2010). Due to the high energy requirements of cardiomyocytes and therefore the high density of mitochondria per cell, injury to the heart would be expected to promote a significant response to MTDs. Another intracellular factor that is released from dead cells and during acute inflammation is high mobility group box 1 (HMGB1), which when located in the nucleus can act as an architectural chromatin-binding factor (Scaffidi et al., 2002). *In vitro*, extracellular HMGB1 has been shown to reduce macrophage efferocytosis of apoptotic neutrophils through binding to phosphatidylserine (Liu et al., 2008), suggesting that HMGB1 could delay engulfment of dying cells near and in the infarct, however, it is yet to be determined how HMGB1 affects clearance of cardiomyocytes by macrophages. Injection of HMGB1 into experimental hearts after coronary artery ligation has been shown to have beneficial effects in the heart when infused 3 weeks post MI (Takahashi et al., 2008). Also, injection of anti-HMGB1 just prior to reperfusion in rats resulted in increased infarct sizes compared to control (Oozawa et al., 2008). Multiple roles of HMGB1 are now emerging, including a regenerative role for accumulation of newly formed myocytes post MI (Limana et al., 2011) and it is becoming evident that this molecule can act at multiple levels, with an apparent overall beneficial effect. Downstream of DAMP and PRR signaling is NF-κB. NF-κB activity is elevated in both myocardial and inflammatory cells in ischemic heart disease (Frantz et al., 2004), however, the overall effect of NF-κB remains incomplete. Deletion of the p50 subunit of the NF-κB complex has been shown to improve heart failure after MI (Frantz et al., 2006), suggesting a role for maladaptive signaling post MI. In addition, transgenic over-expression of NF-κB p65 in myocytes resulted in adverse cardiac remodeling and increased endoplasmic reticulum stress and apoptosis in cardiomyocytes post MI (Hamid et al., 2011), indicating that persistent NF-κB activation exacerbates cardiac remodeling. However, another study reported that NF-κB p50 deletion exacerbates cardiac function post MI, consistent with a cardioprotective role (Timmers et al., 2009). The role of the NF-kB is complex. Though predominantly associated with pro-inflammatory responses, NF-kB has also been linked to the resolution of inflammation. For example, NF-kB activation during inflammation resolution is associated with expression of anti-inflammatory genes and induction of apoptosis (Lawrence et al., 2001). The NF-kB complex includes RelA (p65), RelB, c-Rel, p50, and p52, as well as inhibitory IkB and stimulatory IkBkinse (IKK) regulators. NF-κB can form homodimers or heterodimers depending on its mode of activation. Only p65, c-Rel, and RelB contain transactivation domains, whereas p50 and p52 do not and can act to suppress gene transcription (Vallabhapurapu and Karin, 2009). NF-κB can also be directly regulated by receptors involved in efferocytosis *per se*. For example, in the case of the efferocytosis receptor tyrosine kinase, MERTK, suppression of NF-κB transcriptional activation is directly associated with downstream inflammatory signaling (Tibrewal et al., 2008). Thus, the overall role of NF-κBin heart failure is far from understood and future experiments are required to elucidate both cell-type specific effects (myocardial versus inflammatory) and how the kinetics of NF-κB activation may differentially affect inflammation versus inflammation resolution in the heart.

Though innate inflammatory activation may at certain levels promote adverse effects on the heart after injury, inflammation is nevertheless necessary to clear away dead cardiac tissue and begin inflammation resolution, as described below. Resolution of inflammation is not a passive process and instead relies on biosynthesis of pro-resolving mediators. Many of these mediators are derived from poly-unsaturated fatty acids (PUFA), including lipoxins, E-series resolvins, D-series resolvins, protectins, and, maresins (Serhan and Savill, 2005). One interesting resolvin is Resolvin E1. Resolvin E1, has been shown to promote the efferocytosis of neutrophils *in vitro* and *in vivo* (Schwab et al., 2007). In the context of the heart, Resolvin E1, which is derived from eicosapentaenoic acid, has been shown to directly protect cardiomyocyte cell lines from ischemia-reperfusion injury *in vitro* and in addition, limit infarct size after prophylactic injection (Keyes et al., 2010). Though pro-survival molecules in cardiomyocytes, such as AKT, were up-regulated, further experiments are required to determine mechanism at the causal level. Recently, the receptor for Resolvin E1 was identified as ChemR23, otherwise known as CMKLR1 (Ohira et al., 2010). Future studies utilizing knockout models of this receptor are required during MI. Thus, not only will pathways that suppress acute-phase inflammation be required, but also pathways that target active pro-resolution pathways, potentially downstream of efferocytosis. As one such proof of principle that such an approach is feasible and linked to phagocytic clearance, Harel-Adar et al. (2011) by simulating a macrophage response to an apoptotic cell, were able to modulate the activity of cardiac macrophages to improve infarct repair, post experimental MI. Specifically, the investigators injected phosphatidylserine (PS)-presenting liposomes intravenously to mimic the anti-inflammatory effects of apoptotic cells. Both in a rat model of acute MI, and *in vitro*, PS-liposome uptake by macrophages promoted the secretion of the anti-inflammatory cytokines TGFβ and IL-10. This was accompanied by down-regulation of pro-inflammatory tumor necrosis factor α (TNFα). Thus, an exciting proof of concept that modulation of macrophage pathways related to clearance may have therapeutic application and promote inflammation resolution.

Similar to atherosclerosis, the levels of IL-10 may be important in reducing inflammation post MI. For example, IL-10 deficient mice exhibited increased infarct size and myocardial necrosis associated with elevated neutrophil infiltration (Yang et al., 2000). IL-10 also inhibits inflammation and attenuates left ventricular remodeling after MI via activation of STAT3 and suppression of mRNA stabilizing protein HuR (Krishnamurthy et al., 2009). As further evidence that the type of inflammatory response may dictate post MI repair, Cheng et al. (2005) found IFN-gamma producing T-cells were significantly increased in patients post MI, creating a Th1/Th2 imbalance. Also, in patients with acute MI, significant increases in Th17 cytokines were found concomitant with reduced levels of T-regulatory associated cytokines (Cheng et al., 2008). Finally, monocytes and macrophages secrete growth factors that can promote angiogenesis, specifically through targeting and activating myofibroblasts. Myofibroblasts secrete extracellular matrix and accumulate within the first week after an infarct (van den Borne et al., 2010). These cells are critical for scar formation

and prevention of cardiac dilation. However, too much matrix deposition, particularly at areas remote to the infarct, can also lead to heart failure. Thus, fine-tuned modulation of the immune response post MI appears to be required to promote resolution pathways while suppressing maladaptive/excessive inflammation.

Certainly, the aforementioned example whereby infarct repair was improved after injection of PS-liposomes suggests there is indeed potential for inflammation-modulating agents during myocardial reperfusion, particularly at the level of clearance. Such a strategy may be proven even more efficacious if tested in a population that more closely resembles the advanced age of the average victim of MI. That is, older age is associated with increased mortality after a heart attack. In addition, aging-related defects have been reported to be associated with adverse cardiac remodeling in experimental mice. Specifically, Frangogiannis et al. (2002) showed by both histomorphometric and echocardiographic end points, that older mice exhibit significantly reduced neutrophil and macrophage infiltration after coronary ligation, and in turn, impaired phagocytosis of dead cardiomyocytes. This led to enhanced dilative and poor systolic function (Bujak et al., 2008). Additional analysis revealed reduced collagen deposition and hypertrophic remodeling in these hearts. The reduced inflammation seen in aged mice can also be measured in experimental models that inhibit inflammatory cell recruitment. For example, the effects of reduced macrophage recruitment have been tested in a model of MCP-1/CCL2 deficiency. Lack of MCP-1 is associated with delayed macrophage infiltration into the heart and delayed replacement of injured cardiomyocytes with granulation tissue (Dewald et al., 2005). In this scenario, reduced levels of myeloid cell infiltration was associated with delayed dying cell clearance and impaired healing. However, in the reverse direction, that is, excessive inflammation in the setting of atherosclerotic hyperlipidemia, Nahrendorf et al. (2007) examined the inflammatory response post MI in atherogenic apolipoprotein E deficient mice and discovered that a subset of monocytes, the Ly-6C(hi) and CCR2-recruited subset, were markedly elevated and this also correlated with impaired heart healing (Panizzi et al., 2010). The injured myocardium exhibited elevated inflammatory gene expression of tumor necrosis factor-alpha, myeloperoxidase, and decreased transforming growth factor-beta and a higher abundance of proteases. Previous work from the same group identified two distinct phases of monocyte action post MI. In non-atherosclerotic (i.e., non-apoE-deficient mice), Ly-6C(hi) monocytes were the first to be recruited to the heart and were highly phagocytic. Though increased Ly-6c (hi) monocytosis in apoE deficient mice were detrimental, depletion of Ly-6c (hi) monocytes under non-dyslipidemic, non-apoE-deficient mice resulted in impaired heart healing, indicating a contribution of dyslipidemia to adversely modulateLy-6c (hi) function during heart inflammation. Ly-6C(lo) monocytes enter later and expressed vascular-endothelial growth factor and therefore promoted healing via myofibroblast accumulation, angiogenesis, and deposition of collagen (Nahrendorf et al., 2007).

OXYGEN AND CLEARANCE IN THE HEART

Reduced perfusion to the infarct reduces nutrient availability and therefore stresses cellular metabolism. Low oxygen tensions and nutrient deprivation lead to the induction of hypoxia-inducible transcription factors (HIF) in phagocytes. During normoxia, HIF-1α protein is constitutively degraded. During hypoxia, HIF-1α is stabilized and translocates to the nucleus, where it dimerizes with HIF-1β, and acts as a transcription factor for gene elements encoding hypoxic response elements (Maxwell et al., 1999). HIF-1α mRNA can be detected in myocardial specimens with pathological evidence of acute ischemia within the first day post MI (Lee et al., 2000). In phagocytes, HIF isoforms mobilize and differentially coordinate intracellular signaling that regulate cell migration, glycolysis, cell-survival, and inflammatory cytokine secretion (Cramer et al., 2003). Deficiency of myeloid HIF-1α has been shown to reduce infiltration of leukocytes and improve cardiac function post MI. This reduced infiltration has been linked to the down-regulation of chemokine receptors (Dong et al., 2010). With respect to how oxygen tension affects clearance in the heart, little is known. *In vitro*, hypoxia promotes the phagocytosis of bacteria by macrophages, and this has been linked to p38 MAPK signaling (Anand et al., 2007). Less is known regarding effects of apoptotic cell clearance by macrophages during hypoxia, however, in retinal pigment epithelial cells, hypoxia enhanced efferocytosis concomitant with upregulation of the clearance receptor CD36 (Mwaikambo et al., 2009). In contrast, hypoxia has also been shown to potentiate secretion of factors that can inhibit phagocytosis (Wei et al., 2011). Finally, during reperfusion, the restoration of blood and oxygen can also promote additional myocardial damage and elevated reactive oxygen species as described above. Our understanding of how these factors intersect during ischemia to regulate phagocyte-mediated clearance and inflammation resolution, remain incomplete.

FUTURE DIRECTIONS

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Ischemic heart failure is on the rise, part and parcel with an increase in the aged population, who are at highest risk of cardiovascular disease. In addition, better therapeutics have led to the propensity of patients to survive acute coronary events such as MI (Stewart et al., 2003). Both atherosclerosis and MI are characterized by increased cell death. In the case of atherosclerosis, genetic causation experiments indicate that clearance factors are a required pathway toward reducing inflammation and stabilizing vulnerable plaques. Acute MI can lead to the loss of irreplaceable cardiomyocytes, deleterious ventricular remodeling, and reduced cardiac output. Despite significant advances in current standards of therapy, the prevalence of post MI heart failure remains high. Post MI, the causal demonstration of clearance pathways and how they affect post MI repair directly are still unknown. Thus, the magnitude of how clearance affects the heart is yet to be determined and must be formally tested. However, apoptotic cell death is programmed to lead to compartmentalization and non-phlogistic metabolism of intracellular self-antigens, and additionally, to activate pro-resolving signaling (Serhan and Savill, 2005). That is, given that phagocytosis of necrotic debris and clearance of apoptotic cells are intimately linked to downstream signaling pathways that modulate inflammation resolution and tissue repair, targeting of the innate immune system may be a strategy toward reducing adverse cardiac remodeling that leads to

heart failure. Thus, in our working model (**Figure 1**), we and others propose a need for efficient clearance pathways to promote dying cell engulfment in the heart that are coupled to downstream pro-resolving pathways that also suppress inflammation. In the case of advanced atherosclerosis, enhancing efferocytosis efficiency has previously been proposed as a critical step (Tabas, 2005). In the case of failed inflammation resolution leading to MI, optimizing cardiac repair pathways that lead to efficient cardiac output will also be required to prevent heart failure. Toward these ends, it will be important to elucidate the causal molecular pathways that regulate tissue repair during wound healing. This will include testing the effects of heart failure risk factors, including abnormal metabolism and aging. These approaches will be tested with methods of cell and molecular biology, both *in vitro* and in gene-targeted *in vivo* models. In addition, the effects of clearance during reperfusion injury, such as occurs in the clinical setting will also have to be addressed. By defining the critical molecular pathways required for an optimal immune response, a pathway can be discovered toward promoting inflammation resolution and reducing myocardial necrosis and heart failure. In atherosclerosis, strategies to enhance clearance are currently being tested toward stabilizing plaque (Tabas, 2010). Post MI, not only will therapeutics that potentiate effective clearance be a testable strategy, but it will also be key to find the right balance of inflammation that promotes effective clearance of dying and dead tissue while limiting maladaptive inflammation.

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Non-identical twins – microglia and monocyte-derived macrophages in acute injury and autoimmune inflammation

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The brain has been commonly regarded as a "tissue behind walls." Appearance of immune cells in the brain has been taken as a sign of pathology. Moreover, since infiltrating monocyte-derived macrophages and activated resident microglia were indistinguishable by conventional means, both populations were considered together as inflammatory cells that should be mitigated. Yet, because the microglia permanently reside in the brain, attributing to them negative properties evoked an ongoing debate; why cells that are supposed to be the brain guardians acquire only destructive potential? Studies over the last two decades in the immune arena in general, and in the context of central nervous system pathology in particular, have resulted in a paradigm shift toward a more balanced appreciation of the contributions of immune cells in the context of brain maintenance and repair, and toward the recognition of distinct roles of resident microglia and infiltrating monocyte-derived macrophages.

Keywords: microglia, monocyte-derived macrophages, central nervous system, neurodegeneration, neuroinflammation

DIFFERENTIAL MONONUCLEAR PHAGOCYTE ORIGINS

The mononuclear phagocyte system – a term coined by van Furth et al. (1972) – is known today to comprise monocytes, dendritic cells (DCs), and macrophages, as well as their respective committed bone marrow (BM) progenitors. Mononuclear phagocytes can arise via two exclusive developmental routes (Orkin and Zon, 2008). Most peripheral mononuclear phagocytes are descended from BM-derived cells formed as part of ongoing definitive hematopoiesis. A major breakthrough in defining this developmental pathway was the identification of a clonotypic BM-resident founder cell, termed the macrophage-DC precursor (MDP), that can give rise to peripheral mononuclear phagocytes while having lost granulocyte potential (Fogg et al., 2006). MDPs differentiate within the BM into monocytes (Varol et al., 2007) and into dedicated DC precursors, the pre-DCs, that exit to the circulation to enable the repopulation of peripheral tissue macrophages and DCs, respectively. As opposed to the ephemeral DCs, peripheral macrophage populations are however heterogeneous with respect to their turnover rate, and thus, in their steady state, rely to a varying degree on renewal by BM-derived precursors, e.g., monocyte input (Landsman et al., 2007; Varol et al., 2007). Under inflammatory conditions, most resident microglia are complemented by recruited monocytes that differentiate *in situ* into macrophages (Ajami et al., 2011). However, the contribution of these cells is often transient (Ajami et al., 2011). Moreover, in certain scenarios, such as parasite-driven Th2 inflammation, tissue-resident macrophage populations were recently shown to even expand locally without any monocyte influx (Jenkins et al., 2011).

As exemplified by the brain microglia (Alliot et al., 1999; Ginhoux et al., 2010), mononuclear phagocytes can also arise during early development (around embryonic day 7.5) as a result of primitive hematopoiesis occurring in extra-embryonic tissue (Orkin and Zon, 2008). After seeding the tissue that is their final destination, these primitive macrophage-derived cells can – as is the case for the secluded microglia – maintain themselves through longevity coupled with limited self-renewal. Thus, these compartments remain independent of monocytic input throughout adult life (Ajami et al., 2007; Mildner et al., 2007; Shechter et al., 2009; Ginhoux et al., 2010). This alternative pathway might encode functional specialization. However, to what extent it applies to macrophage populations other than the brain microglia, remains currently unknown and will have to await future fate mapping studies.

THE MYSTERY OF THE ROLE OF MONOCYTE-DERIVED MACROPHAGES FOLLOWING STERILE CNS INJURY

Extensive comparison of the responses of the peripheral and central nervous system (CNS) to axonal injury has revealed that recruitment of blood-derived cells to injured peripheral nerves is more pronounced than to the CNS (Perry et al., 1993). Opinions differed, however, regarding the interpretation of this finding (Rapalino et al., 1998; Schwartz et al., 1999; Shechter et al., 2009; London et al., 2011). Insight into "sterile" (non-infectious) injuries outside the CNS (DiPietro, 1995) and the assumption that recruited cells may be beneficial though their numbers might be insufficient for optimal repair, let to the suggestion that introduction of blood-derived macrophages with the

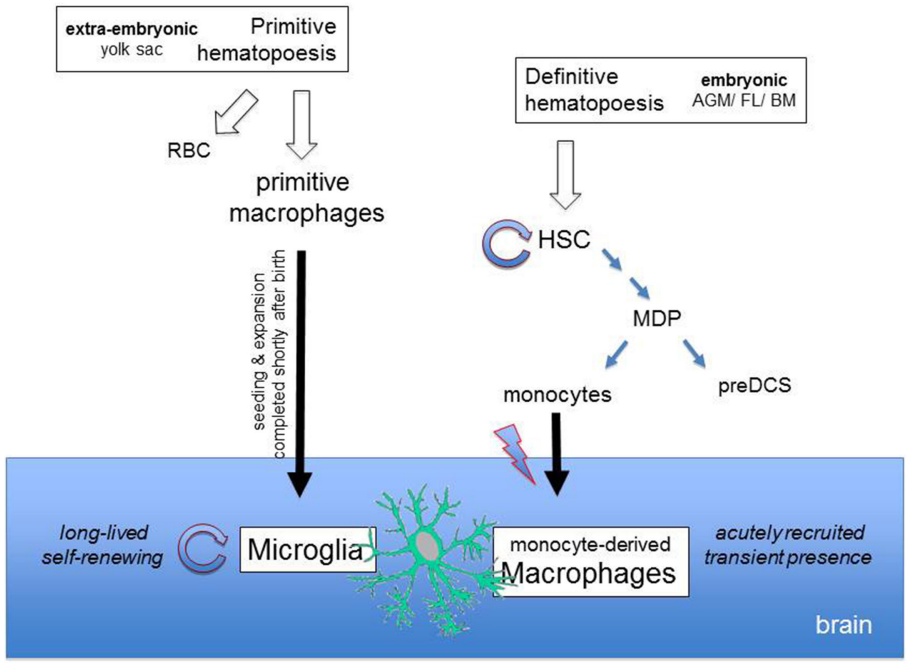

FIGURE 1 | Differential origins of microglia and monocyte-derived brain macrophages. Microglia derive from macrophages that arise from primitive hematopoiesis in the yolk sac, The primitive macrophages seed the developing brain and expand to give rise to the microglia, which subsequently maintains itself through longevity and limited self-renewal. Upon injury, the brain recruits monocytes that differentiate locally into monocyte-derived macrophages and transiently complement the brain mononuclear phagocyte compartment. Monocytes are generated in the BM from macrophage-DC precursors (MDPs) that are constantly replenished from self-renewing hematopoietic stem cells (HSCs) which arose from definitive hematopoiesis.

correct phenotype, at the right time, and to the correct location following CNS injury could promote repair. Indeed, in a model of spinal cord injury, it was demonstrated that "alternatively activated" blood macrophages, when locally transplanted at the margin of a spinal cord lesion, resulted in improved recovery (Rapalino et al., 1998). The success of such macrophage transplantation was found to be dependent on the site of their injection (no effect was found when cells were administered at the center of the lesion or far from its margins), the number of injected cells, and the time elapsed between the injury and the injection (Schwartz and Yoles, 2006). The results of these manipulations were initially puzzling, primarily due to the lack of appreciation of macrophage heterogeneity (Gordon, 2003; Gordon and Taylor, 2005) by neuroscientists (Popovich et al., 1999; Mabon et al., 2000). Since the site of injury becomes spontaneously overwhelmed with locally activated microglia, the idea of deriving therapeutic benefit by adding additional blood-derived macrophages seemed contrary to common logic. Subsequent studies from several laboratories regarding the roles of various macrophage populations further complicated the picture, as the researchers primarily studied macrophages activated *in vitro* using products of bacteria or yeast, assuming that such inflammatory cells mimic strongly activated macrophages. However, activating agents differ not only in their strength of activation, but also in the functional state that they induce; thus, macrophages activated by yeast or bacterial cell wall components, such as zymosan or LPS, do not necessarily represent cells activated following "sterile" injury.

Additional missing pieces in the puzzle of the role of macrophages following CNS injury were the realization that distinct immune activities might be needed following the insult for tissue rescue/protection (Shechter et al., 2009) and restoration/regeneration (Benowitz and Yin, 2010; Benowitz and Popovich, 2011), and that although activated microglia and monocyte-derived macrophages share morphology and phenotypes, they might display distinct activities at critical time points following the insult (Shechter et al., 2009). These facts were belatedly accepted when standard histological or immunohistochemical techniques of limited resolution were complemented by new genetic approaches enabling the tracking of cell origins and fates.

DIFFERENTIAL CONTRIBUTIONS OF ACTIVATED RESIDENT MICROGLIA AND INFILTRATING MONOCYTE-DERIVED MACROPHAGES TO THE RECOVERY FROM ACUTE SPINAL CORD INJURY

As outlined above, it is becoming clear that the resident microglia compartment is derived from primitive macrophages that enter the CNS during embryonic development; this compartment remains independent of monocytic input throughout life (**Figure 1**). The use of chimeric mice, in which monocytes, but not the microglia express a green fluorescent protein, GFP (CX_3CR1^{gfp} > wild type chimeras) allowed us to define the origins of the innate immune cell populations at a site of spinal cord injury. The chimeric mice were prepared by total body irradiation excluding the head, since although microglial cells are radio-resistant and remain strictly

host-derived in BM chimeras, cells of donor marrow origin have been shown to enter irradiated brains but not of head protected brain. In the absence of injury, blood-derived macrophages were found to be excluded from the healthy host brain, as long as the chimeras were created while shielding the head during the conditioning irradiation. Following spinal cord injury, blood-derived GFP^+ myeloid cells were found at the lesion site; yet, the majority of these recruited cells arrived with a delay relative to the initial injury-associated breach of the blood–brain barrier. Secondly and most critically, conditional ablation of the recruited blood-derived macrophages, by virtue of their expression of CD11c and use of the CD11c-DTR system (Jung et al., 2002), worsened the functional motor score of the injured animals and extended the lesion size, while elevating the inflammatory response of the local microglia (Shechter et al., 2009). Thus, the blood-derived macrophages, are recruited to the site of the injury only following insult, and facilitate the termination of the local immune response by displaying an anti-inflammatory activity required for the regulation of the locally activated microglia. Recovery from CNS insults hence seems to comprise consecutive discrete phases, as proposed for peripheral wound repair (Arnold et al., 2007; Nahrendorf et al., 2007). Accordingly, a first response primarily involves resident microglia and possibly also some infiltrating immune cells that "clean" the site of injury, removing dead cells, and debris. This phase is then followed by an active process of immune termination that involves resolving monocyte-derived macrophages, reminiscent of healing of tissues outside the CNS (Gordon and Taylor, 2005; Arnold et al., 2007). Indeed, we found that for blood-derived macrophages to be beneficial at the lesion site, they must express IL-10, the classical anti-inflammatory cytokine known to be associated with alternatively activated macrophages (also known as "M2" or "resolving macrophages"; Shechter et al., 2009). Ablation of the blood-derived macrophages followed by their replacement with macrophages that are defective in IL-10 expression preserves recruitment of cells, but results in impaired overall recovery (Shechter et al., 2009). Similarly, suppressing recruitment of innate immune cells at this stage is counterproductive, and is likely to result in chronic inflammation and insufficient repair (Shechter et al., 2009). Conversely, boosting of monocyte recruitment results in improved recovery (Shechter et al., 2009). Collectively, these results, argue in favor of both microglia and monocyte-derived macrophages as key players in the repair of spinal cord injuries, but with non-redundant activity. The monocyte-derived macrophages display in this scenario a local anti-inflammatory and beneficial role, which is critically dependent upon their expression of interleukin 10.

DIFFERENTIAL CONTRIBUTIONS OF ACTIVATED RESIDENT MICROGLIA AND INFILTRATING MONOCYTE-DERIVED MACROPHAGES TO EXPERIMENTALLY INDUCED AUTOIMMUNE ENCEPHALITIS

Multiple sclerosis and its established mouse model, experimentally induced autoimmune encephalitis (EAE), are characterized by extensive CNS infiltration of both lymphoid and myeloid inflammatory cells (Hickey, 1991). As with the acute injury model, specific contributions or resident microglia and recruited monocyte-derived macrophages have long remained a matter of debate due to the absence of suitable experimental systems. Using a combination of parabiosis and irradiation, Rossi and colleagues recently shed considerable light on this enigma (Ajami et al., 2011). Specifically, their strategy relied on reconstitution of an irradiated parabiont by its shielded parabiosis partner, thereby avoiding transfer of mechanically collected BM, which could contain precursors that are not released into the circulation under physiological conditions. This resulted in efficient replacement of BM and peripheral blood by donor cells, but no myeloid repopulation of the CNS; hence this system enabled monitoring of the dynamics of monocyte-derived macrophages and microglia during the course of active EAE. Interestingly, significant monocyte recruitment via a meningal route was found only in animals that already displayed considerable disease scores (>2). Progression to severe EAE, strongly correlated with the extent of this myelomonocytic CNS infiltrate. Moreover, corroborating an earlier report (Mildner et al., 2009), the use of CCR2-deficient parabionts established monocyte-derived macrophages as active drivers of CNS inflammation.

The study of Ajami et al. (2011) also allowed a glimpse at the dynamic response of the resident microglia. Activation of microglia, as indicated by BrdU incorporation (a measure of proliferation), was observed prior to monocyte recruitment and irrespective of disease progression. During the acute severe EAE disease phase, the microglial compartment transiently expanded but is trimmed, presumably to its original density.

Collectively, also during setting of autoimmune inflammation, non-redundant activities of microglia and monocyte-derived macrophages emerge. Interestingly, the monocyte-derived cells display in this scenario as opposed to the injury model a pro-inflammatory role.

CONCLUDING REMARK

The results summarized above highlight important lessons:

a. That infiltrating monocyte-derived macrophages are not part of the microglia turnover.
b. The functional plasticity of monocyte-derived macrophages, which can adopt opposite fates depending on the context encountered. Thus, while they have a pivotal and beneficial role in sterile injury model, under inflammatory autoimmune disease conditions the recruited monocytes contribute to disease severity.
c. The intriguing and apparently distinct roles of resident microglia, the brain sentinels: on one hand, their essential role in the first phase of recovery from sterile injury, and, on the other hand, their key contribution in the initiation of inflammation autoimmune disease possibly through their interaction with autoreactive $CD4^+$ T cells.

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Oral inflammatory diseases and systemic inflammation: role of the macrophage

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Inflammation is a complex reaction to injurious agents and includes vascular responses, migration, and activation of leukocytes. Inflammation starts with an acute reaction, which evolves into a chronic phase if allowed to persist unresolved. Acute inflammation is a rapid process characterized by fluid exudation and emigration of leukocytes, primarily neutrophils, whereas chronic inflammation extends over a longer time and is associated with lymphocyte and macrophage infiltration, blood vessel proliferation, and fibrosis. Inflammation is terminated when the invader is eliminated, and the secreted mediators are removed; however, many factors modify the course and morphologic appearance as well as the termination pattern and duration of inflammation. Chronic inflammatory illnesses such as diabetes, arthritis, and heart disease are now seen as problems that might have an impact on the periodontium. Reciprocal effects of periodontal diseases are potential factors modifying severity in the progression of systemic inflammatory diseases. Macrophages are key cells for the inflammatory processes as regulators directing inflammation to chronic pathological changes or resolution with no damage or scar tissue formation. As such, macrophages are involved in a remarkably diverse array of homeostatic processes of vital importance to the host. In addition to their critical role in immunity, macrophages are also widely recognized as ubiquitous mediators of cellular turnover and maintenance of extracellular matrix homeostasis. In this review, our objective is to identify macrophage-mediated events central to the inflammatory basis of chronic diseases, with an emphasis on how control of macrophage function can be used to prevent or treat harmful outcomes linked to uncontrolled inflammation.

Keywords: innate immune system, macrophage, oral disease, inflammation, resolution

INTRODUCTION

Inflammation is the physiological response of the body to injury. The inflammatory response can be either acute and of short duration or chronic, which does not resolve and leads to pathology. The major function of innate immune cells most studied during the inflammatory process is the identification and recognition of the injurious and/or foreign substances promoting the defense response. Less acknowledged roles played by the innate immune cells involve the resolution pathways and wound healing, both of which include repair and regeneration of lost or damaged tissues. These are now recognized as highly regulated, active processes rather than passive events (Van Dyke, 2008). Macrophages are actively involved in all phases of inflammation and their role as effector and regulatory cells is now widely recognized. Another interesting and important feature of macrophages is their high level of specialization and tissue specificity. While all tissue-bound macrophages differentiate from circulating monocytes, they acquire distinct characteristics and functions locally due to their response profiles. One of the major factors for this diversity is the complexity of microbial load as well as tissue architecture. Thus, it is no surprise that some of the most sophisticated interactions between the host and parasites also dictate the most evolved phenotypic characteristics of the macrophage. Some examples of this specificity and complexity of macrophage phenotype and function are the Kupffer cells of the liver and macrophages of the lung alveoli where the cells, while similar in appearance, are involved in distinct responses against different pathogens as well as non-pathogenic stimuli.

The oral cavity is one of the most ecologically complex microenvironments in the human body where interactions between the host and microbes define health and disease (Gemmell et al., 1997). The teeth are the only functional hard tissues extending from inside to outside of the human body crossing a series of other hard (i.e., bone) and soft (i.e., connective tissue and epithelia) tissues surrounded by a tight biofilm formed by the richest collection of bacteria outside the colon. Such architecture creates several zones, which work in concert during the inflammatory responses in the mouth. Regulation of immune–inflammatory mechanisms in oral disease is governed in part by patient susceptibility and environmental factors (Seymour, 1991; Seymour and Gemmell, 2001; Uitto et al., 2003). In particular; oral macrophages address these complex requirements for mounting a successful inflammatory response as the cell type at the center of many processes including signaling to resolution of inflammation, healing, and regeneration. In this review, within the context of pathogenic mechanisms, possible clinical outcomes will be

discussed in relation to the inflammatory–immunological changes throughout the disease process. Since most inflammatory diseases of the oral cavity involve the tissues of periodontium, the pathological changes in the periodontal structure will be used as a model to assess the role of the macrophages in oral inflammation and its resolution.

ACTIVATION OF ORAL INFLAMMATION AND THE ROLE OF MACROPHAGES

Typically, there are two common diseases affecting the oral tissues and the health of the supporting structures of a tooth. In the case of *gingivitis*, inflammation is limited to the soft tissues, epithelium, and connective tissue; or in the case of *periodontitis*, inflammatory processes extend to the supporting tissues including the alveolar bone (Page and Schroeder, 1976). In both forms of periodontal inflammation, the pathological consequences are associated with the accumulation of bacteria at the tooth surface leading to a host response generating inflammatory cell infiltration (Socransky and Haffajee, 2005). Since the soft and hard tissues of the oral cavity are part of the same functional and physiological organ, separating the host response to several components is artificial and does not acknowledge the dynamic relationship between the cells, bacteria, and extracellular structures. Likewise, while practical and instructive, the supposition of a linear shift in lesions from acute to chronic is not clear. Recent discoveries defining the pathways of resolution in the inflammatory processes challenge the concepts of compartmentalization and linearity in acute and chronic responses (Serhan, 2010; Pruss et al., 2011). Nevertheless, this is the prevailing paradigm, since the tools for analyses of the events at multiple levels are just being incorporated into oral research (Singh et al., 2011; Hasturk et al., 2012). Based on the preliminary results of studies that use high-throughput measurements to generate a systems-biology approach, the complex nature of host–bacteria interactions in a highly complex environment of the oral cavity is being redefined (Bakthavatchalu et al., 2011; Mishima and Sharma, 2011; Singh et al., 2011). To this end, novel approaches have revealed the orchestrated coupling of activation and resolution phases as well as tissue healing.

Macrophages are central to the coordinated resolution of inflammation and return to tissue homeostasis (Zadeh et al., 1999). During the first step of the inflammatory process directed against microorganisms, bacteria, and their virulence factors (e.g., capsule, lipopolysaccharide, fimbria) trigger receptor-mediated production of cytokines by epithelial cells with simultaneous release of neuropeptides, which cause vasodilation of local blood vessels. Generation of chemoattractant proteins (chemokines) at this stage results in attraction of the first line of defense, the neutrophil, which leave the vessels and migrate to the site of microbial invasion. This step is critical and plays a pivotal role in generation of an effective defense system. Neutrophils are followed by the macrophages. This is the step usually where clinical signs of oral inflammation including bleeding, swelling, and redness of the gingiva are detectable. The inflection can either be confined and cleared by the function of neutrophils and macrophages at this early stage, or expand to include the other cells and structures (Page and Schroeder, 1976). Being myeloid cells of hematopoietic origin (Medzhitov and Janeway, 1997; Janeway and Medzhitov, 2002) the overall role of the macrophages is to limit the pathological changes to the soft tissues or elevate the inflammatory response to the next level. Major functions of macrophages include elimination of invading bacteria, recruitment of other cells to the site of infection, clearance of the excess neutrophils, production of cytokines and chemokines, and activation of the lymphocyte-mediated adaptive immune response. The net outcome of these functions can be either complete resolution with healing, limiting the infection with resultant fibrosis and healing with scar tissue formation, or a failure to clear the infection with establishment of a chronic inflammatory lesion.

In the case that the inflammatory process is prolonged and becomes chronic, destruction of soft and hard tissues including the alveolar bone is observed due to direct tissue destruction mediated by inflammation (McCauley and Nohutcu, 2002; Hasturk et al., 2006; Taubman et al., 2007; Graves, 2008; Li et al., 2011). Macrophages together with neutrophils are responsible of phagocytosis and digestion of microorganisms and foreign substances through surface receptors that recognize and bind certain surface molecules of bacteria such as the lipopolysaccharides (LPS; Medzhitov and Janeway, 1997). These receptors are the key components for distinguishing between the host and the invader and defined as pathogen recognition receptors called toll-like receptors (TLR; Anderson, 2000), which mediate the elimination of the pathogenic microbes through phagocytosis and killing (Wingrove et al., 1992). TLRs regulate apoptosis, inflammation, and immune responses (Anderson, 2000). Evidence supporting a role for TLR-mediated recognition of macrophage function in resolution of inflammation is accumulating providing strong support indicating that this receptor–ligand interaction is key to the homeostatic restoration of the host defense (Duffield et al., 2006; Schif-Zuck et al., 2011). Recently, a group of nucleotide-binding oligomerization domain proteins (NODs) have been described as potential regulators of apoptotic events and nuclear factor κB (NF-κb) activation within the context of pathogen recognition and the inflammatory responses (Inohara and Nunez, 2003). While it is not clear how NODs are involved in oral inflammatory diseases, evidence suggests that they are expressed in gingival cells and may play role in promotion of oral inflammation (Uehara and Takada, 2007; Tang et al., 2011).

The TLR family is the best-characterized class of pathogen recognition receptors. TLRs are unique receptors that recognize molecules, broadly shared by microorganisms, but are distinguishable from the host molecules, referred to as "pathogen-associated molecular patterns (PAMP)." TLRs detect multiple PAMPs, including LPS, bacterial lipoproteins and lipoteichoic acids, flagellin, CpG DNA of bacteria and viruses, double-stranded RNA, and single-stranded viral RNA (Iwasaki and Medzhitov, 2004). To date, 11 different TLRs have been identified (Liu et al., 2000; Takeda et al., 2003; Krutzik and Modlin, 2004; Quesniaux et al., 2004). When TLRs bind to antigens, series of intracellular events are initiated and the process leads to the production of cytokines, chemokines, and antimicrobial peptides (Donati et al., 2009). The binding can be through four different adapters. Each adapter has the potential of producing various cytokines stimulating NF-κB pathway in the nucleus of the cell. Known adapter proteins of TLRs are MyD88, toll–interleukin-1 receptor domain

containing adapter protein (TIRAP), toll–interleukin-1 receptor domain containing adapter-inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM). TLRs also utilize interleukin-1 receptor-associated kinase (IRAK), and TNF receptor-activated factor 6 (TRAF6; Jiang et al., 2000). Different TLRs induce different responses; for example, in dendritic cells, the interaction of TLR 4 and LPS results in the production of pro-inflammatory cytokines such as interleukin-12. TLR-2 and TLR 4 have been shown to be expressed in oral tissue cells. The same TLR can trigger different responses through different intracellular adapter proteins (Alexopoulou et al., 2001; Kaisho and Akira, 2002; Cook et al., 2004; Krutzik and Modlin, 2004; Watters et al., 2007). TLRs 1, 2, 4, 5, and 6 specialize in the recognition of mainly bacterial products that are unique to bacteria and not made by the host. This gives them the specificity to differ the invader from the host (Iwasaki and Medzhitov, 2004). Recognition by the TLR pathway is a crucial phase in inflammation. After recognition, many cytokines are released from various cell types including the macrophages through the NFκB pathway (Uehara and Takada, 2007). After TLR4 activation, MyD88 is recruited to TLR4 through respective Toll/IL-1 receptor (TIR)–TIR interactions (Medzhitov et al., 1998; Muzio et al., 1998; Raschi et al., 2003). MyD88 also contains a death domain (DD), a highly conserved protein-binding domain that facilitates its interaction with another DD-containing signaling molecule, IRAK (Cao et al., 1996). IRAK subsequently undergoes phosphorylation and dissociates from MyD88, interacts with TRAF6, and thereby activates several downstream kinases (Cao et al., 1996; Yamin and Miller, 1997; Aderem and Ulevitch, 2000; Jiang et al., 2000; Swantek et al., 2000; Raschi et al., 2003). Following LPS stimulation, two signaling pathways have been described, the MyD88-dependent and -independent pathways (Akira et al., 2000; Kawai et al., 2001; Sato et al., 2002; Yamamoto et al., 2003). Endotoxin activation of the MyD88-dependent pathway results in rapid NF-κB activation and release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-1β. Endotoxin activation of the MyD88-independent pathway results in rapid activation of interferon regulatory factor 3 (IRF3) leading to beta interferon (IFN-β) release with delayed NF-κB activation (Akira et al., 2000; Kawai et al., 2001; Hoebe et al., 2003). The TLR proteins possess leucine-rich extracellular repeats that recognize the LPS binding protein (LBP)–CD14 complex (Poltorak et al., 2000). The TLR intracellular domain resembles the IL-1β receptor, hence the term TIR homology domain (Medzhitov et al., 1997; Chaudhary et al., 1998; Rock et al., 1998). The TIR domain in the cytoplasmic portion of the molecule is considered essential for triggering activation of mitogen-activated protein kinases (MAPKs) and the transcription factor NF-κB (Means et al., 2000; Akira et al., 2001; Sato et al., 2002). While CD14 is the major LBP on the surface of mononuclear phagocytes, CD14 is not capable of transducing signals across the membrane. A receptor complex comprised of CD14, TLR-2, TLR4, and accessory proteins (MD-2) is necessary for receptor function as well as various kinases, including the three classes of MAPK: extracellular signal-regulated kinase (ERK) 1 and ERK2 (Weinstein et al., 1992), p38 MAPK (Han et al., 1994), and c-Jun N-terminal kinases (JNK; Hambleton et al., 1996). Numerous inflammatory cytokines and mediators are expressed in LPS stimulated macrophages through activation of transcription factors including NF-κB and activator protein-1 (Fujihara et al., 1993; Muroi et al., 1993; Guha and Mackman, 2001). LPS recognition is initiated by LBP, a serum glycoprotein, that first binds to the lipid A moiety of LPS (Schumann et al., 1990; Wright et al., 1990; Gegner et al., 1995). The LPS–LBP complex is then recognized by CD14 (Schumann et al., 1990; Ulevitch and Tobias, 1995; Haziot et al., 1996). Mice with a targeted deletion of the gene encoding CD14 are hyporesponsive to LPS and resistant to the lethal effects of LPS (Haziot et al., 1996). However, mice lacking CD14 are still able to respond to high concentrations of LPS (Wurfel et al., 1997). CD14 is a glycosylphosphatidylinositol-anchored (GPI-anchored) molecule which lacks a cytoplasmic signaling domain, making it incapable of downstream signaling (Haziot et al., 1988). It is not fully clear if the TLR-mediated pathways are directly involved during the oral inflammatory responses including resolution.

We have previously identified moesin as a participant in LPS binding and signal transduction (Tohme et al., 1999). Many physiological and pathophysiological conditions are attributable in part to cytoskeletal regulation of cellular responses to signals. Moesin is an ERM (ezrin, radixin, and moesin) family member and was identified as part of a protein cluster. Moesin was found to be necessary for the detection of LPS, and homozygous moesin knockout mice exhibited a threefold reduction in neutrophil infiltration into LPS injected sites when compared to wild type controls (Amar et al., 2001). Anti-moesin antibody inhibited the release of TNF-α by LPS stimulated monocytes (Tohme et al., 1999), and moesin was also found to be expressed on the surface of differentiated THP-1 cells and primary peripheral blood monocytes. LPS stimulation increased the surface expression of moesin as well as its total protein levels when analyzed by FACS and Western blotting, respectively. Furthermore, moesin was found to co-immunoprecipitate with TLR4 after LPS stimulation (Iontcheva et al., 2004). In moesin mRNA knockdown experiments using antisense mRNA, THP-1 cells no longer responded to LPS (Iontcheva et al., 2004), suggesting a role for moesin in LPS signaling. Using differentiated THP-1 cells, co-immunoprecipitation experiments revealed that moesin and CD14 were associated in the cell membrane in both resting and LPS stimulated cells. TLR4 and MD-2 became associated with moesin and CD14 only after LPS stimulation. These experiments also demonstrated that there was a direct binding between moesin and LPS. Moesin was phosphorylated and mRNA levels of moesin increased significantly after LPS stimulation. During the TLR4-mediated response to LPS, moesin stimulates the NF-κB, p38, and p44/42 MAPK activation (Iontcheva et al., 2004; Zawawi et al., 2010). **Figure 1** shows a model for recognition of LPS involving the dynamic association of multiple molecules, including moesin, forming a cluster that functions as the LPS receptor and an important role in the macrophage-mediated innate immune response and TLR4-mediated pattern recognition in oral inflammatory diseases.

Cytokines and other products of macrophages can also modulate the action, differentiation, and survival of cells outside the immune system, such as the nervous system. The interaction between macrophages and the nervous system relies on the receptor-sensitizing characteristics of cytokines (Opree and Kress, 2000) linked to the discovery of protease-activated receptors

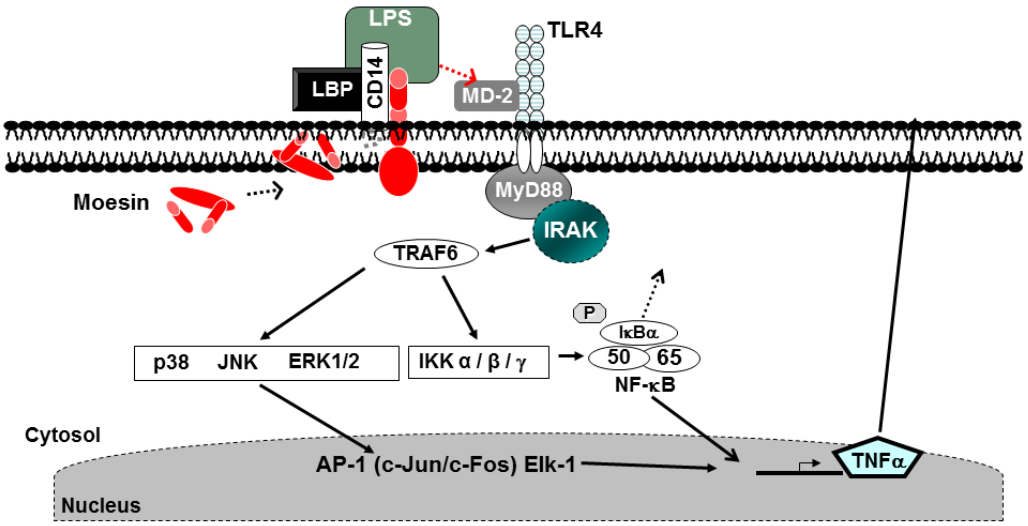

FIGURE 1 | Lipopolysaccharides (LPS) recognition and signaling in macrophages. CD14 and moesin are expressed on the cell membrane in macrophages. LPS stimulation results in phosphorylation of moesin, binding to the TLR4 and MD-2 activating the MyD88. Signaling through this mechanism leads to the production of pro-inflammatory cytokines (Zawawi et al., 2010).

(PARs). Research into the functionality of these receptors has shown that PAR-2 has a particularly important role in disease states associated with chronic inflammation (Vergnolle, 1999). Identification of neuropeptide receptors on immune cells indicates a communication between the immune and neurological systems that possibly results in the modulation of inflammatory response through G-protein-coupled receptors located on the cell membranes or the vanilloid receptor-1 (also named TRPV1), which is shown to be up-regulated in inflammatory bowel disease. These findings suggest a possible role for this receptor in chronic inflammation (McGillis et al., 1991; Yiangou et al., 2001; Tracey, 2002; Lundy and Linden, 2004). Cytokines have been shown to regulate substance P expression and response to LPS (Kessler and Freidin, 1993; Hua et al., 1996). Substance P limits the production of TGF-β by macrophages and induces synthesis of IL-6 (Lieb et al., 1996; Marriott and Bost, 1998). Macrophages can produce substance P when activated with LPS *in vitro* (Lambrecht et al., 1999). The precise mechanism through which these receptor-mediated events might regulate the macrophage response in the oral cavity is not clear; future research is needed to understand their role.

ROLE OF MACROPHAGES IN GINGIVAL INFLAMMATION AND BONE RESORPTION

Macrophages efficiently ingest particulate antigen, express MHC class II molecules and have co-stimulatory activity on T cells. Macrophages can be phenotypically polarized by the microenvironment. The classically activated macrophages (M1) are activated by IFN-γ and LPS, and alternatively activated macrophages (M2) produced in response to IL-4 or IL-13 (Martinez et al., 2009). M2 macrophages have been shown to play role in resolution of inflammation with a reduced capacity to produce cytokines (Bhatavadekar and Williams, 2009). Cytokine and chemokine production by macrophages is a key step in immune response and the inflammation process. Cytokines interact between each other, amplify signaling, modulate cell surface receptors, and perform synergistic or antagonistic interactions on cell function (Balkwill and Burke, 1989). It is not only the presence of one cytokine that regulates the response, but the concentration of the same mediator can also affect the outcome of a response (Gemmell et al., 1997). Their secretion is dependent on the NF-κB in the nucleus of many immune system cells (Baldwin, 1996; Hanada and Yoshimura, 2002). In addition to macrophages, cytokines can be produced by both resident cells such as epithelial cells, fibroblasts and other phagocytes such as neutrophils in the periodontal tissues (Ara et al., 2009). After microbial recognition, cytokines in innate response such as TNF-α, IL-1, and IL-6 are the first to start communication in disease pathogenesis (Garlet, 2010). IL-1β and IL-6 are the signature innate cytokines and have been characteristically associated with inflammatory cell migration, highly produced by the macrophages and involved in osteoclastogenesis processes (Graves et al., 2008; Fonseca et al., 2009). TNF-α is a multi-role cytokine, that has many functions from cell migration to tissue destruction. It induces the up-regulation of adhesion molecules, stimulates the production of chemokines, and is involved in cell migration to infected and inflamed sites (Peschon et al., 1998; Dinarello, 2000; Wajant et al., 2003; Kindle et al., 2006). TNF-α up-regulates the production of other signature pro-inflammatory innate immunity cytokines, such as IL-1β and IL-6 (Okada et al., 1997; Dinarello, 2000; Wajant et al., 2003; Kwan Tat et al., 2004; Garlet et al., 2007; Graves et al., 2008; Musacchio et al., 2009). TNF-α is also correlated with extracellular matrix (ECM) degradation and bone resorption through its positive correlation with matrix metalloproteinases (MMPs) and RANKL expression (Graves and Cochran, 2003; Garlet et al., 2004; Graves et al., 2008). Experimental periodontitis in TNF-α p55 receptor deficient mice was characterized by a significant decrease in MMPs and RANKL expression (Garlet et al., 2007). Thus, in addition to direct actions in bone resorption, macrophage-derived cytokines also interfere

with the coupled bone formation process (Behl et al., 2008). IL-13 is another potent modulator of human monocyte/macrophage function. Monocyte/macrophage cell surface markers, MHC class II and several integrin molecules are up-regulated by IL- 13 (de Waal Malefyt et al., 1993). The monocyte/macrophage related production of the cytokines IL-1α, IL-1β, 1L-6, IL-8, and TNF-α is also inhibited by IL- 13. On the other hand, IL-1 receptor antagonist secretion is enhanced (de Waal Malefyt et al., 1993; Zurawski and de Vries, 1994). Therefore, IL-13, along with IL-4 and IL-10, would appear to have potential anti-inflammatory activity (Zurawski and de Vries, 1994).

In addition to their cell trafficking role, chemokines provide messages leading to other biological processes, such as angiogenesis, cell proliferation, apoptosis, tumor metastasis, and host defense (Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000; Moser et al., 2004; Rot and von Andrian, 2004; Esche et al., 2005). Chemokines are classified into four subfamilies according to the configuration of cysteine residues near the N-terminus. Chemokines engage their receptors. This binding initiates integrin-dependent adhesion, as well as the binding and detachment of cells from their substrate. Chemokines target all types of leukocytes of the innate immune system, as well as lymphocytes of the adaptive immune system (Terricabras et al., 2004). IL-8/CXCL8 is the first cytokine identified to have chemotactic activity. It can be produced by macrophages as well as fibroblasts, epithelial cells, and endothelial cells (Takashiba et al., 1992; Takigawa et al., 1994; Yumoto et al., 1999). IL-8 is a neutrophil chemoattractant. It is detectable in healthy and diseased periodontal tissues and has been associated with subclinical inflammation (Yoshimura et al., 1987; Payne et al., 1993; Mathur et al., 1996). It has direct action on osteoclast differentiation and activity by signaling through the specific receptor, CXCR1 (Bendre et al., 2003). Another crucial chemokine for macrophage function is MCP-1/CCL2, which mediates the recruitment of monocytes/macrophages (Hanazawa et al., 1993; Okamatsu et al., 2004). Together with RANTES/CCL5, MIP-1α/CCL3 may also be involved in the migration of macrophages to oral tissues (Gemmell et al., 2001; Kabashima et al., 2002). CXCR3 and its ligand IP-10/CXCL10 are also expressed in diseased periodontal tissues and associated with higher levels of IFN-γ during the inflammation process (Kabashima et al., 2002; Garlet et al., 2003). CCR4 is found expressed at higher levels in chronic periodontitis and it is associated with higher levels of IL-4 and IL-10 messages in the periodontium (Garlet et al., 2003, 2004).

In addition to recruitment of cells, chemokines are crucial in guiding adaptive immunity cells with a role in bone metabolism. MDC/CCL22, TARC/CCL17, and I- 309/CCL1 have been shown to attract Th2 and Treg cells via binding their CXCR 4 and CXCR 8 receptors (D'Ambrosio et al., 1998; Sallusto et al., 1998; Gu et al., 2000). Chemokines have been recognized as essential signals for the trafficking of osteoblast and osteoclast precursors, and consequently as potential modulators of bone homeostasis (Bendre et al., 2003; Wright et al., 2005). Chemokines are capable of regulating bone metabolism via CCR1, CCR2, CXCR3, and CXCR4 receptors expressed on osteoclast precursors, mature osteoclasts, and osteoblasts. These receptors have the ability to bind many different chemokines such as SDF-1/CXCL12, MIP-1α/CCL3, RANTES/CCL5, MIP-1γ/CCL9, MCP- 1β/CCL2, MCP-3/CCL7, MIG/CXCL9, and CKβ8/CCL23 (Votta et al., 2000; Lean et al., 2002; Okamatsu et al., 2004; Yu et al., 2004; Kwak et al., 2005; Wright et al., 2005; Kim et al., 2006a,b; Yang et al., 2006). IP-10/CXCL10 induces osteoblast proliferation through receptor CCR3 (Grassi et al., 2003; Lisignoli et al., 2004), while SDF-1α/CXCL12 and BCA-1/CXCL13 induce both proliferation and collagen type I mRNA expression in osteoblasts through receptors CCR4 and CCR5 (Lisignoli et al., 2006). In addition to its role in osteoclastogenesis, chemokines also affect osteoclast functions. It has been reported that SDF-1α/CXCL12 increases MMP-9 activity in human osteoclasts, resulting in increased bone resorption activity (Grassi et al., 2004). There is evidence that RANTES/CCL5 can also act on osteoblasts, resulting in chemotaxis and promoting cell survival (Yano et al., 2005). RANKL also induces the production of MCP-1/CCL2, MIP-1_/CCL3, RANTES/CCL5, and MIG/CXCL9 by osteoclasts, suggesting a coupling role, which could contribute to bone resorption (Kim et al., 2006a). Taken together, these studies suggest that macrophage-produced chemokines can effectively contribute to the bone remodeling process by driving osteoblast migration and activation during periodontal wound healing.

An important key mediator of macrophage function is the prostaglandins, which are derived from hydrolysis of membrane phospholipids. Phospholipase A_2, cleaves arachidonic acid, a precursor of a group of small lipids known as eicosanoids, from membrane phospholipids. Eicosanoids generally act as inflammatory agents (Lewis, 1990). Arachidonic acid is metabolized via two enzymatic pathways. The first is the action of lipoxygenases that results in the formation of the hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT). Alternatively, cyclooxygenases (COX) catalyze the conversion of arachidonic acid into prostaglandins, prostacyclins, and thromboxanes. Prostaglandins have 10 sub-classes, of which D, E, F G, H, and I are the most important (Gemmell et al., 1997). Inflamed oral tissues synthesize significantly large amounts of prostaglandins (Mendieta et al., 1985). Prostaglandin E_2 is the most potent stimulator of alveolar bone resorption (Goodson et al., 1974; Dietrich et al., 1975). Within oral lesions, prostaglandin E_2 is mainly localized within macrophage-like cells and secreted when stimulated with bacteria LPS (Loning et al., 1980). Periodontal ligament cells also produce prostaglandin E_2 even at rest. This secretion is enhanced by IL-1β, TNF-α, and parathyroid hormone (Richards and Rutherford, 1988; Saito et al., 1990a,b). Prostaglandin E_2 has a biphasic action on cells; in high doses, it decreases IgG levels but in low doses has the potential to increase them. When combined with IL-4, low doses of prostaglandin E_2 induce a synergistic rise in IgG production, suggesting an immune-regulatory role for prostaglandin E_2 (Harrell and Stein, 1995).

Disruption of the balance between osteoblast and osteoclast activities by bacterial products and inflammatory cytokines constitutes the main underlying causes of inflammation-induced bone loss (Liu et al., 2000). It is shown that LPS of bacteria either directly or through its action on the macrophages is capable of stimulating bone resorption when added to osteoclast precursor cultures containing osteoblasts and/or stromal cells (Iino and Hopps, 1984). In addition to this, TLR and inflammation-induced osteoclastogenesis pathway is the most common pathway related to bone loss (The American Academy of Periodontology Academy

Report, 1999; Pihlstrom et al., 2005). Inflammation-induced and macrophage-mediated bone loss in oral infection involves complex inflammatory signals and cytokine networks regulating osteoclastogenesis, such as RANKL, interleukin-1, interleukin-6, tumor necrosis factor-α, and prostaglandin E_2 have been reported to be significantly associated with this type of tissue destruction (Henderson et al., 2003). Before the discovery of receptor activator of NKκB (RANK), its ligand (RANKL), and its antagonist osteoprotegerin (OPG), the development and formation of osteoclasts were attributed to factors produced by osteoblasts and bone marrow stromal cells (Rodan and Martin, 1981; Martin and Sims, 2005). It is now clear that RANKL, RANK, OPG are the key regulators of bone remodeling, directly involved in the differentiation, activation, and survival of osteoclasts and osteoclast precursors (Anderson et al., 1997; Lacey et al., 1998; Yasuda et al., 1998). RANKL is expressed by osteoblasts, stromal cells, chondrocytes, and other mesenchymal cells. Activated T and B cells can also express RANKL (Theill et al., 2002; Mahamed et al., 2005; Kawai et al., 2006). RANK is expressed by osteoclast progenitors, mature osteoclasts, chondrocytes, monocytes/macrophages, and dendritic cells (Anderson et al., 1997; Hsu et al., 1999). Their decoy receptor OPG is known to be expressed by periodontal tissue cells like fibroblasts and periodontal ligament cells (Liu et al., 2000). Blocking RANKL activity with OPG significantly inhibits bone loss in rheumatoid arthritis, osteoporosis, cancer-related bone metastasis, and diabetes associated alveolar bone destruction (Mizuno et al., 1998; Kong et al., 1999; Honore et al., 2000; Brown et al., 2004; Hofbauer and Schoppet, 2004; Mahamed et al., 2005), confirming the critical role of the RANKL, RANK, OPG triad in osteoclastogenesis. Osteoclastogenesis via RANK, RANKL pathway depends on Macrophage-Colony Stimulating Factor (M-CSF; Tanaka et al., 1993; MacDonald et al., 2005). Pathogens, stress, or pathology influence the production of M-CSF via pro-inflammatory cytokines and have a significant role on the subsequent osteoclast activity where TLR-2 activation up-regulates the expression of M-CSF (Song et al., 2009). LPS from different pathogens can stimulate bone resorption *in vitro* and in animal models as in primary mouse calvarial osteoblasts, the activation of TLR-2 and TLR-6 by LPS causes enhanced expression of RANKL through a MyD88-dependent mechanism (Sato et al., 2004). In mouse calvarial osteoblasts, expression of TLR 4 and TLR-9 results in the activation of NFκB and related to that the increased secretion of TNF-α and M-CSF (Morse et al., 2008). LPS-induced interleukin-1 production through TLR pathway can up-regulate RANKL and inhibit osteoprotegerin expression by osteoblasts resulting in osteoclast formation in a prostaglandin E2-dependent manner. TLR-2 substantially decreases the responses to LPS (Song et al., 2009). LPS directly, or via TLR pathway by stimulating different cell types is capable of inducing osteoclast development and activity. Thus, TLRs could influence the inflammatory response in the bone microenvironment, and may play a critical role in modulating inflammation-induced osteoclastogenesis and bone loss.

Another mechanism underlying macrophage involvement in oral tissue pathologies is the destruction of ECM. Collagenases, along with other MMPs, play an important role in this process. MMPs are a family of structurally related but genetically distinct enzymes that degrade ECM and basement membrane components. Twenty-three enzymes have been classified into collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs, mainly based on the substrate specificity and molecular structure. MMPs are involved in physiological processes such as tissue development, remodeling, and wound healing. MMP activity is controlled by changes in the delicate balance between the expression and synthesis of MMPs and their major endogenous inhibitors, tissue inhibitors of MMPs (TIMPs). It is clear that MMPs are up-regulated in periodontal as well as other types of oral inflammation (Ebert et al., 2005). MMP activation involves tissue and plasma proteinases and bacterial proteinases together with oxidative stress (Henry et al., 2002; Rot and von Andrian, 2004). It is now clear that a broad range of cell types present in the normal and diseased human periodontium such as gingival sulcular epithelial cells, fibroblasts and endothelial cells, monocytes/macrophages, neutrophils, and plasma cells has the ability to express distinct MMPs (Sorsa et al., 1995; Takagi et al., 1995; Kiili et al., 2002; Wahlgren et al., 2002).

Matrix metalloproteinases gene transcription is very low in the healthy periodontal tissue; their secretion is stimulated or down-regulated by various cytokines. The main stimulatory cytokines for MMPs are TNF-α, IL-1, and IL-6. Activated MMPs are capable of activating other MMPs (Visse and Nagase, 2003). There is a close interaction between MMP activation and cytokine function. IL-1β and TNF-α can stimulate MMP-3, MMP-8, and MMP-9 secretions from gingival fibroblasts and MMP-13 in osteoblasts. TGF-βa suppresses MMP-1, MMP-3, and MMP-8 gene transcription but induces MMP-2 and MMP-13 in keratinocytes (Birkedal-Hansen, 1993; Kahari and Saarialho-Kere, 1999; Konttinen et al., 1999). MMP-1 (collagenase-1) has a wide range of substrates. It can digest interstitial collagen, ECM components, and soluble non-matrix mediators (Sorsa et al., 2006). MMP-9 (gelatinase B) is a gelatinolytic enzyme degrading several ECM proteins, including basement membrane-type IV collagen (Lee et al., 1995; Sume et al., 2010; Kantarci et al., 2011). MMP-9 is found to be expressed in epithelial cells; its production can be stimulated by several cytokines such as TNF-α, growth factors such as epidermal growth factor, and by some bacterial products such as LPS (Putnins et al., 1996; Firth et al., 1997). Although MMP-1, MMP-8, and MMP-9 are the main enzymes that are involved in the ECM and base membrane breakdown, other MMPs and their tissue inhibitors (TIMPs) have also been linked to periodontal diseases. MMP-2 (gelatinase A) has been shown to be strongly expressed in inflamed pocket epithelium and to be important in epithelial cell migration (Makela et al., 1999). MMP-13 (collagenase-3) is expressed by the basal cells of the gingival pocket epithelium able to degrade collagens type I, III, and IV as well as fibronectin, tenascin, and some proteoglycans (Kahari and Saarialho-Kere, 1997; Knauper et al., 1997; Uitto et al., 1998). MMP-13 plays an important role in the ability of pocket epithelium to invade periodontal connective tissue. Some oral bacterial species, especially *Fusobacterium nucleatum* were found to induce MMP-13 (Uitto et al., 2003).

RESOLUTION OF ORAL INFLAMMATION AND THE ROLE OF MACROPHAGES

Oral inflammatory diseases can be considered as an adverse outcome of the protection efforts of the host against the invading

pathogens. Inflammation should resolve in a timely manner to prevent tissue injury, and maintain health. The rapid and complete elimination of invading leukocytes from a lesion is the ideal outcome following an inflammatory event (Schwab et al., 2007). Inadequate resolution and failure to return tissue to homeostasis results in neutrophil-mediated destruction and chronic inflammation (Van Dyke and Serhan, 2003). If the host is unable to neutralize the pathogens, then acute inflammation would become chronic with consequences such as destruction of ECM and bone, scarring, and fibrosis (Van Dyke, 2008). Controlling the invasion of the neutrophils can impact the conversion of an acute gingivitis to chronic periodontitis. Scarring and fibrosis in periodontitis prevents the return to homeostasis (Van Dyke, 2007). When tissue injury is mild, necrotic cells will be replaced by new cells by regeneration process. If tissue damage is extensive, the process of healing is repair. When repair takes place, fibrin is not cleared rapidly and efficiently after the acute phase of inflammation and granulation tissue is formed from surrounding tissue compartments. Later phases of repair involve fibroblast-mediated collagen deposition, disappearance of vascular tissues and replacement of these areas by avascular and fibrotic scar tissue (Kumar et al., 2005). The efforts to control inflammation process has been mainly with the use of pharmacologic agents, which act as antagonists for some of the mediators of inflammation (Serhan et al., 2007). The resolution of inflammation previously thought to be a passive event, but greater understanding of the pathways and processes underlying resolution of inflammation has led to the recognition of an active progress where the activation of pro-resolving molecules are needed to neutralize and eliminate inflammatory leukocytes, and thereby prevent pathology (Van Dyke and Serhan, 2003; Van Dyke, 2007; Serhan et al., 2008). Restoration of tissue homeostasis is initiated following an acute inflammatory response that generates lipid mediators of inflammation (Van Dyke, 2008). Various lipid mediators such as eicosanoids, prostanoids, and prostacyclins are produced upon agonist stimulation of G-protein receptors on the cell membrane. Arachidonic acid (AA) plays key role in this process (Kantarci and Van Dyke, 2003) and is metabolized either by a cyclooxygenase (COX)-1 or COX-2-dependent pathway that results in the generation of prostanoids or a 5-lipoxygenase (5-LO)-dependent pathway that results in leukotriene (LT) production.

There is a high concentration of cells containing lipoxygenases, and corresponding pro-inflammatory products, a "class switch" may occur within neutrophils (Levy et al., 2001; Van Dyke, 2007). This class switch gives rise to the synthesis of pro-resolving molecules. One of the active resolution molecules is lipoxins, which are generated late in inflammation when a second lipoxygenase interacts with a lipoxygenase product generated earlier by a different cell (Serhan, 2004). These molecules are synthesized through a series of enzymatic reactions starting with the oxidation of AA by 15 LO through the process of transcellular biosynthesis, resulting in 15-*S*-hyroxy-(*p*)-eicosatetraenoic acid [15-*S*-H(*p*)ETE]. Accordingly, 15-*S*-H(*p*)ETE is further acted on by 5-LO to induce the synthesis of lipoxins, such as lipoxins A_4 (LXA_4) and B_4 (LXB_4; Kantarci and Van Dyke, 2003; Van Dyke and Serhan, 2003). The lipoxins produced act as agonists to stimulate the resolution of inflammation and promote the restoration of tissue homeostasis through a number of mechanisms. These include limiting PMN migration into sites of inflammation, activating monocytes without the generation of a superoxide anion, and stimulating the uptake of apoptotic PMN by macrophages (Serhan et al., 1993; Maddox and Serhan, 1996; Maddox et al., 1997). When the lipoxin pathway is activated and aspirin is present during this synthesis, acetylation of the COX-2 enzyme occurs to inhibit further production of prostanoids from AA metabolism. This alternative pathway will lead to the synthesis of 15-R-H(*p*)ETE transforming to 5(6)-epoxytetraene with the help of 5-LO activity. The next step is the synthesis of 15-epi-LXs or aspirin-triggered lipoxins (ATLs) from 5(6)-epoxytetraene (Van Dyke and Serhan, 2003). 15-epi-LX is a form of native lipoxin and possesses potent pro-resolving properties (Serhan et al., 1995; Claria et al., 1996; Van Dyke and Serhan, 2003).

In addition to the omega-6 derived pro-resolving molecules, resolvins, and protectins are derived from the omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA; Van Dyke, 2007; Serhan and Chiang, 2008). They are able to stimulate anti-inflammatory and pro-resolving pathways similar to the lipoxins, but their binding sites on inflammatory cells differ from each other (Serhan et al., 2004; Van Dyke, 2007; Serhan and Chiang, 2008). Resolvins stimulate the resolution of inflammation through multiple mechanisms, including preventing neutrophil penetration, phagocytosing apoptotic neutrophils to clear the lesion, and enhancing clearance of inflammation within the lesion to promote tissue regeneration (Bannenberg et al., 2005; Hasturk et al., 2007; Schwab et al., 2007). The classic inflammatory eicosanoids (i.e., prostaglandins and leukotrienes), in addition to activating and amplifying the cardinal signs of inflammation, are also responsible for inducing the production of mediators that have both anti-inflammatory and pro-resolution activities, such as the lipoxins, resolvins, and protectins, reinforcing the active nature of the resolution process (Serhan et al., 2008). In humans, the aspirin-tolerant subjects generated both LXA_4 and ATL, but aspirin-intolerant patients proved to have a diminished capacity to generate ATL and LX upon aspirin challenge (Sanak et al., 2000). In an experimental periodontitis rabbit model, animals are protected by LXA_4 and transgenic (TG) rabbits over expressing 15 LO generate enhanced levels of LX, exhibit a reduced inflammatory phenotype, and are protected from bone loss in periodontal disease (Serhan et al., 2003). The treatment with Resolvin-E1 (RvE1) prevented and completely eliminated the signs of inflammation. In the RvE1 treated group, inflammation was completely eliminated; pocket depth was returned to normal and soft tissues returned to healthy levels and appearance (**Figure 2**). Regeneration of new cementum and bone with an organized periodontal ligament was observed (Hasturk et al., 2007). Restoration of crestal bone height, elimination of infrabony defects, and regeneration of new cementum, connective tissue, and bone with an organized periodontal ligament were signs of complete regeneration of tissues to pre-disease levels (Hasturk et al., 2007). Periodontitis was also shown to have a systemic impact elevating the levels of IL-1β and C-reactive protein (CRP) in all animals. Oral topical RvE1 therapy reduced systemic IL-1β and CRP levels. Rabbits treated with RvE1 showed an essentially complete recovery without any signs of local and

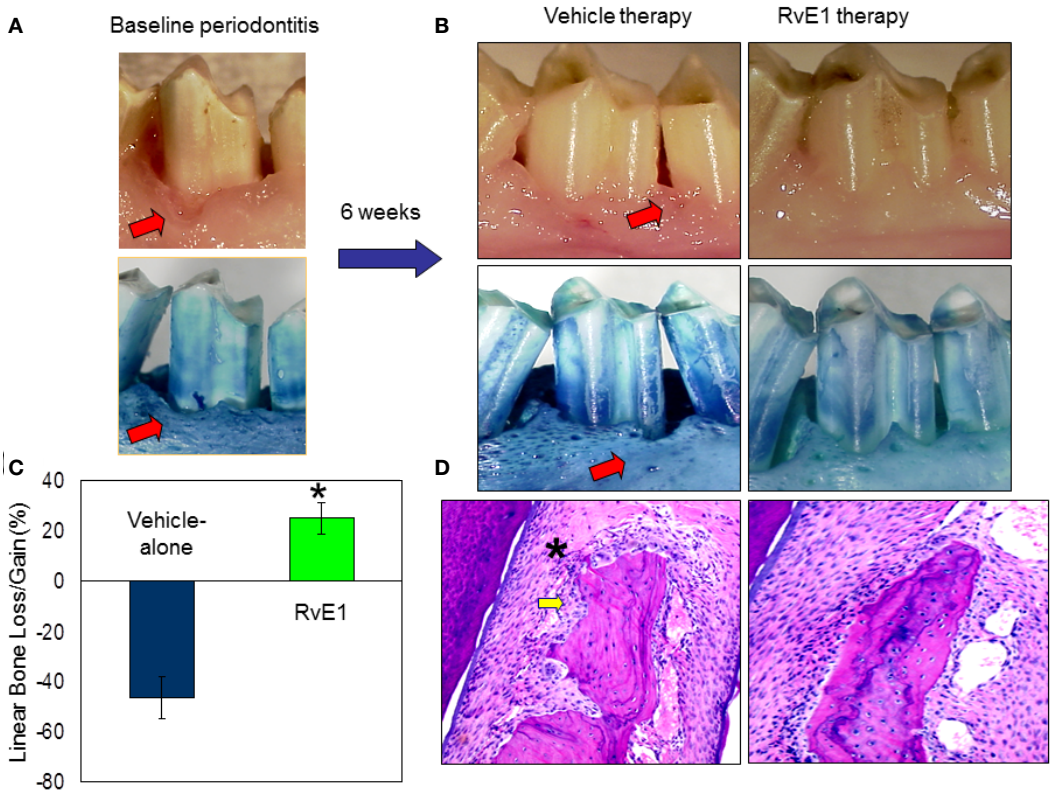

FIGURE 2 | Regulation of inflammation by resolvin-E1 in experimental periodontitis. (A) Periodontal disease was induced by ligature and *Porphyromonas gingivalis* application over 6 weeks in rabbits. Classical characteristic of periodontal disease including tissue and bone loss were observed. **(B)** Sites were treated either with RvE1 (1 mg/ml) or vehicle (ethanol) for an additional 6 weeks. RvE1 treatment did not only stop the disease progression but also reversed the tissue and bone loss and allowed the tissues to reach to a completely healthy state. Vehicle treatment did not have any impact on controlling the disease, conversely the disease continued to progress. **(C)** Histological evaluations confirmed the clinical observations where RvE1 treated sites showed no bone loss and no or minimal inflammatory cell activity. **(D)** Histomorphometric evaluations quantified the bone level changes during these treatments over 6 weeks. While RvE1 treatment resulted in bone gain, vehicle treatment showed worsening and lost more bone as a result of disease progression.

systemic inflammation (Hasturk et al., 2007) suggesting a complete return to tissue homeostasis. This mechanism is most likely regulated through an orchestrated series of events where in addition to neutrophils and lymphocytes, macrophages play the pivotal role.

MACROPHAGES AS A POSSIBLE LINK BETWEEN ORAL AND SYSTEMIC INFLAMMATION

The critical role of the macrophages in inflammatory diseases has been studied extensively in various organ systems in the human body. While the debate over the direction and cross-reactivity of local and systemic inflammation continues regarding which of the local specialized tissues are affected by systemic inflammatory changes and how the specific inflammatory processes in any part of the body have a generalized impact distant to the affected site, it is thought that the relationship is bidirectional (Offenbacher and Salvi, 1999; Amar et al., 2007; Ebersole et al., 2010; Hajishengallis, 2010). Such a dynamic response requires an intricate network of cellular and non-cellular components where macrophages are at the epicenter due to their extensive functional interactions with other cells and processes of inflammation. To this end, research has provided evidence of an oral and systemic connection in several diseases such as diabetes, cardiovascular diseases, and pathological conditions such as pre-term birth (Paquette et al., 1999; Nassar et al., 2007; Offenbacher et al., 2009).

We have previously studied macrophages and their role in the aggravation of inflammation in diabetics and identified critical markers of regulation at cellular signal transduction. These series of studies have demonstrated that oxidative stress plays a substantial role in the pathogenesis of diabetic complications. Superoxide anion is the first molecule generated during the respiratory burst of phagocytes, including macrophages, by NADPH oxidase. Either at rest or after stimulation with PMA or opsonized zymosan (OPZ), monocytes from people with diabetes produced significantly more anion than those from healthy individuals. The increased anion generation was found to be correlated with glycemic control (HbA1c) of patients. To clarify the impact of hyperglycemia on superoxide generation, normal human monocytes were then treated with receptor for advanced glycation end products (RAGE) ligands (advanced glycation end product, AGE protein and S100B) or high glucose media before stimulation. Both RAGE ligands and high glucose concentration increased

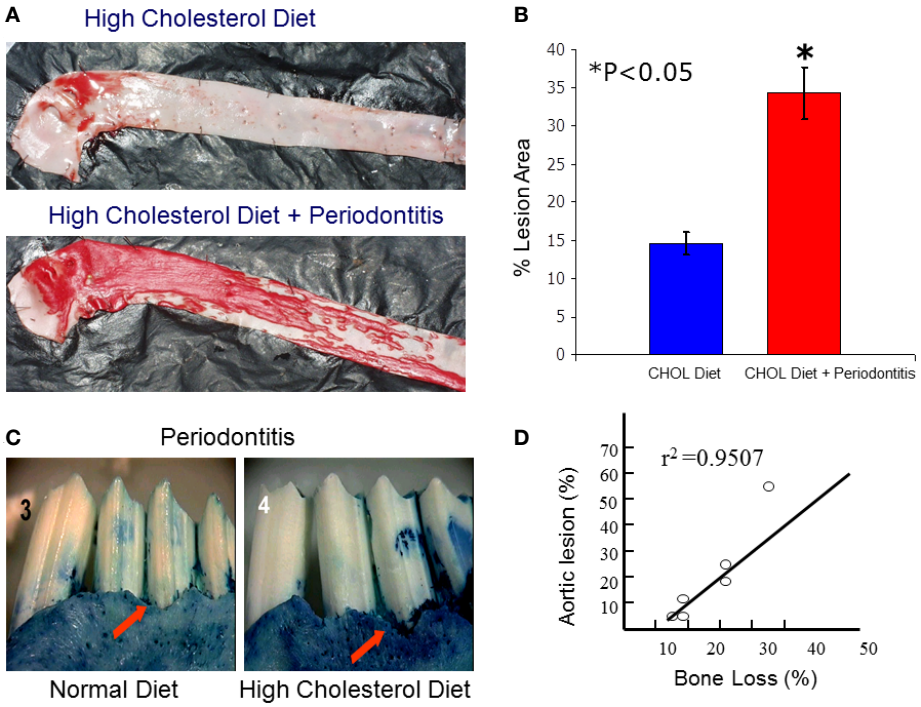

FIGURE 3 | Local periodontal inflammation as a modifier of atherosclerotic changes in aortas of high cholesterol-fed rabbits. **(A)** Atherosclerosis was induced by high cholesterol diet (0.5%) in rabbits over 13 weeks. Simultaneously, periodontal disease was also induced as explained above over a 6-weeks period. At 13 weeks, the aortas dissected en face and stained with Sudan IV for detection of lipid depositions. As a result of high cholesterol diet, rabbits developed early fatty streaks as indicated by Sudan IV stained lipid depositions mainly limited at the aortic arch and thoracic aorta. Rabbits challenged with *P. gingivalis* showed dramatically more and extended level of lipid depositions covering almost entire surfaces of thoracic and abdominal aortas. **(B)** Quantification of lipid covered area clearly showed that local periodontal inflammation significantly increases the atherosclerotic changes induced by cholesterol diet. **(C)** Periodontal disease was also more dramatic in those rabbits received high cholesterol diet suggesting a reciprocal relationship between local and systemic inflammations. **(D)** The severity of bone loss was positively correlated with degree of the fatty streaks (lipid depositions; $r^2 = 0.9501$).

anion generation from human macrophages. Notably, high glucose was associated with correspondingly increased osmotic pressure. This study demonstrated that RAGE ligands can significantly contribute to the hyper-responsive phenotype of diabetic monocytes and macrophages, which might be reversible by blocking RAGE or reducing RAGE ligands by controlling hyperglycemia (Ding et al., 2007a).

Hyperglycemic episodes in diabetes are closely associated with increased oxidative and nitrosative stress, which can trigger the development of diabetic complications. Hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased superoxide anion formation. Superoxide anion interacts with nitric oxide, forming the potent cytotoxin peroxynitrite, which attacks various biomolecules in the vascular endothelium, vascular smooth muscle, and myocardium, leading to cardiovascular dysfunction (Pacher et al., 2005). High concentrations of hydrogen peroxide activate insulin signaling and induce typical metabolic actions of Czech et al. (1974). The pathogenetic role of nitrosative stress and peroxynitrite, and downstream mechanisms including poly(ADP-ribose) polymerase (PARP) activation. PARP activation can also up-regulate various pro-inflammatory pathways which leads to pathological modifications in adhesion molecule expression, angiogenesis, and other processes (Virag and Szabo, 2002).

In order to identify the specific signaling pathway through which the RAGE-mediated functional changes are effected in macrophages in diabetic people, we focused on the enzyme systems, which regulate the oxidative burst in macrophages. To this end, our research has shown that an alteration in the protein kinase C (PKC) family of intracellular enzymes, which plays a crucial role in signaling for a variety of cellular responses of mononuclear phagocytes including phagocytosis, oxidative burst, and secretion, are directly involved in the pathogenesis of the complications of diabetes. The consequences of PKC activation were evaluated by endogenous phosphorylation of PKC substrates with a phospho-specific PKC substrate antibody [pPKC(s)]. Phosphorylation of a 40-kDa protein was significantly increased in mononuclear phagocytes from diabetics as a downstream marker of PKC activation, and its phosphorylated form was found to be associated with the membrane. Through a wide range of techniques including the mass spectrometry, immunoprecipitation, and immunoblotting, we have identified this protein as pleckstrin. Phosphorylation and translocation of pleckstrin in response to the activation of RAGE suggested that pleckstrin was involved in RAGE signaling and AGE-elicited macrophage dysfunction. Suppression of

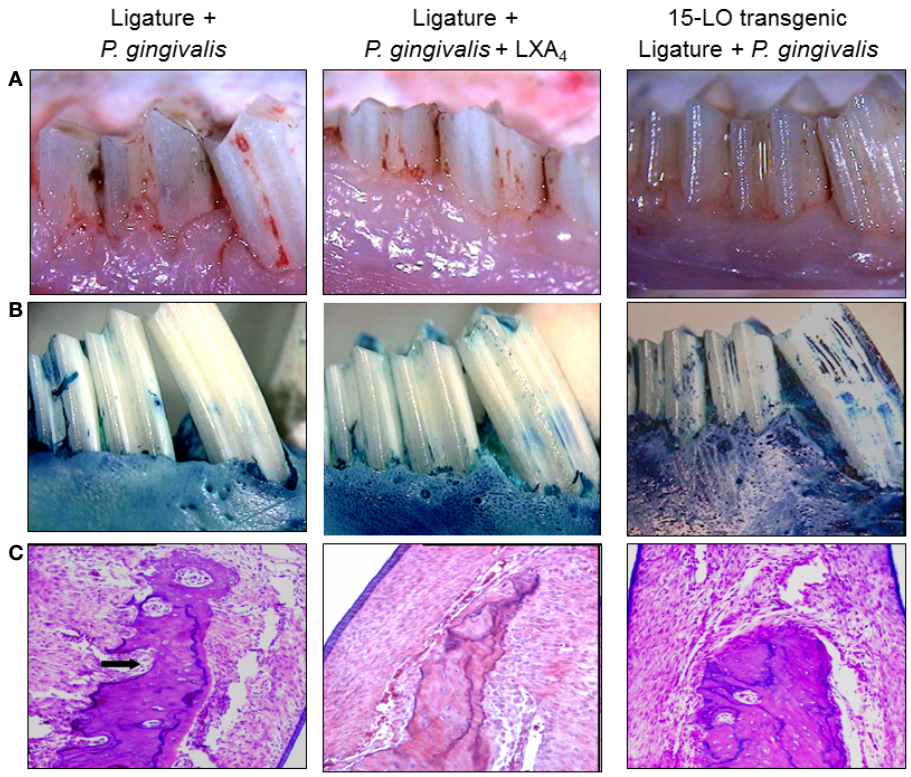

FIGURE 4 | Lipoxin A_4, a resolution phase agonist, conferred similar actions with RvE1 on periodontal tissues challenged by *P. gingivalis* and ligature. (A) Periodontal inflammation was induced in transgenic and non-transgenic rabbits as described elsewhere for 6 weeks. Simultaneously, topical LXA_4 (5–6 μg/site) was applied to the ligated sites in some non-transgenic animals. At 6 weeks, similar to RvE1, Lipoxin A_4 resulted in significant reduction of tissue inflammation as a result of disease initiation. 15 LO overexpressing transgenic rabbits (15 LO-TG) exhibited no inflammation or tissue destruction and were completely protected from periodontal inflammatory changes. **(B)** The defleshed specimens clearly showed the amount of bone loss as a result of the periodontal disease induced by the human oral microorganism, *P. gingivalis* (left panel). *LXA_4* was capable of preventing from these inflammatory changes and bone loss (middle panel), while the 15 LO-TG rabbits were not affected by disease induction, and were completely resistant to the disease (right panel). **(C)** Histological evaluations have confirmed the clinical observations and once again showed a complete protection in 15 LO-TG rabbits from inflammatory changes demonstrated by an unaffected healthy bony architecture (right panel). Topical LXA_4 application protected from the destructive effects of periodontal disease as indicated by histological evaluations (middle panel).

pleckstrin expression with RNAi silencing revealed that phosphorylation of pleckstrin is an important intermediate in the secretion and activation pathways of pro-inflammatory cytokines (TNF-α and IL-1β) induced by RAGE activation. Thus, phosphorylation of pleckstrin up-regulated in diabetic mononuclear phagocytes was in part due to the activation of PKC through RAGE binding, and pleckstrin was a critical molecule for pro-inflammatory cytokine secretion in response to elevated AGE in diabetes in macrophages (Ding et al., 2007b).

Cardiovascular diseases and oral inflammation is also linked through the pivotal role of the macrophages. Epidemiological and recent clinical studies have implicated periodontitis as a risk factor for cardiovascular disease. Leukocytes can affect the vascular endothelial lining and can cause oxidation of low-density lipoprotein (LDL). Monocytes are induced to become macrophages, which take up modified lipoproteins and become lipid-laden "foam cells" (Paigen et al., 1987a,b). The local inflammation is sustained by secreting chemical mediators. Activated macrophages in the atherogenic plaque produce inflammatory cytokines (interferon, interleukin-1, and TNF-α), which induce the production of substantial amounts of interleukin-6. These cytokines are also produced in various tissues in response to infection and in the adipose tissue of patients with metabolic syndrome (Hansson, 2005). Interleukin-6, in turn, stimulates the production of large amounts of acute phase reactants, including CRP, serum amyloid A, and fibrinogen, by the liver (Ridker et al., 2000). CRP, the well-accepted marker of atherosclerotic disease, is shown to activate complement and accounts for LDL uptake by macrophages (Zwaka et al., 2001). The atherosclerotic lesion begins to bulge within the luminal wall and as the lesion progresses; the ECM is degraded by proteolytic enzymes and becomes susceptible to rupture. Thromboses can occur, occluding blood flow to the heart, which may eventually lead to infarction.

While macrophages play a central role in the development of atherosclerosis, specifically in the initial accumulation of cholesterol in the arterial wall (Ross, 1993), it has been suggested that infection and chronic inflammatory conditions such as periodontitis may influence the atherosclerosis process (Haraszthy et al., 2000). *P. gingivalis*, one of the major pathogens involved in periodontitis, has been detected in human atheromas (Deshpande

et al., 1998; Dorn et al., 2000) suggesting that *P. gingivalis* infection may be associated with atherosclerosis. It has been proposed that bacteria or viruses may infect atherosclerotic lesions contributing to the inflammatory process. Distant infections may increase systemic inflammation through the release of toxins (i.e., bacterial LPS) or the leakage of chemical mediators into the circulation (Qi et al., 2003). Although multiple cross-sectional studies have supported these hypotheses by demonstrating a higher incidence of atherosclerotic complications in patients with periodontal disease (Mattila et al., 1989; Arbes et al., 1999) and suggest a strong link between periodontal inflammation and atherosclerosis (DeStefano et al., 1993), these observational studies are far from proving causation as proposed. Experimental animal models where periodontitis and atherosclerosis were developed in the same animal have been recently used to address this challenge. The ApoE-null mouse periodontal disease model was able to demonstrate that experimental induction of periodontal disease by serial inoculations of *P. gingivalis* exacerbated early atherosclerotic lesions (fatty streaks) within 4 months (Lalla et al., 2003). In addition, serum IL-6, aortic VCAM-1, and tissue factor antigen levels were increased in mice with *P. gingivalis* infection. In parallel, our group has shown that *P. gingivalis* induced periodontitis in rabbits dramatically increased lipid deposition in the aortas of cholesterol-fed rabbits compared to high cholesterol diet alone within 13 weeks (Jain et al., 2003). Animals with experimentally induced periodontitis had more extensive accumulations of lipids in the aorta compared to non-periodontitis animals ($P < 0.05$), and there was a positive correlation between the severity of periodontal disease and the extent of lipid deposition ($r^2 = 0.9501$; **Figure 3**). In this study, *P. gingivalis* 16S ribosomal RNA were not found in atheromatous plaques supporting the concept that rather than the bacteria itself, *P. gingivalis* cells or its vesicles released from periodontal lesions into the circulation may deliver virulence factor(s) such as LPS to the arterial wall to initiate or promote foam cell formation by macrophages and contribute to atheroma development (Qi et al., 2003). In a subsequent study, transgenic rabbits overexpressing 15-lipoxygenases and their response to inflammatory challenge were examined. Periodontal disease was initiated by topical *P. gingivalis* application. 15 LO-TG rabbits exhibited markedly reduced bone loss and local inflammation compared to non-transgenic rabbits where a significant amount of tissue destruction was observed (**Figure 4**). Further, application of topical aspirin-triggered lipoxin (LXA_4) to the gingival site dampened the PMN-mediated tissue breakdown and bone loss suggesting that regulation of inflammation can provide an enhanced anti-inflammation status, which results in prevention of periodontal inflammation (Serhan et al., 2003). Overexpression of 15-lipoxygenase type I in transgenic rabbits increases the levels of endogenous lipoxin A_4, which leads to prevention of periodontal inflammation as well as reduction of accelerated inflammatory events that contribute to atherosclerotic changes (Shen et al., 1996; Serhan et al., 2008).

CONCLUSION

Oral inflammatory processes involve microbial etiologic factors induce a series of host responses that mediate an inflammatory cascade of events in an attempt to protect and/or heal the tissues. It is becoming clear that the phenotype of the macrophage is central to determining the fate of the lesion; resolving; or chronic. Since the response of the macrophage is essential to health and disease, it is important to achieve a more complete understanding of the molecular events in this complex system. It is now becoming apparent that innate immune system cells are the determinants of the fate of the tissues and organs and are more than just transient, and their role is not limited to engulfing the invading microbes. Neutrophils and monocyte/macrophages are the key cells of the host response where their role go beyond the "defense" and is involved in the entire armamentarium of tissue homeostasis where protection, healing-repair, and regeneration are encoded.

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