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RESOLUTION OF INFLAMMATION: LEUKOCYTES AND MOLECULAR PATHWAYS AS POTENTIAL THERAPEUTIC TARGETS

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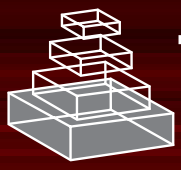

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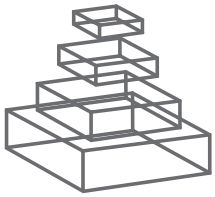

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RESOLUTION OF INFLAMMATION: LEUKOCYTES AND MOLECULAR PATHWAYS AS POTENTIAL THERAPEUTIC TARGETS

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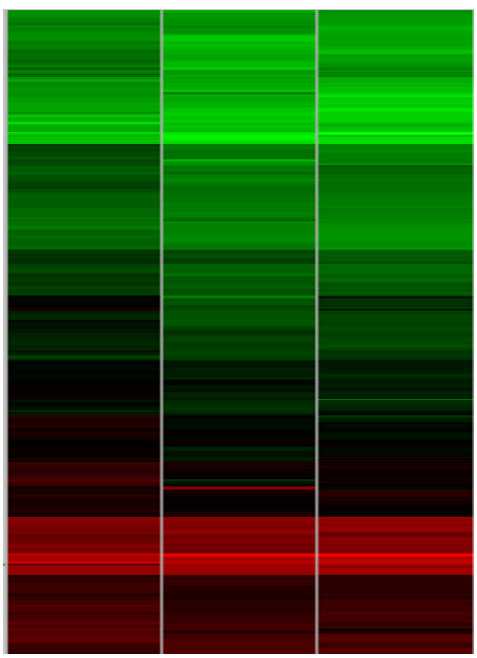

Inflammation is the principal defense mechanism against invading pathogens and host injury. Neutralization of the offending insult ideally prompts resolution of inflammation and restoration of tissue homeostasis. Excessive or dysregulated inflammatory response results in nonresolving inflammation and persisting tissue damage. Ground-breaking work during the past decade has led to the recognition that resolution of inflammation is a tightly-controlled active process that involves generation of a new class of lipid mediators, proteins and autacoids. This Research Topic focuses on articles that discuss the molecular mechanisms governing resolution circuits and the function and fate of leukocytes in inflammation underlying asthma, insulin resistance, metabolic dysfunction, myocardial ischemia/reperfusion, diabetic nephropathy arthritis, multiple sclerosis and sepsis. These articles also identify potential targets for development of innovative therapeutic approaches for the treatment of inflammatory pathologies based on either mimicking or activating endogenous pro-resolution mechanisms.

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Resolution of inflammation: leukocytes and molecular pathways as potential therapeutic targets

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Keywords: inflammation, leukocytes, molecular pathway, therapeutic target, inflammatory response

Inflammation is the principal defense mechanism against invading pathogens and host injury. Neutralization and elimination of the offending insult ideally prompts resolution of inflammation and restoration of tissue homeostasis. Excessive or dysregulated inflammatory responses together with impaired repair processes results in non-resolving inflammation and persisting tissue damage, which is now considered as a critical component of many chronic human diseases. Ground-breaking work during the past decade has led to the recognition that resolution of inflammation is a tightly controlled active process that involves generation of a new class of lipid mediators, proteins, and autacoids. These endogenous molecules possess overlapping but not fully identical biological properties and may act simultaneously or sequentially to regulate processes that are essential for timely and complete resolution of inflammation. Among these mechanisms are sequestering and suppression of generation of pro-inflammatory cytokines, inhibition of inflammatory cell trafficking into inflamed sites, promotion of neutrophil apoptosis, and macrophage polarization into M2 (alternatively activated or pro-resolution) phenotype, and facilitation of removal of apoptotic neutrophils and other cell types via efferocytosis and tissue repair.

During the past years much progress has been made in the identification and characterization of pro-resolution mediators and signaling circuits. Of these, protein mediators, such as annexin A1, and novel classes of lipid mediators, including arachidonic acid-derived lipoxins, and omega-3 polyunsaturated fatty acid-derived resolvins, protectins, and maresins exhibit dual anti-inflammatory and pro-resolution properties. Extensive research has characterized biosynthetic pathways, G-protein-coupled receptors, downstream intracellular signaling pathways, and identified several microRNAs that are involved in the regulation of pro-resolving molecular and cellular circuits. While in inflamed sites neutrophils interact with other cells in their immediate vicinity to produce these lipid mediators, eosinophil granulocytes can also generate pro-resolving mediators from omega-3 polyunsaturated fatty acids, indicating an important role for this type of leukocytes in inflammatory resolution. Resolvins facilitate macrophage polarization into the M2 phenotype. Lipoxins and resolvins interfere with β2 integrin-mediated outside in signaling that in addition to governing leukocyte trafficking also modulates neutrophil apoptosis, another control point in resolving inflammation. Data emerging from a variety of experimental models demonstrated that resolvins and protectins limit adaptive immunity in asthma, attenuate adipose tissue inflammation and metabolic dysfunctions, including type-2 diabetes mellitus-associated insulin resistance and non-alcoholic fatty acid liver disease, and protect the kidney against diabetic nephropathy. The glucocorticoid-regulated protein annexin A1 may act in concert with lipoxins to limit inflammation underlying myocardial ischemia/reperfusion, arthritis, multiple sclerosis, and sepsis. Altering local adenosine signaling affects leukocyte activation and trafficking, and may restore tissue homeostasis in liver injury and rheumatoid arthritis. The nuclear proteins proliferating cell nuclear antigen (PCNA) and myeloid nuclear differentiation antigen (MNDA) play opposing roles in modulating neutrophil apoptosis in patient with sepsis. Targeting the complement C5a receptors C5Ra and C5L2 may attenuate excessive inflammation in sepsis and autoimmune diseases. Intracellular molecular mechanisms have also been implicated in dampening inflammation. The NOD-like receptors NOD1 and NOD2 recognize intracellular pathogens and activate transcriptional responses that would lead to restriction of infection. Suppression of IFNγ production in T lymphocytes by allopurinol may attenuate cell-mediated inflammatory diseases.

This Research Topic focuses on articles that discuss the molecular mechanisms governing the function and fate of leukocytes during inflammation and identify potential targets for development of innovative therapeutic approaches for the treatment of inflammatory pathologies based on mimicking and/or activating endogenous pro-resolution mechanisms.

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Pro-resolving lipid mediators (SPMs) and their actions in regulating miRNA in novel resolution circuits in inflammation

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Unresolved inflammation is associated with several widely occurring diseases such as arthritis, periodontal diseases, cancer, and atherosclerosis. Endogenous mechanisms that curtail excessive inflammation and prompt its timely resolution are of considerable interest. In recent years, previously unrecognized chemical mediators derived from polyunsaturated fatty acids were identified that control the acute inflammatory response by activating local resolution programs. Among these are the so-called specialized pro-resolving lipid mediators (SPMs) that include lipoxins (LX), resolvins (Rv), protectins (PD), and maresins (MaR), because they are enzymatically biosynthesized during resolution of self-limited inflammation. They each possess distinct chemical structures and regulate cellular pathways by their ability to activate pro-resolving G-protein coupled receptors (GPCRs) in a stereospecific manner. For instance, RvD1 controls several miRNAs of interest in self-limited acute inflammation that counter-regulate the mediators and proteins that are involved in inflammation. Here, we overview some of the biosynthesis and mechanisms of SPM actions with focus on the recently reported miR involved in their pro-resolving responses that underscore their beneficial actions in the regulation of acute inflammation and its timely resolution. The elucidation of these mechanisms operating *in vivo* to keep acute inflammation within physiologic boundaries as well as stimulate resolution have opened resolution pharmacology and many new opportunities to target inflammation-related human pathologies via activating resolution mechanisms.

Keywords: resolution, resolvin, protectin, n-3 PUFA, lipoxin

ACUTE INFLAMMATION: A PROTECTIVE HOST RESPONSE THAT CAN TURN HARMFUL

Acute inflammation is a defensive physiological response occurring in vascularized tissues to protect the host against injuries (Majno and Joris, 1996). This formidable ally manifests its important role, for instance, in the early phase after a microbial infection, when it fights against invading pathogens before the adaptive immune system is engaged (Abbas et al., 2011). The characteristic "cardinal signs" of inflammation, described by the Roman physician Celsus in the first century, *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain), are the macroscopic manifestation of changes that occur at molecular and cellular levels in inflamed tissues. Tissue edema is one of the earliest events in the acute inflammatory response that arises from increased vascular permeability of the microvasculature (**Figure 1**). Leukocytes are then recruited at sites of inflammation and traverse postcapillary venules. Polymorphonuclear neutrophils (PMN) are among the first leukocyte responders that accumulate in the inflamed site. As they are the first line of defense of the innate immune system, these cells kill pathogens by engulfing them via phagocytosis and release of microbicidal proteins stored in their intracellular granules and reactive oxygen species into phagolysosomal vacuoles to kill invaders (Majno and Joris, 1996). Next, in experimental acute inflammation, mononuclear cells enter the inflammatory site. They can differentiate into macrophages (MΦs) and clear microbes, cellular debris, and apoptotic PMN by phagocytosis in a non-phlogistic process termed efferocytosis (Honn et al., 1989; Gordon, 2007; Serhan et al., 2007).

Ultimately, the clearance and efflux of phagocytes allow for resolution of the tissue and the return to homeostasis, namely catabasis (**Figure 1**). In order to maintain a healthy status, both the initiation of acute inflammation and its resolution must be efficient. Notably, it is not how often or how extensive an acute inflammatory reaction starts, but how effectively and quickly it resolves that determines whether the battle of inflammation is detrimental or the ideal favorable outcome for the host. Indeed, uncontrolled or unresolved inflammation is now recognized as a major driver of human pathologies, including arthritis, asthma, cancers, and cardiovascular diseases (Serhan, 2004; Serhan and Savill, 2005; Nathan and Ding, 2010). Given the high occurrence of these and many other diseases, understanding how acute inflammation resolves is of wide interest.

This review focuses on the specialized pro-resolving mediators (SPM) that are biosynthesized from essential polyunsaturated

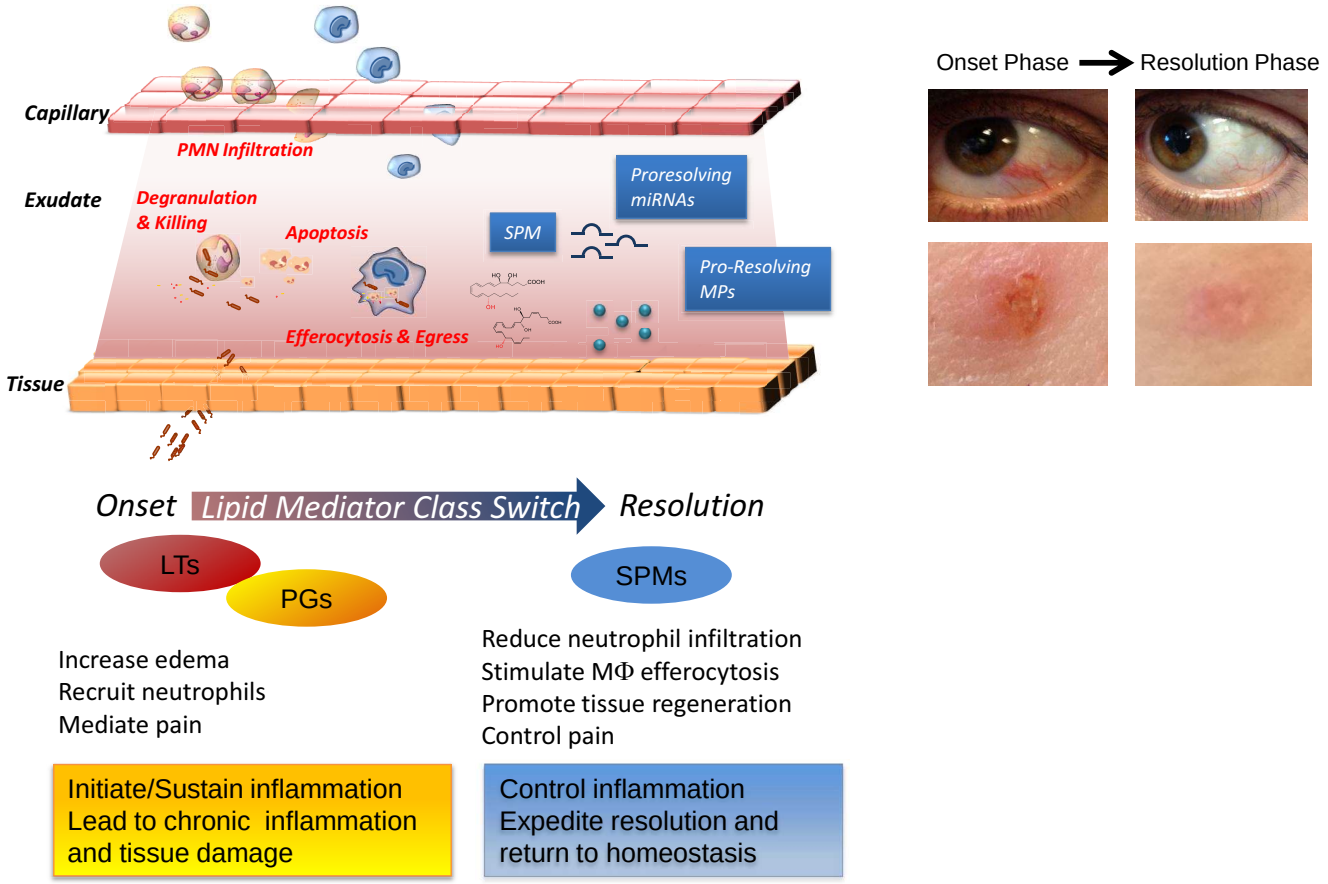

FIGURE 1 | Cellular and molecular mediators in acute inflammation and resolution. Surgical intervention, tissue injuries, or microbial infections in vascularized tissues evoke a rapid acute inflammatory response characterized by a rapid exudate formation with edema, leukocyte infiltration, and serum proteins. Polymorphonuclear leukocytes (PMN) are among the first responders that fight microbes followed by monocytes that differentiate locally into pro-resolving macrophages (MΦs). Efferocytosis of apoptotic PMN and microbes by pro-resolving MΦs and subsequent egress via lymphatics are hallmarks of tissue resolution. Redness and swelling, two of the cardinal signs of inflammation, can be easily appreciated in the example of eye and skin inflammation shown in the right panel. A few days later, inflammation is almost completely resolved and homeostasis re-established. (Pictures were taken using a Lumix SZ7 digital camera). Lipid autacoids prostaglandins (PGs) and leukotrienes (LTs) are classical mediators of the onset phase of inflammation, promoting edema, PMN recruitment, and pain. By sustaining inflammation, PGs and LTs can lead to chronic inflammation and tissue damage. Specialized pro-resolving lipid mediators (SPMs) are biosynthesized within resolving exudates and proved to be very potent in reducing further PMN infiltration, stimulating non-phlogistic MΦ efferocytosis, promoting tissue regeneration, and controlling pain. Recent advances have demonstrated that specific miRNAs and microparticles can possess pro-resolving properties.

fatty acids (PUFAs), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), namely lipoxins (LX), resolvins (Rv), protectins (PD), and maresins (MaR) and on their biosynthetic pathways, receptors, and miRNAs that act to control self-limited inflammation and promote its timely resolution. For readers interested in the biosynthesis of Rv and PD, this subject was recently reviewed in detail in Bannenberg and Serhan (2010), and the confirmation and total organic synthesis in Serhan and Petasis (2011).

RESOLUTION IS AN ACTIVE PROCESS CONTROLLED BY SPM: SELF-LIMITED EXPERIMENTAL SYSTEM

At the histological level, resolution was well described by pathologists for more than 100 years as the time when the neutrophils that infiltrated the inflamed tissue sites leave or are lost from the site (Majno and Joris, 1996). Traditionally, resolution was thought to be a passive process, simply due to the attenuation/dissipation of chemotactic and pro-inflammatory signals. Our results (Serhan et al., 2000a; Levy et al., 2001), followed by those from many others worldwide (reviewed in a consensus report in Serhan et al., 2007) demonstrated that resolution is instead an *active process* orchestrated by special novel chemical mediators that *turn on* biochemical and cellular pathways to enable the return to homeostasis.

Lipid mediators (LM) from PUFA play essential roles in distinct phases of acute inflammation, with prostaglandins (PGs; Flower, 2006; Samuelsson, 2012) and cysteinyl leukotrienes (cysLTs) promoting early increase in vascular permeability and leukotriene (LT) B_4 acting as a potent leukocyte chemoattractant (Samuelsson, 1983). Prostaglandins also contribute to fever and pain

(von Euler, 1973). Chronic inflammation is widely viewed as an excess of pro-inflammatory mediators (**Figure 1**; Nathan and Ding, 2010). Results from our laboratory first demonstrated that the resolution phase is characterized by the active biosynthesis of specific LM that operate as "*resolution agonists*" to a) keep inflammation within physiological boundaries and b) expedite the complete return to homeostasis (**Figure 1**). The identification of this new array of LM was achieved using self-limited or naturally resolving acute inflammation models *in vivo* and a systems approach (Serhan et al., 2000a, 2002). The pharmacologic impact of the Rv and PD was reviewed in (Serhan and Chiang, 2008). This new array of LM is now recognized as a genus of SPM (Serhan and Chiang, 2008) that have two broad functions and are anti-inflammatory and pro-resolving via stimulating multi-level actions. Accruing evidence indicates that failure or disruption of the endogenous pro-resolution pathways governed by SPM can be detrimental and underlie some of the mechanisms of chronic inflammatory diseases (Gilroy et al., 1999; Karp et al., 2004; Schwab et al., 2007; Chan and Moore, 2010). SPM exert their potent dual anti-inflammatory and pro-resolving activities in the low nano- to microgram dose range when added back into experimental inflammatory disease models (Serhan and Chiang, 2008) and provide biotemplates for the design of novel therapeutics currently in clinical trials (see http://Clinicaltrials.Gov Identifier: NCT00799552). Therefore, harnessing these SPM may provide fascinating opportunities in the new and uncharted terrain of resolution pharmacology, with a substantial shift from a depletion pharmacology (i.e., via inhibitors, blockers, antagonists) toward a new approach based on resolution agonists that activate endogenous protective and clearance mechanisms.

Additional chemical mediators are operative in inflamed tissues to switch off leukocyte infiltration and restore their physiological functions. Among these are several cytokines (e.g., TGFβ, IL-10) that accumulate in resolving exudates (Bannenberg et al., 2005); glucocorticoids and the glucocorticoid-induced annexin-1 protein, which tune the inflammatory response and bring about homeostasis (for a recent review see (Perretti and Dalli, 2009)); and the transcription factor NF-κB, which also carries some anti-inflammatory properties (Lawrence et al., 2001). Moreover, inducing PMN apoptosis as well as lymphoid cells while stimulating their prompt removal by MΦs also can promote resolution (Honn et al., 1989; Ariel et al., 2006). Recent results indicate that small inhibitors of cyclin-dependent kinases fulfill this goal (Leitch et al., 2012) as do annexin-1 peptides (Perretti and Dalli, 2009). Therefore, the resolution process can be pharmacologically targeted.

Importantly, resolution is not synonymous with endogenous anti-inflammation. This is because, in order to be considered a "pro-resolver," a chemical and/or molecular entity, in addition to serving as a "stop signal" for neutrophil trafficking and other cardinal signs of inflammation (e.g., swelling, pain), must also stimulate efferocytosis by MΦ, favor the antibacterial activities, and promote tissue repair and regeneration to achieve homeostasis. Along these lines, PGE_2 can have anti-inflammatory properties in certain settings via stimulation of cAMP, but is not acting as pro-resolver since it does not enhance the uptake and clearance of apoptotic cells by MΦs (Kunkel et al., 1981). Also, although cyclooxygenase (COX) inhibitors as well as certain lipoxygenase (LO) inhibitors reduce some of the cellular events of the inflammatory reaction (e.g., edema formation, PMN recruitment, and pain), they dramatically impact the endogenous pro-resolution circuits and may delay or even derange this ideal outcome of acute inflammation and thus are "resolution toxic" (Gilroy et al., 1999; Schwab et al., 2007). In contrast, aspirin and glucocorticoids work synergistically with endogenous pro-resolution pathways (Perretti et al., 2002).

Complete resolution also requires the clearance of the remnants of damaged tissues and activated or apopotic cells, so-called microparticles (MPs). Originally viewed merely as empty vesicles, MPs are now recognized as "specialized shuttles" used by the organism to transfer bioactive molecules from cell to cell. Their role in inflammation and resolution is now being appreciated. Recently, the anti-inflammatory properties of a PMN-derived sub-population of MPs were uncovered, where they appear to signal to activate resolution mechanisms (Gasser and Schifferli, 2004; Dalli et al., 2008; Norling et al., 2011). Mimicking this new endogenous mechanism in resolution, novel human PMN-derived nanoparticles containing AT-RvD1 or a LXA_4 stable analog, termed humanized pro-resolving nanomedicines, were prepared. These SPM-enriched nanohumanized particles possessed beneficial bioactivities in reducing acute inflammation *in vivo*, expediting resolution, and promoting wound healing (Norling et al., 2011). Hence, NPs can serve as mimetics of endogenous pro-resolution pathways (**Figure 1**).

Active resolution includes gene expression regulation of several soluble chemical mediators (e.g., cytokines, chemokines), receptors (e.g., Toll-like receptors), as well as transcription factors. An emerging line of investigation indicates that many genes are under tight control of miRNAs, short non-coding RNA molecules that act as translational repressors of mRNA transcripts (Bartel, 2009). They are involved in many physiological and pathological processes, including cell development, cancer (Iorio and Croce, 2009), and inflammation (Sheedy and O'Neill, 2008; Alam and O'Neill, 2011; O'Neill et al., 2011). Our recent results uncovered roles of miRNAs in self-limited inflammation and in the resolution phase, specifically, RvD1-G-protein coupled receptor-dependent gene networks in resolution of acute inflammation as part of the endogenous circuitry that controls this active process (Recchiuti et al., 2011; Krishnamoorthy et al., 2012).

IDENTIFYING SPM IN RESOLUTION

An unbiased systems approach was taken to identify the endogenous SPM and decode their mechanisms of action in resolution. For this, the murine dorsal air pouch and self-limited acute inflammation was ideal because it permitted isolation of contained inflammatory exudates (Serhan et al., 2000a, 2002) and also enabled direct LM lipidomics of bioactive products, as well as their inactive precursors and further metabolites, proteomics, and analyses of cellular composition of the resolving exudate; namely the natural means by which inflammation returns to resolution and homeostasis. With this systems approach it was also possible to establish the local and temporal dissociation of LM biosynthesis (Bannenberg et al., 2005). For example, upon initiation

of inflammation with TNF-α, there was a typical acute-phase response characterized by rapid PMN infiltration preceded by local generation of both PGs and LTs. Unexpectedly, the eicosanoids undergo what was termed earlier a "class switch" and the profiles of LM made within this milieu switched with time (Levy et al., 2001). Indeed, the potent chemoattractants LT were deactivated and 15-LO required for LX and Rv production was transcriptionally activated (Levy et al., 2001). Notably, the omega-3 essential fatty acids DHA and EPA are precursors of Rv, PD, and MaR, are rapidly carried into the exudates via plasma edema and are then made available for conversion for the congregated exudate cells (Kasuga et al., 2008). Of interest, this *LM class switching* is driven in part by COX-derived PGE_2 and D_2, via transcriptional regulation of enzymes involved in LX biosynthesis (Levy et al., 2001). Hence, the concept that "alpha signals omega," namely the beginning signals the end in inflammation, was introduced by Sir John Savill and one of us to emphasize this finding (Serhan and Savill, 2005) to note that at time zero mediators are biosynthesized that signal to limit PMN influx and terminate the contained acute inflammatory response.

WHAT IS A LIPID MEDIATOR?

To qualify as a LM, a product must be stereoselective in its actions and be produced in amounts that are commensurate with its potency and range of action (Serhan et al., 1996). Along this line, LX, Rv, and PD are present in human serum in pM to nM amount (e.g., LXA_4, ∼1.4 nM; RvD1, ∼50 pM; RvE1, ∼0.5 nM; values from the Serum Metabolome Project; Psychogios et al., 2011; see also Oh et al., 2011 for RvE1 and Oh et al., 2012 for RvE2 values) in human peripheral blood samples from healthy donors. LM lipidomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with informatics permit profiling of closely related compounds and identification of new molecules. Retrograde synthesis, both biogenic and total organic, allows the complete elucidation of chemical structure, stereochemistry, and physical properties, along with the recapitulation of the *in vivo* biosynthetic pathway (for examples see Sun et al., 2007; Serhan et al., 2009). The matching/identification of LM is usually carried out with at least two different instruments and/or mobile phase solvent systems and the criteria to identify a known LM are the following: (a) LC retention time should match by coelution with the LM authentic standard; (b) UV chromophore should match the synthetic and authentic LM (i.e., λ_{max} and band shape); as well as (c) ≥6 diagnostic ions of tandem MS/MS spectrum (**Figure 2**). Also, the LC-MS/MS fragmentation mechanisms for the Rv and PD D1 and related DHA-derived products have been studied using deuterium-labeled compounds that facilitated their identification *in vivo* (Hong et al., 2007).

LIPOXINS

Lipoxin A_4 and B_4 were the first anti-inflammatory LM recognized to possess pro-resolving actions (Serhan et al., 1984a,b; Maddox et al., 1997; Takano et al., 1998; Godson et al., 2000). Although LXs were first isolated and identified in the 1980s in the Samuelsson laboratory (Serhan et al., 1984a), their potent bioactions were uncovered some years later with the identification of the aspirin-triggered LX (ATL; Claria and Serhan, 1995) and the design of ATL stable analogs (Serhan et al., 1995; *vide infra*), when it became clear that they act as "braking signals" of further PMN infiltration (Takano et al., 1998) and as potent stimuli for the non-phlogistic recruitment of monocytes (Maddox et al., 1997) and MΦ efferocytosis (Godson et al., 2000; recently reviewed in Serhan, 2005; Spite and Serhan, 2011). LXs are lipoxygenase interaction products derived from the enzymatic conversion of AA via transcellular biosynthesis during cell–cell interactions occurring during inflammation (Samuelsson et al., 1987). In humans, sequential oxygenation of AA by 15-LO and 5-LO, followed by enzymatic hydrolysis, leads to the biosynthesis of LXA_4 and B_4 in mucosal tissues, such as airways, gastrointestinal tract, and oral cavity (Edenius et al., 1990; Levy et al., 1993; Gronert et al., 1998; **Figure 3** and reviewed in Romano (2010). Blood vessels represent a second site for LX biosynthesis, with the conversion of 5-LO-derived LTA_4 into LXA_4 and B_4 by 12-LO in platelets (Serhan and Sheppard, 1990; Romano and Serhan, 1992; Romano, 2010).

ATL: THE FIRST ASPIRIN-TRIGGERED MEDIATORS

A third LX synthetic pathway is initiated by aspirin, the well-known derivative of salicylates, by acetylation of COX-2. This covalent modification shifts the enzyme activity from endoperoxidase to lipoxygenase-like, and COX-2 converts AA into 15R-HETE, which is a substrate of leukocyte 5-LO for the biosynthesis of 15R-epi-LXA_4 and B_4 (Claria and Serhan, 1995). Hence, among the non-steroidal anti-inflammatory drugs (NSAID), aspirin has the unique capability to "jump start" resolution by its ability to trigger endogenous biosynthesis of so-called "aspirin-triggered" LX (**Figure 3**) Notably, ATL produced *in vivo* in human subjects taking aspirin (Chiang et al., 2004) proved to mediate the local anti-inflammatory actions of low-dose aspirin in healthy individuals (Morris et al., 2009).

SPM BIOSYNTHESIZED FROM OMEGA-3 POLYUNSATURATED FATTY ACIDS: LOCAL MEDIATORS

The essential roles of omega-3 PUFA in health were evident in 1929 (Burr and Burr, 1929), and ω-3, also known as n-3 PUFA EPA and DHA, have beneficial effects in human diseases including potential antithrombotic, immunoregulatory, and anti-inflammatory properties (Iigo et al., 1997; De Caterina, 2011). Also, the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione trial evaluated the effects of ω-3 PUFA supplementation with >11,000 patients surviving myocardial infarction taking >1 g of ω-3 PUFA daily along with recommended preventive treatments including aspirin, and reported a significant benefit with a decrease in cardiovascular death (GISSI-Prevenzione Investigators, 1999). It is believed that the actions of the major lipid of fish oil EPA (C20:5) are based upon (a) preventing conversion of AA to proinflammatory and prothrombotic eicosanoids; (b) serving as an alternate substrate for the five-series LTs that are less potent than four-series LTs; and (c) conversion by COX to three-series prostanoids (i.e., PGI_3) that also maintain antithrombotic actions. These and other explanations offered (Iigo et al., 1997; Calder, 2009; De Caterina, 2011) have not been generally accepted because of the lack of molecular evidence *in vivo* and the high concentrations of ω-3 PUFA required to achieve putative "beneficial actions" in *in vitro* cell culture experiments.

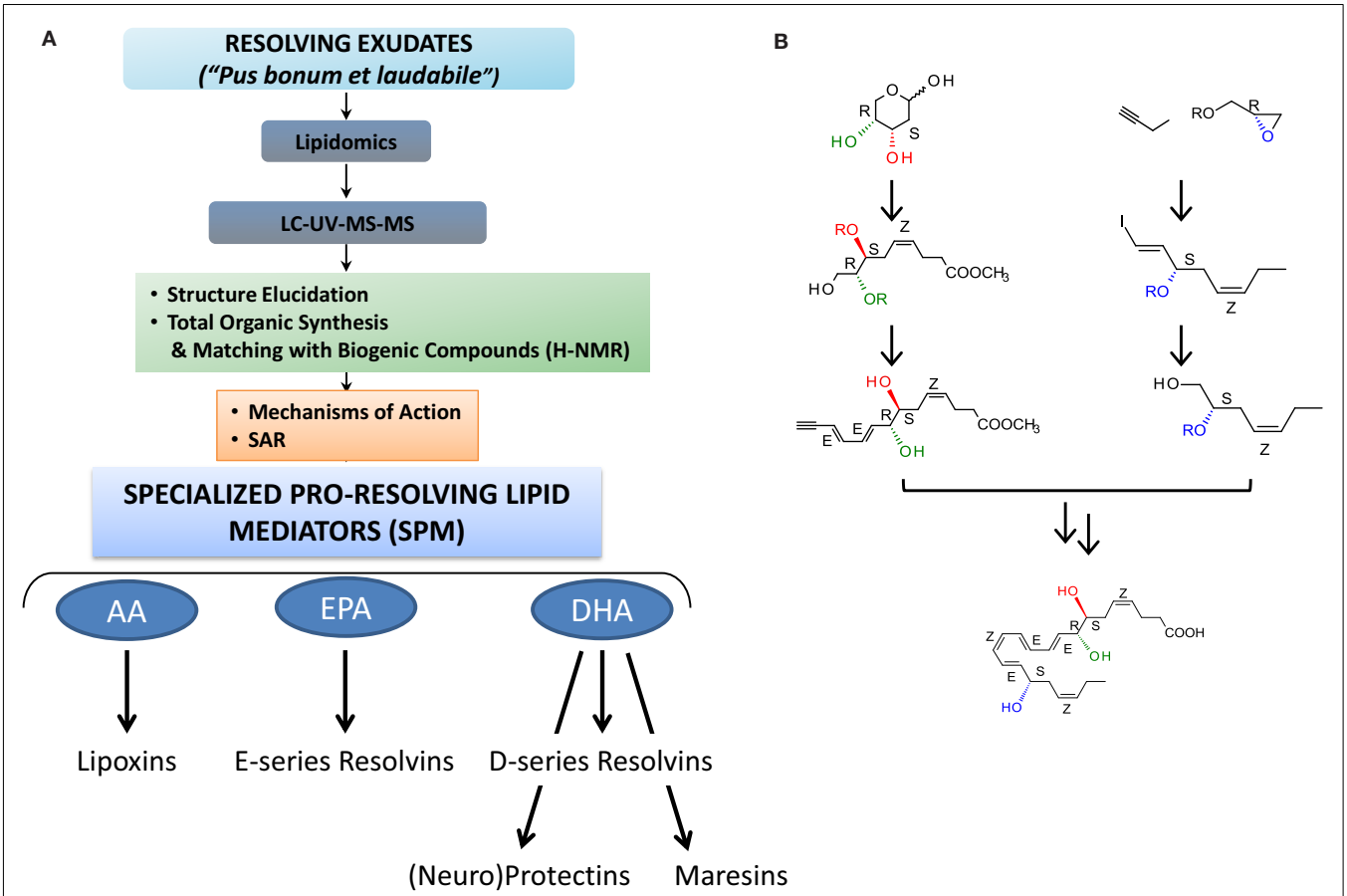

FIGURE 2 | Strategy for functional profiling of SPM in resolving exudates. (A) During self-limited inflammation, murine exudates (a "good and laudable" pus according to ancient physicians; Majno, 1991) as well as human leukocytes biosynthesize SPM, which include the lipoxins, E-series resolvins, D-series resolvins, protectins (neuroprotectin D1), and maresins, which work to keep the inflammatory response within physiological boundaries and help to expedite the return to homeostasis. Functional profiling takes advantage of liquid chromatography-ultraviolet spectrometry-tandem mass spectrometry (LC-UV-MS/MS) for identifying and quantifying SPM. Gas chromatography-mass spectrometry (GC-MS) is also useful to provide additional information together with LC-UV-MS/MS to support structural identification and proposed structures. Retrograde analysis with biogenic synthesis using isolated human cells and total organic synthesis allows the assignment of chirality and double bond geometries using H-NMR with synthetic materials and matching studies (see text; Fiore et al., 1991; Serhan et al., 2000a, 2002, 2006, 2012; Sun et al., 2007; Spite et al., 2009a; Krishnamoorthy et al., 2010 for details). Bioactions of SPM are assessed in both animal models and human cell systems. They must be stereoselective and evident at concentrations/doses that are commensurate with the amount of SPM produced. **(B)** Example of RvD1 stereoselective total organic synthesis (reported in Sun et al., 2007); for further details (see Serhan and Petasis, 2011) for a recent review of organic synthesis.

To address the molecular basis for anti-inflammatory properties of ω-3 fatty acids, an unbiased LC-MS/MS-based informatics approach was developed to identify novel mediators generated from ω-3 precursors during acute inflammation *in vivo*. Using this approach, EPA and DHA were found to be enzymatically converted into novel potent LMs coined Rv, an acronym of *resolution phase interaction products*, because they: (a) are produced during cell–cell interactions occurring in the resolution phase of acute inflammatory response; (b) "stop" further neutrophil entry to sites of inflammation, and (c) reduce exudates (Serhan et al., 2000a, 2002, 2006; Hong et al., 2003; Bannenberg et al., 2005). Rv represented an entirely new family of mediators produced from the ω-3 fatty acids and importantly they appeared during the resolution phase via active biosynthetic processes. The biosynthesis of Rv gives rise to stereospecific local mediators that have potent actions and activate specific receptors.

E-SERIES RESOLVINS

EPA-derived E-series Rv are endogenously biosynthesized *in vivo* in resolving murine exudates and in isolated human cells systems by isolated cells (e.g., endothelial cell-leukocyte interaction) and in whole blood (*vide infra*). The complete stereochemistry of the first member of this family, RvE1, has been established as 5S,12R,18R-trihydoxy-6Z,8E,10E,14Z,16E-EPA (Arita et al., 2005a). For further details on the total organic synthesis (see Serhan and Petasis, 2011). Within vascular endothelial cells, aspirin-acetylated COX-2 converts EPA into 18R-hydro(peroxy)-eicosapentaenoic acid (HEPE), which is rapidly taken up by activated leukocytes (e.g., PMN) and further metabolized into RvE1. Notably, quantitative

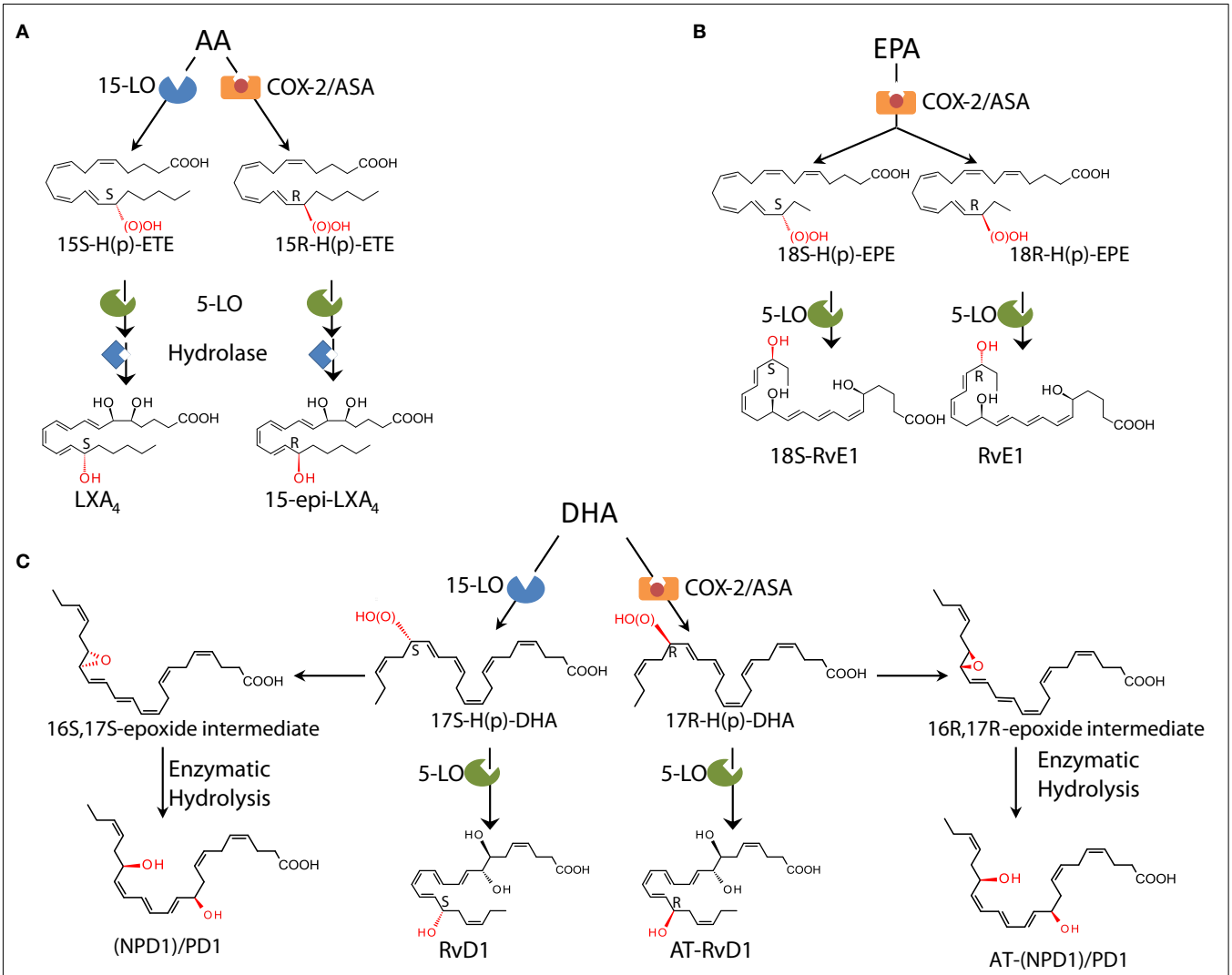

FIGURE 3 | Biosynthetic schemes of SPM. (A) In humans, AA can be converted into 15S-H(p)-ETE through 15-LO and into 15R-H(p)-ETE by aspirin (ASA)-acetylated COX-2. Both intermediates can be further metabolized through 5-LO and enzymatic hydrolysis yielding LXA4 or 15-epi-LXA4. **(B)** E-series resolvins are biosynthesized via conversion of EPA by ASA-acetylated COX-2. Products of these reactions, 18S-H(p)-EPE and 18R-H(p)-EPE, are rapidly taken up by 5-LO and converted to 18S-RvE1 and RvE1. **(C)** The DHA metabolome includes several SPM biosynthesized via 15/5-LO and ASA-acetylated COX-2. Each SPM is biosynthesized via distinct biochemical routes involving stereocontrolled oxygenation, epoxide formation, and enzymatic hydrolysis. The main structures of key SPM and their biosynthetic routes (with precursors and main enzymes involved) are depicted (see text and Serhan and Petasis, 2011 for further details). The complete stereochemistry of each of these SPM is established, total organic synthesis achieved, and bioactions confirmed.

chiral HPLC analysis indicated that the 18*R*-HEPE isomer was dominant to its epimer 18*S*-HEPE in human plasma from healthy subjects taking EPA (Oh et al., 2011). In contrast, human subjects who were administered aspirin before EPA had more 18*S*- than 18R-HEPE, indicating that aspirin might promote 18*S*-HEPE production as well as 18*R*-HEPE from ingested EPA (Oh et al., 2011). This 18*S*-HEPE can also be converted to epimeric RvE1 and RvE2 by human recombinant 5-LO and LTA_4 hydrolase (LTA_4H), known as pro-inflammatory LTB_4-synthesizing enzymes (Oh et al., 2011). RvE1 is also produced *in vivo* through an aspirin-independent pathway via cytochrome P450-driven oxygenation of EPA (Serhan et al., 2000b). Of interest, RvE1 was also found to be produced by *Candida albicans* and appears to be involved in clearance of this organism (Haas-Stapleton et al., 2007). Thus RvE1 has multiple biosynthetic routes. RvE2 (5*S*,18-dihydroxy-EPE) is biosynthesized in resolving exudates and in human whole blood via reduction of 5*S*-hydroperoxy,18-hydroxy-EPE, an intermediate in the biosynthetic pathway of RvE1 (Tjonahen et al., 2006; Ogawa et al., 2009; Oh et al., 2012; **Figure 3**).

D-SERIES RESOLVINS

Earlier investigations using LC-MS/MS lipidomics of resolving exudates from mice given DHA and aspirin provided the first evidence of novel endogenous routes that lead to the formation of 17-hydroxy-containing mediators. Gaining information on how human tissue and cells may produce D-series Rv involved

the *in vitro* recapitulation of biosynthetic pathways using isolated human cells and recombinant enzymes establishing potential origins of novel compounds isolated from resolving exudates *in vivo*. Along these lines, hypoxic human endothelial cell COX-2 converted DHA to 13-hydroxy-DHA, which switched with ASA to 17R-HDHA. Human neutrophils transformed 17R-hydroxy-DHA into two series of di- and trihydroxy products; one initiated via oxygenation at carbon 7 and the other at carbon 4 (Serhan et al., 2002). The conversion of 17*R*-HDHA by human PMNs displayed similar features as those established for the conversion of AA to LTB_4 or LXs as well as the 18*R* series of EPA products. These were termed the "aspirin-triggered" D-series Rv (Serhan et al., 2002). Remarkably, in the absence of aspirin, D-series Rv carrying the 17S-hydroxy group were identified in murine exudates and isolated human cells (Serhan et al., 2002; Hong et al., 2003). The enzymatic pathway leading to the formation of 17*S*- and 17*R*-RvD1 is shown in **Figure 3**. Following the complete organic synthesis, the stereochemistry of 17S-, 17R-RvD1, and RvD2 were established as 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA (17S-RvD1), 7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA (17R-RvD1; Sun et al., 2007), and 7*S*, 16*R*, 17*S*-trihydroxy-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*, 19*Z*-DHA (RvD2; Spite et al., 2009a). Additional members of this family were identified (RvD3–RvD6). Each of these arises by similar biosynthetic routes, but has distinct chemical structures and potentially additional bioactions that are now being unveiled (Chiang et al., 2012). Importantly, both RvE1 and RvD1 were identified in circulating blood of healthy donors by (Psychogios et al., 2011) as part of the Serum Metabolome Project.

THE (NEURO)PROTECTINS

In addition to D-series Rvs, DHA also serves as precursor of a new family of LM characterized by a conjugated triene system and two alcohol groups called PD. The name PD accounts for their protective actions observed in neural tissues and within the immune system, while the prefix neuro PD gives the tissue localization and site of action. The structure of the founding member of this family, PD1, was first disclosed in a report on the isolation and elucidation of Rv (Serhan et al., 2002; Hong et al., 2003), and its complete stereochemistry later established as 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (Serhan et al., 2006). In addition to PD1, several stereo- and positional isomers that also possess lower bioactivity than PD1 were identified in human and mouse tissues. These include 10S,17S-diHDHA, 4*S*,17*S*-diHDHA, 7*S*,17*S*-diHDHA, and 22-hydrox-10,17*S*-docosatriene (a putative inactivation product of PD1; Serhan et al., 2002; Hong et al., 2003). The geometry of the double bonds in PD1, their positions during biosynthesis, and chirality of C10 indicate that PD1 biosynthesis proceeds through a C16(17)-epoxide intermediate and requires specific enzymatic steps to generate the potent bioactive molecule from DHA, as confirmed by the isolation of alcohol trapping products as well as two vicinal diol 16,17-docosatrienes as minor products of PD1 biosynthesis (Hong et al., 2003; Serhan et al., 2006). Recently, a novel aspirin-triggered COX-2 driven pathway was reported that biosynthesizes the 17R-epimeric form of PD1 from DHA (Marcheselli et al., 2003; **Figure 3**). The total organic synthesis and complete stereochemical assignment of AT-PD1 (10R,17R- dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) were recently achieved. Both PD1 and AT-PD1 reduced leukocyte infiltration in murine peritonitis, reduced PMN transmigration with endothelial cells, and enhanced efferocytosis of apoptotic PMN by human MΦ (Serhan et al., 2006). These are the hallmark actions of a SPM carrying both anti-inflammatory and pro-resolving actions demonstrable both *in vitro* and *in vivo*.

MARESINS

MΦs have pivotal roles in orchestrating the return to homeostasis (Gordon, 2007) and biosynthesize SPM that enhance their pro-resolving and homeostatic functions. For example, MΦ ingesting apoptotic cells initiate the biosynthesis of LXA_4, RvE1, and PD1 but not LTB_4 (Freire-de-Lima et al., 2006; Schwab et al., 2007). In addition, a new family of SPM biosynthesized by MΦs was identified (Serhan et al., 2009). Unbiased LM LC-MS/MS-based metabololipidomics during self-limited peritonitis led to the identification of a novel pathway that converted DHA into 14-hydroxy DHA (HDHA). Experiments with 12/15-$LO^{-/-}$ mice or with LO inhibitor confirmed that 14-HDHA production was via a DHA carbon 14 lipoxygenation pathway. Freshly prepared 14-H(p)DHA is rapidly converted by isolated human and mouse MΦ into a new set of bioactive products, whose molecular structure was established (Serhan et al., 2009) and recently confirmed by matching of biogenic material with those prepared by total organic synthesis (Serhan et al., 2012). The major product of this new pathway proved to be 7,14-dihydroxydocosa-4*Z*,8*E*,10E,12Z,16*Z*,19*Z*-hexaenoic acid, denoted MaR (*ma*crophage mediator in *re*solving *in*flammation) 1 (first in the family; MaR1; Serhan et al., 2009). Similar to that of other potent SPM, MaR1 biosynthesis proceeds via an epoxide intermediate, specifically the MaR1 13,14-epoxide intermediate, that was demonstrated by trapping experiment and with ^{18}O-containing molecular oxygen O_2 that opens during enzymatic conversion to MaR1 keeping the double bond geometry and chirality of carbon 7 and 14.

In addition to MaR1 in resolving murine exudates, a novel double dioxygenation product was isolated and identified, 7*S*,14*S*-dihydroxydocosa-4*Z*,8*E*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid (denoted 7*S*,14*S*-diHDHA), formed by consecutive lipoxygenation of 14-HDHA, was also identified using molecular oxygen incorporation, and proved bioactive but less potent in activity than MaR1 in stimulating efferocytosis with human cells (Serhan et al., 2009, 2012). MaR1 and RvE1 are also potent stimulators of organ regeneration using a planaria regeneration system (Serhan et al., 2012). Hence, SPM are primordial molecules that signal from the inflammatory site to generate the aftermath of inflammation and tissue injury.

GPCRs FOR SPM IN ANTI-INFLAMMATION AND RESOLUTION

GPCRs FOR LXs

The first evidence for receptor-mediated actions of LXA_4 arises from studies with Santosh Nigam when he was on sabbatical in the Serhan Lab at BWH in the late 1980s, which demonstrated stimulation of rapid lipid remodeling and pertussis toxin (PTX)-sensitive release of arachidonate in PMN treated with LXA_4 (Nigam et al., 1990). To examine the molecular basis of these actions, synthetic [11,12-^{3}H]-LXA_4 was prepared and used to demonstrate specific and reversible binding to intact human PMN with a $K_d \sim 0.5$ nM. [^{3}H]-LXA_4 binding was stereoselective as LXB_4, LTB_4, 6S-LXA_4, or 11-trans-LXA_4 did not compete for LXA_4 binding, while cysteinyl

LT C_4 and D_4 partially displaced bound labeled LXA_4 (Fiore et al., 1992). Screening of cDNA clones from differentiated HL60 human cells lines led to the identification of formyl peptide receptor like-1, a homolog of formyl receptor, as putative LXA_4 GPCR (Fiore et al., 1994). This receptor has recently been coined ALX/FPR2 by the international nomenclature committee in light of its high affinity for LXA_4 (Ye et al., 2009).

Human FPR2/ALX is highly expressed in myeloid cells and at a lower extent in lymphocytes, dendritic cells, and resident cells (Chiang et al., 2006). Orthologs of the human FPR2/ALX have been identified in mice (Takano et al., 1997) and rats (Chiang et al., 2003). In addition to LXA_4, FPR2/ALX is activated by the glucocorticoid-induced protein annexin-1 and its N-terminal peptides (Perretti et al., 2002), representing the prototype of GPCR able to coordinate anti-inflammatory and pro-resolving activities of both lipid and peptide ligands. Genetic manipulation of ALX/FPR2 and its ortholog in mice has provided evidence for the essential role of this GPCR in controlling immune responses. Indeed, myeloid-driven overexpression of human FPR2/ALX in transgenic mice resulted in a reduced neutrophil infiltration during zymosan-induced peritonitis (Devchand et al., 2003), whereas ALX/FPR2$^{-/-}$ mice have an exacerbated inflammatory phenotype and delayed resolution (Dufton et al., 2010).

More strikingly, ATL and FPR2/ALX expression levels dictate both the magnitude and duration of acute inflammation in humans (Morris et al., 2010). Hence, mechanisms that regulate this expression are of wide interest. Recent results from Simiele et al. (2012) uncovered the molecular basis of ALX/FPR2 transcription machinery, with the identification of the core promoter sequence, the elucidation of transcription factors and epigenetic mechanisms that regulate promoter activity, and the identification of the first inheritable SNP that impairs promoter activity in individuals at high cardiovascular risk. Notably, LXA_4 upregulates ALX/FPR2 levels by activating its promoter, suggesting an additional mechanism by which LXA_4 exerts its bioactivities (Simiele et al., 2012). This is particularly relevant in considering LX roles in stimulating resolution. In addition, earlier studies demonstrated that radiolabeled 15-epi-LXA_4 binds at cysteinyl LT receptor 1 (CysLT1) with equal affinity to LTD_4, providing additional molecular mechanisms for ATL dampening CysLT signals in the vasculature as well as regulating leukocyte trafficking via ALX/FPR2 (Gronert et al., 2001).

GPCRs FOR E-SERIES RESOLVINS

At least two GPCRs are involved in mediating RvE1 actions, namely ChemR23 and BLT1 (Arita et al., 2005a, 2007). RvE1 binding to ChemR23 was assessed with [^{3}H]-labeled RvE1, which was prepared by catalytic hydrogenation from synthetic diacetylenic RvE1. [^{3}H]-RvE1 bound to ChemR23 transfectants with high affinity ($K_d = 11.3 \pm 5.4$ nM) and stereoselectivity, since RvE1 biogenic precursors EPA and 18*R*-HEPE did not compete with [^{3}H]-RvE1. Also, the synthetic peptide fragment (YHSFFFPGQFAFS) derived from human chemerin that was earlier reported to be a ligand for this same receptor (Wittamer et al., 2003) displaced [^{3}H]-RvE1 binding by ∼70% when tested at 10 μM concentration, suggesting that RvE1 and chemerin share recognition sites on ChemR23 (Arita et al., 2005a; Ohira et al., 2009). [^{3}H]-RvE1 specific binding was also demonstrated with membrane fractions isolated from human PMN. Radiolabeled RvE1 bound human PMN with K_d of ∼50 nM and was displaced by homoligand RvE1 ($K_i \sim 34$ nM), LTB_4 ($K_i = 0.08$ nM), and LTB_4 receptor 1 (BLT1) selective antagonist U-75302 ($K_i = 1.5$ nM), but not by the chemerin peptide (Arita et al., 2007). These results strikingly demonstrated that RvE1 binding sites are pharmacologically distinct from ChemR23 on human PMN and prompted us to investigate whether RvE1 binds to LTB_4 receptors.

In these studies, Arita et al. found that [^{3}H]-RvE1 also gave high affinity binding to recombinant BLT1 ($K_d \sim 45$ nM) that was competed by unlabeled LTB_4 ($K_i = 3$ nM). In contrast, BLT2-overexpressing cells did not show [^{3}H]-RvE1 binding at concentrations up to 10 nM. These results clearly demonstrated that RvE1 binds to BLT1 on human PMN and acts as a partial agonist to attenuate LTB_4 incoming signals in both mouse and human leukocytes (Arita et al., 2007).

Profiling for tissue distribution of human ChemR23 showed expression of this GPCR in brain, kidney, cardiovascular, gastrointestinal, and myeloid tissues (Arita et al., 2005a). More recently, direct evidence for ligand-receptor interactions of RvE1 and its epimer 18*S*-RvE1 was provided using ChemR23 and BLT1 β-arrestin cells. In this system, cells were engineered to co-express a β-arrestin protein tagged with an inactive moiety of β-galactosidase enzyme together with a candidate GPCR fused to the short Pro-Link peptide derived from β-galactosidase. In the presence of ligand, in this context RvE1 activates GPCR interacts with β-arrestin, bringing to proximity two inactive portions of β-galactosidase and reconstituting the fully active enzyme. The activity of this enzyme, which is stoichiometrically dependent on GPCR-ligand interaction, is monitored with a chemiluminescence detection system. With ChemR23 β-arrestin cells, 18*S*-RvE1 dose-dependently activated ChemR23 receptor, with EC_{50} (∼6.3 pM) lower than that obtained with RvE1 (∼0.14 nM). 18*S*-RvE1 also antagonized LTB_4-mediated BLT1 activation with higher potency and efficacy than RvE1 in BLT1 β-arrestin cells (Oh et al., 2011). Hence, RvE1 and 18*S*-RvE1 can share the same site(s) of specific binding to human ChemR23 as well as BLT1 and thus suggest location-dependent mechanisms in their signaling capabilities.

RvE2 exerts potent and cell-specific bioactions on human leukocytes (Tjonahen et al., 2006; Oh et al., 2012). Recently, tritium-labeled [^{3}H]-RvE2 was synthesized and gave comparable K_d (∼25 nM) with other SPM in isolated human PMN. In addition, using ChemR23 and BLT1 β-arrestin cells, RvE2 was found to share, at least in part, receptors with RvE1 (Oh et al., 2012).

GPCRs FOR D-SERIES RESOLVINS

RvD1 activates its own GPCR and does not activate ChemR23. RvD1 exerts specific bioactivities on human PMN, namely reduction of F-actin polymerization, that are inhibited by PTX but not cholera toxin, whereas it did not stimulate Ca^{2+} release nor activate cAMP in human PMN (Krishnamoorthy et al., 2010). For the purpose of investigating direct binding of RvD1 to human PMN, [^{3}H]-RvD1 was prepared by catalytic hydrogenation of synthetic [13, 14]-acetylenic RvD1 methyl ester by custom tritiation (American Radiolabel; Krishnamoorthy et al., 2010). This procedure was followed by de-esterification and isolation of the RvD1 label using RP-HPLC. [^{3}H]-RvD1 specifically bound to human PMN with high affinity ($K_d \sim 0.17$ nM) and was competed

by homoligand cold RvD1 (100%) and LXA_4 (∼60%) but not the ALX-ligand annexin-1-derived Ac2-12 peptide. In parallel, [^{3}H]-RvD1 also showed specific binding with human monocytes (Krishnamoorthy et al., 2010). Since RvD1 counteracts TNF-α *in vivo*, luciferase-based reporter system (Arita et al., 2005a) was employed in functional screenings to assess the ability of selected GPCRs (**Figure 4**) to transduce RvD1 signals that block NF-κB activity in response to TNF-α.

Phylogenetically related GPCR linked to inflammation and chemoattraction were overexpressed into HeLa cells together with a reporter vector consisting of NF-κB promoter sequence linked to the luciferase gene. RvD1 significantly reduced TNF-α-stimulated NF-κB response in cells overexpressing either the LX receptor ALX/FPR2 or the orphan, GPR32, but not other GPCRs (e.g., BLT1, BLT2, CB1, GPR-1, FPR, and ChemR23; Krishnamoorthy et al., 2010). Moreover, RvD1 dose-dependently activated ALX/FPR2 and GPR32 in recombinant β-arrestin cells with EC_{50} in the low picomolar range ($EC_{50} \sim 1.2$ pM for ALX/FPR2; 8.8 pM for GPR32; **Figure 5**). In contrast, RvD1 did not activate RvE1 receptor ChemR23, demonstrating the high selectivity of these ligands for their specific GPCR (Krishnamoorthy et al., 2010). In comparison, at equimolar concentrations, RvD1, its epimer AT-RvD1, RvD1-carboxy-methyl ester, and a metabolically more stable analog 17 (R/S)-methyl RvD1-ME activated both ALX/FPR2 and GPR32 with similar potencies and EC_{50}, whereas the biosynthetic precursor native DHA was not active with GPR32 and ALX/FPR2 in this concentration (Krishnamoorthy et al., 2012). Of interest, the known anti-inflammatory ALX/FPR2 agonist compound 43 identified by traditional medicinal chemistry screening also activated GPR32 ($EC_{50} \sim 2.2$ pM) and ALX/FPR2 ($EC_{50} \sim 2.0$ pM) in β-arrestin cells but not the ADP receptor $P2Y_{12}$ (Krishnamoorthy et al., 2010). Hence, RvD1, AT-RvD1, and the derivatives carboxy methyl ester and 17(R/S)-RvD1 directly activate ALX/FPR2 and GPR32, hereafter referred to as RvD1 receptor (DRV1) following the IUPAC recommendations for receptor nomenclature (Brink et al., 2003; **Figure 5**). Overexpression of either ALX/FPR2 or GPR32 in human MΦs gave further enhancement of efferocytosis in response to RvD1, while knockdown of ALX/FPR2 or DRV1/GPR32 determined a decrease in RvD1-stimulated phagocytosis response (Krishnamoorthy et al., 2010).

In keeping with this, Norling et al. (2012) demonstrated that the ability of RvD1 to reduce human PMN-endothelial cell interactions is absolutely dependent on ALX/FPR2 and DRV1/GPR32. Interestingly, actions of low concentration (1 nM) RvD1 were dampened by DRV1/GPR32 blocking antibody, whereas at high concentrations (10 nM) they appeared ALX/FPR2-specific. These two receptors also have distinct responses in an activated cell environment in that upon activation human PMN rapidly mobilized ALX/FPR2 stored in secretory granules, but not DRV1/GPR32, to the cell membrane. In addition, in ALX/FPR2 knockout mice RvD1 did not exert anti-inflammatory (e.g., stop PMN infiltration) nor pro-resolving (e.g., enhancing MΦ efferocytosis) actions (Norling et al., 2012). Hence, specific GPCRs selectively mediate RvD1 actions with ALX/FPR2 being rapidly upregulated in PMN that are exposed to pro-inflammatory stimuli and DRV1/GPR32 possibly conveying more homeostatic functions. With respect to cell and tissue distribution, ALX/FPR2 is present on leukocytes and resident cells (including MΦ, synovial fibroblasts, mesangial cells, endothelial, and epithelial cells; Krishnamoorthy et al., 2010). Human DRV1/GPR32 was identified in peripheral blood leukocytes and arterial and venous tissues using a cDNA array. It is mostly abundant on PMN, monocytes, and macrophages and is also present on vascular endothelial cells (Krishnamoorthy et al., 2010). The murine ortholog of DRV1/GPR32 is currently not known but is present in chimpanzees. Regulatory mechanisms of DRV1/GPR32 are of interest while those of ALX/FPR2 have recently been uncovered (Simiele et al., 2012). Although specific receptors for RvD2, RvD3, and RvD4 have not yet been identified, the stereoselective actions of RvD2 were inhibited by PTX (Spite et al., 2009a), implicating the involvement of GPCRs. More recently, Chiang et al. (2012) reported activation of RvD1-receptor DRV1/GPR32 by RvD5, which is related biosynthetically to RvD1, with the recombinant human DRV1.

GPCRs FOR NEURO (N)PD1/PD1

Biosynthesis of (N)PD1 occurs in neural tissues in response to injury, ischemia-reperfusion, and exposure to β-amyloid peptides (Marcheselli et al., 2003; Mukherjee et al., 2004; Bazan, 2007). In addition (N)PD1 shows protective anti-inflammatory and pro-resolving actions within the immune system (Serhan et al., 2002; Hong et al., 2003). Hence, it was of interest to determine the molecular basis of (N)PD1/PD1 actions. Specific binding of tritium-labeled (N)PD1 obtained by catalytic tritiation of synthetic 15-acetylenic NPD1 methyl ester was demonstrated with both retinal pigment cells (RPE) and human PMN. [^{3}H]-(N)PD1 bound RPE with $K_d \sim 30$ pmol/mg of cell protein. However, at high concentration of radio-ligand (>10 nM), non-specific binding was evident, in line with the highly hydrophobic nature of this compound. In these experiments, competitive binding studies with unlabeled ligand demonstrated 90–100% displacement for the free acid form of cold (N)PD1. In these studies (N)PD1-ME showed a lower affinity for binding sites and ∼74% displacement, while other structurally related omega-3 fatty acid-derived compounds (17*S*-hydroxy-DHA, RvE1, Δ15-*trans*-NPD1, and Δ15-*trans*-NPD1-carboxy methyl ester) gave only minimal or no displacement. Specific binding experiments with [^{3}H]-NPD1 and isolated human PMN proved high affinity specific binding and showed a two-site fit for sites of high and low affinity binding ($K_d \approx 25$ and 200 nM). Two other SPM that bind specific receptors on human PMN, namely LX A_4 (LXA_4) and RvE1, did not compete with [^{3}H]-NPD1 binding on these cells (Marcheselli et al., 2010).

HOW DO THE PRO-RESOLVING MEDIATORS WORK?

By definition, SPM: (a) are generated within the resolution phase; (b) limit leukocyte infiltration; (c) enhance phagocytic activity of pro-resolving MΦ to remove apoptotic cells and/or microbes; (d) stimulate the clearance of PMN from mucosal surfaces and their anti-microbial actions. If a LM fulfills each of these bioactivities, then it belongs to the genus of SPM. At the cellular and molecular levels, SPM display distinct modes of action on PMN and monocyte/MΦs, which can be demonstrated with isolated cells. Each SPM (RvE1, RvE2, and RvD1) stimulates a rapid shape change of human leukocytes that reflects ligand-receptor responses and cytoskeletal events that ultimately limit the PMN to diapedesis

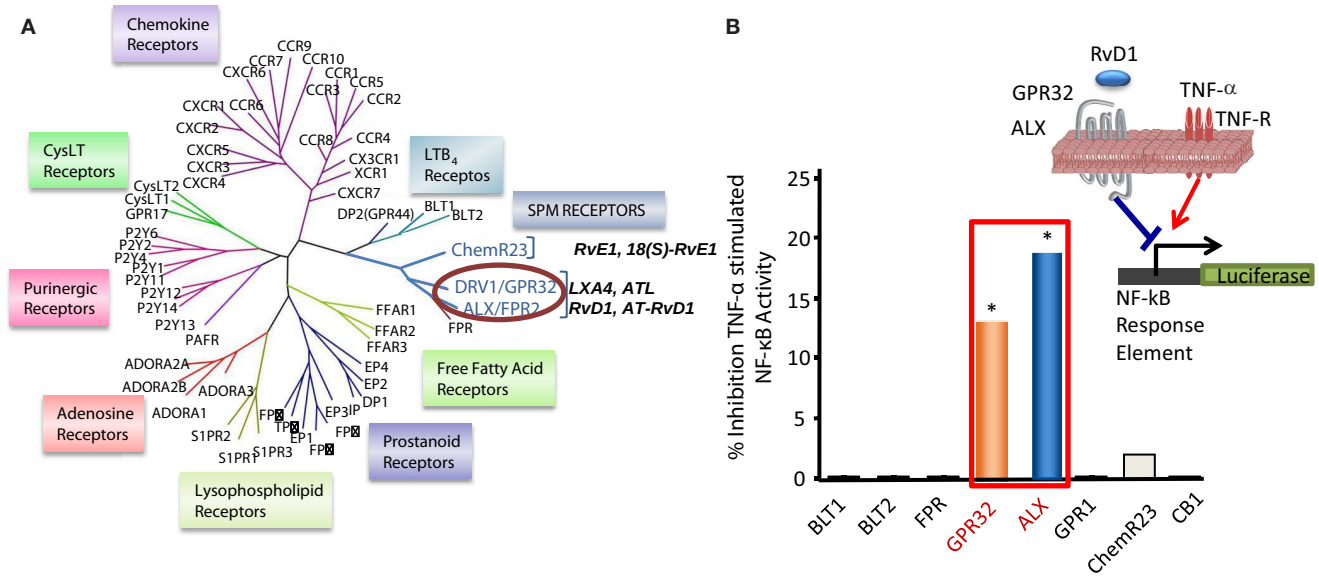

FIGURE 4 | Identification of RvD1 GPCR. (A) Phylogenetic tree shows similarities in the amino acid sequences of human GPCRs closely related to LXA_4, RvE1, and LTB_4 receptors (left panel). Cluster was generated with the ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2). Protein sequences were deduced from the NCBI database and receptor nomenclature followed the IUPAR classification for GPCR. **(B)** For functional screening and identification of RvD1 receptor, GPCRs were cloned in pcDNA3 vector and overexpressed in human HeLa cells cotransfected with pNF-κB luciferase plasmid. Cells were treated with RvD1 and TNF-α (see inset in the right panel). RvD1 reduces TNF-α-stimulated NF-κB activation in DRV1/GPR32 and ALX/FPR2 receptor-overexpressing cells, while cells transfected with other related GPCRs (e.g., BLT1, BLT2, CB1, GPR-1, FPR, and ChemR23) did not significantly inhibit TNF-α-induced NF-κB luciferase activity on addition of the ligand RvD1. The results illustrated are expressed in luminescence units subtracted from pNF-κB and pcDNA3 empty vector (*$P < 0.05$ vs. BLT2-transfected cells).

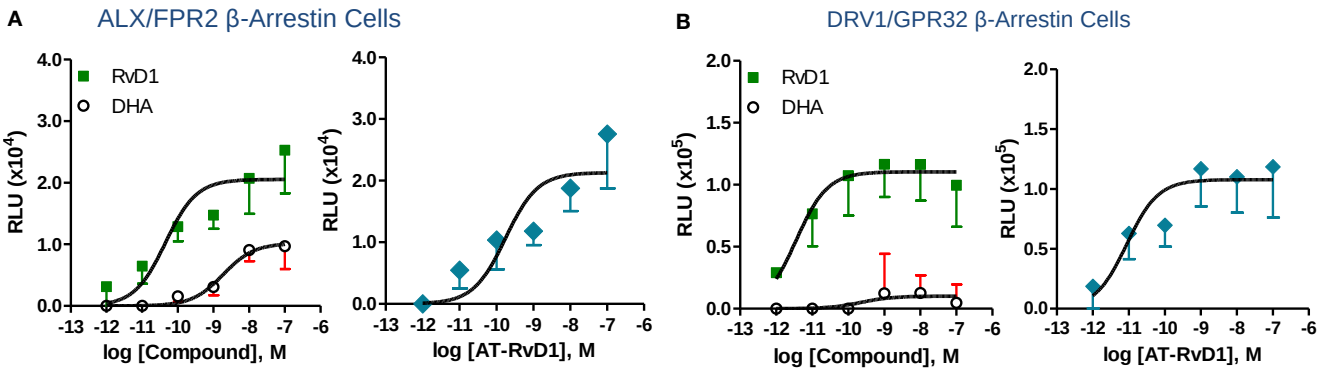

FIGURE 5 | Structure-activity relationship of RvD1, AT-RvD1, and DHA on ALX/FPR2 and DRV1/GPR32 receptors. Activation of ALX/FPR2 and DRV1/GPR32 was determined using the β-arrestin cell system. This system is engineered by stably expressing the target ALX/FPR2 or DRV1/GPR32 tagged with the β-galactosidase Pro-Link peptide. Cells also co-expressed the β-arrestin protein linked to the β-galactosidase EA fragment. In the presence of ligand, activated GPCR interacts with β-arrestin, bringing to proximity the EA and Pro-Link fragments, forming a functional enzyme. β-galactosidase activity is measured by adding the substrate and generating a chemiluminescent signal that is stoichiometrically associated to ligand dependent GPCR activation (see text and Krishnamoorthy et al., 2010 for further details). Dose-response curves show activation of ALX/FPR2 **(A)** and DRV1/GPR32 **(B)** receptors by RvD1, AT-RvD1, but not DHA determined using the β-arrestin cell system. Results are mean (±SEM) from $n = 4$ to 6. RLU, relative luminescence unit.

in vivo, hence reducing inflammation and tissue damage (Dona et al., 2008; Kasuga et al., 2008; Oh et al., 2012) and prepare the macrophages to enhance phagocytosis of both apoptotic cells and microbes (Schwab et al., 2007; Ohira et al., 2009).

The best examples of these pro-resolving cellular mechanisms studied to date are for the Rv ligands RvE1 and RvD1. In the case of RvE1, its direct binding to the human recombinant GPCR denoted ChemR23, an RvE1 receptor, activates the receptor with the subsequent regulation of Akt intracellular signaling pathway and

phosphorylation signaling of proteins that increase phagocytosis (Ohira et al., 2009). In the case of RvD1, the ligand reduces actin polymerization in isolated human PMN in a PTX-sensitive manner (Krishnamoorthy et al., 2010), diminishes surface expression of CD11b involved in leukocyte adhesion to endothelial cells (Krishnamoorthy et al., 2010) as does RvE1 (Dona et al., 2008), evokes a shape change and stops chemoattractant-initiated PMN migration observable at a single cell level with human cells isolated within microfluidic chamber systems (Kasuga et al., 2008). Of note, LXA_4 also stops PMN chemotaxis *in vitro* (reviewed in Serhan, 2005), while it induces rapid activation of small GTPases and redistribution of cytoskeletal proteins (e.g., MYH9, F-actin) in human monocyte-derived MΦs during phagocytosis (Maderna et al., 2002; Reville et al., 2006). Hence, there appears to be a general mechanism on how pro-resolving molecules work to achieve their potent anti-inflammatory and pro-resolution actions, namely via SPM coupling to distinct high affinity receptors that evoke cell-type and specific intracellular pathways.

Besides these general actions, each SPM possesses additional specific activities (see **Table 1**; Fredman and Serhan, 2011). Given the important protective function of acute inflammation to fight infections or dangers from within and the need to safeguard the host from an uncontrolled reaction, it is not surprising that SPM have some bioactions overlapping in target tissues and specific cell types. In addition, the sites of biosynthesis for each SPM and the degree of cell distribution of their GPCRs may underlie selectivity and specificity of the pro-resolving system. In experimental models of inflammation and resolution, SPM proved to be stereoselectively active in the nano- to low microgram dose range to control inflammation, limit tissue damage, shorten resolution intervals, promote healing, and alleviate pain (**Table 1**). In order to define resolution in unbiased, quantitative terms, mathematical resolution indices were introduced for determining the cellular changes in exudates, i.e., T_{max}, time point of maximum PMN infiltration (Ψ_{max}); T_{50}, time necessary to achieve 50% reduction in PMN number (Ψ_{50}) from Ψ_{max}; resolution interval ($R_i = T_{50} - T_{max}$), time interval between T_{max} and T_{50} (Bannenberg et al., 2005). The introduction of resolution indices permits the evaluation of pro-resolution bioactions of endogenous chemical mediators or pharmacological agents (Schwab et al., 2007; Haworth et al., 2008; Navarro-Xavier et al., 2010). These results first demonstrate the possibility to pharmacologically manipulate resolution and stimulate resolution. Along these lines, results from the first human Phase I–II clinical trials demonstrated the safety and efficacy of a Rv analog that reduces both signs and symptoms of dry eye syndrome (http://Clinicaltrials.Gov Identifier: NCT00799552) and have moved forward to Phase III clinical trial with Celtic Therapeutics. Dye eye syndrome is a chronic illness commonly treated with the immune-suppressant cyclosporine, providing evidence that SPM have the potential to treat a broad array of human diseases.

Acute inflammation following tissue injury, surgery, or infections causes pain (Majno and Joris, 1996). Peripheral sensitization of primary sensory neurons is induced by inflammatory mediators released after tissue insults, such as bradykinin, prostaglandins, nerve growth factors (NGF), pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β and IL-6, and pro-inflammatory chemokines (Stein et al., 2009). The contribution of PGE_2 and I_2 led to the use of NSAID (e.g., naproxen, ibuprofen) and selective COX-2 inhibitors as analgesic. Since SPM are potent regulators of acute inflammation and pro-inflammatory mediators (including PGs, TNF-α, and IL-1β), and since COX-2 inhibitors are resolution toxic (Schwab et al., 2007), it was of interest to investigate whether SPM could control inflammation associated and chronic pain. The initial report from Svensson et al. (2007) on the antinociceptive actions of LXA_4 was followed by further studies demonstrating that Rvs, PD, and also MaR1 have potent analgesic activities when administered both locally and systematically (Xu et al., 2010; Huang et al., 2011; Lima-Garcia et al., 2011; Park et al., 2011; Serhan et al., 2012). Notably, the exquisite potent actions of SPM, which proved as effective as morphine and COX-2 inhibitor NS-398 at much lower doses (Xu et al., 2010), occur without altering basal sensitive perception, unlike other anesthetics used to control pain during surgery. Hence it appears possible to resolve pain signaling as well as inflammation.

Complete resolution requires regeneration of destroyed tissues without affecting their functionality as in the case of fibrosis or scarring. Pro-resolving MΦ play key functions in tissue remodeling under both homeostatic (e.g., post parturition) and pathological (e.g., removal of microbes from infected tissues) conditions (Honn et al., 1989; Majno and Joris, 1996; Gordon, 2007). In this regard, SPM are of considerable interest in view of their roles in regulating MΦ activities. For instance, LX, Rv, and PD stimulate the non-phlogistic efferocytosis by MΦ (Godson et al., 2000; Schwab et al., 2007; Hong et al., 2008; Krishnamoorthy et al., 2010; Oh et al., 2011). In addition, RvD1 regulates MΦ accumulation in diabetic obese mice (Hellmann et al., 2011) and reduces arthritic pain (Xu et al., 2010; Lima-Garcia et al., 2011). Failure in the MΦ-driven pro-resolution program can support persistent inflammation associated with many human diseases, such as periodontitis. In keeping with this, recent reports indicate that MΦ from localized aggressive periodontitis have impaired phagocytosis and persistent inflammation that is rescued with RvE1 (Fredman et al., 2011). In addition to enhancing MΦ phagocytosis, MaR1 biosynthesized *in vivo* during tissue injury repair also accelerated tissue regeneration in planaria (*D. tigrina*) after surgical head removal (Serhan et al., 2012). Of note, these actions of MaR1 were inhibited by PTX, indicating the involvement of GPCR and related signaling in this process (Serhan et al., 2012).

miRNAs IN RESOLUTION CIRCUITS

Results from the Serhan laboratory at Brigham and Women's Hospital-Harvard Medical School demonstrated for the first time that SPM are operative in resolution and act locally to control leukocyte trafficking, regulate chemical mediators (e.g., cytokines, chemokines, and lipid autacoids), and expedite the return to homeostasis. Since microRNA (miRNAs) have emerged as the fine tuners of many cellular processes, including immune responses and cancer, it was of interest to investigate whether they also played roles in resolution and SPM-regulated specific miRNA as part of their mechanisms of action. To identify a miRNA signature of resolution in self-resolving exudates, a strategy with resolving exudates from murine peritonitis was used (**Figure 6**). Zymosan A particles from *S. cerevisiae*, a Toll-like receptor 2 and 4 ligand, were injected i.p. and peritoneal exudates collected to

Table 1 | Bioactions of SPM.

SPM	Disease model	Mechanism of action	Reference
Lipoxin A4/ATL	Mouse/dermal inflammation	Inhibits neutrophil recruitment and vascular leakage	Takano et al. (1997)
	Mouse/dorsal air pouch	Inhibits neutrophil recruitment	Clish et al. (1999)
	Rabbit/periodontitis	Reduces PMN infiltration and prevents connective tissue and bone loss	Serhan et al. (2003)
	Mouse/peritonitis	Inhibits neutrophil recruitment and lymphatic removal of phagocytes	Bannenberg et al. (2005) and Schwab et al. (2007)
	Mouse/colitis	Attenuates pro-inflammatory gene expression and reduces severity of colitis, inhibits weight loss, inflammation, and immune dysfunction	Gewirtz et al. (2002)
	Mouse/asthma	Inhibits airway hyper-responsiveness and pulmonary inflammation	Levy et al. (2002)
	Mouse/cystic fibrosis	Decreases neutrophilic inflammation, pulmonary bacterial burden, and disease severity	Karp et al. (2004)
	Mouse/ischemia/reperfusion (I/R)	Attenuates hind limb I/R-induced lung injury. Detachment of adherent leukocytes in mesenteric I/R vessels. Reduces myocardial infarct size and area at risk in myocardial I/R	Scalia et al. (1997) and Chiang et al. (1999)
	Mouse/cornea	Accelerates cornea re-epithelialization, limits sequelae of thermal injury (i.e., neovascularization, opacity) and promotes host defense	Gronert et al. (2005)
	Mouse/angiogenesis	Reduces angiogenic phenotype: endothelial cell proliferation and migration	Fierro et al. (2002)
	Mouse/bone marrow transplant (BMT)	Protects against BMT-induced graft-versus-host diseases (GvHD)	Devchand et al. (2005)
	Rat/glomerulonephritis	Reduces leukocyte rolling and adherence; decreases neutrophil recruitment	Papayianni et al. (1995)
	Rat/hyperalgesia	Prolongs paw withdraw latency, reducing hyperalgesic index, and reduces paw edema	Svensson et al. (2007)
	Rat/pleuritis	Shortens the duration of pleural exudation	Bandeira-Melo et al. (2000)
	Mouse/tumor growth	Suppresses the growth of transplanted tumors in mice; inhibits angiogenesis	Chen et al. (2010)
	Mouse/allograft rejections	Prevents acute rejection of vascularized cardiac and renal allografts	Levy et al. (2011)
	Mouse/arthritis	Inhibits edema formation and PMN influx, reduces TNFα and LTB_4 levels	Conte et al. (2010)
	Rat/acute pancreatitis	Reduces intercellular adhesion molecule 1 (ICAM-1) and NF-κB p65 expression in the pancreas, and expression of ICAM-1 in the lungs	Zhou et al. (2011)
	Zebrafish/mycobacterial infection	Reduces bacterial burden and growth; improves microbial containment by phagocytes	Tobin et al. (2012)
Resolvin E1	Mouse/dorsal air pouch	Inhibits neutrophil recruitment	Serhan et al. (2000a)
	Mouse/peritonitis	Inhibits neutrophil recruitment, regulates chemokine/cytokine production, and promotes lymphatic removal of phagocytes	Arita et al. (2005a), Bannenberg et al. (2005), and Schwab et al. (2007)
	Rabbit/periodontitis	Reduces PMN infiltration, prevents connective tissue and bone loss, promotes healing of diseased tissues, and promotes regeneration of lost soft tissue and bone	Hasturk et al. (2006, 2007)
	Mouse/retinopathy	Protects against neovascularization	Connor et al. (2007)
	Mouse/colitis	Decreases PMN recruitment and pro-inflammatory gene expression; improves survival and reduces weight loss; favors LPS-detoxification through induction of intestinal alkaline phosphatase	Arita et al. (2005b), Campbell et al. (2010), and Ishida et al. (2010)

(Continued)

Table 1 | Continued

SPM	Disease model	Mechanism of action	Reference
	Mouse/asthma	Reduces IL-23 and IL-6, and increases IFNγ and LXA_4 in lungs to dampen airway inflammation; decreases eosinophil and lymphocyte recruitment	Aoki et al. (2008, 2010) and Haworth et al. (2008)
	Mouse/obesity	Regulates adipokines and protects against liver steatosis	Gonzalez-Periz et al. (2009)
	Mouse/inflammatory pain	Inhibits spontaneous pain, and heat and mechanical hypersensitivity	Xu et al. (2010)
	Rat/cardiac ischemia/reperfusion injury	Reduces infarct size	Keyes et al. (2010)
	Mouse/allograft rejections	Prevents acute rejection of vascularized cardiac and renal allografts	Levy et al. (2011)
	Mouse/dry eye	Promotes tear production, corneal epithelial integrity, and decreases in inflammatory inducible COX-2. RvE1 inhibits keratocyte transformation to myofibroblasts and lowers the number of monocytes/macrophages	Li et al. (2010)
	Mouse/herpes simplex virus	Reduces severity of herpes simplex virus-induced ocular lesions, reduces angiogenesis, and stromal keratitis	Rajasagi et al. (2011)
Resolvin D1	Mouse/peritonitis	Inhibits neutrophil recruitment; shortens resolution interval; regulates miRNAs and target genes in resolving exudates; reduces LTB_4, PGD_2, $PGF2_\alpha$, and TXA_2 in peritoneal exudates	Hong et al. (2003), Sun et al. (2007), Spite et al. (2009b), Recchiuti et al. (2011), Krishnamoorthy et al. (2012), and Norling et al. (2012)
	Mouse/*E. coli* (peritoneal) and *S. aureus* (skin) infection	Reduces bacterial titers and hypothermia; increased survival; enhances microbial containment and killing by phagocytes; lowers antibiotic requirement; shortens resolution interval	Chiang et al. (2012)
	Mouse/dorsal air pouch	Inhibits neutrophil recruitment	Serhan et al. (2002) and Hong et al. (2003)
	Mouse/kidney ischemia-reperfusion	Protects from ischemia/reperfusion-induced kidney damage and loss of function; regulates macrophages	Duffield et al. (2006)
	Mouse/retinopathy	Protects against neovascularization	Connor et al. (2007)
	Mouse/inflammatory pain	Inhibits spontaneous pain, heat, and mechanical hypersensitivity; selectively blocks TRPV1 and TRPA1-mediated pain	Xu et al. (2010) and Park et al. (2011)
	Mouse/obesity	Reduces inflammatory cytokines in adipose tissue macrophages; stimulates M2 macrophage differentiation; promotes resolution of adipose tissue inflammation	Titos et al. (2011)
	Mouse/T2 diabetes	Reduces macrophage accumulation in adipose tissue; ameliorates insulin sensitivity	Hellmann et al. (2011)
	Rats/post-operative pain	Reduces post-operative pain, tactile allodynia, and hyperalgesia	Huang et al. (2011)
	Mouse/pain	Attenuates agonist-induced and inflammatory pain behaviors; inhibits TRPA1, TRPV3, and TRPV4 receptors; does not affect basal sensitivity	Bang et al. (2010) and Xu et al. (2010)
	Mouse/acute lung injury	Blocks leukocyte infiltration and reduces cytokine levels in BALF	Wang et al. (2011)
	Mouse/corneal inflammation	Reduces leukocyte infiltration and hemangiogenesis	Jin et al. (2009)

(Continued)

Table 1 | Continued

SPM	Disease model	Mechanism of action	Reference
AT-RvD1	Mouse/colitis	Reduces disease activity index, PMN number, and pro-inflammatory levels	Bento et al. (2011)
		Attenuates pain signals and behaviors by blocking TRPV3	Bang et al. (2012)
	Rats/arthritic pain	Possesses anti-hyperalgesic effects upon systemic administration. Decreases TNF-α and IL-1β production	Lima-Garcia et al. (2011)
Resolvin D2	Mouse/peritonitis	Blocks further PMN infiltration into the peritoneum	Spite et al. (2009a)
	Mouse/sepsis	Prevents hypothermia, decreases bacterial load in the blood and peritoneum, promotes survival	Spite et al. (2009a)
	Mouse/colitis	Improves disease activity index, weight loss, and colonic PMN infiltration. Reduces pro-inflammatory levels	Bento et al. (2011)
(Neuro)Protectin D1	Mouse/peritonitis	Inhibits neutrophil recruitment and regulates chemokine/cytokine production	Bannenberg et al. (2005) and Serhan et al. (2006)
		Promotes lymphatic removal of phagocytes; regulates T-cell migration; enhances CCR5 expression on apoptotic leukocytes	Ariel et al. (2005, 2006) and Schwab et al. (2007)
	Mouse/asthma	Protects from lung damage, airway inflammation, and hyper-responsiveness	Levy et al. (2007)
	Human/asthma	PD1 is generated in human asthmatic patients	Levy et al. (2007)
	Mouse/kidney ischemia/reperfusion	Protects from ischemia/reperfusion-induced kidney damage and loss of function; regulates macrophages	Duffield et al. (2006)
	Mouse/retinopathy	Protects against neovascularization	Connor et al. (2007)
	Rat/ischemic stroke	Inhibits leukocyte infiltration, NF-κB, and COX-2 induction	Marcheselli et al. (2003)
	Human/Alzheimer's disease	Diminished PD1 production in human Alzheimer's disease	Lukiw and Bazan (2008)
	Mouse/liver injury	Protects from necroinflammatory liver injury	Gonzalez-Periz et al. (2009)
	Mouse/Alzheimer's disease	Downregulates inflammatory genes; reduces amyloidogenic Aβ42 cleavage; protects from apoptosis	Zhao et al. (2011)
Maresin-1	Mouse/peritonitis	Blocks PMN infiltration into the peritoneum	Serhan et al. (2009)
	Planaria/tissue regeneration	Stimulates tissue regeneration post surgical damage	Serhan et al. (2012)
	Mouse/pain	Reduces pain	Serhan et al. (2012)

monitor temporal changes in both leukocyte numbers and composition. Rapidly after zymosan injection, leukocytes infiltrated the peritoneal cavity during the onset phase of acute inflammation (4 h), reaching a maximum ($\sim 22.0 \times 10^6$) at ∼12 h and declined at 24–48 h. Differential analysis with flow cytometry confirmed that the majority of leukocytes in exudates at 4 and 12 h were PMN (Ly-6Ghigh/CD11b$^+$; $\sim 10.0 \times 10^6$ cells at 4 h and $\sim 15 \times 10^6$ cells at 12 h). PMN number declined during the resolution phase 24 and 48 h after initiation of peritonitis. Conversely, resident peritoneal MΦs (F4/80$^+$cells), which represent the main leukocyte population in naive mice peritonea (Winyard and Willoughby, 2003), were not detected in exudates at 4 h. Monocytes (Ly-6G^{low}/CD11b^{high} cells) gradually increased at 12 h and differentiated into MΦs, consistent with their pro-resolution functions (Gordon, 2007), reaching ∼60% of exudate leukocytes at 48 h. Because it is important to define resolution in unbiased terms, resolution indices, introduced by Bannenberg et al. (2005), were calculated (i.e., $\Psi_{max} \sim 15.0 \times 10^6$; $T_{max} \sim 12$ h; $\Psi_{50} \sim 7.5 \times 10^6$; $T_{50} \sim 30$ h; $R_i \sim 18$ h; **Figure 7**) to characterize the resolution phase and determine temporal changes in miRNA expression during this time interval. Hierarchical clustering grouped the ∼300 miRNAs examined into distinct clusters based on their relative abundance at the different time intervals, indicating that specific miRNAs are temporally regulated during acute inflammation and its natural self-limited resolution.

In these studies, miR-21, miR-146b, miR-208a, and miR-219 were significantly regulated at 12 and 24 h compared to 4 h (**Figure 7**), suggesting a role in resolution. For instance, miR-21 proved to be critical for the production of anti-inflammatory IL-10 in experimental peritonitis (Sheedy et al., 2010), corroborating our findings. Next, resolution indices were used to pinpoint RvD1 actions in resolution. RvD1, administered in its pro-drug carboxy

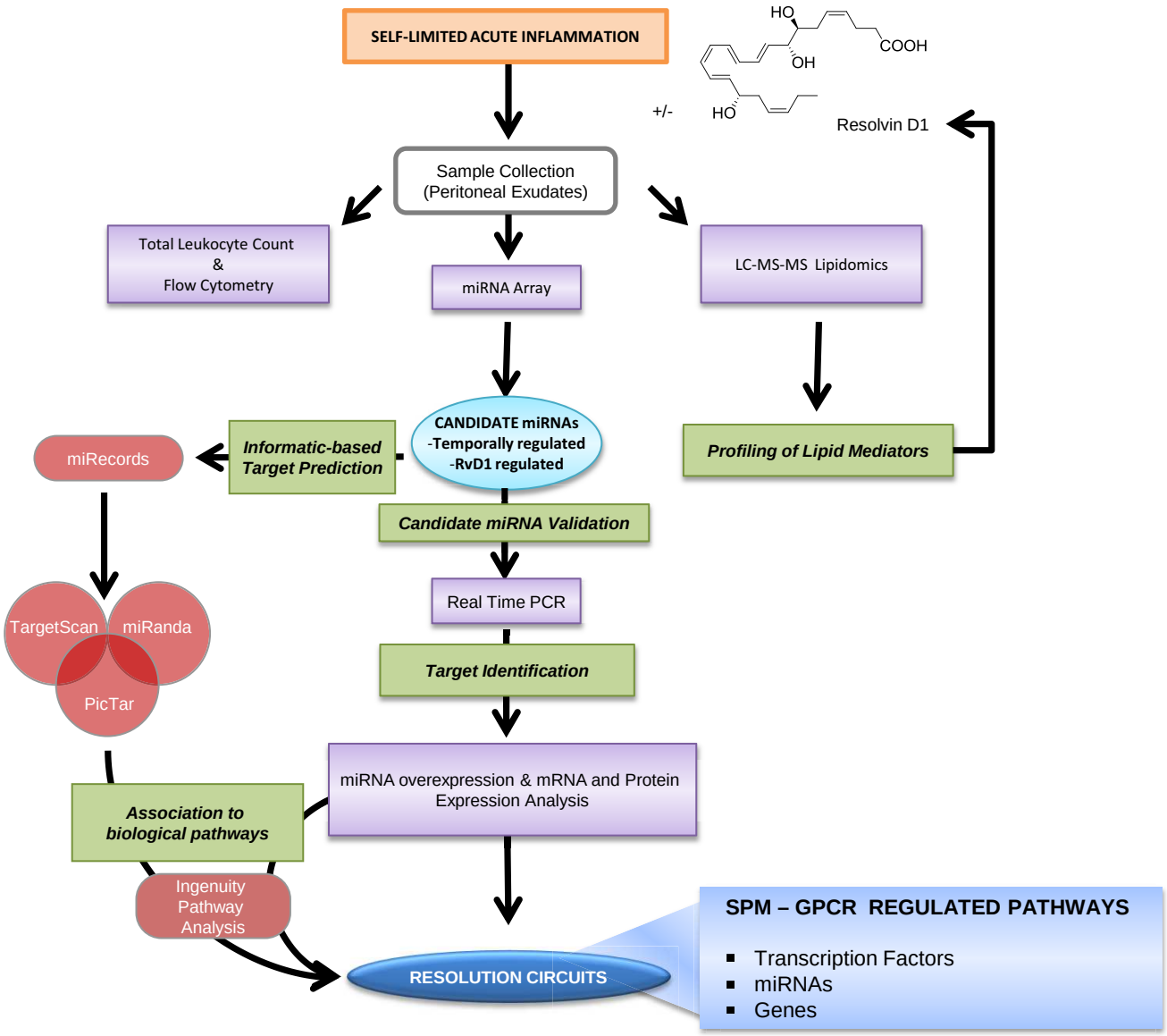

FIGURE 6 | Strategy for identification of RvD1-GPCR circuits. Self-limited zymosan-stimulated mouse peritonitis was used to obtain resolving exudates that were collected 4, 12, 24, and 48 h after zymosan A injection for temporal and differential analysis of leukocyte counts and phenotype, miRNA real-time PCR analysis, and lipidomics as in Recchiuti et al. (2011) and Krishnamoorthy et al. (2012). Candidate miRNAs, defined as those that were temporally and/or RvD1-regulated, were confirmed using bioinformatics and prediction of target genes, real-time PCR, as well as overexpression in human MΦs. The Ingenuity Pathway Analysis Software database was used to identify circuits of molecules and biological functions controlled by RvD1-regulated miRNAs and target genes (see text; Recchiuti et al., 2011 for analysis details).

methyl ester form, significantly lowered the Ψ_{max} (from 15.0 to 11.5×10^6), and accelerated or shortened the R_i by ∼4 h. Quantitative real-time PCR analyses carried out with exudates from zymosan- and zymosan plus RvD1-treated mice revealed that RvD1 temporally controls the specific sets of pro-resolving miRNAs miR-21, miR-146b, miR-208a, and miR-219 (coined *resolution miRs*) in exudates *in vivo*. For translation to humans, actions of RvD1 on these miRNAs were assessed in ALX/FPR2 and DRV1/GPR32-overexpressing isolated human cells, namely MΦ, the master regulators of resolution. Notably, RvD1 also regulated miR-21, -146b, -208a, and 219 in a GPCR-dependent manner. In order to identify target molecules of RvD1-GPCR-regulated miRs, we overexpressed miR-146b, -208a, and -219 in human MΦ for real-time PCR analysis to assess which mRNAs were significantly regulated.

Since miRNAs can regulate mRNAs and protein levels of hundreds of genes involved in biological processes (Bartel, 2009), miR-target genes were clustered using the Ingenuity Pathway Analysis knowledge database based on their physical and/or functional interactions, creating the first identified

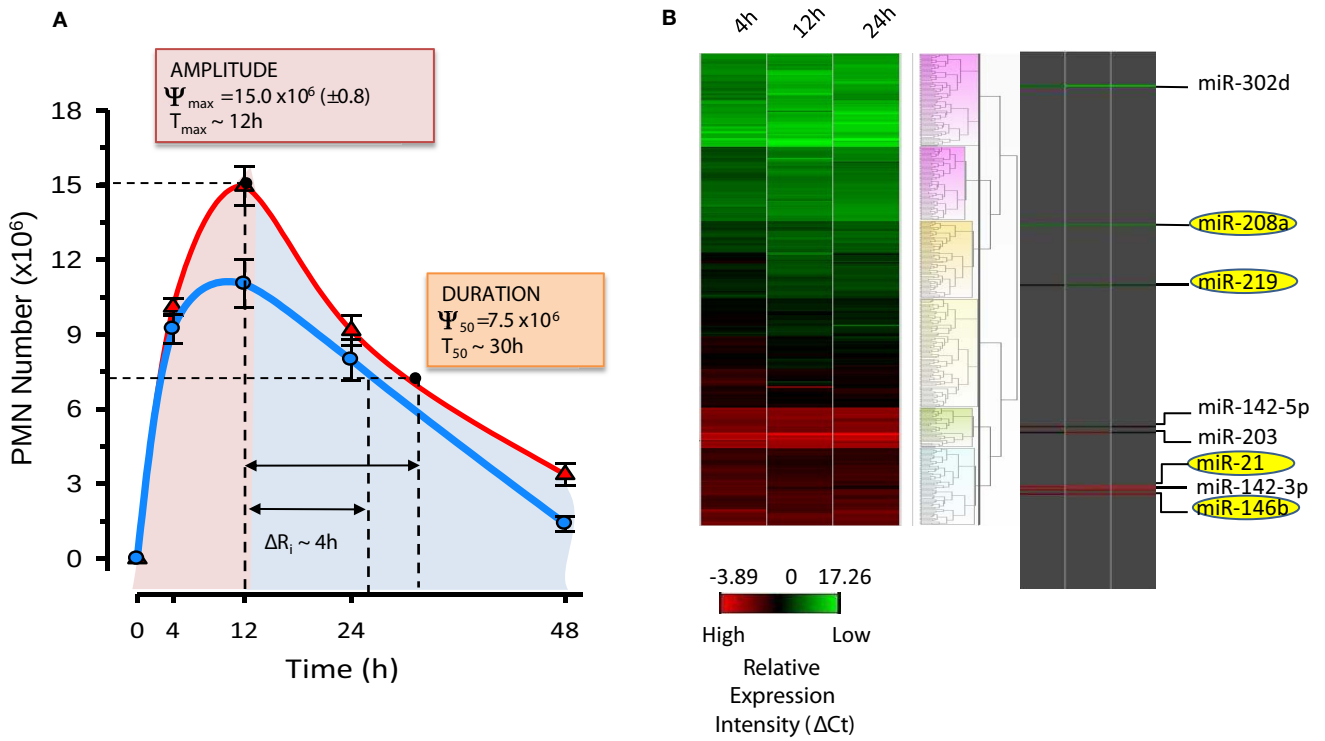

FIGURE 7 | Resolution indices and miRNA signature of resolution. (A) Resolution indices were calculated as in (Bannenberg et al., 2005) using PMN numbers in peritoneal exudates from mice treated with zymosan alone (red line) and zymosan plus RvD1 (blue line) at indicated time intervals: T_{max}, time of maximum PMN infiltration (Ψ_{max}); T_{50}, time to achieve 50% reduction in PMN number (Ψ_{50}) from Ψ_{max}; R_i, resolution interval ($T_{50} - T_{max}$; time interval between T_{max} and T_{50}). RvD1, administered at the beginning of peritonitis, significantly lowers amplitude (i.e., Ψ_{max}) and duration (T_{50}) of PMN infiltration, shortening the R_i by ~4h. **(B)** Heat map cluster represents relative expression of ~300 mouse miRNAs from resolving exudates determined with real-time PCR after normalization with housekeeping small RNAs (snoRNA251, snoRNA202, snoRNA142, and Rnu6). Relative expression intensities are indicated in a green-red color code based on ΔCt expression values ($n = 3$ mice/group). Temporal and RvD1-regulated miRNAs are highlighted.

RvD1-GPCR-regulated gene networks involved in inflammation and resolution. For instance, miR-146 networks 1 and 3 included genes of the NF-κB activation pathway (e.g., IκB kinase and tumor necrosis factor receptor-associated factor 6) and innate response to pathogens (e.g., Toll-like receptors, S100 protein, C-reactive protein, peptidoglycan recognition protein; **Figure 8A**). Several cytokines and chemokines (IL-8, 10, 12, interferon-α and β) belonged to the miR-146b network 2 (**Figure 8A**). NF-κB is a critical transcription factor involved in regulation of cell functions in inflammation and resolution (Lawrence et al., 2001). Of interest, RvD1 in human monocytes reduces the nuclear translocation of NF-κB, TNF-α induced phosphorylation of IκB (Recchiuti et al., 2011), counteracts NF-κB activation in ALX/FPR2 and DRV1/GPR32 recombinant cells (Krishnamoorthy et al., 2010), dampens acute inflammation in murine dorsal air pouches evoked by local administration of TNF-α (Serhan et al., 2002), and down-regulates IKK levels in murine peritonitis (Recchiuti et al., 2011). Therefore, regulation of miRNAs and the TNF-α-NF-κB axis seems to be a key component in the RvD1-GPCR downstream signaling network.

The miRNA miR-208a downregulates CD14, CD40 ligand, PGI2 receptor, thromboxane A2 receptor, and programmed cell death (Recchiuti et al., 2011; **Figure 8B**), a tumor suppressor molecule that acts as a translational repressor of IL-10 (Sheedy et al., 2010), consistent with the existence of a seed region for miR-208a in the 3′ UTR of PDCD4. Notably, in self-limited peritonitis, RvD1 reduced PDCD4 and increased IL-10 production, providing *in vivo* correlates of RvD1-miR-dependent gene regulations.

The miR-219 network includes CD14 and 5-LO (**Figure 8C**), a key enzyme for the biosynthesis of LTs, and SPMs. In addition, a significant reduction in 5-LO protein levels and LTB_4 production was found in human MΦ overexpressing the RvD1-regulated miR-219, translating findings in mouse peritonitis to the human MΦ cell system (Recchiuti et al., 2011). Endogenous regulatory mechanisms of 5-LO are of wide interest given the important roles of LTs, LXs, and Rvs in inflammation and resolution (Samuelsson, 1983; Serhan et al., 2002). In addition to transcriptional regulation by cytokines and growth factors (Radmark et al., 2007), miR-219 can provide a rapid mean to balance the abundance of 5-LO protein in cells under dynamic conditions such as during inflammation.

RvD1 actions were also tested in genetically engineered mice in order to obtain further evidence of ALX/FPR2-dependent action. Transgenic mouse colonies in which human ALX/FPR2 gene was placed under control of a CD11b promoter that drives transgene expression in mature murine myeloid cells were created

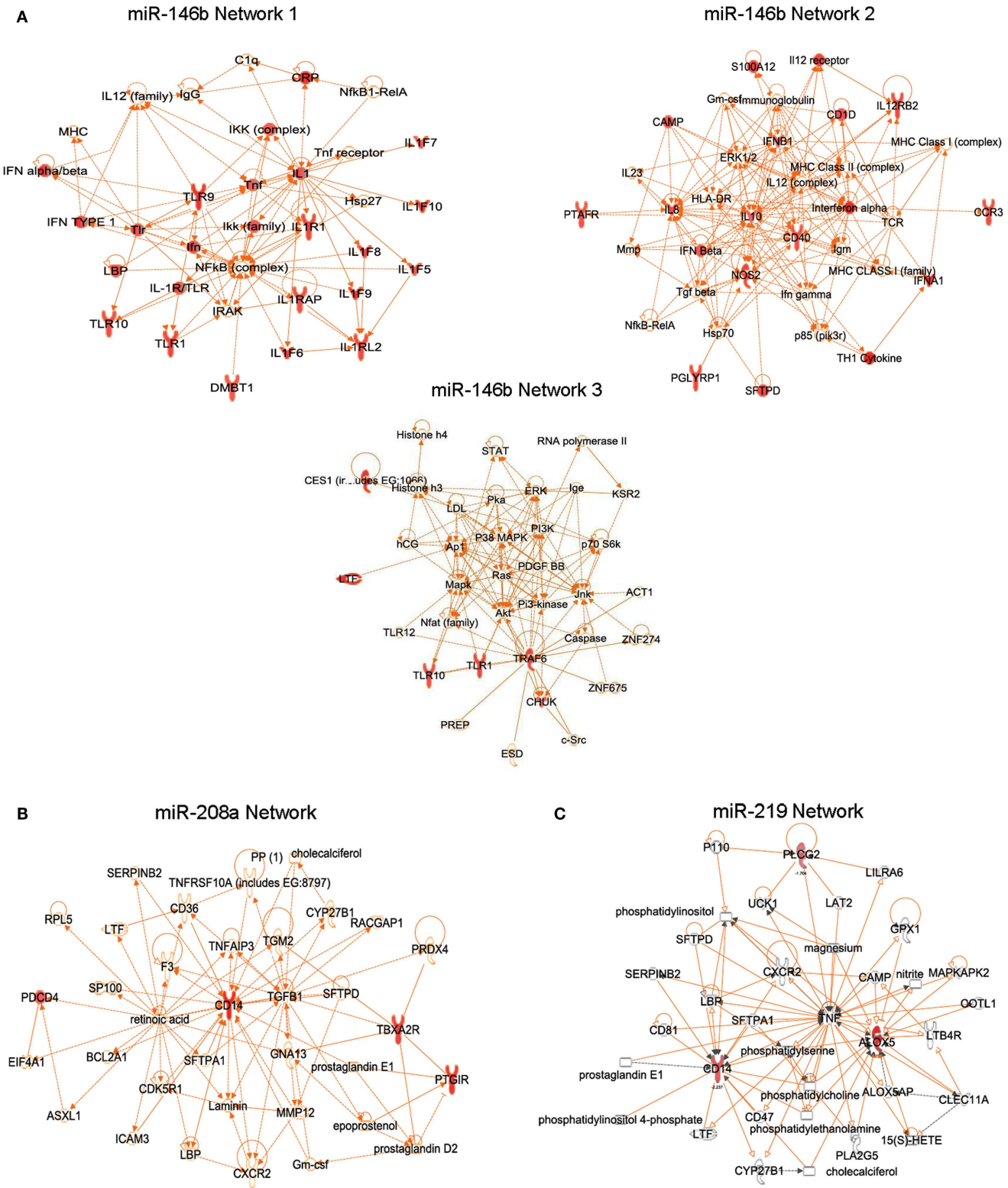

FIGURE 8 | Translation of RvD1-dependent miRNA circuits in human cells. RvD1-GPCR gene networks connecting target genes of miR-146b **(A)**, miR-208a **(B)**, and miR-219 **(C)**. Genes that are significantly down regulated in each network are indicated in red.

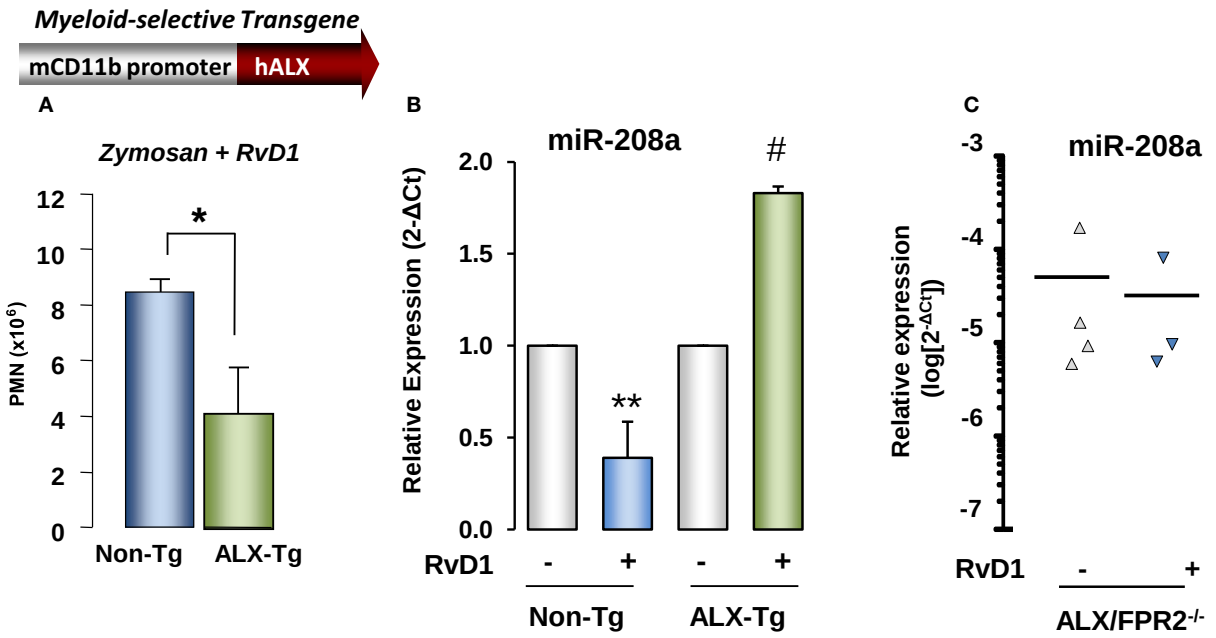

FIGURE 9 | Evidence for ALX/FPR2-RvD1-dependent miRNA pathways *in vivo*. Reduction in **(A)** PMN infiltration by RvD1 is significantly higher in myeloid-driven human ALX-transgenic (Tg) mice challenged with zymosan (1 mg/mouse, i.p., 24 h) than non-Tg littermates. Results are mean ± SEM from three mice/group (*$P < 0.05$ vs. non-Tg). **(B)** Relative expression of miR-208a determined in resolving exudates 24 h after zymosan alone or zymosan plus RvD1 administration in hALX-Tg or non-Tg littermates (mean ± SEM, $n = 3$ mice/group; **$P < 0.05$ vs. non-Tg zymosan group; #, $P < 0.05$ vs. hALX-Tg zymosan group). **(C)** miR-208a expression in peritoneal exudates (24 h) from ALX/FPR2 knockout mice treated with zymosan alone or zymosan plus RvD1.

(Devchand et al., 2003). Interestingly, total peritoneal leukocytes at 24 h peritonitis were significantly lower in ALX/FPR2 transgenic mice treated with zymosan alone compared to non-transgenic littermates, and RvD1 administration resulted in a further significant decrease (∼53% reduction) in peritoneal exudate leukocyte numbers in ALX/FPR2 transgenic mice compared to non-transgenic littermates (**Figure 9**). Quantitative PCR analysis of miRNAs isolated from peritoneal lavages collected 24 h post injection of zymosan showed that RvD1 significantly upregulated identified resolution miRs miR-208a and miR-219 *in vivo* (Recchiuti et al., 2011), whereas it does not appear to regulate miR-21, miR-146b, and miR-302d (**Figure 9**). To further investigate ALX/FPR2-dependent actions of RvD1 *in vivo*, it was next tested whether RvD1 can regulate some of these miRNAs in ALX/FPR2 knockout mice, where LXA_4 did not regulate leukocyte trafficking (Dufton et al., 2010). In ALX/FPR2$^{-/-}$ mice, RvD1 did not regulate PMN infiltration, consistent with recent results (Norling et al., 2012), nor did it significantly alter miR-208a or miR-219 expression at 24 h (**Figure 9**).

Taken together, these results indicate that RvD1 controls leukocyte infiltration and specific resolution phase miRs in acute inflammation in an ALX/FPR2-dependent manner in mice and DRV1/GPR32-dependent in isolated human cells.

SUMMATION AND CONCLUSION

It is now eminently clear that the acute inflammatory response of the host is a highly coordinated defensive response and its complete resolution is the ideal outcome. This ideal outcome is achieved in self-limited inflammation by activation of endogenous resolution programs that are governed in part by resolution phase local chemical mediators. Excessive inflammation can lead to chronic disorders and host damage. LM from AA, such as PGs and LTs, can amplify the inflammatory response, and PGE_2 and D_2 initiate the "class switch" that leads to the biosynthesis of LXA_4, the first member of the new genus of SPM. SPM include several families of LM such as E-and D-series Rv (neuro)PD, and the youngest family, the MaR, which are biosynthesized from essential ω-3 fatty acids. Of interest in experimental animal models of disease, each of the SPM can accelerate the resolution process when administered *in vivo,* and their bioactions are highly stereospecific, GPCR-mediated, and exerted at low doses. SPM also reduce pain and promote tissue regeneration, the ultimate goal of complete return to homeostasis. Results from the first human clinical Phase I and II trial with a Rv analog appear encouraging and can open new opportunities for resolution pharmacology based on endogenous mediators to terminate inflammation and treat inflammation-related diseases. We trust that more human trials will be launched to test the notion that stimulating resolution mechanisms can improve disease and health status.

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Emerging roles of eosinophils and eosinophil-derived lipid mediators in the resolution of inflammation

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Acute inflammation and its resolution are essential processes for tissue protection and homeostasis. Once thought to be a passive process, the resolution of inflammation is now shown to involve active biochemical programs that enable inflamed tissues to return to homeostasis. The mechanisms by which acute inflammation is resolved are of interest, and research in recent years has uncovered new endogenous anti-inflammatory and pro-resolving lipid mediators (i.e., lipoxins, resolvins, protectin, and maresin) generated from polyunsaturated fatty acids (PUFAs). This review presents new insights into the cellular and molecular mechanisms of inflammatory resolution, especially the roles of eosinophils, and a series of omega-3 PUFA-derived anti-inflammatory lipid mediators that they generate.

Keywords: resolution of inflammation, lipid mediator, eosinophils, lipidomics, lipoxygenase, resolvins

INTRODUCTION

Inflammation is a defensive response to injury and infection, but excessive or inappropriate inflammation contributes to a range of acute and chronic human diseases. Acute local inflammation in healthy individuals is self-limited and resolves by means of an active termination program (Gilroy et al., 2004; Serhan and Savill, 2005). The mechanisms that regulate the progression and resolution of inflammation remain of interest. Lipid mediator metabolomics of self-resolving inflammatory exudates recently uncovered a new family of potent anti-inflammatory and pro-resolving mediators. These include arachidonic acid (AA)-derived lipoxins, eicosapentaenoic acid (EPA)-derived E-series resolvins, docosahexaenoic acid (DHA)-derived D-series resolvins, protectin, and maresin (Bannenberg and Serhan, 2010). In inflammatory exudates, lipid mediators change in the course of acute inflammation and resolution (Levy et al., 2001; Bannenberg et al., 2005; Blaho et al., 2009; Yamada et al., 2011). In this review, we provide an overview of the novel cellular and molecular components involved in lipid mediator class switching during resolution of inflammation, especially eosinophils and the lipid mediators that they generate.

INFLAMMATORY RESPONSE AND THE RESOLUTION OF INFLAMMATION

Inflammation is a host defense mechanism that is characterized by the movement of serum proteins and leukocytes from the blood to the extravascular tissue. The acute inflammatory response is characterized by the initial recruitment of neutrophils, followed by the recruitment of monocytes that differentiate into macrophages (**Figure 1**). Many mediators coordinate the initial events of acute inflammation. Lipid mediators such as prostaglandins (PGs) and leukotrienes (LTs), cytokines, and chemokines coordinately regulate vascular permeability and leukocytes infiltration (Larsen and Henson, 1983). Once the noxious materials are removed via phagocytosis, the inflammatory reaction must be resolved to maintain homeostasis. The resolution of acute inflammation is an active process that is controlled by endogenous pro-resolving mediators. These factors switch off leukocyte trafficking to the inflamed site, reverse vasodilation, and vascular permeability, and promote the clearance of inflammatory cells, exudates, and tissue debris, thereby leading to the restoration of homeostasis to the inflamed tissue. AA-derived lipoxin A_4 (LXA_4) was the first PUFA-derived mediator found to have anti-inflammatory and/or pro-resolving activities (Godson et al., 2000; Serhan, 2005). Nanomolar concentrations of LXA_4 inhibit polymorphonuclear leukocyte (PMN) entry into inflamed tissues in animal models (Colgan et al., 1993). LXA_4 is synthesized by the actions of both 15-lipoxygenase (15-LOX) and 5-LOX, and phosphorylation of 5-LOX at S663 was recently shown to convert the enzyme to a robust 15-LOX, which can stimulate production of LXA_4 in cells which do not express 15-LOX (Gilbert et al., 2012). Also, omega-3 fatty acids EPA and DHA are precursors of endogenous anti-inflammatory and/or pro-resolving mediators. Using an unbiased lipidomics approach and the enzymatic oxygenation of omega-3 fatty acids, Serhan and collaborators identified families of novel bioactive mediators derived from EPA and DHA. These include EPA-derived E-series resolvin (RvE1, E2; Serhan et al., 2000; Arita et al., 2005; Tjonahen et al., 2006), DHA-derived D-series resolvin (RvD1-6; Serhan et al., 2002; Sun et al., 2007; Spite et al., 2009; Chiang et al., 2012), neuroprotectin/protectin (NPD1/PD1; Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2006), and maresin (MaR1; Serhan et al., 2009, 2012). These lipid mediators promote resolution via enhanced macrophage clearance of apoptotic PMNs, chemokines, cytokines, and microbial products (Ariel et al., 2006; Schwab et al., 2007). In the course of acute inflammation and

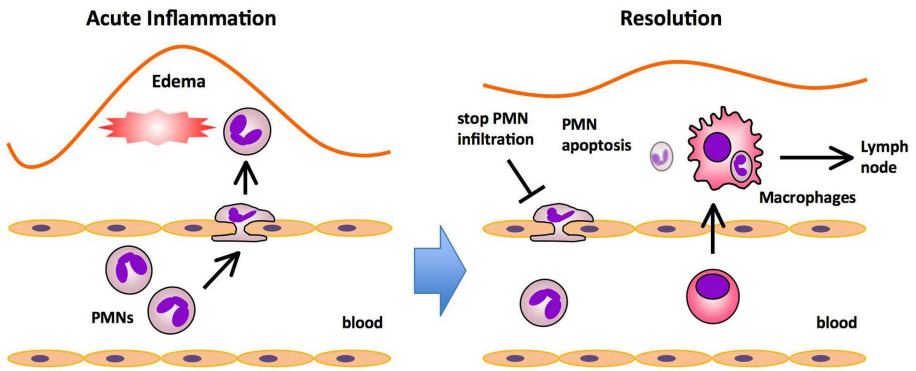

FIGURE 1 | Major processes in acute inflammation and resolution. The initiation phase of acute inflammation is characterized by the rapid infiltration of polymorphonuclear neutrophils (PMNs) followed by the infiltration of monocytes that mature into macrophages, and edema formation in response to injury. PMNs provide the first line of immune defense by migrating to sites of injury and neutralizing invading microorganisms or noxious materials by phagocytosis. In the resolution phase, PMNs undergo apoptosis and are ingested by macrophages that emigrate rapidly from the inflamed site to the draining lymph nodes (DLNs).

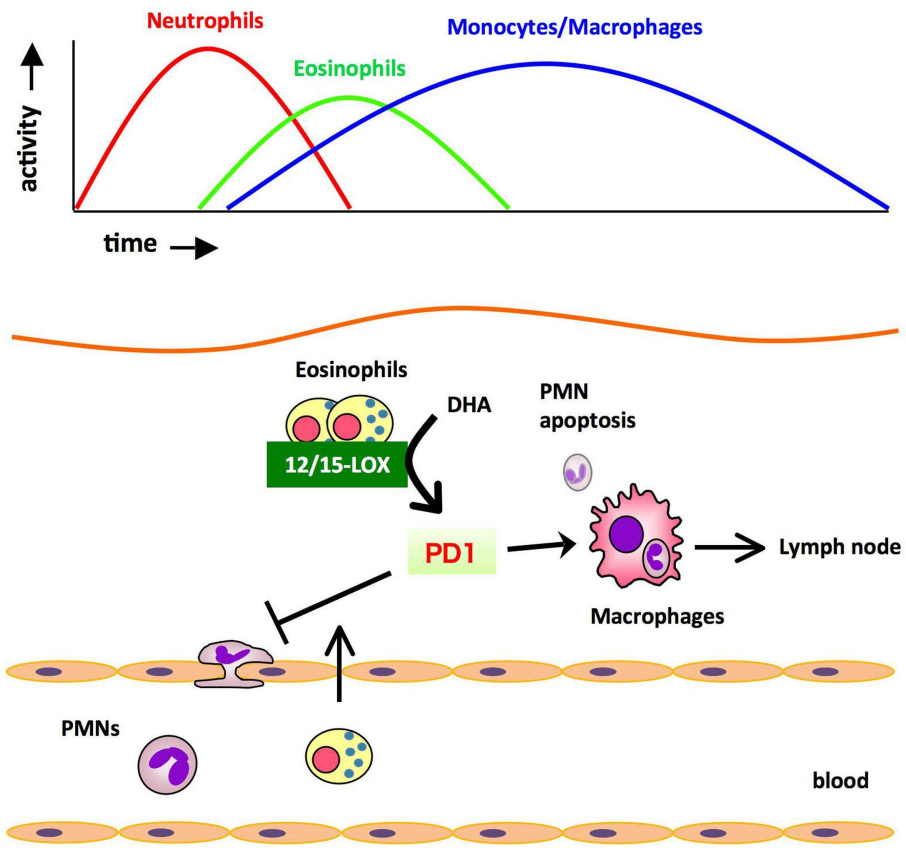

FIGURE 2 | Eosinophils are recruited to the inflamed loci and promote resolution of inflammation by producing anti-inflammatory and pro-resolving lipid mediators. During the resolution phase of self-resolving acute peritonitis model, eosinophils are recruited to the inflammatory site, where they locally produce pro-resolving lipid mediators such as PD1 via 12/15-LOX-initiated biosynthesis. These mediators block PMN infiltration and/or promote the clearance of phagocytes carrying engulfed zymosan from the inflammatory site to DLNs.

resolution, changes of cellular composition or cell–cell interactions are accompanied by a switch of lipid mediator profiles in exudates (Serhan, 1997, 2007). The temporal switch in lipid mediator class is an active process that they underscore the ability of inflammatory cells to trigger the self-limited response of acute inflammation.

NOVEL ROLES OF EOSINOPHILS IN PROMOTING THE RESOLUTION OF ACUTE INFLAMMATION

The profile of lipid mediators during inflammation-resolution was determined with a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based lipidomic analysis. In murine zymosan-induced peritonitis, the maximal levels of 5-LOX products such as leukotriene B_4 (LTB_4) were observed in the initiation phase, and subsequently decreased during the resolution. In comparison, the levels of 12/15-LOX products such as protectin D1 (PD1) were low at the initiation of inflammation, then gradually increased during the resolution phase. The DHA-derived lipid mediator PD1 is biosynthesized via a 12/15-LOX-mediated pathway that converts DHA into a 10,17-dihydroxy-containing bioactive molecule (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2006). PD1 promotes resolution by counter regulating PMN influx and stimulating macrophage ingestion of apoptotic PMNs, and increasing phagocyte clearance into DLNs (Schwab et al., 2007). Therefore, the major cellular components of PD1 biosynthesis in the resolution phase were of interest. Recently we identified eosinophils as major PD1-producing cells in the resolution phase of zymosan-induced peritonitis (Yamada et al., 2011). Eosinophils are multifunctional leukocytes that have been implicated in the pathogenesis of numerous inflammatory processes, including parasitic infections and allergic diseases. However, the roles of eosinophils in acute inflammation and resolution are unclear. To determine the role of eosinophils in the resolution of inflammation, an anti-IL-5 monoclonal antibody (Corry et al., 1996) was administered to deplete eosinophils from mice challenged with zymosan. *In vivo* depletion of eosinophils resulted in an increased number of PMNs and a reduced number of phagocytes leaving the inflamed peritoneum to the draining lymph nodes (DLNs), both of which showed a resolution deficit. The LC-MS/MS-based lipidomic analysis revealed that the amounts of 12/15-LOX-derived mediators including PD1 were dramatically decreased in eosinophil-depleted mice, whereas the amounts of COX and 5-LOX derived products did not differ between the two groups. Adoptive transfer of wild type eosinophils, but not eosinophils from 12/15-LOX knockout mice, successfully restored the resolution phenotype. Also, administration of PD1 restored the resolution phenotype. These results indicate that eosinophils are recruited to the inflamed loci during the resolution phase, where they locally produce anti-inflammatory and pro-resolving lipid mediators such as PD1 via a 12/15-LOX-initiated biosynthetic route, which contribute to resolution (**Figure 2**).

Eosinophils are circulating granulocytes that typically mature in the bone marrow, and can be recruited to sites of immunological or inflammatory responses. Triggering of eosinophils by engagement of receptors for cytokines, immunoglobulins, or complement can lead to the secretion of an array of cytokines [IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and transforming growth factors (TGF-α/β)], chemokines (RANTES and eotaxin-1), lipid mediators [platelet-activating factor (PAF), and leukotriene C_4 (LTC_4)], and cytotoxic granule cationic proteins [major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN; Gleich and Adolphson, 1986; Kita, 1996]. These mediators are generally considered to be involved in the eosinophil-mediated defense against parasitic infections. Also, locally accumulated eosinophils are considered to be involved in the pathogenesis of allergic diseases such as asthma. Recently, eosinophils were shown to promote alternatively activated macrophages in an IL-4 and IL-13 dependent manner, which in turn improved glucose metabolism (Wu et al., 2011). Eosinophils in the resolution phase may modulate macrophage phenotype, and thereby promote resolution of acute inflammation.

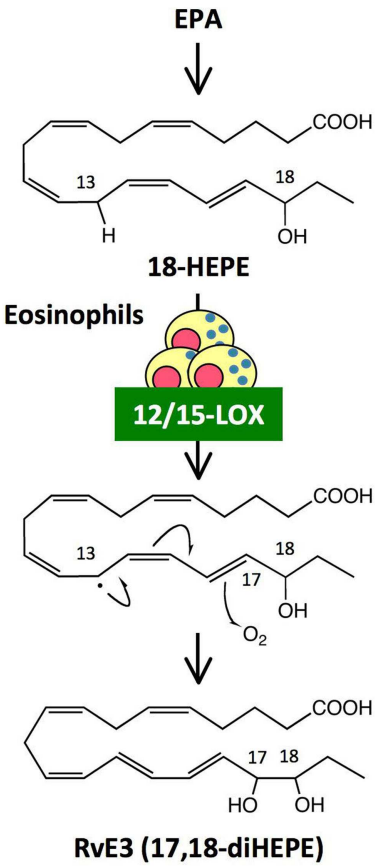

FIGURE 3 | Generation of RvE3, a novel anti-inflammatory lipid mediator, by eosinophils. RvE3 was identified by unbiased target lipidomics. While other E-series resolvins such as RvE1 and RvE2 are biosynthesized by human PMNs via the 5-LOX pathway, RvE3 is generated by eosinophils via the 12/15-LOX pathway from a common precursor, 18-HEPE. It is likely that hydrogen abstraction from C13 by 12/15-LOX induced a stereospecific oxygen insertion at C17, leading to the formation of RvE3. RvE3 displayed a potent anti-inflammatory action by limiting PMN infiltration in zymosan-induced acute peritonitis.

A NOVEL EOSINOPHIL-DERIVED LIPID MEDIATOR WITH ANTI-INFLAMMATORY PROPERTIES

Omega-3 PUFAs such as EPA and DHA are enriched in fish oils, and have beneficial effects in many inflammatory disorders including cardiovascular disease, arthritis, colitis, and asthma. Omega-3 PUFAs have been proposed to act via several mechanisms, such as by preventing conversion of omega-6 PUFA arachidonic acid to pro-inflammatory eicosanoids, and by being converted to potent anti-inflammatory mediators such

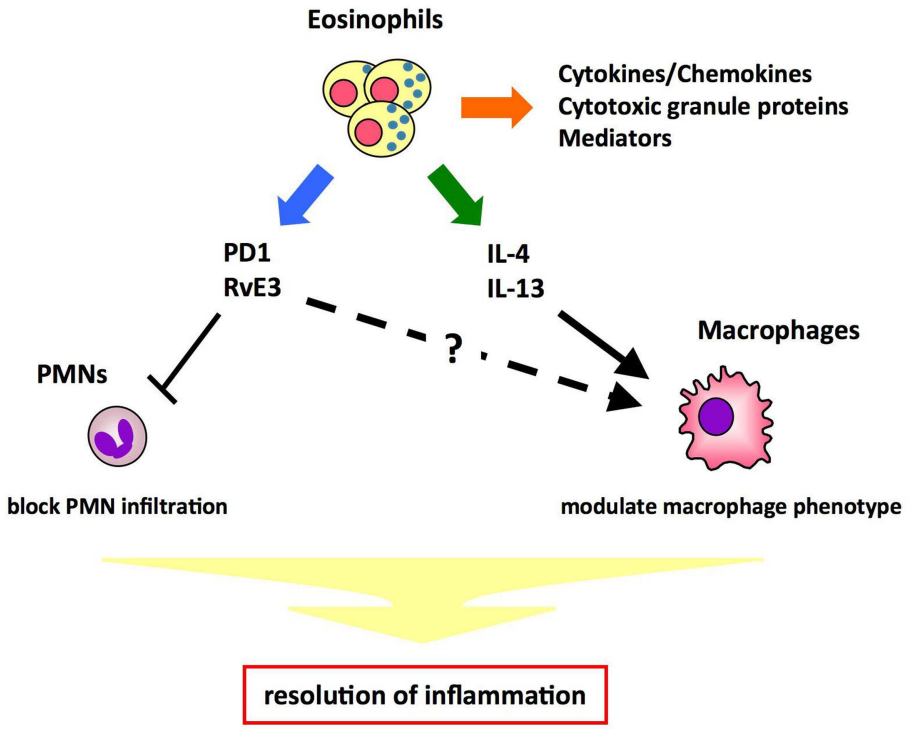

FIGURE 4 | Proposed mechanisms of eosinophils' pro-resolving function. Eosinophils play a role in host defense by releasing cytotoxic granule proteins, cytokines/chemokines, and mediators. In addition, eosinophils maintain metabolic homeostasis by promoting alternative macrophage activation in an IL-4- and IL-13- dependent manner. Thus, it is proposed that eosinophils promote resolution of inflammation by blocking PMN infiltration and/or modulating macrophage phenotype through cytokines (e.g., IL-4, IL-13) and/or lipid mediators (e.g., PD1, RvE3).

as resolvins. RvE1 [5S,12R,18R-trihydroxy-eicosapentaenoic acid (EPE)] and RvE2 (5S,18R-dihydroxy-EPE) are biosynthesized by human PMNs via the 5-LOX pathway from a common precursor, 18-hydroxy eicosapentaenoic acid (18-HEPE; Serhan et al., 2000; Arita et al., 2005; Tjonahen et al., 2006; Oh et al., 2011). Analyses by unbiased target lipidomics using LC-MS/MS recently showed that eosinophils converted 18-HEPE into novel 8,18-dihydroxy-EPE (8,18-diHEPE), 11,18-diHEPE, 12,18-diHEPE, and 17,18-diHEPE (Isobe et al., 2012). Among those, 17,18-diHEPE, termed RvE3, displayed potent anti-inflammatory activity by blocking PMN infiltration in acute peritonitis. Unlike RvE1 and E2, both of which are biosynthesized by PMNs via the 5-LOX pathway, RvE3is biosynthesized via the 12/15-LOX pathway, which is highly expressed in eosinophils (Isobe et al., 2012; **Figure 3**). As mentioned above, eosinophils are recruited to the inflamed loci and promote resolution of inflammation. Therefore, RvE3 may, at least in part, contribute to the eosinophils' function to regulate acute inflammation and resolution. 12/15-LOX is also expressed in tissue resident macrophages, dendritic cells, mast cells, and airway epithelial cells (Kühn and O'Donnell, 2006). 12/15-LOX deficiency leads to progressive atherosclerosis (Merched et al., 2008), exacerbation of arthritis, and inflammatory joint destruction (Krönke et al., 2009), reduced corneal re-epithelialization (Gronert et al., 2005), and a decline of self tolerance (Uderhardt et al., 2012). Cells expressing 12/15-LOX might be involved in regulating inflammatory responses by locally producing lipid mediators such as RvE3.

PERSPECTIVES

Eosinophils are known to be involved in allergic diseases and host protection against parasites through the release of cytokines/chemokines, mediators, and cytotoxic products. Here we provide the first evidence that eosinophils act as specific pro-resolving cells that are recruited and switched on during the resolution phase of acute peritonitis. Resolution of inflammation is a highly regulated and coordinated process that involves the suppression of PMN migration, macrophage recruitment, phagocytosis and clearance of apoptotic cells, and tissue debris. In innate immune responses, the macrophage phenotype is critical in determining whether the inflamed site resolves or progresses to chronic inflammation. Resolution phase macrophages express markers such as mannose receptor (MMR) and CD36, which are typical of alternatively activated macrophages (Fernandez-Boyanapalli et al., 2010). A recent study indicates that eosinophils promote alternative macrophage activation in an IL-4 and IL-13 dependent manner (Wu et al., 2011). Therefore, it is likely that eosinophils promote resolution of inflammation by blocking PMN infiltration and/or modulating macrophage phenotype through cytokines and/or lipid mediators (**Figure 4**). Detailed characterization of eosinophils in the resolution phase will provide insights into the molecular mechanisms for resolution of inflammation.

Failure of acute inflammation to adequately resolve might contribute to the development of chronic inflammation and tissue dysfunction. Indeed, some of the most common and difficult to treat diseases are linked to excessive, uncontrollable, or

chronic inflammation, including cardiovascular disease, rheumatoid arthritis, periodontal disease, asthma, diabetes, and inflammatory bowel disease (IBD), as well as neurological disorders such as Alzheimer's disease and age-related macular degeneration (AMD).

The studies summarized here raise the possibility that modulating eosinophil number and function, or using endogenous mediators released from eosinophils in the resolution phase could provide novel therapies for many inflammatory diseases.

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Annexin A1 and the regulation of innate and adaptive immunity

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Inflammation is the body's way of defending itself against noxious stimuli and pathogens. Under normal circumstances, the body is able to eliminate the insult and subsequently promote the resolution of inflammation and the repair of damaged tissues. The concept of homeostasis is one that not only requires a fine balance between both pro-inflammatory mediators and pro-resolving/anti-inflammatory mediators, but also that this balance occurs in a time and space-specific manner. This review examines annexin A1, an anti-inflammatory protein that, when used as an exogenous therapeutic, has been shown to be very effective in limiting inflammation in a diverse range of experimental models, including myocardial ischemia/reperfusion injury, arthritis, stroke, multiple sclerosis, and sepsis. Notably, this glucocorticoid-inducible protein, along with another anti-inflammatory mediator, lipoxin A_4, is starting to help explain and shape our understanding of the resolution phase of inflammation. In so doing, these molecules are carving the way for innovative drug discovery, based on the stimulation of endogenous pro-resolving pathways.

Keywords: annexin A1, inflammation, innate immunity, adaptive immunity, formyl peptide receptor

INTRODUCTION

Inflammation is essential for the body to maintain homeostasis and recover from tissue injury or noxious stimuli. However, a key step in the inflammatory response is the resolution phase. Overly aggressive or prolonged inflammation, which fails to resolve, can lead to tissue destruction (Serhan and Chiang, 2008). The resolution of inflammation was once thought to be a passive process, but is now deemed to be very much an active phenomenon, and one that is tightly controlled by endogenous "pro-resolving" mediators. Resolution is accepted as one of the four major outcomes for acute inflammation, the others being progression to chronic inflammation, scarring, and fibrosis (Cotran et al., 1999; Kohli and Levy, 2009).

Over the years, interest has focused on anti-inflammatory mediators that have pro-resolving properties. In particular, endogenous anti-inflammatory molecules, such as the glucocorticoid-regulated protein annexin A1 (AnxA1), have particular appeal for drug discovery programs, based on the contention that drugs founded on endogenous anti-inflammatory molecular pathways could mimic their pro-resolution effects while potentially having fewer side effects than existing therapeutic agents.

This review will focus on AnxA1, its history, mechanism of action and its role in both innate and adaptive immunity and how this 37 kDa protein has potential in drug discovery.

THE HISTORY OF ANNEXIN A1

In the late 1970s, a new protein was discovered and characterized by its ability to quash eicosanoid generation by affecting phospholipase A_2 (PLA_2) activity. The action on arachidonate and eicosanoid release *in vitro*, e.g., the inhibition of PGE_2 and LTB_4 release by monocytes and neutrophils (Parente et al., 1984) was accompanied by an inhibitory effect in experimental models of inflammation *in vivo* (e.g., TXA_4 release from perfused guinea pig lungs; Cirino et al., 1987). Different names were proposed for this new protein, the molecular weight of which ranged from 15 to 40 kDa: macrocortin (because it was isolated from peritoneal exudates from glucocorticoid-treated rats), renocortin (released rat renal medulla cells; Russo-Marie and Duval, 1982), or lipomodulin (released from isolated neutrophils; Hirata, 1981), but it was decided that it should be termed lipocortin 1. It was accepted that these three proteins were functionally identical and all active fragments of the same precursor, thus it was agreed that a uniform name should be chosen: lipocortin (Di Rosa et al., 1984). In 1986, lipocortin was cloned (Wallner et al., 1986) and the sequence was termed lipocortin 1. Today, the name AnxA1 has been agreed on as a more appropriate choice, due to the ability of this protein to "annex" phospholipid membranes. Following on from this, AnxA1 has been shown to mimic the anti-inflammatory effect of glucocorticoids in number of *in vitro* and *in vivo* studies (the gene encoding AnxA1 is located on chromosome 19q24).

Annexin A1 is a 37-kDa member of the annexin superfamily of calcium- and phospholipid-binding proteins, of which there are currently 13 members. It consists of 346 amino acids and is made of four repeated sequences, which are arranged around a core [which represents the large majority ($\geq$80%) of the protein], giving the protein a "doughnut" shape. At low Ca^{2+} concentrations, the N-terminal domain is embedded within the pore, but elevations in [Ca^{2+}] ($\geq$1 mM, e.g., as in plasma or other biological fluids) expose this region and may thereby influence the biological activity of the protein (Rosengarth et al., 2001a,b). All members

of the annexin superfamily comprise of a core domain made up of four similar repeats (six repeats in the case of AnxA6), each approximately 70 amino acids long. The N-terminal region of each member of the annexin superfamily is unique and as such represents its own fingerprint, and confers biological activity. It is, however, still unknown as to whether there is any close regulation among members of the annexin family. It is likely that is the case, e.g., in the case of AnxA2, cells appear to require it as a structural/scaffolding protein that stabilizes and/or regulates the dynamics of certain membrane domains. Thus, it is probable that AnxA2 shares its activity with other annexins (Rescher and Gerke, 2004).

BIOLOGY OF AnxA1

Within peripheral blood cells, under resting conditions, AnxA1 is mainly expressed in subcellular granules of neutrophils, eosinophils, and monocytes, with small amounts expressed in specific subsets of lymphocytes (Goulding et al., 1990; Morand et al., 1995; Rescher and Gerke, 2004; Spurr et al., 2011). T cells and mast cells express the protein, although B cells express it at low levels, and platelets do not (Cirino et al., 1987; Morand et al., 1995; Oliani et al., 2000; Rescher and Gerke, 2004). Cell differentiation (such as monocytes maturing into macrophages) tends to be associated with higher levels of expression of AnxA1, as demonstrated in studies showing that levels of AnxA1 expression are lower in monocytes relative to those in macrophages from the same donor (Perretti and Flower, 1996).

AnxA1 is undetectable in plasma, but is found in many tissues, including the lung, bone marrow, and intestine, at concentrations $<$50 ng/ml, with the highest levels in seminal fluid (150 μg/ml).

AnxA1 AND THE INNATE IMMUNE RESPONSE

THE ROLE OF AnxA1

Since its discovery, AnxA1 has been shown to be capable of modulating a number of biological events, including both acute (Gavins et al., 2003) and chronic (Goulding et al., 1998) inflammation, ischemia/reperfusion injury (D'Amico et al., 2000; La et al., 2001; Gavins et al., 2003), pain (Marchand et al., 2005), fever (Lim and Pervaiz, 2007), intracellular vesicle trafficking (Gerke et al., 2005), arachidonic acid release (Croxtall et al., 2000), leukocyte migration (Williams et al., 2010), and tissue growth and apoptosis (Petrella et al., 2005; Scannell and Maderna, 2006). AnxA1 may also play a role in the regeneration of skeletal muscle tissue by stimulating the migration of satellite cells via the modulation of myoblast cell differentiation, which in turn causes skeletal muscle differentiation (Hawke and Garry, 2001; Bizzarro et al., 2012). In addition, AnxA1 has a valuable role in inhibiting the negative feedback effects of glucocorticoids on the release of corticotrophin (ACTH) and hypothalamic-releasing hormones (Buckingham et al., 2006), and also affects a number of mediators that are involved in the inflammatory response, including cyclo-oxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS; Minghetti et al., 1999; Ferlazzo et al., 2003; Perretti and Dalli, 2009).

Evidence indicates that AnxA1 may also play an important role in tumor development and progression, with AnxA1 levels being up- and down-regulated in different cancers, e.g., the loss of AnxA1 expression in prostate cancer correlates with an early onset of tumorigenesis (Xin et al., 2003). The fact that this protein also contains phosphorylation sites that can be phosphorylated by a number of proliferative signaling molecules, including PKC and EGF receptor tyrosine kinase, suggests that AnxA1 may also have a role in signaling pathways that are important in cancer (Alldridge et al., 1999; Hsiang et al., 2006). However, further work is required to define the actions of AnxA1 in this setting.

AnxA1 AND CELL RECRUITMENT AND MIGRATION

AnxA1 has been shown to induce a number of effects relating to the adhesion and migration of leukocytes, processes that represent fundamental steps in the development of the inflammatory response. These include induction of L-selectin shedding by neutrophils, and detachment of adherent leukocytes from the endothelium (**Figure 1**). These actions contribute to the ability of AnxA1 to restrict leukocyte transmigration and recruitment during inflammation. AnxA1 has also been shown to reduce α4β1 integrin-dependent monocyte adhesion and migration (Cote et al., 2010). This anti-migratory capacity extends to other cell types such as endothelial cells (Cote et al., 2010). However, in contrast to its predominantly inhibitory effects on these processes, a recent study demonstrated that a novel cleavage product from the C terminus of AnxA1, released by activated neutrophils, acts to promote neutrophil transmigration by promoting clustering of intracellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface around migrating neutrophils (Williams et al., 2010).

EXTERNALIZATION OF AnxA1

In order for AnxA1 to exert its anti-inflammatory effects, it must be externalized by its cellular sources. However, AnxA1 lacks a signal peptide (similar to other proteins such as interleukin-1; Muesch et al., 1990; Christmas et al., 1991), and as such, cannot be exported through the classical secretory pathway, but rather by exocytosis. Upon cellular activation, AnxA1 is released from its storage site and translocates to the membrane, where it is secreted via different pathways, depending upon the cell type involved. In macrophages, the ATP-binding cassette (ABC) transporter system is responsible for the secretion of AnxA1 (Wein et al., 2004). In the neutrophil, AnxA1 is stored in gelatinase granules, and upon neutrophil-activating events, such as adhesion to the endothelial cell surface, it is rapidly mobilized to the outside leaflet of the plasma membrane (Perretti et al., 1996). In this location, the intact 37 kDa AnxA1 undergoes conformational change (Rosengarth et al., 2001a,b), exposing the N-terminal region, resulting in a structure which evidence indicates is the active form of AnxA1. In addition AnxA1 can be cleaved into a 33-kDa fragment. Several enzymes have been suggested to cause the cleavage, including elastase (Rescher et al., 2006), a metalloprotease (proposed to cleave the first seven amino acids of the AnxA1 terminus; Movitz et al., 1999), and proteinase 3 (Vong et al., 2008). It is still unknown whether the cleavage process occurs (1) to allow AnxA1 to act as a pro-drug via the production of a bioactive fragment, or (2) to produce homeostasis by limiting the action of AnxA1 (Perretti and Gavins, 2003; Pederzoli-Ribeil et al., 2010). Supporting the latter concept, a modified recombinant form of AnxA1 resistant to proteinase 3-mediated cleavage has been shown to have longer-lasting

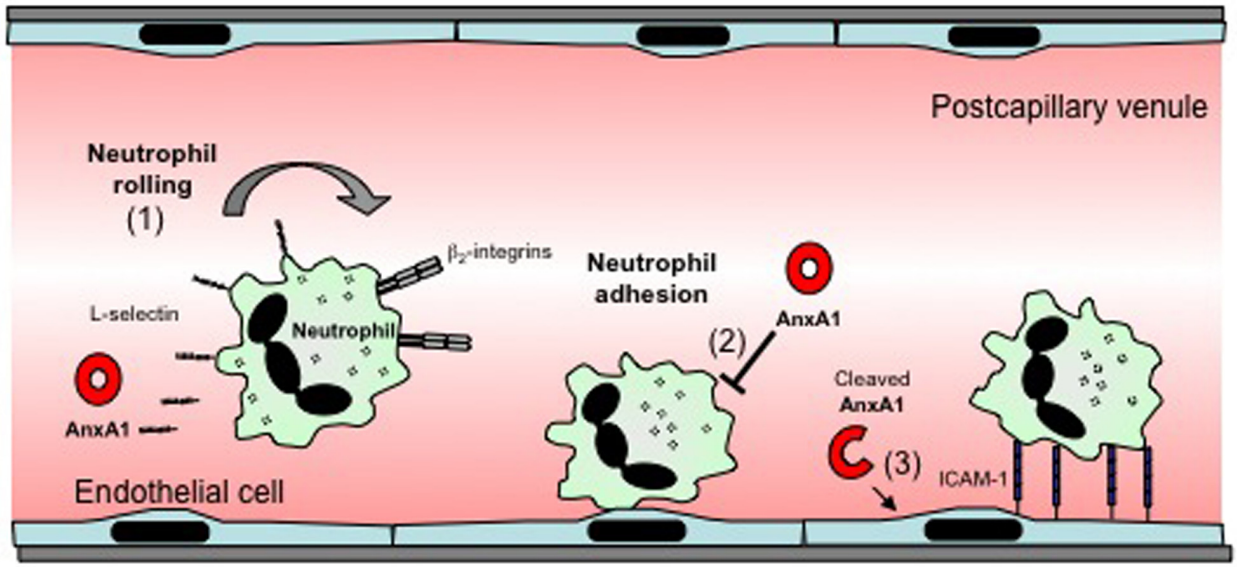

FIGURE 1 | Effects of AnxA1 on leukocyte–endothelial cell interactions. The effects of AnxA1 on leukocyte endothelial cell interactions include: (1) induction of L-selectin-shedding, which may reduce leukocyte rolling. (2) Inhibition of leukocyte adhesion or detachment of already adherent leukocytes, an effect associated with reduced transmigration. (3) A novel cleaved form of AnxA1 has been reported to act on endothelial cells to promote ICAM-1 clustering around transmigrating neutrophils, facilitating transmigration.

effects on neutrophil adhesion *in vivo*, relative to native AnxA1 (Pederzoli-Ribeil et al., 2010). In addition, of interest, in the case of the neutrophil, it has recently been shown that AnxA1 externalization can occur without interaction with an endothelial monolayer indicating that cellular adhesion to the endothelium is not required for release of AnxA1 (Vong et al., 2008).

Microparticle release may be an additional alternative mode of AnxA1 release from neutrophils. Microparticles are small vesicles released from activated cells, and neutrophil-derived microparticles have been shown to be rich in AnxA1 (Dalli et al., 2008). Furthermore, neutrophil-derived microparticles have been shown to inhibit neutrophil–endothelial cell interactions under flow *in vitro*, an effect dependent on AnxA1 present in the microparticles. These observations indicate that AnxA1-containing microparticles may be a critical source of functionally-relevant AnxA1 produced by neutrophils.

MECHANISMS OF ACTION OF AnxA1

Until 2000, the way in which AnxA1 mediated its cellular effects remained unclear. However, a seminal paper by Gerke and colleagues demonstrated that formyl peptide receptor (FPR) antagonists [butyloxycarbonyl (Boc) derivatives] blocked the anti-migratory effects of both intact AnxA1, and the AnxA1-derived peptide Ac2-26, on human neutrophils, as well as modulating the effects of AnxA1 on calcium flux and L-selectin shedding (Walther et al., 2000). These *in vitro* effects were mirrored *in vivo* using $Fpr1^{-/-}$ mice, which displayed an attenuation of the inhibitory actions of AnxA1 and peptide Ac2-26 in a model of peritonitis (Perretti et al., 2001). Together, these studies were the first to demonstrate a role for FPRs in mediating the cellular effects of AnxA1.

THE FORMYL PEPTIDE RECEPTORS

Evidence now indicates that AnxA1 mediates most of its cellular effects via interaction with FPRs (**Figure 2**). The FPRs are a family of seven transmembrane domain, G protein-coupled receptors, that are expressed mainly in mammalian phagocytic leukocytes, where they serve to induce responses to various ligands, including the bacteria-derived peptide, fMLF (Ye et al., 2009). Three FPR receptors exist in the human, namely FPR1, FPR2/ALXR (which shares 69% amino acid sequence homology with FPR1, and is also known as the LXA_4 receptor) and FPR3 (which shares 56% amino acid similarity to FPR1 and 72% to FPR2/ALXR; Brink, 2003; Ye et al., 2009; Gavins, 2010). The receptor story in the mouse is rather more complicated with the gene cluster having undergone differential expansion. It is now agreed that *Fpr1*, the murine ortholog of human *FPR1*, is 77% identical to human *FPR1* (Brink, 2003; Ye et al., 2009; Gavins, 2010) and *Fpr3* is 73% identical to human *FPR2/ALXR*. Fpr2 binds fMLF with low affinity (Brink, 2003; Ye et al., 2009; Gavins, 2010). *Fprs3*, *4*, *6*, and *7* appear not to have direct counterparts in the human genome (Yang et al., 2004; for further review of these receptors, see Brink, 2003; Ye et al., 2009; Gavins, 2010).

The FPRs are primarily coupled through pertussis toxin-insensitive G proteins ($G_I\alpha_2$, $G_I\alpha_3$) to the activation of phospholipase C and ultimately release Ca^{2+} from intracellular stores (Wenzel-Seifert et al., 1999). This release of Ca^{2+} causes the opening of the store-operated Ca^{2+} channel in the plasma membrane allowing further Ca^{2+} influx into the cell (Ye et al., 2009). Other intracellular signaling effects of ligand binding to FPRs include tyrosine kinase-mediated phosphorylation of PLA, PLD, and members of the MAP kinase family (Brink, 2003; Ye et al., 2009; Gavins, 2010), and signaling through Cdc42 to activate

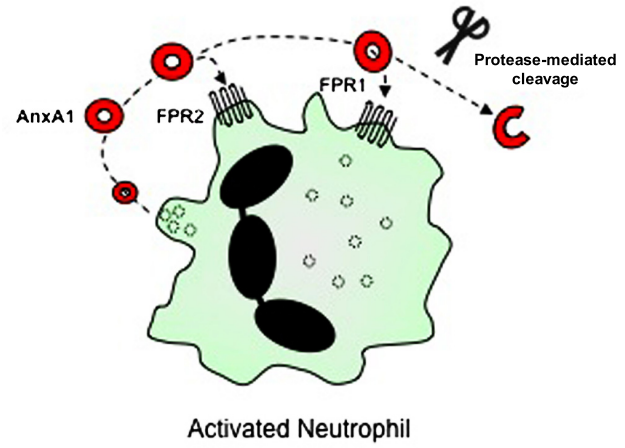

FIGURE 2 | Cellular receptors for AnxA1. AnxA1 is released from granules of activated neutrophils into the extracellular space, where it is then able to bind to G protein-coupled receptors including FPR2/ALXR and FPR1, thereby mediating cellular effects via autocrine (and paracrine) pathways. In addition, proteases released from activated neutrophils can cleave full-length AnxA1, although the purpose of this process (for AnxA1 to act as a pro-drug, or whether it is a catabolic event terminating its action) is still under debate.

Rac- and ARP2/3-dependent pathways leading to actin nucleation (VanCompernolle et al., 2003).

FPRs AND AnxA1

Upon binding to the FPRs on neutrophils, AnxA1 induces responses such as L-selectin shedding and detachment from the endothelium (Perretti et al., 1996; Lim et al., 1998; Gavins et al., 2003). It has also been shown to cause desensitization of the receptor toward the fMLF stimulus (D'Acquisto et al., 2008). Evidence indicates that much of the AnxA1 that mediates this effect comes from the leukocyte itself (D'Acquisto et al., 2008). This autocrine/paracrine effect of AnxA1, which inhibits the process of leukocyte transmigration, has been suggested specifically for leukocytes (Maderna et al., 2005), but it is likely that it occurs in a number of different cells, including macrophages (Babbin et al., 2006) and epithelial cells (Solito et al., 2000).

The specific member(s) of the FPR family mediating the effect of AnxA1 and its mimetic peptides appear to be not only tissue-specific, but also different depending upon whether full-length AnxA1 or AnxA1-derived peptide is used (Yang et al., 2004; Gavins et al., 2005a,b). For example, in a model of peritoneal inflammation induced by zymosan A, the anti-migratory effect of peptide Ac2-26 was absent in Fpr1 null mice, whereas the response to whole protein was not (Perretti et al., 2002). In the murine mesentery, both Fpr1 and Fpr2 appear to be involved, with the latter receptor being more functionally involved in the detachment of leukocytes from the endothelium (Gavins et al., 2003).

Further evidence of an interaction between AnxA1 and FPRs came from immunoprecipitation studies in which neutrophil-derived AnxA1 could be immunoprecipitated with FPR2/ALXR when the leukocytes were adherent to endothelial monolayers (Solito et al., 2003). These data were supported by ligand-binding studies using transfected cells stably expressing members of the FPR family.

With respect to formylated mitochondria proteins, studies have reported that mitochondrial-derived FPR1 ligands function as chemotactic damage-associated molecular pattern molecules (DAMPs, also known as or alarmins or danger signals; McDonald et al., 2010; Reddy and Standiford, 2010). DAMPs have pro-inflammatory activity and are released or generated after injury, thus activating the innate immune system (Chen and Nunez, 2010; Rock et al., 2010). To our knowledge, no studies have shown an involvement of AnxA1 in modulating the inflammatory response to injury associated with formylated mitochondria proteins.

It is worth pointing out that LXA_4 and its analogs have opposing effects to AnxA1 and its mimetic peptides, despite both interacting with FPR2/ALXR on peripheral blood leukocytes. Whereas AnxA1 and its peptides (Walther et al., 2000; Jozsef et al., 2002; Solito et al., 2003) cause L-selectin shedding by both neutrophils and monocytes *in vitro*, LXA_4 and its analogs increase basal cell surface levels of L-selectin (Gavins et al., 2005b). These observations may be due to ligand-specific conformation that may occur with the same receptor, resulting in ligand-specific signal transduction responses that yield specific cellular effects unique to each particular ligand (Gavins et al., 2005a).

AnxA1$^{-/-}$ MOUSE

The development of the AnxA1$^{-/-}$ mouse (generated by homologous recombination, with a transgenic gene that disrupted the endogenous AnxA1 gene, and a LacZ gene under the control of the AnxA1 promoter; Hannon et al., 2003) has led to a greater understanding of the roles played by AnxA1 in inflammation, and has demonstrated that, in general, where AnxA1 is absent, inflammation is exacerbated and prolonged. This has important significance in exploiting the biological properties of AnxA1 in development of novel anti-inflammatory agents. AnxA1$^{-/-}$ mice have a heightened inflammatory response as displayed by increased leukocyte transmigration (Chatterjee et al., 2005), higher levels of inflammatory markers in a model of localized joint inflammation (Reddy and Standiford, 2010), increased neurological deficit in a stroke model (Gavins et al., 2007) and delayed repair in a model of colitis (Babbin et al., 2008). *In vitro*, neutrophils from these mice have a greater propensity for chemotaxis and higher CD11b expression. In addition, consistent with these observations, absence of AnxA1 also leads to increased inflammation and in some cases a higher mortality in life-threatening inflammation-associated conditions, e.g., stroke (Reddy and Standiford, 2010). In many of these inflammatory conditions and situations, the administration of exogenous AnxA1 is able to rescue the phenotype in AnxA1$^{-/-}$ mice (Gavins et al., 2003).

PRO-INFLAMMATORY ACTIONS OF AnxA1

In contrast to much of the research on AnxA1 and innate immunity, some studies have pointed to the capacity of this protein and its cleavage products to mediate pro-inflammatory actions. For example, as described earlier, Williams et al. (2010) found that that a novel cleavage product of AnxA1 promotes neutrophil transmigration via effects on endothelial ICAM-1. Similarly, a peptide from the N-terminal domain of AnxA1 has

been found to promote leukocyte chemotaxis via FPR family members (Ernst et al., 2004). AnxA1 has also been found to be released from rheumatoid arthritis synovial fibroblasts (RASF) following TNF-mediated activation, and to promote RASF matrix metalloproteinase-1 secretion (Tagoe et al., 2008). Furthermore, the absence of AnxA1 has recently been reported to be without effect in a T cell-independent, antibody transfer model of arthritis, indicating that under some conditions, the role of AnxA1 is minimal (Patel et al., 2012). These studies demonstrate the multifaceted nature of the actions of this intriguing molecule and its cleavage products, and highlight the necessity for detailed studies in the complex *in vivo* environment in order to fully understand their actions in inflammatory responses.

AnxA1 AND THE ADAPTIVE IMMUNE RESPONSE

As already described, much of the research on AnxA1 has focused on its effects in forms of inflammation mediated by neutrophils and monocyte/macrophages. However, a growing body of evidence indicates that AnxA1 also modulates the adaptive immune response and tissue injury in models of inflammation induced by activation of the adaptive immune system (D'Acquisto et al., 2008). A notable difference between these sets of observations is that studies of innate responses routinely report that AnxA1 mediates anti-inflammatory effects, while data emerging from studies on the role of AnxA1 in the adaptive immune response have been much less consistent. In this section, we will examine these studies and summarize the evidence regarding the actions of AnxA1 in the adaptive response.

POSITIONING AnxA1 IN THE DEVELOPMENT OF THE ADAPTIVE RESPONSE

To understand the potential actions of AnxA1 in adaptive immunity, i.e., responses mediated via antigen recognition by T cells, B cells, and antibody, it is important to understand the typical steps in the development of an adaptive immune response. After emerging from the thymus, naïve T cells migrate to peripheral lymphoid organs, where they can undergo activation upon exposure to cognate antigen presented via dendritic cells. Similarly, immature B cells migrate from the bone marrow into the periphery and subsequently undergo antigen-dependent activation promoting their maturation into antibody-secreting cells. This initial phase is termed the activation or sensitization phase. As a result of these activation processes, the immune response is primed to react rapidly to re-exposure to the same antigen. This antigen-specific "effector response" commonly occurs in peripheral tissues in response to local re-application of the same cognate antigen, resulting in a long-lived inflammatory response at the site of exposure. Examples of experimental models used to investigate the mechanisms of this process include experimental autoimmune encephalomyelitis (EAE), antigen-induced arthritis, and dermal contact hypersensitivity, in which the effector phases target the brain, joint, and skin, respectively (Liu et al., 1998; Reddy and Standiford, 2010; Deane et al., 2012). The complexity of these multi-step processes allows numerous opportunities for AnxA1 to exert effects.

It is reasonable to assume that for AnxA1 to participate in development of the adaptive response, that the key cellular players would express AnxA1 and/or FPR2/ALXR. As such, it was recognized many years ago that AnxA1 is expressed constitutively by T cells, although at ∼25% of the levels expressed in neutrophils (Goulding et al., 1990; Morand et al., 1995; Perretti and Flower, 1996; Paschalidis et al., 2009; Spurr et al., 2011). In addition, while unstimulated T cells have been shown to express FPR2/ALXR at low or negligible levels, following stimulation they increase surface expression of FPR2/ALXR within 30 min, maintaining elevated expression for several hours (D'Acquisto et al., 2007a). In addition, T cells release AnxA1 following activation of the T cell receptor (TCR; D'Acquisto et al., 2007b). Recent studies have performed more detailed analyses of the expression of AnxA1, and its receptor, in a range of T cell subsets. This work reveals that $CD4^+$ T cells express slightly more AnxA1 than $CD8^+$ T cells, predominantly intracellularly. Further analysis of the $CD4^+$ subsets demonstrated that activated and memory cells express more AnxA1 than naïve cells, both intracellularly and on the cell surface. FPR2/ALXR is also expressed at a higher level in post-activation T cells, although the scale of increase is smaller relative to that of AnxA1 (Spurr et al., 2011). B cells also express intermediate levels of AnxA1 but low levels of FPR2/ALXR in the absence of stimulation (Spurr et al., 2011). Dendritic cells have also been shown to constitutively express and release AnxA1 (Huggins et al., 2009). Together these findings raise the possibility that AnxA1 may have important actions on these cell types.

AnxA1 AND SUPPRESSION OF THE ADAPTIVE RESPONSE

Early studies of the actions of AnxA1 ("lipomodulin") provided evidence that AnxA1 has the capacity to promote the development of anti-inflammatory regulatory T cells. This effect, detected using thymocyte-based suppression assays, was complex in that it occurred under moderate stimulatory conditions, but was reversed in response to strong T cell stimulation via high concentration Con A (Hirata and Iwata, 1983). Nevertheless, this study was interpreted to indicate that at least under some activating conditions, AnxaA1 promotes the generation and/or maturation of "suppressor" T cells. These findings were consistent with AnxA1 acting to limit T cell-dependent responses under some conditions. Parallel findings were reported by Gold et al. (1996), who observed suppression of T cell proliferation in response to exogenous AnxA1, using antigen-stimulated rat T cell lines. Similarly, proliferation and activation of peripheral blood mononuclear cells (PBMC) from atopic individuals were found to be inhibited in the presence of exogenous AnxA1-derived peptides, Ac2-26 and antiflammin-2 (AF-2; aa246–254; **Figure 3**; Kamal et al., 2001). The use of PBMC in this study made it unclear which leukocyte subsets were involved in the response. However, these cells were stimulated with peptide antigens known to induce T cell responses in the donors (house dust mite allergen – Der p; purified protein derivative of *Mycobacterium tuberculosis* – PPD), ensuring that stimulation occurred in an antigen-specific, T cell-dependent manner. While this did not exclude actions of AnxA1 in other leukocyte subsets present in the PBMC preparation, it ensured that the T cell was the primary target of the activation.

This work was extended into the *in vivo* setting by Yang et al., who examined $AnxA1^{-/-}$ mice in an antigen-induced model of

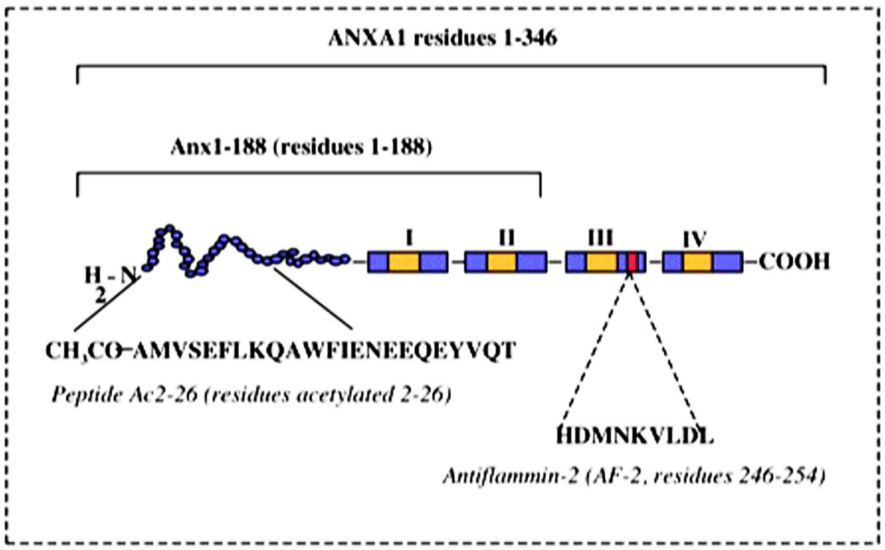

FIGURE 3 | Peptide structure of AnxA1. Schematic representation of AnxA1 and peptides derived from the primary sequence.

arthritis mediated by initial sensitization to mBSA, and subsequent local intra-articular mBSA challenge (Reddy and Standiford, 2010). In this study, absence of AnxA1 was associated with increased joint inflammation, consistent with AnxA1 acting to limit this adaptive response. Interestingly, in parallel with this result, antigen-specific IgG levels were reduced in AnxA1$^{-/-}$ mice, despite the exacerbation of inflammation. This finding raises the intriguing possibility that under some circumstances, AnxA1 has the opposing effect on B cell function and generation of humoral immunity as it does on T cell-dependent inflammation. More recently, the role of AnxA1 was examined in an ovalbumin (OVA)-induced model of airways hyperresponsiveness (Ng et al., 2011). Similar to the arthritis study, the absence of AnxA1 was associated with evidence of increased allergic inflammation in the OVA-challenged lung, including increased eosinophil recruitment, IL-4 production, and airways dysfunction. However, this study revealed unanticipated complexities in the actions of AnxA1. Firstly, despite the exacerbation of inflammation in AnxA1$^{-/-}$ mice, antigen-induced activation of MAP kinase and NFκB pathways in lungs of these animals were markedly blunted relative to that in wild-type mice. Furthermore, the absence of AnxA1 was associated with exacerbated airway reactivity in naïve mice, i.e., mice those had not undergone sensitization to antigen. These findings pointed to unidentified actions of AnxA1 in pulmonary physiology, presumably unrelated to its actions in the immune system.

Together these studies provide evidence that AnxA1 acts to limit inflammation in models associated with activation of the adaptive immune system. However, the molecular mechanisms of these effects, and the range of cellular targets of AnxA1, remain to be fully characterized. Furthermore, as these experiments predominantly focused on whole animal approaches, they did not determine whether AnxA1 mediated these effects directly in T cells or other cells involved in development of the adaptive response. Given the broad range of actions of AnxA1, it is conceivable that the absence of AnxA1 from cells in the target tissue with key roles in the effector response, potentially including endothelial cells or other effector leukocyte populations such as neutrophils, may have contributed to the exacerbation of inflammation in the absence of AnxA1. This is particularly important in the context of an unexpected association between exacerbated inflammation and defective T cell activation.

AnxA1 AND ACTIVATION OF THE Th1 RESPONSE

In contrast to the work described above, a further group of studies has provided evidence that AnxA1 promotes T cell activation and T cell-dependent inflammation. In initial studies, D'Acquisto et al. (2007b) examined the effects of recombinant AnxA1 on T cells undergoing anti-CD3/CD28 activation, observing increased proliferation and IL-2 production in response to AnxA1. The effect of AnxA1 was most evident in T cells undergoing sub-maximal stimulation, while AnxA1 alone was insufficient to activate T cells. This effect was associated with increased TCR-dependent signaling, as demonstrated by activation of AP-1, NFκB, and NFAT. T cell activation also rapidly increased surface expression of FPR2/ALXR and exteriorization and release of AnxA1, and increased phosphorylation of ERK and Akt, signaling pathways downstream of FPR2/ALXR.

Numerous studies have demonstrated that following activation, CD4^{+} T cells differentiate down distinct lineages (Th1, Th2, Th17) defined by the profile of the cytokines they produce. Moreover, *in vivo* the composition of these subsets determines the phenotype of the resultant effector response (Iwakura et al., 2008; Zhu and Paul, 2010). D'Acquisto et al. (2007b) investigated the effect of AnxA1 on T cell differentiation and observed that exogenous AnxA1 favored generation of the IFNγ-producing Th1 subset, while inhibiting development of the IL-4-producing Th2 subset. To test if this subset "skewing" also occurred *in vivo*, they examined

the Th1-dominant collagen-induced model of arthritis (CIA) and observed that exogenously administered AnxA1 induced exacerbation of joint inflammation associated with increased lymphocyte production of the key Th1 cytokine, IFN-γ. In this experiment, recombinant AnxA1 was administered twice daily commencing immediately after immunization. The presence of exogenous AnxA1 at these early stages of immunization in parallel with the significant enhancement of disease severity suggests that T cell differentiation was an important target of AnxA1 in this model. These findings support the hypothesis that AnxA1 alone does not directly activate T cells, but enhances activation and Th1 differentiation in the context of conventional T cell activation.

In complementary studies, the same group examined responses of T cells from AnxA1$^{-/-}$ mice (D'Acquisto et al., 2007a). Notably, AnxA1$^{-/-}$ T cells were found to undergo a significantly higher rate of basal proliferation relative to wild-type T cells. However, following non-specific activation, AnxA1$^{-/-}$ T cells displayed significant reductions in proliferation and cytokine production relative to comparably-activated wild-type cells (D'Acquisto et al., 2007b). These findings indicate that the actions of AnxA1 differ according to the state of T cell activation. Consistent with previous work, in activated cells the absence of AnxA1 was associated with reduced activation of signaling pathways downstream of the TCR and Fpr2/ALXR (AP-1, NFκB, NFAT, MAP kinase, Akt; Ng et al., 2011). Mirroring the effect of exogenous AnxA1 on Th1 development, the absence of AnxA1 favored development of IL-4/IL-13-generating Th2 cells (D'Acquisto et al., 2007b). In addition, AnxA1$^{-/-}$ T cells produced less IL-17, suggesting that AnxA1 also supports development of the Th17 CD4^{+} T cell phenotype. Data from a Th2-dependent model of allergic peritonitis supported this idea in that AnxA1$^{-/-}$ mice showed increased effector phase leukocyte recruitment, most prominently of eosinophils. These studies provide evidence that the actions of AnxA1 on T cell activation are subset-specific, promoting development of the Th1 and Th17 subsets, while inhibiting development of the Th2 response.

This work was extended to the examination of T cell-mediated inflammation of the central nervous system (Paschalidis et al., 2009). Using the myelin oligodendrocyte glycoprotein (MOG)-induced model of EAE, Paschalidis et al. (2009) observed a mild protection from clinical disease in AnxA1$^{-/-}$ mice, most prominently in the later phase of the disease. This was associated with reduced antigen-specific recall proliferation and IL-2 production, and reduced T cell production of Th1 and Th17 cytokines in AnxA1$^{-/-}$ mice.

Together this body of work indicates that endogenous AnxA1 acts to restrict Th2 development, favoring development of Th1- and Th17-mediated responses. These findings raise the possibility of AnxA1 being a therapeutic target in autoimmune diseases characterized by inappropriate activity of Th1/Th17 subsets.

AnxA1 AND THE ADAPTIVE RESPONSE – WHERE TO FROM HERE?

Taken together, some inconsistencies between these studies remain, indicating that further work is required to clarify the actions of AnxA1 in T cell-mediated responses. One of the fundamental questions that emerges is, via what mechanism does AnxA1 promote Th1/Th17 development while inhibiting Th2 development? To this end, there is a growing body of evidence that control of T cell activation via modulation of T cell signaling is a key component of this action of AnxA1. In T cells exposed to a range of stimuli, AnxA1 has been found to modulate activation of Akt and ERK MAP kinase, pathways that are central to the TCR-mediated response (D'Acquisto et al., 2007a,b; Paschalidis et al., 2009). Further work is required to determine how these intracellular effects control the polarization of CD4^{+} T cells during development of the immune response. An additional aspect that remains unclear is what are the actions of AnxA1 in different cell types? In none of the *in vivo* studies described has the absence of AnxA1 been restricted to T cells. As such, it is not possible to attribute changes in the resultant *in vivo* response to effects of AnxA1 specifically in T cells. Studies of this nature are critical in that it is beyond doubt that the actions of AnxA1 extend well beyond T cell activation. As already described, numerous studies have demonstrated that AnxA1 acts to restrict recruitment of innate leukocyte subsets, targeting key events in the microvasculature. These leukocytes are critical "responder cells" in the development of T cell-mediated inflammation. Therefore, in addition to its effects on T cell activation, inhibition or absence of AnxA1 *in vivo* has the potential to dramatically modulate the effector response in the periphery. In addition, while early studies provided evidence of an effect of AnxA1 on regulatory T cell function (Hirata and Iwata, 1983), this concept remains to be fully explored. Similarly, unanticipated actions of AnxA1, possibly in non-immune cells, as exemplified by the observation of altered basal lung function in AnxA1$^{-/-}$ mice (Ng et al., 2011) require further investigation. These complexities will be best addressed in studies in which AnxA1 deficiency is restricted to antigen-specific T cells, regulatory T cells, dendritic cells, or non-immune cells.

EFFECT OF EXOGENOUS AnxA1 AND AnxA1-DERIVED PEPTIDES

Much of the interest in AnxA1 as a potential therapeutic has stemmed from results of its use as an exogenous anti-inflammatory agent in *in vivo* models of inflammation. For example, human recombinant AnxA1 has been shown to exert anti-inflammatory effects in a carrageenan-induced edema model of inflammation in the rat paw (Wu et al., 1995). However, a great deal of this work has examined the effects of peptides derived from AnxA1. From a protein of over 300 amino acids, several small peptides derived from the N-terminal region of AnxA1 retain much of its biological activity. These peptides, termed Ac2-26, Ac2-12, or Ac9-25 (constructed with an acetyl-blocked N-terminus for stability and delay of proteolytic degradation) have all been reported to retain the majority of the effects of the full-length AnxA1 protein in a number of different *in vitro* and *in vivo* systems.

The anti-inflammatory effects of peptide Ac2-26 have been demonstrated in numerous models including ischemia/reperfusion injury in both the rat (D'Amico et al., 2000; La et al., 2001) and mouse (Gavins et al., 2003), the mouse air-pouch and rat paw edema models of inflammation (Cirino et al., 1993; Perretti et al., 1993), and in models of neutrophil and monocyte trafficking (Szabó et al., 1997; Bandeira-Melo et al., 2005). The wide-ranging effects of peptide Ac2-26 were clearly demonstrated

in a model of pleurisy in the rat, in which the peptide inhibited mast cell degranulation, plasma protein leakage, and accumulation of both neutrophils and eosinophils (Teixeira et al., 1998). It is important to note that the evidence is consistent with peptide Ac2-26 mediating these anti-inflammatory effects by impacting on several distinct mechanisms. For example, in ischemia/reperfusion of the heart, AnxA1 modulated inflammation via effects on blood-borne cells (La et al., 2001; Gavins et al., 2005a), as well as by having direct effects on cardiomyocytes (Ritchie et al., 2003).

One of the most important techniques employed in teasing out the effects of pharmacological doses of AnxA1 and its mimetic peptides on the inflammatory cascade has been intravital microscopy (IVM). Directly imaging the microvasculature during an inflammatory response has been critical in demonstrating the ability of exogenously administered AnxA1 to reduce the capacity of leukocytes to adhere to and migrate through inflamed post-capillary venules (Lim et al., 1998; Gavins et al., 2003). Exogenous administration of AnxA1 to mice following clamping and release of the superior mesenteric artery to induce ischemia/reperfusion injury, resulted in an anti-inflammatory effect that was associated with the detachment of neutrophils from the endothelium (Gavins et al., 2003). This demonstration of an effect of AnxA1 on leukocyte recruitment is further supported by *in vitro* studies, which demonstrate that AnxA1 inhibits firm adhesion of neutrophils to human umbilical vein endothelial cells under flow conditions. However in contrast, the AnxA1-derived peptide, Ac2-26, only reduces leukocyte capture and rolling without affecting firm adhesion (Hayhoe et al., 2006) or altering increased vascular permeability (Cirino et al., 1989; Gavins et al., 2003). These findings provide evidence that the actions of peptide Ac2-26 do not entirely overlap with those of full-length AnxA1.

The examination of the therapeutic efficacy of AnxA1 has recently been extended to an OVA-induced model of antigen-induced airways inflammation (Perretti et al., 1993) This study examined the effect of a cell-permeable form of AnxA1, administered as an exogenous anti-inflammatory agent to wild-type mice during the sensitization phase of the model (Lee et al., 2012). Administration of AnxA1 conjugated to a "Tat" cell-penetrating peptide, during the latter stages of OVA sensitization, alleviated inflammation, cytokine production, airways hyperresponsiveness, and OVA-specific IgE production. Notably, while the Tat-conjugated form of AnxA1 was therapeutically effective, native AnxA1 administered in a similar fashion did not significantly reduce disease parameters. This finding suggests that the capacity of Tat-AnxA1 to enter target cells was critical in achieving its anti-inflammatory effect in this setting.

IMPACT OF AnxA1 IN DRUG DISCOVERY

The identification of the mechanism of actions of AnxA1 (notwithstanding the unresolved question of whether the role of FPR2/ALXR is pro-inflammatory or anti-inflammatory; Perretti and Dalli, 2009), in parallel with the attractive concept of developing novel therapeutic agents based on mimicking specific endogenous pathways, has lead to an increase in drug discovery programs within this area. The ultimate aim of treatments based on AnxA1 is to retain the anti-inflammatory properties of glucocorticoids that signal for the resolution of inflammatory events, while avoiding the highly detrimental potential metabolic side effects of long-term use of exogenous glucocorticoids (Perretti and Gavins, 2003).

One strategy for this would be to identify new chemical entities from biologically-active peptide sequences from the N-terminus of AnxA1. However, from a drug discovery point of view, small molecules are a more attractive proposition due to their more attractive pharmacokinetic properties. Thus, the identification of the receptor by which AnxA1 mediates its biological effects (i.e., via FPR2/ALXR) has stimulated a great deal of interest. For example, Amgen have developed a program to identify small chemical entities that are specific for FPR2/ALXR (Perretti and Dalli, 2009). In addition, novel computer modeling approaches are now being used to identify ligands that are specific for members of the FPR family, in several cases for FPR2/ALXR (Shemesh et al., 2008; Hecht et al., 2009). The ultimate aim of this work is to identify a low molecular weight compound with the capacity to interact with FPR2/ALXR to mediate a comparable profile of anti-inflammatory effects to that of AnxA1

CONCLUSION

Inflammatory disease affects a huge number of patients worldwide. In many cases, the therapeutic approaches in use for these patients have not changed for the last 30 years. Treatments such as exogenous glucocorticoids remain a first-line therapy for prevalent diseases such as rheumatoid arthritis. Indeed their efficacy in inhibiting inflammation ensures they remain a favored therapeutic modality, despite their well-established highly detrimental metabolic side effects. The next level of sophistication however, is to investigate the mechanisms of action of glucocorticoids in these patients, and to learn more about the endogenous pathways glucocorticoids mobilize and interact with to inhibit inflammation. AnxA1 is a prime example of this approach. The investigation of the mechanisms of action of AnxA1, as well as those of its breakdown products and receptors, holds great promise for the development of more specific novel therapies which mimic the anti-inflammatory effects of glucocorticoids, while potentially avoiding the negative effects of glucocorticoid use. AnxA1 has consistently been found to play an inhibitory role in innate forms of inappropriate inflammation. Therefore novel AnxA1-derived therapeutics are more likely to be immediately applicable in conditions such as ischemia/reperfusion injury, where the innate immune system plays a leading role. In contrast, given the previously-described inconsistency in the reported actions of AnxA1 in T cell-mediated immunity, this remains premature for forms of inflammation mediated by the adaptive immune system. However, given more detailed research in this area, AnxA1-derived therapeutics may also eventually find use in specific forms of T cell-mediated disease.

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Regulation of inflammation by adenosine

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Adenosine, a purine nucleoside generated by the dephosphorylation of adenine nucleotides, is a potent endogenous physiologic and pharmacologic regulator of many functions. Adenosine was first reported to inhibit the inflammatory actions of neutrophils nearly 30 years ago and since then the role of adenosine and its receptors as feedback regulators of inflammation has been well established. Here we review the effects of adenosine, acting at its receptors, on neutrophil and monocyte/macrophage function in inflammation. Moreover, we review the role of adenosine in mediating the anti-inflammatory effects of methotrexate, the anchor drug in the treatment of Rheumatoid Arthritis and other inflammatory disorders.

Keywords: monocytes, macrophages, adenosine, adenosine receptors, neutrophils

INTRODUCTION

The nucleoside adenosine is a potent physiologic and pharmacologic regulator that is produced by cells in response to stress by breakdown of adenosine triphosphate (ATP) (Hasko and Cronstein, 2004; Sitkovsky et al., 2004; Hasko et al., 2005, 2008). ATP is broken down both intracellularly and extracellularly to generate adenosine (**Figure 1**). Intracellular adenosine is exported from cells via equilibrative nucleoside transporters or during apoptosis or necrosis. ATP and its degradation product adenosine diphosphate (ADP) are released from cells through a variety of mechanisms, including membrane damage, through connexin/pannexin and other channels, and through protein or hormone-transporting vesicles. When ATP and ADP are released, the phosphate groups of extracellular ATP and ADP are sequentially hydrolyzed, first by ecto-nucleoside triphosphate diphosphorylases (NTPDases, including CD39) and then by ecto-5′-nucleotidase (Ecto-5′NTase, CD73) (Yegutkin, 2008).

Extracellular levels of adenosine can rise from low nanomolar to micro-molar concentrations in response to stress (Hasko et al., 2008). Adenosine regulates cell function through ligation of adenosine receptors, which consist of a family of four cell surface 7-transmembrane receptors (A_1R, A_{2A}R, A_{2B}R, and A3) (Linden, 2001). The activation of A_1 and A_3 receptors leads to decreased intracellular cyclic adenosine monophosphate (Gallardo-Soler et al., 2008) levels by coupling to pertussis toxin-inhibited G_i-coupled signal transduction proteins. A_{2A} receptors are $G\alpha_S$- or $G\alpha_{olf}$-linked receptors that activate adenylyl cyclase, increase cAMP, and activate protein kinase A (PKA) and Epac1/2which activate their own signaling cascades to regulate cellular function. Interestingly, A_{2A} receptors can also signal in a G protein-independent manner. A_{2B} receptors can signal through both $G\alpha_S$ and Gq proteins.

ADENOSINE AND THE SIGNS OF INFLAMMATION

Classically, inflammation is characterized by rubor (redness), tumor (swelling), calor (heat), and functio laesa (loss of function). These manifestations of inflammation result principally from vascular dilatation and leakage and, although a large number and variety of mediators are involved in inflammation it is likely that adenosine, released at sites of tissue injury, plays a role in the pathogenesis or regulation of these signs. Adenosine, acting primarily at A_{2A} receptors, has long been known to be a potent vasodilator (Drury and Szent-Gyorgi, 1929) and this is the basis for use of adenosine and adenosine A_{2A} receptor agonists for pharmacologic stress testing. Thus, it is likely adenosine release at inflamed sites contributes to the erythema (rubor) and resulting heat loss (calor) associated with inflammation. Interestingly, diminished production of adenosine leads to dramatic vascular leakage resulting from diminished activation of adenosine A_{2B} receptors on the vascular endothelium (Thompson et al., 2004; Eckle et al., 2008) suggesting that the adenosine released at inflamed sites diminishes the swelling (tumor) that is so prominent at inflamed sites.

ADENOSINE INHIBITS RECRUITMENT AND ACTIVATION OF NEUTROPHILS

Neutrophils are recruited to inflamed sites by a combination of chemokines and adhesive interactions between leukocytes and the vascular endothelium. Adenosine diminishes inflammation by diminishing leukocyte recruitment; adenosine inhibits stimulated neutrophil adhesion to the vascular endothelium

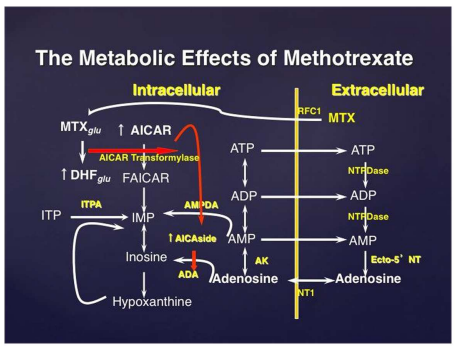

FIGURE 1 | The effect of methotrexate on adenosine release. Methotrexate is actively transported into the cell where it is polyglutamated; MTX polyglutamate is a potent inhibitor of AMP deaminase. Accumulation of AICAR, an intermediate metabolite in de novo purine biosynthesis, leads to enhanced release of adenine nucleotides which are released into the extracellular space and converted to adenosine. MTXglu, methotrexate polyglutamates; DHFglu, dihydrofolate polyglutamates; AICAR, aminoimidazole carboxamidoribonucleotide; FAICAR, formyl AICAR; RFC1, reverse folate carrier 1; ADA, adenosine deaminase; AK, adenosine kinase; NTPDase, nucleoside triphosphate phosphohydrolase; ecto-5′NT, ecto-5′nucleotidase.

(Cronstein et al., 1986) and neutrophil-mediated injury to the endothelium. Adenosine receptor stimulation diminishes neutrophil adhesion to the endothelium by inhibiting both selectin- and integrin-mediated adhesive events (Cronstein et al., 1992; Bullough et al., 1995; Bouma et al., 1996; Sullivan et al., 2004). Presumably these same mechanisms apply to recruitment of other cell types to inflamed sites as well. Although having noted these anti-inflammatory effects of adenosine it has recently been reported that neutrophils release ATP which is converted to adenosine extracellularly and the adenosine binds to A_3 receptors to promote chemotaxis and loss or inhibition of A_3 receptors markedly reduces leukocyte recruitment to sites of bacterial infection (Chen et al., 2006). Other studies suggest that the A_3 receptor-mediated effects on neutrophil recruitment are more selective and may not be important for recruitment to other chemoattractants (Montesinos et al., 2006).

Adenosine diminishes stimulated neutrophil production of oxygen radicals and other potentially deleterious mediators (Reviewed in Taylor et al., 2005). Moreover, adenosine, acting primarily at A_{2A} receptors inhibits phagocytosis of particles (Reviewed in Taylor et al., 2005). Although cAMP mediates many of the downstream effects of adenosine A_{2A} receptors via activation of PKA there have been reports that cAMP-PKA-independent mechanisms mediate inhibition of neutrophil activation by adenosine A_{2A} receptors.

ADENOSINE AND CLASSICAL MACROPHAGE ACTIVATION

Macrophages are best known for initiating an effective innate immune response against microbes by recognizing pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) (Pozzi et al., 2005). Following phagocytosis, macrophages destroy most micro-organisms. By producing diverse molecules and presenting antigens to T cells, macrophages in addition to dendritic cells, orient the adaptive immune response leading to the expansion and differentiation of lymphocytes specific for invaders or cancer cells (Gordon and Taylor, 2005; Preynat-Seauve et al., 2006).

Macrophages comprise a heterogeneous population of cells, and show bewildering functional plasticity in response to dynamic micro-environmental cues. Macrophage heterogeneity arises as macrophages differentiate from immature monocyte precursors or yolk-sac macrophages (Mills et al., 2000; Kuroda et al., 2002; Mosser, 2003; Murray and Wynn, 2011). In a conscious parallel with T helper (Th)1 and Th2 lymphocytes, macrophages have been classified into M1 and M2 phenotypes. M1 or "classical" activation of macrophages is induced by toll-like receptor (TLR) agonists, either with or without the Th1 cytokine interferon (IFN)-γ, and results in an inflammatory phenotype characterized by expression of a series of inflammatory cytokines and chemokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1-β, IL-6, IL-12, and macrophage inflammatory protein (MIP)-1α (Mosser and Edwards, 2008; Biswas and Mantovani, 2010). M1 macrophages are strong promoters of Th1 immune responses (Hasko et al., 2000) and have anti-proliferative and cytotoxic activities, which result from their ability to produce reactive oxygen and nitrogen species, such as hydrogen peroxide, superoxide, nitric oxide (NO), and peroxynitrite, and pro-inflammatory cytokines.

The role of adenosine in regulating classical macrophage activation has been studied in detail. As such, adenosine has been shown to be a broad inhibitor of the pro-inflammatory consequences of classical macrophage activation. The anti-inflammatory effects of adenosine on M1 macrophages include suppression of cytokine/chemokine production (Hasko et al., 1996, 1998, 2007; Szabo et al., 1998; Xaus et al., 1999; Sipka et al., 2005, 2007; Ryzhov et al., 2008; Koscso et al., 2012) and NO production (Csoka et al., 2007a; Ryzhov et al., 2008). In contrast to the suppressive effect of adenosine on the production of pro-inflammatory mediators, adenosine augments production of the anti-inflammatory cytokine IL-10 by M1 macrophages. The current consensus is that the regulatory effects of adenosine on M1 macrophages are mediated predominantly by A_{2A} receptors (Hasko et al., 1996, 2009; Pinhal-Enfield et al., 2003; Nemeth et al., 2005; Kreckler et al., 2006; Csoka et al., 2007b; Chen et al., 2009; Wilson et al., 2009; Belikoff et al., 2011). For example, using A_{2A} receptor deficient mice combined with pharmacologic approaches, it has been shown that adenosine inhibits TNF-α, IL-6, and IL-12 release and augments IL-10 production by lipopolysaccharide (LPS)- or bacteria-activated macrophages mostly through A_{2A} receptors (Nemeth et al., 2005; Hasko et al., 2007; Kara et al., 2010a). Although, A_{2B} receptors have been overshadowed by A_{2A} receptors as the primary adenosine receptors shaping the function of M1 macrophages, there is growing evidence that A_{2B} receptors can also become operational in regulating M1 macrophage function. In this context, we recently showed that A_{2B} receptors but not A_{2A} receptors augment LPS-induced IL-10 production by RAW264.7 macrophages, which express high levels of A_{2B} receptors and low levels of A_{2A} receptors (Pinhal-Enfield et al., 2003). Moreover, adenosine can suppress LPS-induced TNF-α production even in A_{2A} receptor deficient mice, and this effect is mediated by A_{2B} receptors (Kara et al., 2010a). A_1 adenosine receptors and

A_3 receptors are expressed at much lower levels on the surface of macrophages and their role in regulating macrophage function remains incompletely understood (Hasko et al., 1996).

Studies utilizing A_1 receptor deficient mice recently showed that A_1 receptors play a critical role in osteoclast development from monocytic precursors (Merrill et al., 1995). Interestingly, A_1 receptors also regulate fusion of human peripheral blood monocytes into giant cells *in vitro* as well although the mechanism for this regulation has not been fully established (Merrill et al., 1997; Hasko and Pacher, 2012). Genetic studies have yet to confirm the role of A_3 receptors in governing macrophage function (Nelms et al., 1999).

ADENOSINE AND ALTERNATIVE MACROPHAGE ACTIVATION

M2 or "alternatively activated" macrophages were originally described as macrophages induced by the Th2 cytokines IL-4 and IL-13. The effects of IL-4 and IL-13 on macrophages partially overlap because they use dimeric receptors that share the IL-4 receptor (IL-4R)α subunit. In contrast, the differences between signaling by IL-4 and IL-13 stem from the fact that while IL-4 is able to activate both the IL-4Rα/common γ chain and IL-4Rα/IL-13Rα dimers, IL-13 can only activate the latter complex. The intracellular signaling pathways are incompletely characterized and involve members of the Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) family, especially STAT6. In addition to STAT6 (Gray et al., 2005), recent studies have identified CCAAT-enhancer-binding protein (C/EBP)β (Pauleau et al., 2004; Albina et al., 2005; Ruffell et al., 2009), cAMP response element-binding protein (CREB) (Odegaard et al., 2007), peroxisome proliferator-activated receptor (PPAR)γ (Gallardo-Soler et al., 2008; Satoh et al., 2010; Szanto et al., 2010), IFN regulatory factor (IRF)4 (El Chartouni et al., 2010; Liao et al., 2011), Krüppel-like factor 4 (KLF4) (Takeda et al., 2010), hypoxia-inducible factor-2 (HIF-2) (Pello et al., 2012), and c-MYC (Sica and Mantovani, 2012) as contributors to the transcriptional response driving M2 macrophage activation. Hallmark M2 markers include arginase-1, tissue inhibitor of metalloproteinases (TIMP)-1, macrophage galactose-type C-type lectin (mgl)-1, IL-4Rα, Ym1, and resistin-like molecule (RELM)α (Anthony et al., 2006). Increasingly, activation of multiple markers is used to unequivocally identify M2 macrophages in the context of responses to different antigens (Chen et al., 2012).

M2 macrophages are elicited following infection with multicellular parasites, and can lead to an inflammatory response qualitatively different from and capable of downregulating harmful Th1-type inflammatory responses. Recent studies have suggested that increased M2 macrophage arginase activity during helminth infections is an important element in the control and expulsion of worms (Hesse et al., 2001; Gordon, 2003; Edwards et al., 2006). M2 macrophages contribute to the control of inflammation and can mediate enhanced wound healing through arg-1-mediated production of collagen and insulin-like growth factor 1 (IGF-1), and by contributing to the clearance of cellular debris through scavenger receptors (Gordon, 2003; Anthony et al., 2006). The immunoregulatory/protective, rather than tissue damaging, role of M2 macrophages is also exemplified by the fact that they are abundant in healthy tissues that are associated with naturally immune suppressed states, such as placenta, lung, and other immunologically privileged sites (Noel et al., 2004). In contrast to their protective effects in acute inflammation, it has been proposed that M2 macrophages activated by IL-4 and IL-13 during asthma and chronic obstructive pulmonary disease contribute significantly to airway remodeling and lung fibrosis leading to lung dysfunction (Mantovani et al., 2004; Van Ginderachter et al., 2006; Martinez et al., 2008; Csoka et al., 2012). Additionally, M2 macrophages have also been shown to be hijacked by tumor cells to function as suppressors of anti-tumor T cell responses and stimulators of tumor angiogenesis (Martinez et al., 2008; Koroskenyi et al., 2011). Based on these observations, M2 macrophages have been proposed as an emerging therapeutic target for a variety of disease states.

We have recently discovered that adenosine strongly promotes IL-4/IL-13-induced M2 macrophage activation *in vitro*, as indicated by upregulation of the arginase-1, TIMP-1, and mgl-1 (Barczyk et al., 2010). Our studies, utilizing both pharmacological approaches and macrophages from adenosine receptor deficient and wild type (WT) mice, indicate that A_{2B} adenosine receptors, and to a lesser degree other adenosine receptors, are required for mediating the stimulatory effect of adenosine on IL-4-induced M2 macrophage activation. Our data also indicate that the stimulatory effect of adenosine receptor activation on M2 macrophage development is mediated by the transcription factor C/EBPβ, but not STAT6 or CREB (Barczyk et al., 2010).

While the designation M2 usually denotes macrophages activated by IL-4 or IL-13, M2 macrophages can also be induced by other anti-inflammatory stimuli, which include immune complexes, and heterogeneous deactivating mediators such as apoptotic cells, glucocorticoids, and IL-10 (Kular et al., 2012). Thus, IL-4/IL-13-activated macrophages are also called M2a, immune complex-activated macrophages are referred to as M2b, and macrophages activated by apoptotic cells, glucocorticoids, and IL-10 are termed M2c. In macrophages phagocytosing apoptotic cells, adenosine released endogenously activates A_{2A} receptors and inhibits the generation of the pro-inflammatory chemokines MIP-2 and cytokine-induced neutrophil-attracting chemokine (KC) (Kara et al., 2010b). Moreover, glucocorticoids promote survival of anti-inflammatory monocytes via upregulation and autocrine activation of A_3 adenosine receptors (Mediero et al., 2012a). Together, in macrophages exposed to apoptotic cells and glucocorticoids adenosine switches macrophage phenotype from pro-inflammatory to anti-inflammatory (Nelms et al., 1999).

ADENOSINE AND MYELOID/MONOCYTE-DERIVED SYNCYTIAL CELLS (OSTEOCLASTS AND GIANT CELLS)

Myeloid precursors can, in response to M-CSF and RANKL, differentiate into osteoclasts, multinucleated giant cells that mediate bone resorption (Mediero et al., 2012b). Studies utilizing A_1 receptor deficient mice recently showed that A_1 receptors play a critical role in osteoclast development from monocytic precursors and bone resorption (Ernst et al., 2010; McNally and Anderson, 2011). In contrast to A_1 adenosine receptors, A_{2A} receptors inhibit osteoclast differentiation and function (Deaglio et al., 2007) and A_{2A} receptor stimulation has been shown to inhibit wear particle-induced osteolysis, a form of inflammatory bone destruction

Table 1 | Cellular expression of adenosine receptors.

	A_1 receptor	A_{2A} receptor	A_{2B} receptor	A_3 receptor
Neutrophils		Diminished adhesion, activation		Stimulates chemotaxis
M1 macrophages		Inhibit phagocytosis, inflammatory cytokine production, increase IL-10	Stimulates IL-10	
M2 macrophages		Stimulates M2 macrophage differentiation Production of pro-angiogenic factors		
Lymphocytes		Inhibits TH17 differentiation Stimulates T_{Reg} differentiation		
Osteoclasts and giant cells	Promotes osteoclast differentiation Stimulates giant cell formation	Inhibits osteoclast differentiation		

resulting from particulates shed from joint prostheses (Chalmin et al., 2012).

Similar to osteoclasts, in response to IFN-γ or other stimuli, monocytes will fuse to form multinucleated giant cells, hallmarks of responses to foreign bodies and such diseases as Sarcoidosis (Semenza, 2010). Interestingly, A_1 receptors also regulate fusion of human peripheral blood monocytes into giant cells *in vitro* as well although the mechanism for this regulation has not been fully established (Merrill et al., 1997; Hasko and Pacher, 2012). Genetic studies have yet to confirm the role of A_3 receptors in governing macrophage function (Nelms et al., 1999).

ADENOSINE AND T CELLS

Forkhead box P3 (FOXP3)-expressing regulatory T (Treg) cells are crucial in the maintenance of immunological self-tolerance and in the regulation of immune responses. CD39 and CD73 are expressed on the surface of $Foxp3^+$ Tregs and are increasingly used as markers of Tregs (Leibovich et al., 2002). Deaglio et al. (2007) and Adair (2005) showed that CD39 and CD73 on the surface of Tregs produce adenosine, which mediates a substantial portion of the anti-inflammatory and immune regulatory effects of Tregs by engaging A_{2A} receptors on effector T cells. More recently, Th 17 cells were also shown to express CD39 and CD73, which, by producing adenosine, suppress both $CD4^+$ and $CD8^+$ T cell effector functions (Hasko et al., 2009). The expression of both CD39 and CD73 on Th17 cells was upregulated by IL-6 and TGF-β, factors that are crucial for Th17 cell development (Ramanathan et al., 2009).

ADENOSINE AND THE ANGIOGENIC SWITCH IN MACROPHAGES

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and is crucial for the differentiation of endothelial cells during vasculogenesis, and for the outgrowth of new capillaries from pre-existing blood vessels (Ramanathan et al., 2007). VEGF is thus an important component of tissue repair, and is critical for the resolution of inflammation and wound healing. Macrophages are prominent producers of VEGF during the resolution of inflammation and wound healing. There is a plethora of evidence demonstrating that adenosine promotes angiogenesis, in a large part, by increasing macrophage VEGF production (Murphree et al., 2005; Csoka et al., 2007b; Ernens et al., 2010). It has been shown that adenosine stimulation of A_{2A} receptors on TLR-activated macrophages results in a switch from the production of inflammatory cytokines such as TNF-α and IL-12, to the production of anti-inflammatory and angiogenic factors, including IL-10 and VEGF (Ohta and Sitkovsky, 2001; Gessi et al., 2010) and we termed this process angiogenic switch (Csoka et al., 2007b). This model provides a sequential pathway whereby macrophages initially mediate inflammation through TLR-dependent activation to an M1 phenotype, but are then switched into an angiogenic phenotype by adenosine generated in response to hypoxia/ischemia within the wound area. In addition, the initial activation of macrophages by TLR agonists, which markedly induce expression of adenosine A_{2A} and A_{2B} adenosine receptors, primes these macrophages to respond to increased local levels of extracellular adenosine (Chan and Cronstein, 2010; Gessi et al., 2010). It is noteworthy that while A_{2A} (Csoka et al., 2007b) and A_{2B} (unpublished data) receptors mediate the angiogenic switch in murine macrophages, the adenosine-mediated increase in VEGF secretion by human monocytes is mediated by A_{2A}, A_{2B}, and A_3 receptors (Varani et al., 2009, 2011).

ADENOSINE AND ADENOSINE RECEPTORS IN INFLAMMATORY DISEASES

From its initial identification as an anti-inflammatory ligand adenosine was thought to be an important endogenous feedback regulator of inflammation and tissue injury. Nonetheless, the first actual demonstration that adenosine, acting at A_{2A} receptors, was an endogenous anti-inflammatory agent had to wait until the development of adenosine receptor knockout mice. Liver injury in response to concanavalin A, a model for viral hepatitis, was markedly enhanced in the absence of adenosine A_{2A} receptors (Khoa et al., 2006), consistent with the hypothesis that, at least in the liver, increased adenosine release at inflamed sites suppresses inflammation and inflammatory injury.

However, making use of adenosine as an anti-inflammatory agent has remained more of a challenge due to the myriad other effects of adenosine acting at A_{2A} and other adenosine receptors, e.g., hypotension. Interestingly, it is now clear that low-dose methotrexate, the anchor drug for the treatment of rheumatoid

arthritis, mediates its anti-inflammatory effects via promotion of adenosine release at inflamed sites (Reviewed in Khoa et al., 2001). The "immunosuppressive" effects of methotrexate-mediated inhibition of T cell proliferation are unlikely to account for the effects of methotrexate administered at doses well below those required to inhibit cellular proliferation (15–20 mg/week) and methotrexate is usually accompanied by folic acid or folinic acid supplementation to prevent toxicity without diminishing efficacy. The effects of adenosine, described above, on lymphocyte function provide a better explanation for the immunosuppressive effects of methotrexate, as used to treat Rheumatoid Arthritis (Khoa et al., 2001). Because adenosine receptor expression is increased on leukocytes in patients with Rheumatoid Arthritis (Levy et al., 2006; Hesdorffer et al., 2012), most likely the result of exposure of these cells to high concentrations of TNFα, a cytokine previously demonstrated to increase adenosine A_{2A} receptor expression and function (Smail et al., 1992; Thammavongsa et al., 2009), it is likely that these patients are "primed" to respond to increased adenosine concentrations resulting from methotrexate therapy.

ADENOSINE AND AGING

Immunologic and inflammatory responses are blunted at the beginning and end of life leading to increased susceptibilities to infection for neonates and the elderly. Recent work has suggested that adenosine and its receptors play a role in suppressing responses to infection. Levy et al. (2006) have reported that monocyte/macrophages from neonates are much more sensitive to adenosine A_3 receptor-mediated suppression of inflammatory responses (TNF production) than those from adults. In contrast, lymphocytes from the elderly release increased amounts of adenosine leading to suppression of T cell responses (Hesdorffer et al., 2012).

ADENOSINE IS A VIRULENCE FACTOR PRODUCED BY PATHOGENS

It is not uncommon for invasive pathogens to take advantage of mammalian mechanisms for suppression of host responses to promote spread or survival of the infecting organism. Thus, it was not surprising to find that *Candida albicans* hyphae release adenosine which suppresses neutrophil-mediated killing of the organism (Smail et al., 1992). More recent studies have demonstrated that *Staphylococcus aureus* also produce adenosine to avoid killing by the host as well (Thammavongsa et al., 2009). Thus, adenosine, produced by invasive organisms can promote spread of the organism by suppressing host killing of the bacteria.

CONCLUSION

Adenosine is a potent endogenous anti-inflammatory agent that regulates the function of inflammatory cells via interaction with specific receptors expressed on these cells (**Table 1**). Already known as an endogenous regulator of inflammation, adenosine also mediates the anti-inflammatory effects of methotrexate, one of the most widely used anti-inflammatory drugs.

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Conflict of Interest Statement: Bruce Cronstein, Intellectual Property: Patents on use of adenosine A2A receptor agonists to promote wound healing and use of A2A receptor antagonists to inhibit fibrosis. Patent on use of adenosine A1 receptor antagonists to treat osteoporosis and other diseases of bone. Patent on use of adenosine A2A agonists to promote bone regeneration. Patent on use of anti-netrin-1 antibodies for the treatment of bone disease. Patent on use of adenosine A2A agonists and A1 antagonists to inhibit wear part.

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Modulation of neutrophil apoptosis and the resolution of inflammation through β_2 integrins

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Precise control of the neutrophil death program provides a balance between their defense functions and safe clearance, whereas impaired regulation of neutrophil death is thought to contribute to a wide range of inflammatory pathologies. Apoptosis is essential for neutrophil functional shutdown, removal of emigrated neutrophils, and timely resolution of inflammation. Neutrophils receive survival and pro-apoptosis cues from the inflammatory microenvironment and integrate these signals through surface receptors and common downstream mechanisms. Among these receptors are the leukocyte-specific membrane receptors β_2 integrins that are best known for regulating adhesion and phagocytosis. Accumulating evidence indicate that outside-in signaling through the β_2 integrin Mac-1 can generate contrasting cues in neutrophils, leading to promotion of their survival or apoptosis. Binding of Mac-1 to its ligands ICAM-1, fibrinogen, or the azurophilic granule enzyme myeloperoxidase suppresses apoptosis, whereas Mac-1-mediated phagocytosis of bacteria evokes apoptotic cell death. Mac-1 signaling is also target for the anti-inflammatory, pro-resolving mediators, including lipoxin A_4, aspirin-triggered lipoxin A_4, and resolvin E1. This review focuses on molecular mechanisms underlying Mac-1 regulation of neutrophil apoptosis and highlights recent advances how hierarchy of survival and pro-apoptosis signals can be harnessed to facilitate neutrophil apoptosis and the resolution of inflammation.

Keywords: neutrophils, Mac-1, apoptosis, lipoxins, resolvins, myeloperoxidase, phagocytosis, resolution of inflammation

INTRODUCTION

Neutrophils form the first line of defense against invading pathogens or tissue injury. They are rapidly recruited to the sites of infection/injury and play a prominent role in the initiation and progression of the inflammatory response. Their many defense mechanisms that destroy invading pathogens are also capable of inflicting damage to the surrounding tissue (Nathan, 2006). Under certain conditions, these harmful consequences became dominant and prolong inflammation. Once the pathogens are cleared, neutrophils are thought to undergo constitutive apoptosis (Savill et al., 2002). This process renders neutrophils unresponsive to extracellular stimuli, allows their recognition and removal by macrophages (Savill et al., 2002; Gilroy et al., 2004; Nathan and Ding, 2010) and limits their potentially harmful actions. Neutrophil accumulation in inflamed tissues is a balance of their recruitment and removal. Conversely, effective resolution of inflammation requires cessation of neutrophil recruitment as well as timely removal of emigrated neutrophils from the site of inflammation. Apoptosis, which ensures that neutrophils are securely marked for disposal, emerged as a control point in resolving inflammation (Filep and El Kebir, 2009; Fox et al., 2010; Perretti, 2012). Pro-survival and pro-apoptosis signals from the inflammatory milieu can, however, influence the execution of the constitutive death program, thereby profoundly affecting the fate of neutrophils and the outcome of the inflammatory response (reviewed in Filep and El Kebir, 2010; Fox et al., 2010; Geering and Simon, 2011).

β_2 integrins are leukocyte-specific adhesion molecules that govern neutrophil adhesion and transmigration across the activated endothelium and phagocytosis of pathogens (Ross, 2000; Ley et al., 2007). Growing evidence demonstrates that outside-in signaling through β_2 integrins can generate contrasting cues in neutrophils, leading to promotion of their survival or apoptosis. This review will focus on the hierarchy of these signaling circuits and the underlying molecular mechanisms, and will discuss how interference with β_2 integrin signaling could be harnessed for promoting neutrophil apoptosis to enhance the resolution of inflammation.

NEUTROPHIL APOPTOSIS: A CONTROL POINT FOR THE RESOLUTION OF INFLAMMATION

NEUTROPHIL APOPTOSIS DURING HOMEOSTASIS AND INFLAMMATION

Mature neutrophils are terminally differentiated cells that have the shortest lifespan among leukocytes in the circulation. Neutrophil lifespan is generally thought to be in the range of 8–20 h, though recent data with *in vivo* labeling suggest a lifespan of 5.4 days under physiological conditions in humans (Pillay et al., 2010). Aged neutrophils die by constitutive (or spontaneous) apoptosis. This mechanism is essential to maintain the balance of cellular homeostasis under physiological conditions (Cartwright et al., 1964; Coxon et al., 1996). Apoptosis renders neutrophils unresponsive to extracellular stimuli and leads to expression of "eat-me" signals, so that neutrophils can be recognized and removed by macrophages in the spleen and bone marrow and Kupffer cells in the liver (Savill

et al., 1989, 2002). In mice, these three organs contribute equally to removal of senescent neutrophils (Furze and Rankin, 2008).

During inflammation, extending the lifespan of neutrophils during transendothelial migration and at the sites of infection is critical for efficient destruction of pathogens (Watson et al., 1997; Savill et al., 2002; Nathan, 2006). Once this is accomplished, neutrophils may undergo necrosis, apoptosis, NETosis (neutrophil extracellular trap cell death) (Brinkmann et al., 2004; Fuchs et al., 2007), or autophagy (Remijsen et al., 2011) with the type of death profoundly affecting the outcome of the inflammatory response.

Apoptotic neutrophil death *in situ* has multiple pro-resolution actions. In addition to becoming unresponsive to agonists and stopping production of inflammatory mediators, apoptotic neutrophils can sequester cytokines (Ariel et al., 2006; Ren et al., 2008) and their phagocytosis by macrophages induces macrophage polarization from a pro-inflammatory (M1) to a pro-resolution (M2) phenotype (Fadok et al., 1998). M2 macrophages secrete mediators, such as IL-10 and TGFβ, which mediate resolution and tissue repair (Ariel and Serhan, 2012; Sica and Mantovani, 2012). Interestingly, injection of large quantities of apoptotic neutrophils protected mice against endotoxin shock (Ren et al., 2008).

In non-resolving inflammation, neutrophils persist at the inflamed site and are liable to cause tissue destruction (Nathan and Ding, 2010; Soehnlein, 2012). Neutrophil recruitment may occur normally or may become excessive, but neutrophils persist as a result of delayed apoptosis or decreased clearance by macrophages (Haslett, 1999; Savill et al., 2002). The abnormal host response creates a persistent inflammatory microenvironment with ongoing release of inflammatory mediators and damage-associated molecular patterns (Nathan and Ding, 2010; Serhan, 2011).

NEUTROPHIL APOPTOSIS IN HUMAN DISEASE

The tight regulation of neutrophil death is also evident under pathological conditions, though it is often difficult to decide whether prolonged survival or apoptosis is most favorable from the host's perspective. Consistently, both accelerated and delayed neutrophil apoptosis could have severe pathological consequences. For example, infections with the opportunistic pathogen *Pseudomonas aeruginosa* (Allen et al., 2005), influenza virus A (Colamussi et al., 1999), or HIV (Elbim et al., 2009) as well as autoimmune diseases, such as systemic lupus erythematosus (Courtney et al., 1999) shorten neutrophil lifespan by accelerating apoptosis, leading to impaired antimicrobial defenses and increased susceptibility to recurrent infections. Intracellular pathogens may use apoptotic neutrophils as a Trojan horse to infect and propagate in macrophages (Laskay et al., 2008; Rupp et al., 2009). On the other hand, delayed neutrophil apoptosis appears to be a component of the pathophysiology in patients with inflammatory diseases, including acute respiratory distress syndrome (ARDS) (Matute-Bello et al., 1997; chronic pulmonary obstructive disease (COPD) (Brown et al., 2009), viral pneumonia (Lindemans et al., 2006), sepsis (Ertel et al., 1998), burn (Chitnis et al., 1996), acute coronary artery disease (Garlichs et al., 2004), rheumatoid arthritis (Wong et al., 2009), and cystic fibrosis (McKeon et al., 2008), and frequently correlates with disease severity and outcome.

DISTINCT MOLECULAR FEATURES OF NEUTROPHIL APOPTOSIS

A complex network of intracellular death and survival pathways regulates neutrophil apoptosis and the balance of these circuits would ultimately determine the fate of neutrophils. Since neutrophils undergo apoptosis even in the absence of any extracellular stimuli, this type of death is called spontaneous or constitutive programed cell death. However, under most conditions, neutrophils receive both pro-survival and pro-apoptosis cues, and the net effect is likely determined by the balance of these signals. Neutrophil apoptosis shares many morphological features with apoptosis in other cell types; however, it involves distinct molecular mechanisms in executing the cell death program. Predominant expression of the anti-apoptotic protein myeloid cell leukemia-1 (Mcl-1), restricted function of mitochondria to apoptosis, ROS production, release of proteases from azurophilic granules, and unusual roles for nuclear proteins are hallmarks of regulation of apoptosis in neutrophils.

Mcl-1 REGULATION OF NEUTROPHIL SURVIVAL AND APOPTOSIS

Spontaneous neutrophil apoptosis rely upon the balance of pro- and anti-apoptotic members of the Bcl-2 family. Mature human neutrophils constitutively express the pro-apoptotic Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (Bad), Bcl-2 homologous antagonist/killer (Bak), Bcl-2 homology-3 (BH-3)-interacting domain death agonist (Bid), Bcl-2 interacting protein (Bim), and Bcl-2 interacting killer (Bik) as well as the anti-apoptotic Bcl-2 homolog Mcl-1, and to a much lesser extent A1 and Bcl-X_L, but not Bcl-2 (Akgul et al., 2001; Moulding et al., 2001). The pro-apoptotic Bcl-2 homologs have relatively long half-lives and their cellular levels change very little during exposure of neutrophils to agents that either accelerate or delay apoptosis. Genetic deletion of *bim* or *bax/bak* results in increased neutrophil numbers in mice (Lindsten et al., 2000). Mcl-1 contains a PEST domain [rich in proline (P), glutamic acid (E), serine (S), and threonine (T)], which facilitates its proteasomal degradation, resulting in a very short half-life (Edwards et al., 2004). Mcl-1 levels closely correlate with neutrophil survival kinetics (Moulding et al., 1989; Hamasaki et al., 1998; Leuenroth et al., 2000; Kato et al., 2006; Dzhagalov et al., 2007). Survival of myeloid cells decreases following treatment with antisense oligonucleotides against Mcl-1 (Moulding et al., 2000). Myeloid lineage-specific knockout of *mcl-1* reduces neutrophil numbers by accelerating apoptosis (Dzhagalov et al., 2007; Steimer et al., 2009). Recent data indicate that Mcl-1 levels drop in advance of apoptosis, even in the presence of caspase inhibitors (Wardle et al., 2011), indicating that Mcl-1 functions as a regulator and a downstream target of caspase activation. On the other hand, unchanged or even increased Mcl-1 expression has been detected in neutrophils of patients with Crohn's disease (Catarzi et al., 2008) or severe sepsis (Fotouhi-Ardakani et al., 2010). Mcl-1 promotion of neutrophil survival is thought to involve heterodimerization with and neutralization of Bim or Bak in the mitochondrial outer membrane (Reed, 2006; Brenner and Mak, 2009), resulting in maintenance of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and prevention of release of pro-apoptotic proteins.

Mature neutrophils contain a low number of mitochondria that may have a role restricted to apoptosis (Maianski et al.,

2004). Thus, mitochondrial respiration in mature neutrophils is low and mitochondria generate only small amounts of ATP by oxidative phosphorylation (Maianski et al., 2004). The mitochondrial poison cyanide does not affect neutrophil function. Nevertheless, neutrophil mitochondria maintain a transmembrane potential, forms a complex network that plays a role in chemotaxis, phagocytosis, and triggering apoptosis (Fossati et al., 2003). Mitochondria contains pro-apoptotic proteins cytochrome c, second mitochondria-derived activator of caspases (Smac)/DIABLO (direct IAP-binding protein with low pl), apoptosis-inducing factor (AIF), and endonuclease G (Saelens et al., 2004). Loss of $\Delta\Psi_m$ evokes release of these proteins. Cytochrome c and Smac appear to be required for optimal caspase-3 activation (Altznauer et al., 2004). Loss of $\Delta\Psi_m$ precedes development of apoptotic morphology in neutrophils undergoing constitutive (Maianski et al., 2004) and induced apoptosis.

ROLE OF REACTIVE OXYGEN SPECIES AND REDOX BALANCE

Neutrophils generate high amounts of ROS by NADPH oxidase in response to soluble stimuli as well as following phagocytosis of bacteria in order to destroy invading pathogens (Nauseef, 2007). High amounts ROS can inflict damage to the surrounding cell and evoke necrosis. ROS also function as intracellular signaling molecules. The intrinsic or mitochondrial pathway of apoptosis is likely initiated through ROS generation (Kasahara et al., 1997; Maianski et al., 2004; Xu et al., 2010), though the source(s) of ROS in aging non-activated neutrophils is still unknown. Ligation of the cell surface death receptors, TNF, Fas, or TRAIL (TNF-related apoptosis-inducing ligand) receptors triggers the formation of the death-inducing signaling complex (DISC), which through downstream adaptor proteins, such as Fas-associated death domain (FADD) leads to activation of NADPH oxidase and cleavage of caspase-8 (Green, 2000). Likewise, phagocytosis of opsonized microorganisms through complement and/or Fcγ receptors evokes ROS-mediated activation of caspase-8 and subsequently caspase-3, leading to neutrophil apoptosis (Watson et al., 1996; Perskvist et al., 2002; DeLeo, 2004). Consistent with these findings, patients with chronic granulomatous diseases that lacks functional NADPH oxidase exhibit increased neutrophil viability (Fadeel et al., 1998) and reduced neutrophil apoptosis following ingestion of bacteria (Coxon et al., 1996). The effects of ROS are balanced by neutrophil antioxidant defenses, including catalase, superoxide dismutase, and glutathione. These proteins become depleted during *ex vivo* culture of neutrophils parallel with development of apoptotic morphology (Watson, 2002; Melley et al., 2005). Loss of GSH by chloramines or GSH depletion during activation of the respiratory burst predisposes to apoptosis (Melley et al., 2005).

REGULATION OF APOPTOSIS BY GRANULAR AND NUCLEAR PROTEINS

Certain granular and nuclear proteins have also been implicated in the modulation of the cell death program. For example, cathepsin D is released from the azurophilic granules during apoptosis and may contribute to activation of caspase-3 through processing of caspase-8 and Bid (Conus et al., 2008). Consistently, pharmacological or genetic inhibition of cathepsin D results in delayed neutrophil apoptosis. Mature neutrophils constitutively express the cyclin-dependent kinases CDK1, CDK2, and CDK5 (Rosales et al., 2004; Rossi et al., 2006). Culture of neutrophils with R-roscovitine, a non-selective inhibitor of cyclin-dependent kinases enhances apoptosis likely through down-regulation of Mcl-1 expression (Rossi et al., 2006; Leitch et al., 2010). Unlike in other cell types, proliferating cell nuclear antigen (PCNA) is expressed in the cytoplasm of mature neutrophils and is bound to pro-caspases, resulting in suppression of neutrophil apoptosis (Witko-Sarsat et al., 2010). Conversely, decreased cytoplasmic PCNA expression resulted in augmented neutrophil apoptosis. In contrast, during constitutive apoptosis, another nuclear protein myeloid nuclear differentiation antigen (MNDA) is cleaved by caspases and accumulates in the cytoplasm, where it promotes proteasomal degradation of Mcl-1 and subsequently collapse of mitochondrial transmembrane potential (Fotouhi-Ardakani et al., 2010). Bacterial constituents and platelet-activating factor prevent cytoplasmic MNDA accumulation parallel with preservation of Mcl-1 and suppression of apoptosis (Fotouhi-Ardakani et al., 2010).

SUPPRESSION OF APOPTOSIS: INTRACELLULAR SURVIVAL PATHWAYS

Although apoptosis is a default fate of neutrophils, in the inflammatory microenvironment, neutrophils are likely exposed to various pro-survival signals, including granulocyte macrophage colony stimulating factor (GM-CSF) (Colotta et al., 1992; Lee et al., 1993), leukotriene B_4 (Lee et al., 1999), C5a (Lee et al., 1993), the acute-phase reactants C-reactive protein (Khreiss et al., 2002), and serum amyloid A (El Kebir et al., 2007; Christenson et al., 2008) as well as bacterial constituents LPS (Colotta et al., 1992; Lee et al., 1993) and bacterial DNA (József et al., 2004). Multiple kinase pathways are involved in determining the fate of neutrophils. For example, GM-CSF activates the Jak2/STAT and phosphoinositide-3-kinase (PI3K)/Akt pathways, leading to preservation of Mcl-1 expression and retardation of apoptosis (Klein et al., 2000; Epling-Burnette et al., 2001). PI3K generates PtdIns(3,4,5)P3, which also influences NF-κB and cAMP-response-element-binding protein (CREB) and thus may generate additional pro-survival signals (Ward et al., 2004). Many inflammatory mediators also activate the MAPK/ERK pathway that, in turn, inhibits the intrinsic pathway of apoptosis (Filep and El Kebir, 2010; Geering and Simon, 2011). ERK 1/2 and Akt phosphorylate Bad and Bax, leading to dissociation of phosphorylated Bad and Bax from the anti-apoptotic protein Mcl-1 (Akgul et al., 2001; El Kebir et al., 2007). Concomitant activation of Akt and ERK appears to be required for suppression of neutrophil apoptosis, and transient activation of Akt without ERK activation may not be sufficient to delay the death program. Contradictory results have been reported for p38 MAPK; its action on neutrophil survival may be stimulus and/or context-specific (reviewed in Filep and El Kebir, 2010). For example, pro-survival function of p38 MAPK may include phosphorylation, and therefore inactivation of caspase-3 and caspase-8 (Alvarado-Kristensson et al., 2003). In other studies, constitutive or TNF-induced neutrophil apoptosis was found to be associated with phosphorylation of p38 MAPK (Khreiss et al., 2002; El Kebir et al., 2007). Activation of p38 MAPK by sodium salicylate is associated with reduced Mcl-1 expression and acceleration of apoptotic cell death (Derouet et al., 2006).

NEUTROPHIL β_2 INTEGRINS MODULATE LIFE AND DEATH DECISIONS

β_2 INTEGRIN ACTIVATION AND FUNCTION

The β_2 integrin ($\alpha\beta$) family consists of LFA-1 (leukocyte function-associated antigen 1, CD11a/CD18), Mac-1 (CD11b/CD18, $\alpha_M\beta_2$ integrin, ITAM antigen), p150,95 (CD11c/CD18, $\alpha_X\beta_2$ integrin, ITAX antigen), and $\alpha_d\beta_2$ (CD11d/CD18, ITAD antigen). The β_2 integrins are in an inactive (low affinity) conformation on circulating leukocytes. Leukocyte agonists trigger inside-out signaling that through activation of Rap1 (reviewed in Evans et al., 2009) induces conformational changes that reflect the intermediate and high affinity states of Mac-1 (Xiong et al., 2001; Luo et al., 2007). Ligand occupancy, but not integrin clustering promotes switchblade-like extension of the Mac-1 extracellular domain and separation of the α_M and β_2 subunit cytoplasmic tails, structural hallmarks of integrin activation (Lefort et al., 2009). These lead to enhanced affinity for binding their ligand and/or regulation of avidity. Integrin activation is a complex and tightly regulated process which involves displacement of inhibitory proteins from the integrin cytoplasmic tail followed by targeting integrin activators or activator complexes, such as talin, kindlins, integrin-linked kinase, and migfilin (Kim et al., 2011). β_2 integrins contribute to diverse neutrophil functions critical for innate immunity. Activated β_2 integrins mediate leukocyte adhesion and transmigration across the endothelium through interactions with ICAM-1 on the activated endothelial cells (Ley et al., 2007; Abram and Lowell, 2009). Mac-1 also mediates other neutrophil adhesion-dependent functions, including binding to fibrinogen (Pluskota et al., 2004), immune complexes, and platelets (through Gp1b) (Mayadas and Cullere, 2005) and suppression of T cell proliferation (Pillay et al., 2012). Mac-1 and CD11c/CD18 are specific receptors for complement iC3b and mediate efficient phagocytosis of complement-opsonized targets, though they can also recognize many pathogens directly (Ross, 2000). Mac-1 functionally cooperates with other surface receptors, including TNF receptor, FcγRs, Toll-like receptor 2 (TLR2), and CD14 (Ehlers, 2000; Ross, 2000; Kobayashi et al., 2002; Salamone et al., 2004).

Mac-1-MEDIATED PRO-SURVIVAL SIGNALING

Outside-in signaling through Mac-1 could generate contrasting cues for neutrophils in a context-dependent fashion (**Figure 1**). Transendothelial migration of neutrophils (Watson et al., 1997; Yan et al., 2004) or neutrophil adherence to Mac-1 ligands, ICAM-, fibrinogen, and plasminogen, prolongs their lifespan by delaying apoptosis (**Table 1**). Binding of ICAM-1 to Mac-1 induces activation of the PI3k/Akt survival pathway (Whitlock et al., 2000). Fibrinogen-mediated suppression of neutrophil apoptosis also depends on Akt in addition to activation of NF-κB and the MAPK/ERK pathway (Rubel et al., 2003). Crosslinking activated Mac-1 with anti-Mac-1 antibody (Fab fragments) or clustering inactive Mac-1 in neutrophils in suspension signals survival cues through activation of Akt and ERK (Whitlock et al., 2000). Soluble fibrinogen activates neutrophils, as assessed by upregulation of Mac-1 expression and elevation of intracellular calcium concentration (Rubel et al., 2003; Pluskota et al., 2008), indicating that Mac-1-mediated adhesion *per se* is not a prerequisite for generation of survival signals. Engagement of both Mac-1 subunits with these ligands is a prerequisite for induction of pro-survival signals (Pluskota et al., 2008). Consistently, angiostatin, derived from plasminogen and neutrophil inhibitory factor (NIF), which interact primarily with the α_M subunit do not trigger phosphorylation of ERK 1/2 and Akt and do not rescue neutrophils from constitutive apoptosis (Pluskota et al., 2008).

Heparin also binds to Mac-1. Immobilized heparin can mediate leukocyte adhesion (Diamond et al., 1995), whereas unfractionated soluble heparin was reported to inhibit binding of fibrinogen

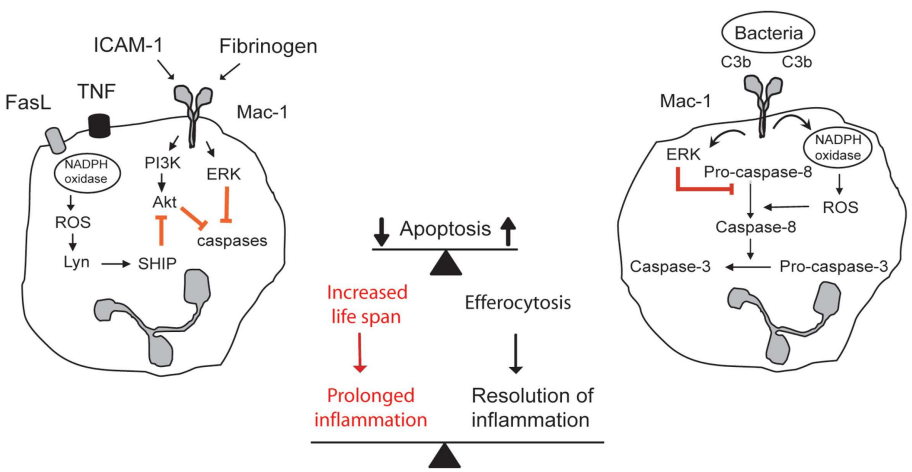

FIGURE 1 | Contrasting outside-in signals through Mac-1 modulates survival and death decision in neutrophils. Aging neutrophils undergo constitutive apoptosis triggered by collapse of mitochondrial function. Engagement of Mac-1 with its ligands ICAM-1, fibrinogen, or MPO delays apoptosis by generating survival cues through activation of the PI3K/Akt and MEK/ERK pathways. Additional stimulation of death receptors with TNF or FasL evokes release of ROS, which through activation of lyn and SHIP leads to inhibition of Akt. Mac-1-mediated phagocytosis of opsonized bacteria evokes ROS production by NADPH oxidase, leading to ROS-mediated activation of caspase-8 and inhibition of ERK, and acceleration of the cell death program. Apoptotic neutrophils are recognized and phagocytosed by macrophages (efferocytosis), thus contributing to inflammatory resolution. Extended neutrophil longevity contributes to aggravation and prolongation of the inflammatory response.

Table 1 | Selective regulation of neutrophil apoptosis by different ligands of Mac-1.

Ligand	Action(s)	Effect on apoptosis	Reference
ICAM-1	Mediates PMN adhesion and transmigration	Suppresses apoptosis	Watson et al. (1997) Whitlock et al. (2000) Yan et al. (2004)
Fibrinogen	Precursor of fibrin initiates coagulation	Suppresses apoptosis	Rubel et al. (2001) Rubel et al. (2003) Pluskota et al. (2004, 2008)
Plasminogen	Precursor of plasmin initiates fibrinolysis	Suppresses apoptosis	Pluskota et al. (2008)
Angiostatin	Inhibits angiogenesis	No effect	Pluskota et al. (2008)
Myeloperoxidase	Bacterial killing upregulates Mac-1 expression induces MPO release	Suppresses apoptosis	El Kebir et al. (2008) Lau et al. (2005)
Heparin	Anticoagulant		
Immobilized	mediates leukocyte adhesion	Induces apoptosis	Diamond et al. (1995)
Soluble	inhibits binding of fibrinogen	Not known	Manaster et al. (1996)
Soluble, low molecular weight		No effect	Peters et al. (1999) Erduran et al. (1999) Brown et al. (2012)
Opsonized bacteria	Phagocytosis destruction of bacteria	Induces apoptosis	Coxon et al. (1996) Watson et al. (1999) Arroyo et al. (2002) Zhang et al. (2003) Perskvist et al. (2002) DeLeo (2004)

MPO, myeloperoxidase; PMN, polymorphonuclear granulocytes.

and complement iC3b to Mac-1 (Peters et al., 1999). Inconsistent reports have been published on the effect of heparin on neutrophil lifespan. Unfractionated heparin was reported to induce apoptosis (Manaster et al., 1996), whereas low molecular weight heparin did not affect neutrophil apoptosis (Erduran et al., 1999; Brown et al., 2012).

In the presence of TNF or anti-Fas activating antibody, crosslinking Mac-1 with activating antibodies promotes neutrophil apoptosis (Whitlock et al., 2000). Activation of TNF receptor or Fas results in NADPH oxidase-mediated ROS generation and extracellular ROS release, leading to activation of SHIP [Src-homology 2(SH2)-containing inositol 5-phosphatase], which hydrolyzes PI3K products through the Src kinase Lyn (Gardai et al., 2002). This would lead to decreased Akt phosphorylation. Although Lyn was reported to exert anti-apoptotic actions in granulocytes (Daigle et al., 2002), exogenous H_2O_2 can activate Lyn independently of adhesion (Yan and Berton, 1996). ROS, most likely H_2O_2 that was released extracellularly might diffuse back into cells to suppress the PI3K/Akt survival signal (Zhu et al., 2006; Xu et al., 2010). Whether ROS affect signaling pathways leading to activation of PI3K or through activation of PTEN (phosphatase and tensin homolog) is still uncertain. PTEN converts phosphatidylinositol 3,4,5-triphosphate to phosphatidylinositol-4,5-diphosphate, thus preventing activation of Akt. PTEN-null neutrophils live longer than wildtype neutrophils (Zhu et al., 2006). In contrast, crosslinking Mac-1 with clustering non-activating antibodies or recombinant ICAM-1 results in partial attenuation of Fas-triggered apoptosis through sustained ERK activation and elevation in reduced glutathione (GSH) levels (Watson et al., 1997; Whitlock et al., 2000). The biological significance of differences in neutrophil responses to Mac-1 activating versus clustering antibodies remains to be investigated. TNF promotion of neutrophil apoptosis evoked by immune complexes or zymosan partially depends on Mac-1 (Salamone et al., 2004), suggesting functional cooperation of Mac-1 with Fcγ or zymosan receptors (Ehlers, 2000; Ross, 2000).

While most studies investigated Mac-1 signaling, ligation of LFA-1 may also generates contrasting cues for neutrophils. LFA-1-deficient mice exhibit neutrophilia and enhanced resistance to *L. monocytogenes* without changes in spontaneous apoptosis (Miyamoto et al., 2003). Similar to Mac-1, ligation of LFA-1 during transendothelial migration suppresses caspase-3 activation and thus delays apoptosis in neutrophils (Yan et al., 2004). In contrast, activation of ICAM-3 with a monoclonal antibody that recognizes an ICAM-3 epitope that binds its ligand LFA-1 was found to induce apoptosis (Kessel et al., 2006).

Mac-1-MEDIATED ACCELERATION OF APOPTOSIS

Phagocytosis of opsonized bacteria or other targets accelerates apoptosis in neutrophils, also referred to as phagocytosis-induced cell death or PICD (Coxon et al., 1996; Watson et al., 1999; Perskvist et al., 2002; DeLeo, 2004). Mac-1-mediated phagocytosis evokes

NADPH-dependent ROS generation within the phagolysosomes (Karlsson and Dahlgren, 2002) and is thought to contribute to killing bacteria (Nauseef, 2007) as well as to triggering PICD (Watson et al., 1996; Zhang et al., 2003). Consistently, patients with chronic granulomatous disease, who have low level of NADPH oxidase due to genetic mutations in $gp91^{phox}$ or other phox genes, suffer from recurrent infections (Heyworth et al., 2003) and their neutrophils do not undergo PICD (Coxon et al., 1996).

Mac-1-mediated phagocytosis evokes generation of NADPH oxidase-derived ROS, which, in turn, leads to activation of caspase-8 and subsequently caspase-3 (Arroyo et al., 2002; Zhang et al., 2003). ROS, most likely hydroxyl radicals and H_2O_2 (Watson et al., 1996; Perskvist et al., 2002) released within phagosomes might leak and trigger PICD. Superoxide released extracellularly is rapidly dismutated to H_2O_2, which might diffuse back to the cell to affect the redox status and signaling pathways. As caspase-8 activation is a signature of death receptor-mediated apoptosis, Mac-1 has been suggested to function as a death receptor, even though it does not contain a recognized death effector domain (Mayadas and Cullere, 2005). It should be noted that many stimuli that generate varying amounts of NADPH oxidase-derived ROS do not trigger neutrophil death. For example, GM-CSF enhances ROS production upon yeast phagocytosis, but inhibits PICD (Zhang et al., 2003). Thus, the amount, nature, and intra- or extracellular release of NADPH oxidase-derived ROS would likely determine their pro-apoptosis potential. PICD occurs despite phagocytosis-induced activation of the MAPK/ERK pathway (Zhang et al., 2003), indicating that ROS triggered pro-apoptosis signals effectively override survival cues. In contrast, GM-CSF evokes a more robust ERK phosphorylation in phagocytosing neutrophils, leading to generation of strong competing survival signals that shift the life-death balance toward survival (Zhang et al., 2003).

MYELOPEROXIDASE: A LIGAND FOR Mac-1 AND SURVIVAL SIGNAL FOR NEUTROPHILS

An unexpected ligand for Mac-1 is myeloperoxidase (MPO), the most abundant granule enzyme in neutrophils (Schultz and Kaminker, 1962; Borregaard and Cowland, 1997). MPO, MPO-generated reactive oxidants, hypochlorous acid (HOCl) in particular and diffusible radical species have been implicated in the elimination of microbes (Klebanoff, 2005; Nauseef, 2007; Davies et al., 2008) as well as in inflicting tissue damage (Klebanoff, 2005; Winterbourn, 2008; Arnhold and Flemming, 2010). Non-activated neutrophils bind to MPO-coated surfaces (Johansson et al., 1997) or "free" circulating MPO through Mac-1 (Lau et al., 2005). Increased MPO association with neutrophil membrane was detected in blood from patients with inflammatory diseases, including sepsis, ischemia-reperfusion, or acute coronary syndromes compared with healthy controls (Lau et al., 2005). Membrane-bound MPO correlates with plasma MPO levels, indicating up-regulation of MPO export toward the plasma membrane as well as a potential for binding of free MPO to the neutrophil surface.

MPO binding to Mac-1 on human neutrophils leads to increased tyrosine phosphorylation (Lau et al., 2005), phosphorylation of p38 MAPK (Lau et al., 2005; El Kebir et al., 2008), ERK 1/2 and PI3K (El Kebir et al., 2008), and activation of NF-κB (Lau et al., 2005). Activation of p38 MAPK induces phosphorylation of $p47^{phox}$, the cytoplasmic regulatory subunit of NADPH oxidase (Babior, 2004), leading to superoxide formation (Lau et al., 2005), and regulates NF-κB-mediated transcription of genes involved in the acute inflammatory response (Park et al., 2003). Intriguingly, MPO also upregulates surface expression of Mac-1 on neutrophils (Lau et al., 2005; El Kebir et al., 2009) through yet unidentified molecular mechanisms. MPO binding to Mac-1 induces release of elastase and MPO from the azurophilic granules (Lau et al., 2005). These findings are consistent with the central role of Mac-1-mediated outside-in signaling in degranulation (Harris et al., 2000), and imply an autocrine/paracrine mechanism for amplifying neutrophil responses to MPO (**Figure 2**) (El Kebir et al., 2008, 2009).

MPO, independent of its catalytic activity, rescues neutrophils from constitutive apoptosis through simultaneous activation of ERK 1/2 and Akt, Mcl-1 accumulation and suppression of the mitochondrial pathway of apoptosis (El Kebir et al., 2008). Consistently, MPO prevents cytochrome c release from the mitochondria and subsequent activation of caspase-3 (El Kebir et al., 2008). MPO-induced phosphorylation of p38 MAPK does not generate a survival signal, for pharmacological blockade of p38 MAPK retards neutrophil apoptosis both in the absence and presence of MPO (El Kebir et al., 2008). In contrast to these findings, MPO was found to mediate apoptosis in HL60 leukemia cells (Wagner et al., 2000; Kanayama and Miyamoto, 2007). While the involvement of Mac-1 in the pro-apoptosis action of MPO has not been elucidated, Mac-1 may play opposing roles in determining the fate of primary and leukemia cells likely by shifting the balance of pro-survival and pro-apoptosis cues. It is intriguing that MPO can prolong the lifespan of neutrophils, the predominant source of this enzyme and that function-blocking monoclonal anti-Mac-1 antibodies can almost completely prevent MPO-induced activation (Lau et al., 2005; El Kebir et al., 2008) and suppression of constitutive apoptosis of human neutrophils *in vitro* (El Kebir et al., 2008).

MYELOPEROXIDASE PROLONGS NEUTROPHIL LIFESPAN AND DELAYS RESOLUTION OF INFLAMMATION

Acute elevation of plasma MPO levels to levels similar to those detected in patients with inflammatory vascular diseases (Brennan et al., 2001; Baldus et al., 2003) results in prolongation of the lifespan of rat neutrophils through suppression of apoptosis as assayed *ex vivo* (El Kebir et al., 2008). MPO also suppresses neutrophil apoptosis in a mouse model of carrageenan-induced lung injury and delays spontaneous self-resolution of pulmonary inflammation (El Kebir et al., 2008). Thus, combined administration of carrageenan and MPO evokes persisting lung injury/inflammation with few airway neutrophil exhibiting signs of apoptosis even 5 days post-injection, when pulmonary inflammation is almost completely resolved in the lungs of carrageenan-injected mice. The effects of MPO closely resemble those of zVAD-fmk, a pan-caspase inhibitor, which aggravates and prolongs carrageenan-elicited acute pleurisy (Rossi et al., 2006) and lung inflammation (El Kebir et al., 2008). MPO-deficient mice exhibit lower pulmonary bacterial colonization, reduced lung injury, and greater survival following intraperitoneal injection of *Escherichia coli* compared

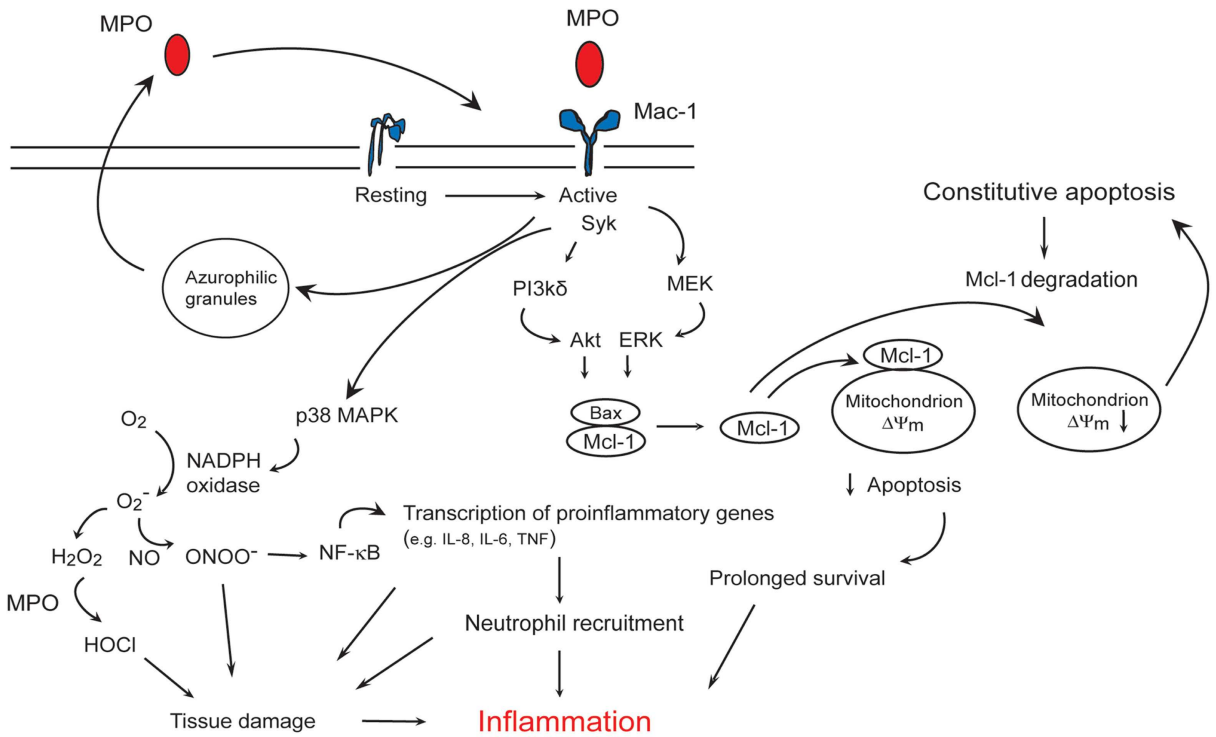

FIGURE 2 | Myeloperoxidase (MPO)-Mac-1 self-amplifying circuit promotes neutrophils survival and inflammation. MPO binding to Mac-1 results in p38 MAPK-mediated NADPH oxidase, and PI3K/Akt and MEK/ERK-mediated preservation of the anti-apoptotic protein Mcl-1, leading to suppression of apoptosis. MPO also triggers MPO release from the azurophilic granules, and upregulates Mac-1 expression, thereby forming an autocrine/paracrine self-amplifying circuit. MPO catalyzed formation of HOCl, activation of NF-κB-regulated transcription of pro-inflammatory genes, recruitment of neutrophils, and prolongation of neutrophil longevity contribute to tissue damage and inflammation.

with wild type mice (Brovkovych et al., 2008). MPO deficiency also reduces ischemia/reperfusion-induced renal dysfunction and neutrophil accumulation in mice, but fails to abrogate apoptosis during early phases of reperfusion (Matthijsen et al., 2007). Intriguingly, MPO-deficient mice exhibit elevated baseline pulmonary inducible NO synthase expression and NO production that may partially compensate for the lack of HOCl-mediated bacterial killing (Brovkovych et al., 2008). The mechanism(s) responsible for upregulation of inducible NO synthase as well as the impact of enhanced NO production on the resolution of lung inflammation remains to be explored. Absence of MPO-derived oxidant production during *E. coli* septicemia in MPO-null mice is consistent with reduced lung injury and mortality. Further studies are required to investigate whether MPO deficiency could affect the lifespan of emigrated or circulating neutrophils, and whether changes in neutrophil longevity could contribute to protection against lung injury in this model of sepsis.

TARGETING Mac-1 SIGNALING FOR THERAPEUTIC INDUCTION OF NEUTROPHIL APOPTOSIS

Accumulating experimental and clinical data suggest a causal relationship between neutrophil apoptosis and outcome of inflammation. Apoptosis of emigrated neutrophils has multiple pro-resolution actions. It renders neutrophils unresponsive to agonists and apoptotic neutrophils stop producing and releasing pro-inflammatory mediators. Apoptotic leukocytes sequester cytokines (Ariel et al., 2006; Ren et al., 2008) and phagocytosis of apoptotic cells induces macrophage polarization from a pro-inflammatory to a pro-resolution phenotype (Fadok et al., 1998; Ariel and Serhan, 2012; Sica and Mantovani, 2012). Injection of large number of apoptotic neutrophils protects mice against LPS-induced shock (Ren et al., 2008). Recent studies identified several classes of molecules for therapeutic induction of apoptosis in neutrophils for enhancing the resolution of inflammation.

LIPOXINS INHIBIT MPO SIGNALING THROUGH Mac-1 AND REDIRECTS NEUTROPHILS TO APOPTOSIS

The pivotal role of MPO in host defense and tissue injury makes it an attractive target for drug development. Indeed, while a number of promising compounds have been developed to inhibit the enzymatic activities of MPO, only limited information is available on their mechanisms of MPO inhibition and biological activities (reviewed in Malle et al., 2007). Targeting MPO signaling through Mac-1 has emerged as an alternative approach to counter the non-enzymatic activities of MPO.

Lipoxin A_4 (LXA_4) and aspirin-triggered 15-epi-LXA_4 are typically generated by transcellular biosynthesis at sites of inflammation (Serhan et al., 2008; Serhan, 2011). In the aspirin-triggered pathway, acetylation of cyclooxygenase at Ser^{530} by aspirin (Clària and Serhan, 1995) or S-nitrosylation at Cys^{526} by atorvastatin

(Birnbaum et al., 2006) catalyzes the conversion of arachidonate to 15R-HETE that can be converted by neutrophils and other cells to 15-epi-LXA_4 and 15-epi-LXB_4. LXA_4 and 15-epi-LXA_4 possess potent anti-inflammatory and pro-resolution actions predominantly through affecting the function of leukocytes. Lipoxins stimulate recruitment of monocytes and inhibit neutrophil trafficking and accumulation in inflamed tissues (reviewed in Serhan et al., 2007; Serhan et al., 2008). These actions are, in part, mediated through down-regulation of leukocyte Mac-1 expression (Fiore and Serhan, 1995; Filep et al., 1999). Accumulation of PGE_2 at inflammatory sites induces a lipid mediator class switching from a predominantly 5-lipoxygenase activity to a 15-lipoxygenase activity generating LXA_4 parallel with the resolution of inflammation (Levy et al., 2001) Thus, initiation of an inflammatory response would also activate subsequent pro-resolution mechanisms (Serhan and Savill, 2005).

Lipoxins exert multipronged actions to counter neutrophil responses to MPO. Down-regulation of Mac-1 expression on neutrophils and inhibition of neutrophil adhesion and transendothelial migration are important components of the anti-inflammatory activities of LXA_4 and 15-epi-LXA_4 (Serhan et al., 2008). 15-epi-LXA_4 also prevents MPO-induced up-regulation of Mac-1 expression and MPO release, thereby interrupting MPO-mediated autocrine/paracrine loop for perpetuation of the inflammatory response (**Figure 3**) (El Kebir et al., 2009). 15-epi-LXA_4 inhibition of NADPH oxidase-derived superoxide generation (Levy et al., 1999) and subsequent formation of $ONOO^-$ (József et al., 2002) result in reduced NF-κB activation and transcription of pro-inflammatory cytokines, such as IL-8 (József et al., 2002).

Lipoxins themselves do not appear to interfere with the apoptotic machinery in neutrophils, whereas they can override the potent outside-in Mac-1-mediated survival signal and redirect neutrophils to apoptosis *in vitro* (El Kebir et al., 2009). 15-epi-LXA_4 attenuates MPO-evoked ERK and Akt-mediated phosphorylation of the pro-apoptotic protein Bad and decreases Mcl-1 expression, critical events in enhancing neutrophil apoptosis. Non-phosphorylated Bad associates with Mcl-1 and prevents its anti-apoptotic actions (Reed, 2006). These would aggravate mitochondrial dysfunction, ultimately leading to caspase-3-mediated cell death (El Kebir et al., 2009; Wardle et al., 2011).

Treatment of mice with 15-epi-LXA_4 at the peak of inflammation enhances resolution of carrageenan plus MPO-induced and *E. coli* septicemia-associated acute lung injury and improves the survival rate (El Kebir et al., 2009). 15-epi-LXA_4 reduces pulmonary neutrophil accumulation with concomitant increases in the percentage of apoptotic neutrophils in the airways, facilitates recruitment of monocytes/macrophages and phagocytosis of apoptotic neutrophils and other cells (El Kebir et al., 2009), consistent with tissue repair (Godson et al., 2000; Mitchell et al., 2002). Furthermore, LXA_4 released at sites of inflammation protects macrophages from apoptosis (Prieto et al., 2010). The beneficial actions of 15-epi-LXA_4 can be prevented in the presence of a pan-caspase inhibitor, indicating the importance of neutrophil apoptosis in inflammatory resolution. Recent results indicate that aspirin or lovastatin reduction of acid aspiration-induced lung inflammation is, in part, mediated through stimulation of synthesis of 15-epi-LXA_4 (Fukunaga et al., 2005; Planaguma et al., 2010). The direct effect of lovastatin on neutrophil apoptosis remains, however, to be investigated. Both aspirin and sodium salicylate promote neutrophil apoptosis and enhance their phagocytosis by macrophages in thioglycollate-induced peritonitis (Negrotto et al., 2006). A recent study reported that serum amyloid A acting through the formyl-peptide receptor 2/lipoxin receptor (FPR2/ALX) can overwhelm anti-inflammatory signaling by LXA_4 to mediate exacerbation of glucocorticoid refractory lung inflammation in patients with chronic obstructive pulmonary disease (Bozinovski et al., 2012).

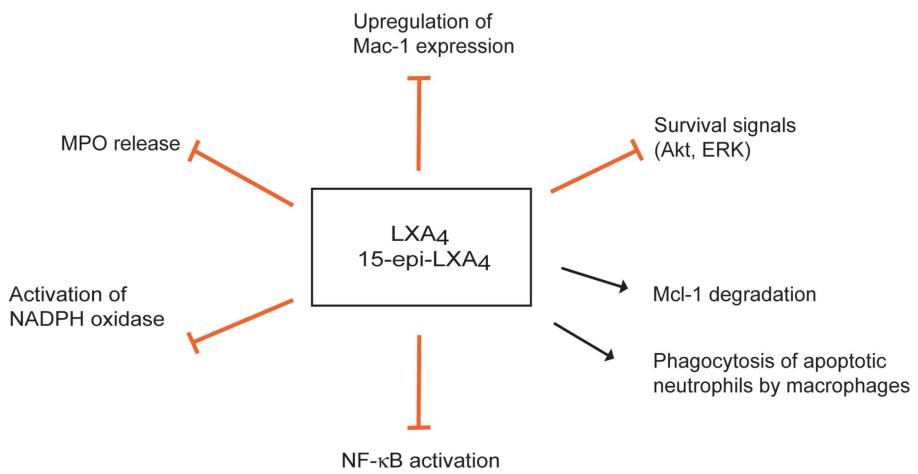

FIGURE 3 | Multipronged actions of lipoxins to inhibit the myeloperoxidase (MPO)-Mac-1 circuit. Lipoxin A_4 (LXA_4) and aspirin-triggered 15-epi-LXA_4 predominantly through FPR2/ALX attenuate MPO-stimulated degranulation, upregulation of surface expression of Mac-1, and superoxide formation, thus effectively interrupting this loop. LXA_4 and15-epi-LXA_4 redirect neutrophils to apoptosis by overriding the powerful survival signals from MPO through inducing loss of expression of Mcl-1 and aggravating mitochondrial dysfunction. Lipoxins also enhance phagocytosis of apoptotic neutrophils by macrophages.

RESOLVIN E1 PROMOTES PHAGOCYTOSIS-INDUCED NEUTROPHIL APOPTOSIS

Resolvin E1 is synthesized from the ω-3 polyunsaturated fatty acid eicosapentaenoic acid during the resolution phase of acute inflammation with leukocyte 5-lipoxygenase playing a pivotal temporal role in the biosynthesis pathway (Serhan et al., 2000; Oh et al., 2011). RvE1 binds to the ChemR23 and (as a partial agonists/antagonist) the leukotriene B_4 (LTB_4) receptor BLT1 (Arita et al., 2005; Oh et al., 2011) and exhibits potent anti-inflammatory and pro-resolution activities. Thus, RvE1 inhibits neutrophil recruitment, facilitates efferocytosis (Serhan et al., 2002, 2008; Ohira et al., 2010; Oh et al., 2011; Serhan and Petasis, 2011), promotes mucosal surface clearance (Campbell et al., 2007), and induces generation of LXA_4 (Haworth et al., 2008).These potent pro-resolution actions were also demonstrated in various experimental models, including peritonitis (Oh et al., 2011), polymicrobial sepsis (Schwab et al., 2007), and bacterial pneumonia (Seki et al., 2010) Moreover, studies on ChemR23-null mice demonstrated an endogenous anti-inflammatory role for ChemR23 (Cash et al., 2008).

Recent results indicate that RvE1 can modulate neutrophil apoptosis (El Kebir et al., 2012). While at low nanomolar concentrations, RvE1 *per se* does not affect the constitutive death program in neutrophils, it enhances Mac-1-mediated phagocytosis of complement-opsonized bacteria and yeast, leading to increased ROS generation by NADPH oxidase and subsequent activation of caspase-8 and caspase-3 (El Kebir et al., 2012). RvE1 also attenuates ERK and Akt-mediated survival cues generated by MPO and decreases Mcl-1 expression, thereby reinforcing the shift toward apoptosis (El Kebir et al., 2012). These actions of RvE1 are predominantly mediated via BLT1 *in vitro*, indicating that resolution mechanisms may also be activated via this type of LTB_4 receptor. In contrast, RvE1 stimulation of phagocytosis of live *E. coli* and apoptotic neutrophils by macrophages leads to a macrophage phenotype switch without evoking apoptosis (Arita et al., 2005; Schwab et al., 2007; Oh et al., 2011). Since in macrophages RvE1 signals via ChemR23 (Ohira et al., 2010), RvE1 may exert different pro-resolution actions via distinct receptors, and concurrent activation of these circuits may be critical for optimal resolution. The neutrophil apoptosis-promoting action of RvE1 was also evident in experimental models of acute respiratory distress (ARDS) and pneumonia (El Kebir et al., 2012), in which MPO has been implicated in mediating lung injury. RvE1 administered at the peak of inflammation, promoted apoptosis in neutrophils *in situ*, enhanced recruitment of monocytes to the airways, and facilitated clearance of apoptotic neutrophils and other cells and tissue repair (El Kebir et al., 2012), consistent with the original properties defining RvE1 actions (Serhan et al., 2008). Efficient resolution of acute lung inflammation is intimately linked to apoptosis of neutrophils within the airways, as the pan-caspase inhibitor zVAD-fmk prevented RvE1-induced dramatic reduction in the number of infiltrating neutrophils (El Kebir et al., 2012) and aggravated lung injury likely due to persisting presence of neutrophils. Eicosapentaenoic acid is also a substrate for acetylated COX-2, which generates aspirin-triggered resolvins that shares anti-inflammatory actions of native resolvins (Spite and Serhan, 2010). These would raise the possibility that resolvin-triggered phagocytosis-induced neutrophil apoptosis could contribute to the beneficial actions of aspirin.

OTHER APPROACHES TO INDUCE NEUTROPHIL APOPTOSIS *IN VIVO*

Given the complexity of pathways involved in the regulation of neutrophil apoptosis, a number of agents have been identified that could shift the balance of survival and pro-apoptosis cues toward apoptosis. More importantly, some of these agents have been found to possess pro-resolution properties in diverse models of inflammation (see also **Figure 4**).

At the light of its pre-eminence as a key survival protein, Mcl-1 is an attractive target for therapeutic induction of apoptosis. The cyclin-dependent kinase inhibitor R-roscovitine (Seliciclib or CYC202) accelerates degradation of Mcl-1 and inhibits transcriptional activity in neutrophils by preventing cyclin-dependent kinase (CDK) 7 and 9-mediated phosphorylation of RNA polymerase II (Leitch et al., 2012), thereby inducing apoptosis in inflammatory cells *in vitro* (Rossi et al., 2006; Leitch et al., 2012). Consistently, treatment of with R-roscovitine enhances resolution of pleuritis and bleomycin-induced lung injury (Rossi et al., 2006) and pneumococcal meningitis in mice (Koedel et al., 2009), coinciding with increased numbers of apoptotic neutrophils in the airways and cerebrospinal fluid, respectively. R-roscovitine induces apoptosis in neutrophils from patients with cystic fibrosis, whereas the selective CFTR inhibitor $CFTR_{Inh172}$ does not affect constitutive apoptosis in neutrophils from healthy volunteers (Moriceau et al., 2010). Suppressed neutrophil apoptosis in cystic fibrosis is likely not simply a consequence of chronic infection. Since cystic fibrosis is associated with enhanced formation of MPO-generated oxidants, it would be interesting to know whether MPO is one of the yet unidentified modulatory factors intrinsic to CF.

The pro-resolution mediator annexin A1 binds to FPR2/ALX, which is also a receptor for LXA_4 and 15-epi-LXA_4, and accelerates neutrophil apoptosis in various murine models of inflammation by decreasing survival signals and Mcl-1 expression (Solito et al., 2003; Perretti and D'Acquisto, 2009; Perretti, 2012). A recent study reported that annexin A1 in inflammatory exudates promotes active resolution and augments neutrophil apoptosis in LPS-induced pleurisy in mice (Vago et al., 2012). Intriguingly, annexin A1-deficient mice exhibit an exaggerated inflammatory response and reduced Mac-1 expression on neutrophils (Hannon et al., 2003). Whether the pro-resolution actions of annexin A1 also involve modulation of signaling through Mac-1 remain to be explored. The phosphodiesterase 4 inhibitor rolipram has also recently been found to promote neutrophil apoptosis and resolution of LPS-induced pleurisy in mice by decreasing PI3K/Akt survival signaling and Mcl-1 expression (Sousa et al., 2010).

Another possibility is induction of caspase activity, the major effectors of apoptosis. Although selective caspase activators are currently not available, recent studies demonstrated the pro-resolving action of the death receptor ligand TRAIL, which may function as a physiological brake to limit the inflammatory

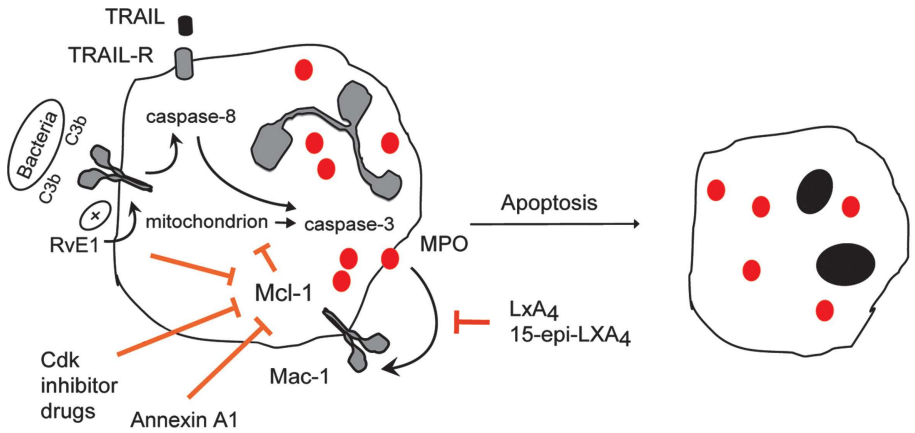

FIGURE 4 | Therapeutic induction of neutrophil apoptosis with demonstrated pro-resolution actions *in vivo*. Treatment of experimental animals with CDK inhibitor drugs (e.g., R-roscovitine), 15-epi-LXA_4, RvE1, rTRAIL, or annexin A1 enhance the resolution of inflammation by promoting apoptosis of neutrophils that have emigrated into tissues in various models of inflammation. Roscovitine through yet unidentified mechanisms, 15-epi-LXA_4 through attenuating MPO-triggered Mac-1-mediated survival signaling and annexin A1 through dampening intracellular survival signaling down-regulate expression of the key survival protein Mcl-1. RvE1 enhances phagocytosis-induced apoptosis, leading to activation of caspase-8 and suppression of Mcl-1.rTRAIL activates caspase-8.

response (Leitch et al., 2011). Thus, TRAIL-deficiency in mice is associated with delayed neutrophil apoptosis and exaggerated inflammatory response (Falschlehner et al., 2009; McGrath et al., 2011). Conversely, rTRAIL was found to facilitate neutrophil apoptosis through inducing activation of caspase-8 both *in vitro* and *in vivo* (Renshaw et al., 2003; McGrath et al., 2011). Consistent with enhanced neutrophil apoptosis, treatment with rTRAIL accelerated resolution of LPS-induced lung injury and zymosan-induced peritonitis in TRAIL-deficient mice (McGrath et al., 2011). Up-regulation of TRAIL has also been implicated in mediating TLR4 signaling through IFN-β to promote neutrophil apoptosis and limiting lung inflammation in a mouse model of ARDS (Leu et al., 2011).

Targeting pro-survival pathways to promote resolution has also been investigated at the level of MAPK and NF-κB signaling. As discussed above, pharmacological blockade of ERK 1/2 and/or PI3K prevents GM-CSF or MPO-induced neutrophil survival *in vitro*. The efficacy of the MEK/ERK inhibitor PD98059 in the resolution of inflammation was also demonstrated in the carrageenan-induced pleurisy model in rats (Sawatzky et al., 2006). NF-κB blockers have potential anti-inflammatory actions (Gosh and Hayden, 2008) and may also affect resolution. An oligonucleotide decoy to NF-κB was found to enhance neutrophil apoptosis and phagocytosis by macrophages in a rat model of chronic inflammation (Maiuri et al., 2004). Increased apoptosis correlated with increases in p53 or Bax expression and decreases in Bcl-2 protein expression. Systemic injection of a cell-permeable form of IκBα (Tat-srIκBα chimera) reduced leukocyte trafficking into the pleural cavity and increased caspase-3 activity and apoptosis in emigrated cells (Blackwell et al., 2004). In contrast, the Tat-srIκBα chimera produced only marginal reductions in neutrophil migration and apoptosis if administered locally (Blackwell et al., 2004), indicating that the route and timing of administration may be critical for exerting beneficial actions. In another study, selective NF-κB inhibitors failed to affect neutrophil accumulation in the pleural cavity (Sousa et al., 2010).

CONCLUSION

Intensive research during the past decade has revealed that inflammation does not terminate spontaneously; rather resolution is a tightly controlled active process. During the past decade, a number of novel mediators, including lipids, peptides, and proteins, and signaling circuits have been identified. Timely and efficient removal of migrated neutrophils requires these cells to undergo apoptosis. A growing body of evidence supports an important role for neutrophil apoptosis as a critical control point for the outcome of inflammation. Neutrophil surface receptors, including the adhesion molecule Mac-1 integrates opposing cues that modulate life and death decisions and therefore the outcome of inflammation. Outside-in signaling through Mac-1 is also a target for endogenous molecules, such as lipoxins, resolvin E1, and synthetic compounds to counter pro-survival and/or induce pro-apoptosis signals. Interfering with Mac-1 function may have two important benefits: inhibition of neutrophil trafficking into the inflamed site and acceleration of neutrophil clearance from inflamed tissues through the process of efferocytosis. Indeed, results from experimental models demonstrate that redirecting neutrophils to apoptosis and facilitating their clearance by macrophages are essential for enhancing resolution of acute inflammation. While clinical trials with these compounds remain distant, therapeutic induction of neutrophil apoptosis at the inflammatory site hold promise as a powerful pro-resolving intervention and may fulfill urgent, yet unmet clinical needs to prevent the deleterious consequences of inflammation.

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NOD1 and NOD2 signaling in infection and inflammation

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Sensing intracellular pathogens is a process mediated by innate immune cells that is crucial for the induction of inflammatory processes and effective adaptive immune responses against pathogenic microbes. NOD-like receptors (NLRs) comprise a family of intracellular pattern recognition receptors that are important for the recognition of damage and microbial-associated molecular patterns. NOD1 and NOD2 are specialized NLRs that participate in the recognition of a subset of pathogenic microorganisms that are able to invade and multiply intracellularly. Once activated, these molecules trigger intracellular signaling pathways that lead to the activation of transcriptional responses culminating in the expression of a subset of inflammatory genes. In this review, we will focus on the role of NOD1 and NOD2 in the recognition and response to intracellular pathogens, including Gram-positive and Gram-negative bacteria, and on their ability to signal in response to non-peptidoglycan-containing pathogens, such as viruses and protozoan parasites.

Keywords: NOD1, NOD2, RIPK2, intracellular pathogens, innate immunity

INTRODUCTION

The immune system is able to recognize a large variety of microorganisms and their molecules through different receptors expressed by innate immune cells, such as macrophages, neutrophils, NK cells, and dendritic cells. The initial recognition of pathogenic microorganisms is critical for the generation of an appropriate acquired immune response. This process occurs through the interaction of microbial- or damage-associated molecular patterns (MAMPs or DAMPs, respectively) with the specific pattern recognition receptors (PRRs) present on the host cell surface or in the cytosolic compartment. Among the PRRs, the Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like helicases (RLRs), and AIM2-like receptors (ALRs) have been extensively investigated and play important roles as the major PRRs, forming the first line of defense against infectious agents (reviewed in Kawai and Akira, 2009; Schroder and Tschopp, 2010; Bonardi et al., 2012).

The TLRs are a group of transmembrane receptors that are able to recognize a large variety of MAMPs from different pathogenic microorganisms and induce the activation of the innate immune system (Janeway and Medzhitov, 2002). In humans, 10 functional TLRs have been identified, and a large amount of data demonstrates that TLRs may also work as sensors for self/endogenous molecules or "alarmins" that contribute to inflammatory processes and may be important for the maintenance of host homeostasis (reviewed in Kawai and Akira, 2009).

About 15 years ago, another family of PRRs was identified in humans. Proteins from this family contain a nucleotide-binding and oligomerization domain (NOD) and were named NLRs (Inohara et al., 1999, 2000; Girardin et al., 2001; Hoffman et al., 2001; Albrecht et al., 2003). The NLR family includes 22 members identified in humans and more than 30 in mice (Ting et al., 2008; Schroder and Tschopp, 2010).

The most studied NLRs belong to the NLR*C* and NLR*P* subgroups. The former is composed of receptors containing a *P*yrin domain in the amino-terminal region of the protein; these receptors are usually involved in the activation of caspase-1 and the assembly of the inflammasome, a molecular platform that has been reviewed elsewhere (Kanneganti et al., 2007; Schroder and Tschopp, 2010; Shaw et al., 2010). The NLR*C* subgroup is composed of receptors containing a *C*ard domain in the amino-terminal region and includes members such as NOD1 and NOD2 that play important roles in pathogen recognition and the activation of immune responses. NOD1 and NOD2 are encoded by the *card4* and *card15* genes, respectively, and are involved with the recognition of peptidoglycan moieties from Gram-positive and Gram-negative bacteria (Inohara et al., 2001; Chamaillard et al., 2003; Girardin et al., 2003a,b,c). Nevertheless, increasing numbers of recent reports suggest that NOD1 and NOD2 have important functions in non-bacterial infections. Whether NOD1 and NOD2 sense other structures and microbes or participate only as signaling partners is still unclear.

In this review, we will focus on functional aspects of the NOD1 and NOD2 proteins and discuss recent findings related to their roles in microbial recognition and the induction of inflammatory responses that lead to the restriction of infections with bacterial and non-bacterial pathogenic microbes.

STRUCTURE AND SIGNALING OF NOD1 AND NOD2

Structurally, NLRs are multi-domain proteins that contain an N-terminal Caspase Recruitment Domain (CARD) that associates with downstream signaling molecules, a centrally located nucleotide-binding oligomerization domain (NBD or NACHT), and a C-terminal leucine-rich repeat domain (LRR) or sensor domain (Proell et al., 2008; Schroder and Tschopp, 2010). NLRs vary in their N-terminal effector domains (PYD, CARD, BIR, and unclassified). Based on the domains present in this region, the NLRs are classified in two subgroups: NLR*C*s (*C*ARD), and NLR*P*s (*P*YRIN). The NLR members NOD1 and NOD2 belong

to the NLRC subgroup as they contain an amino-terminal CARD domain and share the two common domains (NBD and LRR). NOD1 contains a single CARD domain, whereas NOD2 has two (Ogura et al., 2001). The carboxy terminal LRR domain is predicted to mediate protein–protein interactions and function as the regulatory domain. NOD1 and NOD2 contain multiple LRRs, a motif that has been linked to resistance to infection and is found in TLRs and plant R proteins (reviewed by Murray, 2005).

The idea that NOD1 and NOD2 function as intracellular receptors for bacterial peptidoglycan fragments emerged from studies using the over-expression of NOD1 and NOD2 and an NF-κB reporter in HEK293T cells (Inohara et al., 2001; Chamaillard et al., 2003; Girardin et al., 2003a,b,c). Further studies demonstrated that NOD1 activity was triggered by D-glutamyl-meso-diaminopimelic acid (DAP), which is found in Gram-negative and a few Gram-positive bacteria, including *Listeria* and *Bacillus* (Chamaillard et al., 2003; Hasegawa et al., 2006). In contrast, NOD2 activation was triggered by muramyl dipeptide (MDP), a peptidoglycan motif widely distributed among both Gram-positive and Gram-negative bacteria (Girardin et al., 2003b,c).

Until recently, the direct binding of NOD1 and NOD2 to their respective ligands, DAP and MDP, had not been demonstrated in a physiological milieu. However, the direct binding of MDP to NOD2 has recently been reported, suggesting the first biochemical evidence for a direct interaction between NOD2 and MDP (Grimes et al., 2012). In addition to NOD2 activation, different groups have reported that MDP is involved in the activation of other NLRs, including NLRP3 (Martinon et al., 2004; Pan et al., 2007) and NLRP1 (Hsu et al., 2008). The putative activation of NLRP3 and NLRP1 by MDP leads to the production and secretion of IL-1β, an important proinflammatory cytokine (Martinon et al., 2004; Hsu et al., 2008). Although it has been demonstrated that MDP triggers the production of cytokines, chemokines, nitric oxide (NO), and reactive oxygen species, several studies have shown that MDP alone is only weakly immunostimulatory (Parant et al., 1995; Wolfert et al., 2002; Pauleau and Murray, 2003; Kobayashi et al., 2005; Uehori et al., 2005; Kinsner et al., 2006; Moreira et al., 2008a). MDP has been shown to act synergistically with TLRs; the addition of MDP in combination with TLR agonists, such as lipoteicoic acid (LTA), LPS, and peptidoglycan, triggers a robust inflammatory response, including the release of proinflammatory cytokines such as IL-1β and IL-6 (Wolfert et al., 2002; Kim et al., 2007; Natsuka et al., 2008). As expected, the synergistic effect of MDP with TLR agonists is dependent on NOD2, but the molecular mechanisms responsible for this phenomenon are still not known. It is possible that TLR stimulation facilitates the internalization of MDP, a process that is required for NOD2 activation under physiological conditions.

Although the biological roles of DAP and MDP in the activation of NOD1 and NOD2 have been described, the mechanism underlying their internalization to the cytosol remains poorly understood. Recent studies using an HEK293 transfection system demonstrated that DAP and MDP reach the cytoplasm by endocytosis, in a clathrin-dependent manner. Moreover, the cytosolic internalization of the ligands was pH-dependent and occurred prior to the acidification mediated by the vacuolar ATPase (Lee et al., 2009). However, it remains to be determined whether this process also occurs in primary cells such as macrophages, which do not show robust activation in response to DAP or MDP alone (Parant et al., 1995; Wolfert et al., 2002; Pauleau and Murray, 2003; Kobayashi et al., 2005; Uehori et al., 2005; Kinsner et al., 2006; Moreira et al., 2008a).

The current model of NLR signaling proposes that upon specific recognition of their ligands, the NBD domains oligomerize and initiate the recruitment of interacting proteins leading to the interaction of the CARD domain with the CARD-containing kinase RIPK2 (also called RIP2/RICK) through a homotypic CARD–CARD interaction (Kobayashi et al., 2002; Park et al., 2007; Nembrini et al., 2009). This is accomplished via the recruitment of the E3 ubiquitin ligase TRAF6 to RIPK2, followed by TRAF6 autoubiquitination, polyubiquitination of RIPK2, and the ubiquitination-dependent signaling and activation of the TAK1 complex. The activated TAK1 complex promotes the K63-type polyubiquitination of IKK-β, culminating in the degradation of the nuclear factor kappa-B (NF-κB) repressor IκB, the translocation of NF-κB to the nucleus, and the transcription of proinflammatory genes (Hasegawa et al., 2008; **Figure 1**). RIPK2 is critical for the induction of NF-κB activation by NOD1 and NOD2, although the molecular details concerning how the NOD/RIPK2 complex stimulates NF-κB activation are only partially understood. In addition to NF-κB, NOD1, and NOD2 can activate the p38, ERK, and JNK mitogen-activated protein kinases (MAPKs; Pauleau and Murray, 2003; Kobayashi et al., 2005; Park et al., 2007). In addition, it was recently demonstrated that members of the inhibitor of apoptosis protein (IAP) family of proteins, such as XIAP (Krieg et al., 2009), cIAP1, and cIAP2 (Bertrand et al., 2009), interact with RIPK2. Most important, it was demonstrated that cIAP1 and cIAP2 function as E3 ubiquitin ligases responsible for the polyubiquitination of RIPK2, a process that is essential for the induction of NF-κB activation by NOD1 and NOD2 (Bertrand et al., 2009; **Figure 1**).

In addition to the bona fide interaction with RIPK2, NOD1, and NOD2 have been reported to interact with other NLRs that are important for caspase-1 activation. NOD2 was shown to specifically and directly interact with NLRP1, NLRP3, and NLRP12, whereas NOD1 interacts only with NLRP3 (Hsu et al., 2008; Wagner et al., 2009).

NOD1 AND NOD2 EXPRESSION AND SIGNALING REGULATION

NOD1 is widely expressed in many cell types, whereas NOD2 has been found in macrophages (Ogura et al., 2001), dendritic cells (Tada et al., 2005), paneth cells (Ogura et al., 2003), keratinocytes (Voss et al., 2006), epithelial intestinal cells (Hisamatsu et al., 2003), lung epithelial cells (Uehara et al., 2007), oral epithelial cells (Uehara et al., 2008), and osteoblasts (Marriott et al., 2005). NOD1 and NOD2 expression can be induced by different stimuli, such as live and heat-killed bacteria (Pudla et al., 2011); TLR ligands, IFN-γ, and TNF-α (Rosenstiel et al., 2003; Kim et al., 2007, 2008).

Little is known about how the NOD1 and NOD2 signaling pathways are regulated and at which step(s) of the cascade regulation occurs. One study showed that the ubiquitination of RIPK2 induced by MDP appears to be regulated by A20, a

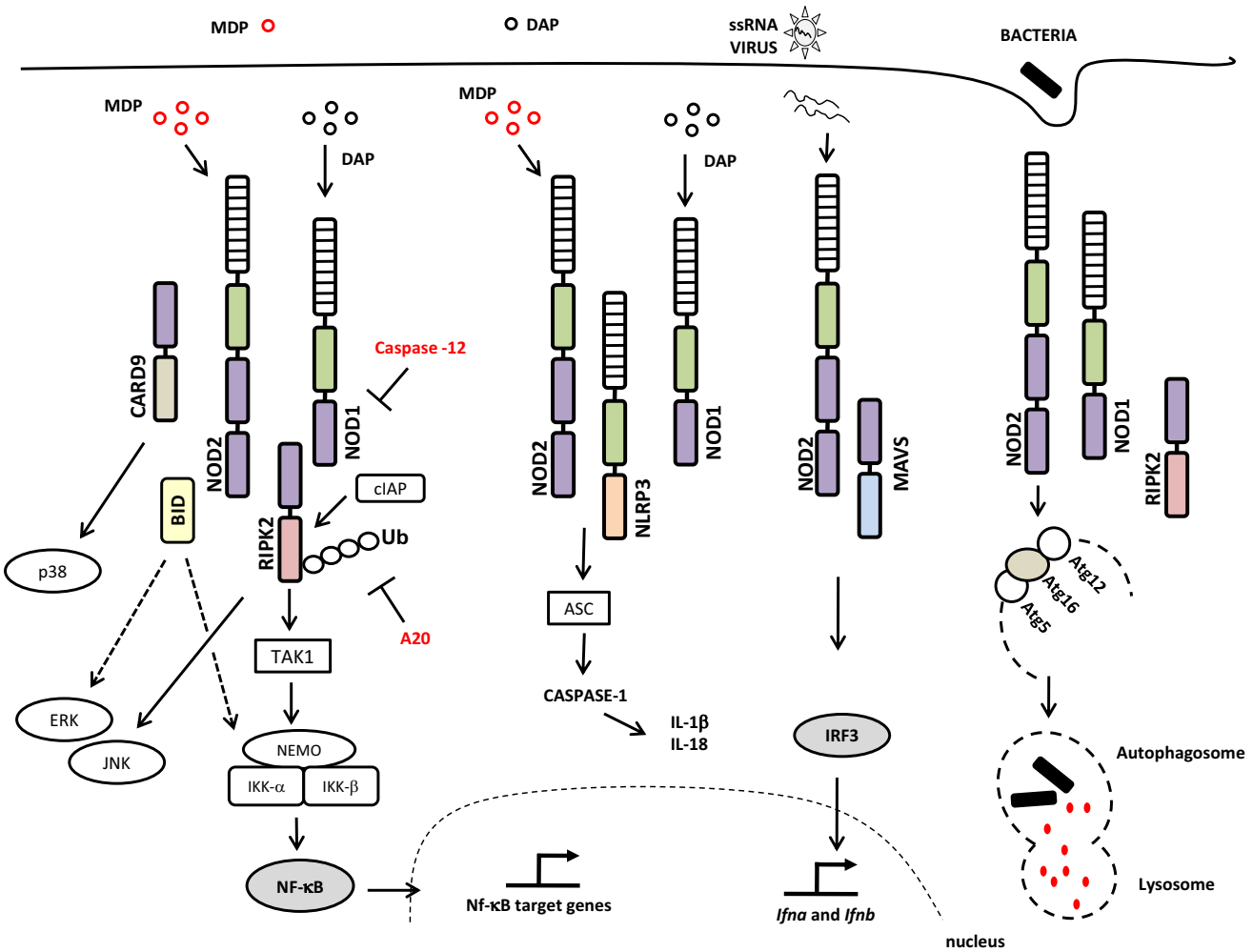

FIGURE 1 | NOD1 and NOD2 signaling pathways and interaction partners. The canonical adaptor protein required for the activation of the signaling pathways downstream of NOD1 and NOD2 is the CARD-containing kinase RIPK2, a protein that interacts with NOD1 or NOD2 via homotypic CARD–CARD interactions leading to the activation of NF-κB and MAPKs. NOD/RIPK2 signaling can be inhibited by caspase-12 and/or A20. NOD1 and NOD2 proteins may also interact with the NLRP3 inflammasome, which leads to caspase-1 activation and IL-18 and IL-1β production. Viral ssRNA triggers a signaling pathway that is dependent on NOD2 and MAVS, leading to the activation of IRF3 and the induction of type I interferon. NOD1 and NOD2 activate the autophagy machinery through their interactions with ATG16L1 protein. Abbreviations include: NOD1, nucleotide-binding oligomerization domain 1; NOD2, nucleotide-binding oligomerization domain 2; RIPK2, receptor-interacting serine/threonine-protein kinase 2; CARD, caspase recruitment domain; JNK, c-Jun N-terminal kinases; TAK1, transforming growth factor-beta-activated kinase 1 TAK1; cIAP, inhibitor of apoptosis protein; NEMO, NF-κB essential modulator; IKK-γ, inhibitor of nuclear factor kappa-B kinase-gamma; IKK, I-kappa-B kinase; Ub, ubiquitinated; A20, ubiquitin-modified enzyme; ERK, extracellular-signal-regulated kinases; ASC, apoptosis-associated speck-like protein containing a CARD; IL-1β, interleukin-1; IL-18, interleukin-18; ssRNA, single-stranded RNA; MAVS, mitochondria anti-virus signaling protein; IFR3, interferon regulatory factor 3; Atg, autophagy-related gene; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB. Specific Domains of NOD1 and NOD2 are indicated: NBD or NACHT (green rectangle); LRR (hatched rectangle); CARD (purple rectangle).

ubiquitin-modifying enzyme. A20-deficient cells exhibit amplified responses to MDP, including increased RIPK2 ubiquitylation, prolonged NF-κB signaling, and increased production of proinflammatory cytokines. The same phenotype was observed *in vivo* when A20-deficient mice were stimulated with MDP (Hitotsumatsu et al., 2008). Another study showed that caspase-12, an enzyme that modulates caspase-1 activation, binds to RIPK2, displacing TRAF6 from the signaling complex, a process that leads to the inhibition of ubiquitin ligase activity and consequently the blunting of NF-κB activation (LeBlanc et al., 2008).

Interestingly, several studies have shown that NOD1 and NOD2 bind/interact with different intracellular molecules that may positively or negatively regulate their signaling pathway. These studies suggest the existence of a highly complex network of protein–protein interactions underlying the biological functions of NOD1 and NOD2 (**Table 1**).

Table 1 | Molecules reported to directly interact with NOD1 and/or NOD2.

Molecule	NOD1	NOD2	Function/model	Reference
RIPK2	+	+	Activation of NF-κB and MAPK/mice and human	Inohara et al. (2000), Kobayashi et al. (2002) and reviewed in (Inohara et al., 2002)
NLRP1		+	Inflammasome signaling/mice and human*	Hsu et al. (2008), Wagner et al. (2009)
NLPR3		+	Inflammasome signaling/human*	Wagner et al. (2009)
NLRP12		+	Inflammasome signaling/human*	Wagner et al. (2009)
Erbin		+	Negative regulator of NOD2 signaling/human*	McDonald et al. (2005)
CENT-1β	+	+	Negative regulator of NOD2 signaling/human*	Yamamoto-Furusho et al. (2006)
Rac1GTPase		+	Negative regulator of NOD2 signaling/human*	Eitel et al. (2008)
RIG-1		+	Negative regulator of NOD2 signaling/human*	Morosky et al. (2011)
JNK-binding protein (JNKBP1)		+	Negative regulator of NOD2 signaling/mice and human*	Lecat et al. (2012)
GRIM-19		+	Positive regulator of NOD2 signaling/human*	Barnich et al. (2005)
CARD9		+	Positive regulator of NOD2 signaling/mice and human*	Hsu et al. (2007)
Vimentin		+	Positive regulator of NOD2 signaling/human*	Stevens et al. (2012)
BID	+	+	Signaling for activation of ERK and NF-κB/mice and human*	Yeretssian et al. (2011)
Atg16L1	+	+	Induction of autophagy/mice and human*	Travassos et al. (2010)
			Induction of autophagy/human*	Cooney et al. (2010)
			Regulation of autophagy/human*	Homer et al. (2012)

**Phenotypes detected in human cells and/or in mammalian cells transfected with human molecules.*

Positive regulators of NOD1 and NOD2 signaling have also been described. A study showed that GRIM-19, a protein involved in cell death, binds to NOD2 and is required for NF-κB activation (Barnich et al., 2005). Other molecules, such as the CARD-containing adaptor protein 9 (CARD9), operate downstream of NOD2 to trigger the RIPK2-independent activation of p38 and JNK (Hsu et al., 2007; **Figure 1**).

The NOD1 and NOD2 proteins have been reported to interact with the apoptotic pathway through additional mechanisms, aside from the role of IAP proteins in the NOD1 and NOD2 signaling pathway (Bertrand et al., 2009; Krieg et al., 2009). Yeretssian et al. (2011) demonstrated that BID, a BLC2 family protein, interacts directly with NOD1, NOD2, and the IκB kinase (IKK) complex in a process that is important for NF-κB and ERK activation, suggesting a non-apoptotic role for BID (Yeretssian et al., 2011). This study used bone marrow-derived macrophages (BMDMs) from *Bid*$^{-/-}$ mice, which failed to activate NF-κB and ERK and were unable to secrete inflammatory cytokines upon stimulation with NOD ligands. In addition, *Bid*$^{-/-}$ mice were unable to trigger cytokine production *in vivo* after challenge with NOD ligands (Yeretssian et al., 2011). In contrast, a study performed by Nachbur and colleagues demonstrated that *Bid*-deficient mice had the same phenotype as wild-type mice upon stimulation with NOD ligands. Moreover, the activation of NF-κB and ERK were similar in both *Bid*$^{-/-}$and wild-type mice, suggesting that BID is not essential for NOD signaling (Nachbur et al., 2012). It is possible that differences in the gut microbiota associated with the animals affect their responses to NOD ligands. Additional studies, including co-housing experiments, will be required to clarify the role of BID in NOD signaling and to determine whether NOD proteins interfere with BID-dependent apoptosis.

Negative regulators of NOD signaling may reduce NOD1 or NOD2 signaling by inhibiting their interaction with other molecules in the pathway. One example is ERBIN, an LRR-containing protein that binds to NOD2, inhibiting MDP-induced signaling (McDonald et al., 2005). NOD1 and NOD2 signaling may also be inhibited by Centaurin β-1 (CENTβ1), a GTPase-activating protein that is a member of the ADP-ribosylation family and colocalizes with NOD1 and NOD2 in the cytoplasm of intestinal epithelial cells. Over-expression of CENTβ1 inhibited NOD1- and NOD2-dependent NF-κB signaling (Yamamoto-Furusho et al., 2006). Rac1 GTPase and retinoic-acid induced gene-1 (RIG-I) have also been shown to interact with NOD2 and inhibit its signaling (Eitel et al., 2008; Morosky et al., 2011).

NOD1 AND NOD2 IN CHRONIC DISEASES

Studies have revealed that some individuals with familial Crohn's disease, a chronic inflammatory condition of the gut, have mutations in the *Card15* gene encoding NOD2 (Hugot et al., 2001; Ogura et al., 2001), but not NOD1 (Zouali et al., 2003). The most common *Card15* mutation associated with Crohn's disease is an L100fs frameshift insertion at nucleotide 3020 (3020insC) in the LRR region of NOD2 (Hugot et al., 2001). Although significant, only a small percentage of Crohn's disease patients harbor this *Card15* mutation (Hugot et al., 2001; Ogura et al., 2001; Lesage et al., 2002). In addition, mutations in the NBD region (R334W, R334Q, and L469F) have been associated with another inflammatory disorder known as Blau syndrome (Miceli-Richard et al., 2001). However, it is still poorly understood how polymorphisms in the NOD2 LRR (L100fs) or NBD (R334W, R334Q, and L469F) domains contribute to Crohn's disease and Blau syndrome, respectively. Some current ideas about NOD2 polymorphisms in Crohn's disease patients were previously reviewed and discussed elsewhere (Schroder and Tschopp, 2010). NOD2 polymorphisms have also been associated with a variety of human inflammatory

diseases, such as atopic eczema (Weidinger et al., 2005), arthritis (Joosten et al., 2008; Vieira et al., 2012), atopic dermatitis (Macaluso et al., 2007), sarcoidosis (Kanazawa et al., 2005), and possibly asthma (Hysi et al., 2005; Duan et al., 2010), and endometrial (Ashton et al., 2010) or prostate cancer (Kang et al., 2012). More recently, it was demonstrated that human NOD2 polymorphisms were also associated with increased susceptibility to infectious diseases, such as leprosy (Zhang et al., 2009; Berrington et al., 2010a), and tuberculosis (Austin et al., 2008; Azad et al., 2012).

NOD1 AND NOD2 FUNCTIONS IN THE RESPONSE TO BACTERIAL INFECTION

The initial studies regarding the functional properties of NOD1 and NOD2 as intracellular receptors for the recognition of peptidoglycan and live bacteria were performed using *in vitro* models. These studies showed that NOD1 senses a substantial variety of Gram-negative bacteria while NOD2 senses Gram-positive and Gram-negative bacteria (**Table 2**).

The role of NOD1 and NOD2 in detecting specific microbial products *in vitro* was further demonstrated in mutant mice through the targeted deletion of these genes. Initial studies on NOD2 function in mice were performed using *Nod2* knockout mice created through an insertion mutation in the first exon encoding the first part of the second CARD domain (Pauleau and Murray, 2003). Surprisingly, these mice did not show evident intestinal dysfunction, even though BMDMs obtained from these mice did not respond to MDP stimulation, thus confirming the loss-of-function of NOD2 (Pauleau and Murray, 2003). Later, two other NOD2 transgenic mice were engineered: one with an insertion causing a frameshift mutation in the final LRR domain, corresponding to one of the most frequent mutation observed in familial Crohn's disease patients (Maeda et al., 2005), and another with a loss-of-function insertion in the *Card15* locus (Kobayashi et al., 2005). As reported by Pauleau and colleagues, the transgenic mice constructed by Maeda et al. and Kobayashi et al. show no intestinal pathology under normal housing conditions (Pauleau and Murray, 2003).

Table 2 | Functions of NOD1 and/or NOD2 in host responses to pathogenic microbes.

Microrganism	Model/protein	Reference
PROTOZOA		
Toxoplasma gondii	Mouse/NOD2	Shaw et al. (2009)
Trypanosoma cruzi	Mouse/NOD1	Silva et al. (2010)
Plasmodium berghei ANKA	Mouse/NOD1 and NOD2	Finney et al. (2009)
BACTERIA		
Bacillus anthracis	Mouse/NOD2	Hsu et al. (2008)
	Mouse/NOD1 and NOD2	Loving et al. (2009)
Borrelia burgdorferi	Human cells and mouse/NOD2	Oosting et al. (2010)
Burkholderia pseudomallei	Human cell line and mouse/NOD2	Pudla et al. (2011)
Campylobacter jejuni	Human cell line/NOD1	Zilbauer et al. (2007)
Chlamydophila pneumoniae	Human cell line/NOD1	Opitz et al. (2005), Shimada et al. (2009)
Chlamydia trachomatis and *Chlamydia muridarum*	Human cell line and mouse/NOD1	Welter-Stahl et al. (2006)
Clostridium difficile	Mouse/NOD1	Hasegawa et al. (2011)
Citrobacter rodentium	Mouse/NOD2	Kim et al. (2011b)
Escherichia coli entero-invasive	Human cell line/NOD1	Kim et al. (2004)
Haemophilus influenzae	Mouse/NOD1	Zola et al. (2008)
Helicobacter pylori	Human cell line, mouse, and clinical study/NOD1	Watanabe et al. (2011)
Helicobacter hepaticus	Mouse/NOD2	Biswas et al. (2010), Grubman et al. (2010)
Legionella pneumophila	Human cell line and mouse/NOD1 and NOD2	Shin et al. (2008), Berrington et al. (2010b), Frutuoso et al. (2010)
Listeria monocytogenes	Human cell line/NOD1	Opitz et al. (2006)
Mycobacterium tuberculosis	Human cells/NOD2	Brooks et al. (2011)
	Mouse/NOD2	Divangahi et al. (2008)
Porphyromonas gingivalis	Human cells/NOD1 and NOD2	Uehara et al. (2008)
Propionibacterium acne	Human cells and clinical study/NOD1 and NOD2	Tanabe et al. (2006)
Pseudomonas aeruginosa	Human cell line and murine fibroblast/NOD1	Travassos et al. (2005)
Salmonella enterica	Mouse/NOD1	Le Bourhis et al. (2009)
	Mouse/NOD1 and NOD2	Geddes et al. (2010), Keestra et al. (2011)
Shigella flexneri	Human cell line/NOD1	Girardin et al. (2001)
Streptococcus pneumoniae	Human cell line/NOD 2	Opitz et al. (2004)
VIRUS		
Respiratory syncytial virus (RSV)	Mice/NOD2	Sabbah et al. (2009)
Murine norovirus-1	Mouse/NOD1 and NOD2	Kim et al. (2011a)

Although *Nod2*$^{-/-}$ mice did not show any inflammatory phenotype in the gut, several reports demonstrated their increased susceptibility to bacterial infections. For *Listeria monocytogenes* infection, NOD2 was shown to be important for restricting bacterial multiplication because *Nod2*$^{-/-}$ mice orally infected with *L. monocytogenes* showed decreased production of β-defensins and an increased bacterial burden (Kobayashi et al., 2005; Kim et al., 2008).

Similar features were observed in *Nod1*$^{-/-}$ mice infected with *Helicobacter pylori*; NOD1 was shown to be important for the recognition of peptidoglycan translocated from the bacterial cell to the host cell cytoplasm through the *cag* type IV secretion system. This feature accounts for NOD1-dependent responses that generate resistance against infection (Viala et al., 2004; Boughan et al., 2006).

In a pulmonary model of infection with *Chlamydophila pneumoniae*, NOD2, and RIPK2 were found to be critical for host responses; *Nod2*- and *Ripk2*-deficient mice infected with *C. pneumoniae* exhibited impaired production of nitric oxide and chemokine (C-X-C motif) ligand 1 production and delayed neutrophil recruitment to the lungs (Shimada et al., 2009). Defective recruitment of neutrophils to the intestines was also observed in *Nod1*$^{-/-}$ mice infected with *Clostridium difficile,* possibly due to the impairment of CXCL1 production. The increased mortality of the *Nod1*$^{-/-}$ mice was accompanied by impaired *C. difficile* clearance and increased bacterial translocation from the intestines to other organs, a process that resulted in elevated levels of microbiota-derived endotoxin and IL-1β in the sera of *Nod1*$^{-/-}$ mice (Hasegawa et al., 2011).

Some authors have reported that both NOD1 and NOD2 function in a synergistic fashion to tune the appropriate responses to certain pathogens. For example, NOD1 and NOD2 double-deficient mice showed a significant reduction in the production of inflammatory cytokines and an increase in the bacterial colonization of the mucosal tissue in a *Salmonella* model of colitis. These phenotypes were not observed in *Nod1*$^{-/-}$ or *Nod2*$^{-/-}$ single knockouts (Geddes et al., 2010). The same group further demonstrated that NOD1 and NOD2 were crucial for the induction of mucosal Th17 responses during the early stages of infection with *Citrobacter rodentium* and *S. enterica* Typhimurium (Geddes et al., 2011). Of note, this response was dependent on the intestinal microbiota, a concept that may flourish in this field within the next few years.

Cooperation between NOD1 and NOD2 was also reported in a murine model of *Bacillus anthracis.* Mice deficient for both NOD1 and NOD2 were more susceptible to lethal challenge with *B. anthracis* and produced lower levels of proinflammatory molecules when compared with single knockouts (Loving et al., 2009). A similar phenomenon was observed after pulmonary infection of mice with *Legionella pneumophila,* an intracellular bacterial pathogen that has been used as an excellent model for investigating bacterial recognition by innate immune receptors (Vance, 2010; Massis and Zamboni, 2011). NOD1 or NOD2 single knockout mice effectively restricted the replication of *L. pneumophila* in the lungs; in contrast, RIPK2-deficient mice were less efficient at clearing pulmonary bacteria (Frutuoso et al., 2010). Additional investigation using *Ripk2*$^{-/-}$ mice indicated that the NOD/RIPK2 pathway cooperates with TLR signaling to restrict bacterial growth in mouse lungs; RIPK2/MyD88 double-deficient mice were significantly more susceptible to *L. pneumophila* infection compared with MyD88 single knockout mice (Archer et al., 2010).

Another study performed with *L. pneumophila* indicated that NOD1 and NOD2 drive distinct inflammatory responses (Berrington et al., 2010b). *Nod1*$^{-/-}$ mice showed impaired bacterial clearance and neutrophil recruitment to the alveolar space and decreased production of proinflammatory cytokines when compared with wild-type mice. In contrast, increased levels of lung neutrophils and proinflammatory cytokine production were observed in *Nod2*$^{-/-}$ infected mice. However, at later stages of infection, both *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice produced significantly increased levels of proinflammatory cytokines in the lungs (Berrington et al., 2010b). Collectively, these studies indicate that although NOD1/NOD2/RIPK2 signaling is not critical for host resistance against *Legionella pneumophila*, both NOD1 and NOD2 participate in the recognition of these bacteria *in vivo* (Archer et al., 2010; Berrington et al., 2010b; Frutuoso et al., 2010).

Regardless of the roles of NOD1 and NOD2 in the immune response to certain microbes, for some bacterial pathogens, such as *Coxiella burnetii* and *Brucella abortus*, NOD1 and NOD2 play no role in restricting bacterial replication in murine models of infection (Benoit et al., 2009; Oliveira et al., 2012). This may also be the case for other pathogenic bacteria that bypass and/or are refractory to NOD1- and NOD2-mediated immunity.

NOD1 AND NOD2 IN RESPONSE TO NON-PEPTIDOGLYCAN-CONTAINING PATHOGENS

As reviewed so far, the role of NOD1 and NOD2 in sensing bacterial products has been intensively investigated. Moreover, the use of *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice supported the key role of these molecules in restricting bacterial infection (**Table 2**). However, recent reports indicate that NOD1 and NOD2 also play a role in host responses against protozoan parasite infections.

Nod2$^{-/-}$ mice failed to clear *Toxoplasma gondii* infection and succumbed at similar rates to *MyD88*$^{-/-}$ mice. NOD2 was shown to be involved in a T cell-intrinsic function rather than being active in macrophages and DCs (Shaw et al., 2009). Moreover, *Nod2*$^{-/-}$ mice failed to trigger IFN-γ production and to induce the differentiation of Th1 lymphocytes (Shaw et al., 2009). However, these observations were not corroborated by studies performed by three independent groups (Caetano et al., 2011). The reason for these discrepancies maybe related to variations in the bacterial microbiota present in the guts of the NOD2-deficient mice. Further studies, including those involving co-housing and the use of germ-free mice, will be required to address this issue. The T cell-intrinsic defect in *Nod2*$^{-/-}$ mice could explain why these mice are partially defective at generating antigen-specific antibodies even in the absence of NOD2 ligands (Moreira et al., 2008a). Nonetheless, the role of NOD2 signaling in CD4+ T cells requires additional investigation.

Studies performed with other intracellular protozoan parasites, such as *Plasmodium berghei* ANKA and *Trypanosoma cruzi*, indicated that NOD2 was not required for host protection against these parasites (Finney et al., 2009; Silva et al., 2010). In contrast, NOD1 was associated with host resistance against *T. cruzi* infection

in vivo (Silva et al., 2010). BMDMs from *Nod1*$^{-/-}$ mice showed impaired induction of NF-κB-dependent products in response to infection and failed to restrict *T. cruzi* infection in the presence of IFN-γ. Despite normal cytokine levels in the sera, *Nod1*$^{-/-}$ mice were highly susceptible to *T. cruzi* infection in a similar manner to *MyD88*$^{-/-}$ and NO synthase 2 (iNOS)-deficient mice. This study indicated that NOD1-dependent responses accounted for host resistance to *T. cruzi* infection via cytokine-independent mechanisms (Silva et al., 2010).

Interestingly, in addition to the detection of bacteria and protozoa, NOD2 has an important role in virus recognition during experimental infection (Sabbah et al., 2009). NOD2, but not NOD1, was shown to facilitate IRF3 activation and the production of type I IFN in response to single-stranded RNA (ssRNA) or infection with respiratory syncytial virus (RSV; Sabbah et al., 2009). The authors showed that ssRNA and RSV, which does not contain peptidoglycan, activated NOD2 through a mechanism that was dependent on MAVS (mitochondrial antiviral signaling protein) and led to the activation of IRF3. Another recent study demonstrated that bacteria-infected mice co-stimulated with poly I:C or IFN-γ or co-infected with murine norovirus-1 dramatically augmented NOD1 and NOD2 signaling and expression and the production of proinflammatory cytokines. This response was attenuated in NOD1/NOD2 double knockout or RIPK2-deficient mice. The crosstalk between NOD1/NOD2 and type I IFN signaling may somehow facilitate bacterial recognition; however, it also induces harmful effects in the virally infected host (Kim et al., 2011a). These data contribute to our understanding of the lethal effects of host co-infection by two pathogens that are not normally lethal in singly infected hosts (Jamieson et al., 2010).

Although these initial reports indicated that NOD1 and NOD2 might be important in responses to non-bacterial pathogens, further studies will be required to address the roles of NOD1 and NOD2 in the recognition of pathogens that lack peptidoglycan moieties. In this context, it is important to determine whether NOD1 and NOD2 act as bonafide receptors for these pathogens or whether they take part in signaling pathways triggered by other innate immune molecules.

NOD1 AND NOD2 IN AUTOPHAGY INDUCTION

Recent reports have shown that NOD1 and NOD2 are associated with the induction of macroautophagy, a highly conserved degradation system in which specific cell components, including damaged organelles or proteins, are engulfed into a double-layered membrane structure for further degradation (reviewed in Lu and Walsh, 2012). Autophagy is also considered an immunologically regulated process and represents an innate defense mechanism that can control the replication of intracellular pathogens, including *Mycobacterium tuberculosis* and others (Gutierrez et al., 2004; Ogawa et al., 2011). Both NOD1 and NOD2 were shown to recruit ATG16L1 to the plasma membrane at the site of bacterial entrance to initiate autophagy (Travassos et al., 2010). In addition, NOD2 agonists induced autophagy in DCs in a RIPK2-, ATG5-, and ATG7-dependent manner (Cooney et al., 2010). In fact, polymorphisms in the ATG16L1 gene are known to be a risk factor for the development of Crohn's disease in humans (Naser et al., 2012). NOD-dependent autophagy induction occurs after cell stimulation with peptidoglycan or live *Shigella flexneri* and is independent of RIPK2 (Travassos et al., 2010). Nonetheless, additional studies have reported that RIPK2 is necessary for the NOD2-mediated induction of autophagy (Cooney et al., 2010; Anand et al., 2011; Homer et al., 2012). The studies performed by Travassos et al. and Cooney et al. were the first to suggest that NOD1 and NOD2 function as a molecular scaffold for the autophagy machinery and may thereby act as nucleation sites for autophagy initiation (Cooney et al., 2010; Travassos et al., 2010).

More recently, different groups have reported a role for NOD1 and/or NOD2 in the induction of autophagy in response to several pathogens including *S. Typhimurium*, *M. tuberculosis*, adherent-invasive *E. coli*, and *L. monocytogenes* (Anand et al., 2011; Homer et al., 2012; Juarez et al., 2012; Lapaquette et al., 2012).

NOD1 AND NOD2 IN ADAPTIVE IMMUNITY

Although NOD1 and NOD2 are associated with innate immune responses, several reports have demonstrated their involvement in the induction of adaptive immune responses. In fact, for certain infections, impairment of NOD1 and NOD2 function interferes with both innate and adaptive immune responses (Divangahi et al., 2008; Shaw et al., 2009).

NOD2 stimulation with MDP triggers an antigen-specific immune response with a Th2-type polarization profile, characterized by the production of IL-4 and IL-5 by T cells and IgG1 antibody responses (Magalhaes et al., 2008). Other studies have suggested that NOD1 is important for T cell priming and antibody production. NOD1 stimulation with its agonist alone was sufficient to drive a Th2 antigen-specific immune polarization. NOD1-deficient mice showed a reduced frequency of IFN-γ − producing$CD4^+$ and $CD8^+$ T cells and decreased antibody production, suggesting that NOD1 was required for optimal T and B cell responses (Fritz et al., 2007); a similar effect was also described for NOD2 (Shaw et al., 2009).

NOD2 was also found to be critical for the induction of both Th1- and Th2-type responses following co-stimulation with TLR agonists (Magalhaes et al., 2008). The lack of NOD2-dependent Th2 differentiation in a subset of Crohn's disease patients might explain how the Th1-mediated inflammation at the intestinal mucosa contributes to the pathogenesis of the disease (Magalhaes et al., 2008). However, the use of MDP as an adjuvant is controversial because MDP alone is a weak adjuvant compared with TLR agonists (Magalhaes et al., 2008). Therefore, MDP may be inefficient at triggering adequate adaptive immune responses, as previously reported (Moreira et al., 2008a).

REGULATION OF IMMUNE RESPONSES BY NOD1 AND NOD2

It is possible that NOD2 has a regulatory function for innate immune responses, acting as a transducer modifier as previously suggested (Murray, 2005). Watanabe and colleagues demonstrated that mixed splenocyte cultures from *Nod2*$^{-/-}$ mice produced high levels of IL-12 upon stimulation with PGN, and a similar phenotype was observed *in vivo* when *Nod2*$^{-/-}$ mice were injected with PGN (Watanabe et al., 2004). Intact NOD2 signaling inhibited the TLR2-driven activation of NF-κB, particularly the NF-κB subunit c-Rel. Moreover, NOD2 deficiency or the presence of a Crohn's disease-like *Card15* mutation increased

the TLR2-mediated activation of NF-κBc-Rel and Th1 responses (Watanabe et al., 2004). Thus, *Card15* mutations may lead to disease by causing excessive Th1 responses. This finding was corroborated by the fact that BMDMs from *Nod2*$^{-/-}$ mice produced less IL-10 upon stimulation with PGN purified from *Streptococcus pneumoniae*, suggesting that NOD2 may have a regulatory effect on IL-10 production (Moreira et al., 2008b). The reduction of IL-10 production would lead to increased production of IL-12, thereby contributing to the excessive inflammation observed in Crohn's disease patients (Moreira et al., 2008b). In fact, it was recently demonstrated that the Crohn's-disease-associated NOD2 mutation suppresses human IL-10 transcription by inhibiting the activity of the nuclear ribonucleoprotein hnRNP-A1 (Noguchi et al., 2009). The NOD2 3020insC mutation blocks p38 phosphorylation of hnRNP-A1, which impairs hnRNP-A1 binding to the IL-10 locus in peripheral blood mononuclear cells from Crohn's disease patients (Noguchi et al., 2009). These findings are consistent with the previous suggestion that NOD2 may interfere with the production of IL-10 (Moreira et al., 2008b), a cytokine that is important for the regulation of inflammatory processes.

In another mouse model, a NOD2 mutation potentiated NF-κB activity and IL-1β processing, suggesting that NOD2 may act as a positive regulator of NF-κB activation and IL-1β secretion, thereby increasing the intestinal inflammation observed in Crohn's disease patients (Maeda et al., 2005). The mechanism by which mutations in the *Card15* gene influence the chronic inflammation status observed with Crohn's disease is still poorly understood. Because signaling via mutated NOD2 proteins leads to defective activation of NF-κB, one hypothesis is that mutations causing deficient NF-κB-dependent Th1 responses increase susceptibility to gut infections. This idea is supported by recent findings showing that wild-type NOD2, but not the mutant variants found in humans, can mediate autophagy, thereby allowing the clearance of bacterial pathogens that reach the host cell cytoplasm.

CONCLUDING REMARKS

The NOD1 and NOD2 proteins play a remarkable role in host immune responses. Despite their undisputed importance for host defense, the specific mechanisms underlying their functions are yet to be determined. Although it is clear that these molecules are able to sense bacterial cell wall components and pathogens, their unique role as intracellular "receptors" is still a matter of debate. Although direct binding of MDP to NOD2 has recently been demonstrated (Grimes et al., 2012), alternative functions for the NODs, such as regulation of signal transduction systems have been proposed (Murray, 2005; Strober et al., 2006), thus corroborating the idea that NOD1 and NOD2 are multifaceted proteins. As mentioned in this review, NOD2 may interfere with IL-10 production and act as a regulatory molecule rather than an inflammatory inducer. The roles of NOD1 and NOD2 in the induction and resolution of inflammatory processes are largely unknown. A more comprehensive understanding of the functions of NOD1 and NOD2 in mammalian immunity may allow the use of new pharmacological interventions to either boost or reduce inflammatory responses against pathogenic microbes.

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Resolvin D1 and resolvin E1 promote the resolution of allergic airway inflammation via shared and distinct molecular counter-regulatory pathways

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Resolvins are generated from omega-3 fatty acids during inflammatory responses in the lung. These natural mediators interact with specific receptors to decrease lung inflammation and promote its resolution in healthy tissues. There are several lung diseases of chronic inflammation that fail to resolve, most notable asthma. This common disorder has a lifetime prevalence of nearly 10% and is characterized, in part, by chronic, non-resolving inflammation of the airway. Pro-resolving mediators are generated during asthma; however, their biosynthesis is decreased in severe and uncontrolled asthma, suggesting that the chronic, adaptive inflammation in asthmatic airways may result from a resolution defect. This article focuses on recent insights into the cellular and molecular mechanisms for resolvins that limit adaptive immune responses in healthy airways.

Keywords: resolution, resolvins, inflammation, lung, asthma

INTRODUCTION

The recent identification of specialized mediators that promote tissue resolution from acute inflammation and injury has opened a new window for discovery of cellular and molecular mechanisms governing chronic inflammation and adaptive immunity. Chronic "unresolved" inflammation is a pathologic response that is associated with several common human diseases for which there is no cure. Asthma is an exemplary illness with chronic inflammation that has a lifetime prevalence of nearly 1 in 10 in Western countries (Fanta, 2009). The chronic inflammation in asthma consists of airway infiltration of eosinophils and T-lymphocytes with increased levels of pro-phlogistic cytokines and lipid mediators (Busse and Lemanske, 2001). Of interest, many patients with uncontrolled lung inflammation, including clinically severe asthma, display a defect in the generation of specialized pro-resolving mediators (Levy et al., 2005, 2007; Planaguma et al., 2008; **Table 1**), consistent with a failure to establish sufficient protective counter-regulatory pathways in the asthmatic lung.

Catabasis is a healthy host tissue response to noxious stimuli. The literal definition of catabasis refers to a military retreat and the term has been adopted for use to describe the resolution process of returning an inflamed or injured tissue to homeostasis after the "battle" of inflammation. Catabasis requires well orchestrated cellular responses in which soluble mediators appear to play critical roles (Serhan, 2007). For resolution (Majno, 1996), restitution of endothelial and epithelial cell barrier integrity is necessary to prevent continued edema formation. Additional granulocyte recruitment is blocked and those granulocytes that have infiltrated the tissue undergo programmed cell death. The apoptotic cells are then cleared, principally by macrophages. The phagocytes also clear tissue microbes and debris, and structural cells re-establish organ function. One family of mediators for these pro-resolving cellular actions is the resolvins, which are enzymatically derived from the omega-3 fatty acids eicosapentaenoic acid (i.e., E-series resolvins) and docosahexaenoic acid (i.e., D-series resolvins; Serhan et al., 2000, 2002). Together with the lipoxins, protectins, and maresins, the resolvins comprise a new genus of endogenous, specialized pro-resolving mediators (Serhan, 2007). These mediators serve as agonists at select receptors to transduce their cell type specific pro-resolving actions (Serhan, 2007). Defects in resolvin signaling pathways can be resolution "toxic" in model systems, leading to conversion of acute inflammatory responses to more chronic pathologic inflammation (Schwab et al., 2007), supporting a potential link for defective resolvin signaling to chronic inflammatory diseases. Of interest, there appears to be population heterogeneity in resolution mechanisms. Randomly

Abbreviations: ALX/FPR2, lipoxin A_4 receptor/formyl peptide receptor 2; AT-RvD1, aspirin-triggered-resolvin D1 (7*S*,8*R*,17*R*-trihydroxy-docosa-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-hexaenoic acid); AT-RvD2, aspirin-triggered-resolvin D2 (7*S*,16*R*,17*R*-trihydroxy-docosa-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-hexaenoic acid); AT-RvD3, aspirin-triggered-resolvin D3 (4*S*,11*R*,17*R*-trihydroxy-docosa-5,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid); AT-RvD4, aspirin-triggered-resolvin D4 (4*S*,5*R*,17*R*-trihydroxy-docosa-6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid); BALF, bronchoalveolar lavage fluid; BLT1, LTB_4 receptor; CMKLR1, chemokine receptor-like 1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-UV-MS/MS, liquid chromatography-ultraviolet spectrometry-tandem mass spectrometry; LOX, lipoxygenase; LTB_4, leukotriene B_4 (5*S*,12*R*-dihydroxy-eicosa-6*Z*,8*E*,10*E*,14*Z*-tetraenoic acid); LXA_4, lipoxin A_4 (5*S*,6*R*,15*S*-trihydroxy-eicosa-7*E*,9*E*,11*Z*,13*E*-tetraenoic acid); PD1, protectin D1 (10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid); RvD1, resolvin D1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid); RvD2, resolvin D2 (7*S*,16*R*,17*S*-trihydroxy-docosa-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-hexaenoic acid); RvD3, resolvin D3 (4*S*,11,17*S*-trihydroxy-5*E*,7*E*,9*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid); RvD4, resolvin D4 (4*S*,5,17*S*-trihydroxy-6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid); RvE1, resolvin E1 (5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid); RvE2, resolvin E2 (5*S*,18*R*-dihydroxy-8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid; SAA, serum amyloid A; TGF-β, transforming growth factor-beta.

Table 1 | Uncontrolled lung inflammation – A defect in specialized pro-resolving mediators.

Pro-resolving mediator	Disease	Finding	Reference
Lipoxin A_4	Aspirin-exacerbated respiratory disease	Aspirin-tolerant asthmatics generate more lipoxins than aspirin-intolerant asthmatics	Sanak et al. (2000), Celik et al. (2007), Yamaguchi et al. (2011)
	Severe asthma	Diminished lipoxin biosynthesis in severe asthma	Levy et al. (2005), Vachier et al. (2005), Celik et al. (2007), Planaguma et al. (2008), Bhavsar et al. (2010), Wu et al. (2010), Fritscher et al. (2012)
	Bronchoconstriction in asthma	Protects against bronchoprovocation by either LTC_4 or exercise	Tahan et al. (2008), Christie et al. (1992)
	Asthma exacerbation	Decreased lipoxin levels in exhaled breath during exacerbation	Hasan et al. (2012)
	Cystic fibrosis	Decreased generation and actions in cystic fibrosis	Karp et al. (2004), Yang et al. (2012), Chiron et al. (2008), Mattoscio et al. (2010)
	Scleroderma lung disease	Decreased lipoxin levels in BALFs	Kowal-Bielecka et al. (2005)
Resolvin E1	Cystic fibrosis	Decreased resolvin E1 levels in cystic fibrosis with a relationship to lung function	Yang et al. (2012)
Protectin D1	Asthma exacerbation	Decreased Protectin D1 in uncontrolled asthma	Levy et al. (2007)

selected healthy individuals display significant differences in the pace of resolution for acute exudative inflammation with segregation of these apparently healthy subjects into discrete cohorts of rapid and delayed resolvers (Morris et al., 2010) that may be partially explained by genetic variability, which has recently been linked to inflammatory disease (Simiele et al., 2011). In health, the conversion of acute to chronic airway inflammation is prevented by endogenous pro-resolving mechanisms for tissue catabasis.

Intrinsically linked to innate immune responses, the development of adaptive immunity is essential to host defense, but unregulated adaptive inflammation can also lead to disease, including autoimmune disorders, transplant rejection, and allergy. As part of a series of review articles exploring the research theme of "Resolution of inflammation: leukocytes and molecular pathways as potential therapeutic targets," this article will focus on the regulation of adaptive inflammatory responses by resolvins, in particular shared and distinct counter-regulatory mechanisms for resolvin E1 and resolvin D1 in allergic inflammation.

RESOLVINS AND THEIR RECEPTORS IN THE LUNG

Eicosapentaenoic acid is an essential fatty acid that can be enzymatically converted to E-series resolvins, including resolvin E1, resolvin E2, and resolvin E3, during inflammation in mammals and fish (Serhan et al., 2000; Isobe et al., 2012). These mediators display stereoselective and cell type specific actions. Resolvin E1 (5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid) can transduce its biological actions by interacting with specific G-protein coupled receptors, namely chemokine-like receptor 1 (CMKLR1) and leukotriene B_4 receptor 1 (BLT1; Arita et al., 2005a, 2007). RvE1 serves as an agonist for CMKLR1, which is expressed in macrophages, dendritic cells (DCs), natural killer (NK) cells, and other T cells, and RvE1 serves as a receptor antagonist at BLT1 for leukotriene B_4. BLT1 is expressed on granulocytes, T cells, and macrophages. Resolvin E1 and resolvin E2 (5*S*,18*R*-dihydroxy-6*E*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid) are generated via the actions of 5-lipoxygenase (ALOX5) from a common precursor 18-hydroxyeicosapentaenoic acid (18-HEPE) with two parallel stereospecific pathways (Arita et al., 2005a; Tjonahen et al., 2006; Oh et al., 2012). Resolvin E3 [17,18(*R*/*S*)-dihydroxy-5*Z*,8*Z*,11*Z*,13*E*,15*E*-eicosapentaenoic acid] is distinct from RvE1 and RvE2 because it is generated via the actions of 12/15-lipoxygenase (ALOX12/15; Isobe et al., 2012). Since ALOX5 and ALOX12/15 are generally compartmentalized into distinct leukocyte classes, neutrophils (ALOX5) appear to play significant roles in RvE1 and RvE2 generation, while eosinophils (ALOX12/15) are significant in RvE3 biosynthesis.

Docosahexaenoic acid is another essential omega-3 fatty acid that can be enzymatically converted to resolvins. In a lipoxygenase-dependent manner, DHA is transformed to D-series resolvins, including resolvin D1–D6, during inflammation (Serhan, 2007). These mediators also display stereoselective and cell type specific actions. Resolvin D1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) transduces its biological actions by interacting with specific G-protein coupled receptors, including the lipoxin A_4 receptor ALX/FPR2 and, in humans, GPR32 (Sun et al., 2007; Krishnamoorthy et al., 2010, 2012). RvD1 serves as an agonist for both of these receptors. ALX/FPR2 is broadly expressed in many cells types, including most leukocytes as well as structural cells, such as airway epithelial cells (Chiang et al., 2006). GPR32 is expressed on phagocytes (Krishnamoorthy et al., 2010). There are also aspirin-triggered 17*R* D-series resolvins (AT-RvD1–4) that are generated in human and murine tissues, including lung, and AT-RvD1 can also interact with ALX/FPR2 and GPR32 receptors (Krishnamoorthy et al., 2012). Of note, in an aspirin-independent manner, cytochrome P450 enzymes, which are abundant in the lung, can also convert DHA to 17*R*-hydroxy-DHA that can serve as a precursor for 17*R*-RvD1 (i.e., AT-RvD1), so the presence of aspirin is not required for AT-RvD1 generation.

Relatively little information is currently available on most of these recently identified E-series and D-series resolvins regarding their actions during lung inflammation; however, recent evidence has identified important roles for resolvin E1 and resolvin D1 and their cellular targets in promoting the resolution of lung inflammation (Aoki et al., 2008; Haworth et al., 2008, 2011; Seki et al., 2010; Bilal et al., 2011; Wang et al., 2011; Eickmeier et al., 2012; El Kebir et al., 2012; Rogerio et al., 2012), including allergic airway responses (Aoki et al., 2008; Haworth et al., 2008, 2011; Bilal et al., 2011; Rogerio et al., 2012).

EXPRESSION OF CMKLR1 – CELL TYPE, LUNG TISSUE, ASTHMA

CMKLR1 is highly expressed in immature plasmacytoid DCs and at lower levels in myeloid DCs, macrophages, and NK cells (Arita et al., 2005a; Parolini et al., 2007). The CMKLR1 signaling pathway is structurally and functionally conserved between human and mouse. In a model of zymosan induced peritonitis, CMKLR1 deficient mice, exhibit increased inflammation, indicating that this receptor is important for counter-regulatory signaling (Cash et al., 2008). Both RvE1 and chemerin can interact with CMKLR1 and display potent anti-inflammatory properties in LPS-induced acute lung inflammation in mice, reducing neutrophil infiltration and inflammatory cytokine release in a CMKLR1-dependent manner (Luangsay et al., 2009).

The expression of CMKLR1 in plasmacytoid DCs suggests an important role in anti-viral immunity. When wild-type and CMKLR1 knock-out mice are infected by pneumonia virus of mice, the CMKLR1 deficient mice display higher mortality and morbidity, alteration of lung function, delayed viral clearance and increased neutrophilic infiltration. The CMKLR1 deficient mice have a lower recruitment of plasmacytoid DCs and a reduction in type I interferon production. Recruitment of plasmacytoid DCs via CMKLR1 contributes to adaptive immune responses and viral clearance, but also enhances the inflammatory response. Anti-inflammatory pathways involving CMKLR1 expressed by non-leukocytic cells in the lung also contribute to the increased morbidity/mortality in CMKLR1 deficient mice (Bondue et al., 2011).

Of interest, CMKLR1 signaling in acute lung inflammation appears context dependent. In a separate model of acute lung inflammation, cigarette smoke-induced lung inflammation was attenuated in CMKLR1 deficient mice with decreased levels of inflammatory chemokines and inflammatory cells. In addition, the infiltration of leukocytes persists for 14 days after cessation of smoke exposure in this model in wild-type mice, but the CMKLR1 deficient mice have a marked decrease in lung T cells at this time point (Demoor et al., 2011). Together, these findings indicate that the RvE1 receptor CMKLR1 is expressed in the lung by both leukocytes and structural cells and CMLKR1 signaling plays pivotal roles in the regulation of innate and adaptive immune cell activation in the lung.

The recruitment of CMKLR1-expressing leukocytes to the lung is regulated during inflammatory responses. Following acute LPS-induced lung inflammation, NK cells expressing CMKLR1 are recruited to the airways in a CCRL2-dependent manner. Engagement of CCRL2 on endothelial cells initiates adhesion of CMKLR1-expressing lymphoid cells through an $\alpha(4)\beta(1)$ integrin/VCAM-1-dependent mechanism. CCRL2 expression by endothelial cells is regulated by cell activation, so CMKLR1-dependent lymphocyte adhesion to endothelial cells can be targeted to sites of inflammation, including inflamed lung (Monnier et al., 2012).

EXPRESSION OF ALX/FPR2 – CELL TYPE, LUNG TISSUE, ASTHMA

ALX/FPR2 is expressed in several types of leukocytes (Chiang et al., 2006), including neutrophils (Fiore et al., 1992, 1994), monocytes (Maddox and Serhan, 1996), eosinophils (Levy et al., 2002), myeloid progenitors (Stenke et al., 1991), NK cells (Ramstedt et al., 1987; Haworth et al., 2011), and activated T cells (Ariel et al., 2003), as well as resident cells such as macrophages (Godson et al., 2000), synovial fibroblasts (Sodin-Semrl et al., 2000), and intestinal epithelial cells (Gronert et al., 1998). ALX/FPR2 is expressed in murine and human lung (Planaguma et al., 2008; Rogerio et al., 2012), airway epithelial cells (Bonnans et al., 2003, 2006), and alveolar macrophages (Rogerio et al., 2012). As early as 2 h after acute lung injury or inflammation, ALX/FPR2 expression increases in mucosal epithelial cells (Bonnans et al., 2006). Counter-regulatory signaling via ALX/FPR2 has been demonstrated *in vivo* using ALX/FPR2 deficient mice (Dufton et al., 2010) and transgenic mice that express human ALX/FPR2 directed by a component of the myeloid CD11b promoter (Devchand et al., 2003). ALX/FPR2 deficient mice have more marked inflammatory responses with increased leukocyte adherence and emigration into inflamed tissue after ischemia-reperfusion injury and after carrageenan-induced paw edema. In addition, ALX/FPR2 knock-out mice display increased sensitivity to arthrogenic serum and fail to resolve from this chronic inflammatory arthritis (Dufton et al., 2010). Also of note, human ALX/FPR2-transgenic mice have decreased inflammatory responses and are protected from the development of allergic airway inflammation with markedly decreased eosinophil activation and tissue accumulation (Levy et al., 2002). In asthma, ALX/FPR2 receptor expression is regulated in a cell type specific manner with decreases in peripheral blood neutrophil and eosinophil expression in this chronic inflammatory condition (Planaguma et al., 2008).

Recently, in subjects with chronic obstructive pulmonary disease, serum amyloid A (SAA) was identified as a biomarker for acute exacerbations (Bozinovski et al., 2008). SAA can also interact with ALX/FPR2 receptors, and unlike RvD1 or LXA_4, the SAA-ALX/FPR2 interactions are pro-inflammatory (Bozinovski et al., 2012). Because plasma levels of SAA are at least two-log orders higher than LXA_4 during acute exacerbations (Bozinovski et al., 2012), the pro-inflammatory SAA-ALX/FPR2 signaling can overwhelm the pro-resolving mediator protective signaling at this receptor. The balance of ALX/FPR2 ligands during asthma and the influence of corticosteroids is a subject of on-going investigation.

ALLERGIC AIRWAY RESPONSES – AN EXPERIMENTAL MODEL OF ADAPTIVE IMMUNITY AND ASTHMA

Animal models have not been developed that fully resemble human asthma, but they are quite useful for investigation of adaptive immunity and asthma traits. To model allergic airway

inflammation, animals are first sensitized to an allergen and then challenged by respiratory tract exposure to the same allergen (Kips et al., 2003; Corry and Irvin, 2006; Pichavant et al., 2007; Zosky and Sly, 2007). Roles for representative family members of D-series resolvins and E-series resolvins have been determined using a model in which chicken ovalbumin (OVA) serves as an allergen for in-bred mice. The animals are sensitized by intraperitoneal injection of OVA combined with the adjuvant aluminum hydroxide to initiate a strong Th2 phenotype (Aoki et al., 2008; Haworth et al., 2008, 2011; Bilal et al., 2011; Rogerio et al., 2012). In sensitized mice, OVA aerosol challenge on four consecutive days leads to adaptive inflammation consisting of predominantly eosinophils and T-lymphocytes, in particular in medium to small airways and alveoli (Levy, 2010). There is also perivascular inflammation. Antigen-induced responses also increase airway mucus metaplasia and hyper-responsiveness (Levy, 2010). To determine the extent of the airway hyper-responsiveness, methacholine is administered via inhalation while the mice are intubated and sedated on a ventilator circuit. A dose response curve is constructed for methacholine-initiated changes in lung resistance.

In most instances, the airway inflammation of asthma in humans does not resolve completely; however, in healthy airways, inhalation of potential allergens or provocative stimuli leads to an acute inflammatory response that is self-limited. Several classes of natural anti-inflammatory mediators, including resolvins, have been identified in inflamed airways (Bilal et al., 2011; Eickmeier et al., 2012). Because the clinical presentation of asthma is after the disease has already developed, more recent research has focused on the natural factors that promote resolution of allergic airway responses and identification of potential disease mechanisms that counter these endogenous, protective signals to perpetuate inflammation and potentially maladaptive airway responses. In the murine model of allergic airway responses described above, the cessation of OVA aerosol challenge leads to self-limited lung inflammation with resolution of the adaptive immune responses within 1–2 weeks (Haworth et al., 2008). Investigation of the resolution phase of allergic airway responses has uncovered several pro-resolving molecular and cellular mechanisms for adaptive airway inflammation (Levy et al., 2007; Haworth et al., 2008, 2011; Rogerio et al., 2012). NK cells were recently assigned important roles for clearance of antigen-specific T cells. During the natural resolution phase of allergic airway inflammation, eosinophils, and T cells decrease markedly concomitant with an increase in the numbers of NK cells in the lung and associated mediastinal lymph nodes (Haworth et al., 2011). These resolution NK cells also acquire cell surface markers, including NKG2D, consistent with NK cell activation (Haworth et al., 2011). The timely resolution of allergic airway inflammation is prolonged when NK cells are depleted, blocked from interacting with target cells, or inhibited from migrating to the lung, leading to a persistence of airway eosinophils and antigen-specific CD4 (Haworth et al., 2011) T cells (Haworth et al., 2011). Lung macrophages also serve important pro-resolving roles in the clearance of allergic airway inflammation. After the respiratory tract is exposed to allergen, lung macrophages display a significant capacity for clearance of airway antigen that was introduced during aerosol challenge (Rogerio et al., 2012). These recent findings identify important pro-resolving roles for innate lung tissue lymphocytes and macrophages in the regulation of adaptive immune responses.

ACTIONS OF RvD1 AND RvE1 IN ALLERGIC AIRWAY INFLAMMATION

LC-MS/MS-based lipido-metabolomic analyses of inflamed murine lung reveals picogram quantities of RvD1 and RvE1 that can be increased several fold with increased substrate availability (Bilal et al., 2011; Eickmeier et al., 2012). In asthma, airway mucosal epithelial cells have depleted stores of docosahexaenoic acid (Freedman et al., 2004) and lower levels of 17-hydroxy-DHA and protectin D1 in exhaled breath condensates compared with healthy control subjects (Levy et al., 2007). Recently, the potential beneficial actions of resolvins in experimental models of airway mucosal inflammation have been reported.

Lung expression of the RvD1 receptor ALX/FPR2 is induced *in vivo* in allergic airway inflammation (Levy et al., 2002). When RvD1 is given to OVA-sensitized mice just prior to OVA aerosol challenge, the development of allergic airway responses is significantly decreased (Rogerio et al., 2012). In particular, RvD1 markedly decreases eosinophils and levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluids (BALFs), consistent with a dominant effect of the mediator on the development of Th2 adaptive inflammation. Regulation of these cytokines by RvD1 is associated with a significant increase in lung IκBα, suggesting decreased activation of NF-κB. Airway mucus metaplasia is also decreased by RvD1 with a more modest effect on airway hyper-responsiveness to methacholine. BALF levels of the counter-regulatory mediators IL-10 and LXA_4 are not increased by RvD1 administration, indicating non-redundant anti-inflammatory signaling circuits for these mediators.

Potent regulation by RvD1 and AT-RvD1 of the allergen-driven accumulation of eosinophils was linked to significant decrements in BALF IL-5, eotaxin, and LTB_4 (Rogerio et al., 2012). In many asthmatics, a Th2 cytokine gene expression signature is induced (Woodruff et al., 2007), including IL-5, which is an important cytokine for the recruitment and activation of eosinophils, especially in conjunction with eotaxins (Busse and Lemanske, 2001). IL-5, eotaxin, NF-κB activation (Yang et al., 1998), and LTB_4 (Terawaki et al., 2005) can all increase airway eosinophilia. In murine allergic airway inflammation, RvD1 and AT-RvD1 decreased each of these mediators of eosinophil activation and accumulation. Eosinophils may also play important roles in airway remodeling and can generate the pro-fibrotic growth factor TGF-β1 (Wong et al., 1991). The RvD1 and AT-RvD1 mediated decrease in eosinophils and TGF-β levels (Rogerio et al., 2012) suggest additional beneficial actions for these mediators in preventing chronic airway remodeling. Further study in models of chronic inflammation is needed to address this potential tissue protective role for these D-series resolvins.

When administered after acute airway inflammation is established, RvD1 significantly and rapidly decreases the allergic lung inflammation within 1 h (Rogerio et al., 2012). Because RvD1 is subject to rapid inactivation in the lung, it is impact is transient (Rogerio et al., 2012), so when RvD1 is given daily for three consecutive days, there is only a modest decrease in the BALF

eosinophil resolution interval over the subsequent week (Rogerio et al., 2012). Of note, an equivalent dose and administration of AT-RvD1 (∼0.005 mg/kg) provides significantly greater pro-resolving actions than RvD1, including a marked decrease in the BALF eosinophil resolution interval by more than 50% – an approximate doubling of the pace of resolution! Both of these D-series resolvins are agonists at ALX/FPR2 receptors albeit with different binding kinetics (Perretti et al., 2002; Krishnamoorthy et al., 2010, 2012; Norling et al., 2012). In addition, the metabolism of these epimers is distinct (Sun et al., 2007). In the presence of lung macrophages, AT-RvD1 has a decreased rate of metabolic inactivation relative to RvD1 (Rogerio et al., 2012). RvD1 and AT-RvD1 are diastereomers, differing only in stereochemistry at carbon 17 (reviewed in Serhan et al., 2008). This change in stereochemistry for AT-RvD1 provides a significant increase in the mediator's half-life *in vivo*, secondary to resistance to metabolic inactivation by eicosanoid oxidoreductases (Sun et al., 2007; Krishnamoorthy et al., 2012).

AT-RvD1 displays potent pro-resolving actions on molecular and cellular inflammatory responses. During resolution of allergic airway responses, BALF levels of IL-17, eotaxin, TARC, TGF-β, and LTB_4 are significantly decreased by AT-RvD1 administration (Rogerio et al., 2012). For tissue catabasis after antigen challenge, it is essential to clear the allergen from the lung. Lung macrophages play critical roles in this catabatic process (Thornton et al., 2012). AT-RvD1 increases the macrophage phagocytosis index for OVA *in vitro* and *in vivo* (Rogerio et al., 2012). By promoting more rapid allergen clearance by lung macrophages, AT-RvD1 accelerates the pace of resolution of allergic airway responses, namely adaptive airway inflammation, mucus metaplasia, and hyper-responsiveness to methacholine.

Resolvin E1 is also a potent anti-inflammatory and pro-resolving mediator for allergic airway responses. Similar to RvD1, RvE1 can prevent the development allergic airway responses in this murine model of asthma (Levy et al., 2002, 2007; Haworth et al., 2008). When RvE1 is administered intravenously, it potently inhibits the induction of allergic airway inflammation (Aoki et al., 2008; Haworth et al., 2008) and when given during the resolution phase of inflammation, RvE1 accelerates the clearance of airway inflammation, mucus metaplasia, and hyper-reactivity to methacholine (Haworth et al., 2008, 2011). These RvE1-mediated bronchoprotective actions during resolution are multi-pronged, including inhibition of Th17 effector lymphocytes, engagement of activated NK cells and increased generation of interferon-gamma (IFN-γ) and LXA_4 (Haworth et al., 2008, 2011). After cessation of allergen exposure, the timely resolution of allergic airway responses is governed by regulation of the Th17 pathway (Haworth et al., 2008). This is in sharp contrast to the pivotal roles for Th2 cytokines during the development of allergic lung inflammation (*vide supra*). IL-17 can be generated by several cell types in asthmatic lung, including Th17 cells that are a subset of $CD4^+$ T helper cells characterized by the production of IL-17 and whose population expansion and survival depends upon IL-23. IL-17 has been linked to the pathogenesis of many inflammatory diseases, is present in the airways of asthmatic patients and can induce lung inflammation, airway hyper-reactivity and mucus production (Chen et al., 2003; Haworth et al., 2008; Al-Ramli et al., 2009; Alcorn et al., 2010; Lajoie et al., 2010). Similar to RvD1 and AT-RvD1, RvE1 also regulates IL-17 to promote resolution of allergic airways responses.

Identification of an important role for IL-17 in the persistence of lung inflammation, in particular during allergic airway inflammation, is supported by several lines of evidence. Transgenic expression of IL-17 in airway epithelial cells induces airway eosinophil and lymphocyte infiltration and structural changes with mucus metaplasia (Park et al., 2005). Mice deficient for the IL-17 receptor are protected from allergen-induced airway inflammation (Schnyder-Candrian et al., 2006), and in humans, asthmatic subjects have higher levels of BALF and sputum IL-17 (Molet et al., 2001; Barczyk et al., 2003; Schnyder-Candrian et al., 2006). Indicative of its importance in the lung, IL-17 may have dual roles in the regulation of allergic airway inflammation, as it can also inhibit T_H2 immune responses in model systems (Schnyder-Candrian et al., 2006). In promoting resolution, RvE1 significantly decreases both IL-17 and allergic airway responses (Haworth et al., 2008), supporting a relationship between IL-17 and persistent airway inflammation.

In addition to IL-17, BALF levels of IL-23 are also decreased by administration of RvE1. IL-23 is also involved in the pathobiology of chronic inflammatory diseases, including colitis, encephalitis, psoriasis, rheumatoid arthritis, and cancer (Langrish et al., 2005; Chan et al., 2006; Langowski et al., 2006; Yago et al., 2007), and IL-23 is critical for the survival of T_H17 cells (Langrish et al., 2005; Bettelli et al., 2006). IL-23 induces the release of pro-inflammatory chemokines from eosinophils, which express the IL-23 receptor (Cheung et al., 2008). Moreover, IL-23 deficient mice are protected in models of chronic inflammation, such as colitis (Yen et al., 2006). Of note, RvE1 also protects against the development of colitis in model systems (Arita et al., 2005b; Hudert et al., 2006). Thus, regulation of IL-23 by RvE1 appears critical to its pro-resolving actions in mucosal inflammation.

As mentioned above, NK cells express the RvE1 receptor CMKLR1 (Arita et al., 2005a; Parolini et al., 2007). NK cell depletion impairs RvE1's protective actions for the timely resolution of adaptive inflammation (Haworth et al., 2011). RvE1 regulates NK cell homing, increases clearance of antigen-specific $CD4^+$ T cells and increases NK cell cytotoxicity. Circulating and lung NK cell numbers increase with RvE1 administration, consistent with a role for RvE1 in NK cell transit through the inflamed lung to its associated mediastinal lymph nodes. RvE1 induces CXCL9 expression in the lung and mediastinal lymph nodes. Antibody-mediated inhibition of CXCL9-CXCR3 interactions blocks NK cell infiltration into inflamed lung and lymph nodes, and delays resolution of adaptive airway inflammation. These findings indicate that RvE1 can regulate tissue chemokines to target NK cell homing to the lung for catabasis. The influence of D-series resolvins and ALX/FPR2 signaling on NK cell functional responses is an area of active investigation.

Current anti-inflammatory therapeutic strategies for asthma include corticosteroids, CysLT1 receptor antagonists and anti-IgE antibody (Fanta, 2009). While little is known regarding the influence of these approaches on the actions of resolvins, their independent signaling pathways suggest that the resolvins

would complement existing therapeutics. Corticosteroids share the resolvins' anti-inflammatory actions on eosinophils and T cells; however, they do not share resolvins' pro-resolving actions for macrophages and NK cells. CysLT1 receptor antagonists and anti-IgE antibody target specific pro-phlogistic pathways and would not be expected to interfere with the resolvins' mechanisms of action. Regarding pharmacological considerations for the resolvins, these mediators carry potent actions in sub-nanomolar concentrations *in vitro* (reviewed in Serhan, 2007) and at doses of ∼0.005 mg/kg *in vivo* (Haworth et al., 2008; Rogerio et al., 2012). As with the Fat-1 transgenic mouse (Bilal et al., 2011), increasing the tissue levels of omega-3 fatty acids can increase resolvin formation and offer protection from allergic airway responses; however, dietary supplementation is less potent than direct administration of resolvins (Seki et al., 2010).

SHARED AND DISTINCT PRO-RESOLVING MECHANISMS FOR RvE1 AND RvD1/AT-RvD1 IN ADAPTIVE INFLAMMATION

RvE1 and RvD1/AT-RvD1 are agonists at distinct pro-resolving receptors, yet share many similar properties in the regulation of adaptive inflammation in the lung (**Figure 1**, **Table 2**). These mediators decrease recruitment of lung eosinophils, lymphocytes, and macrophages during adaptive immune responses and decrease allergic airway responses, including mucus metaplasia and hyper-responsiveness to methacholine. In addition, these specialized pro-resolving mediators share many similarities in the regulation of lung inflammatory peptide and lipid mediators. Despite these commonalities, the RvE1 and RvD1/AT-RvD1 pro-resolving pathways are not entirely redundant. In addition to their distinct

Table 2 | Shared and distinct pro-resolving mechanisms for RvE1 and RvD1/AT-RvD1 during murine adaptive inflammation.

Receptors	RvE1 CMKLR1 BLT1	RvD1/AT-RvD1 ALX/FPR2	LXA_4 analog ALX/FPR2 CysLT1
ALLERGIC AIRWAY RESPONSES			
Inflammation			
Eosinophils	Decreased	Decreased	Decreased
Lymphocytes	Decreased	Decreased	Decreased
NK Cells	Increased		
Macrophages	Decreased	Increased	
Mucus metaplasia	Decreased	Decreased	Decreased
MCh ED200	Increased	Increased	Increased
CYTOKINES			
IL-4	No change	No change	
IL-5	No change	Decreased	
IL-6	Decreased		
IL-10	No change	No change	
IL-13	No change	No change	
IL-17	Decreased	Decreased	Decreased
IL-23	Decreased	Decreased	No change
IL-27	Decreased		
Interferon-gamma	Increased	No change	Decreased
CHEMOKINES			
Eotaxin		Decreased	
TARC		Decreased	
LIPID MEDIATORS			
LTB_4	Decreased	Decreased	
CysLTs	No change		
LXA_4	Increased	No change	

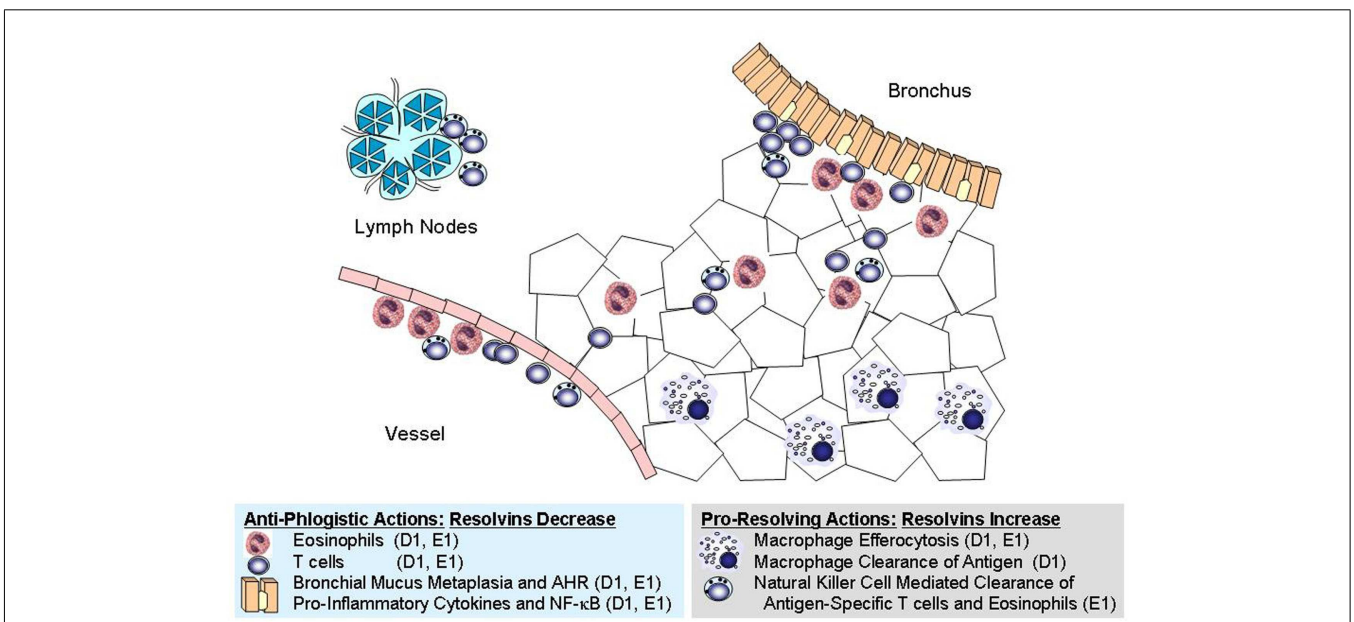

FIGURE 1 | Cellular targets for resolvins in allergic inflammation. Shared and distinct anti-phlogistic and pro-resolving actions are illustrated for RvD1 (D1) and RvE1 (E1). These natural mediators have independent signaling pathways initiated via interactions with distinct receptors, yet they share many overlapping properties at the cellular and tissue levels.

receptors, there are clear differences between RvE1 and RvD1/AT-RvD1 in the regulation of BALF levels of IL-5, IFN-γ, and LXA_4 during resolution (Haworth et al., 2008, 2011; Rogerio et al., 2012). While there is no data on concomitant administration of RvE1 and RvD1 in this model, co-administration of RvE1 and a bioactive LXA_4 stable analog, which, like RvD1/AT-RvD1, interacts with ALX/FPR2 receptors, provides additive pro-resolving actions, yet there are important differences in their mechanisms. Both RvE1 and the LXA_4 analog decrease BALF levels of IL-17, but distinct from RvE1, the LXA_4 analog does not inhibit IL-23 production or increase IFN-γ levels (Haworth et al., 2008). These findings of shared and distinct points of counter-regulation for RvE1 and the LXA_4 stable analog indicate the presence of independent pro-resolving signaling circuits, likely mediated by CMKLR1 and ALX/FPR2 respectively, which in this model of adaptive immunity converge on the regulation of IL-17 to promote catabasis (**Table 2**). Of interest, when administered during the upstroke of allergic inflammation, both RvD1 and RvE1 are also potent regulators of the development of airway hyper-responsiveness to methacholine, mucus metaplasia, eosinophil accumulation, and T_H2 cytokine mediator release (e.g., IL-13; Haworth et al., 2008, 2011). Regulation of IL-13 by resolvins during induction of adaptive inflammation is distinct from their actions when given during resolution. When given after the adaptive inflammation is already established, these resolvins do not lead to significant changes in BALF levels of IL-13; however, both RvD1 and RvE1 lead to marked decreases in BALF levels of IL-17. While the mechanisms that induce adaptive inflammation (i.e., T_H2 cytokines) are distinct from those linked to persistent mucosal inflammation (i.e., IL-17 and IL-23), the protective actions of resolvins include regulation of both of these important families of inflammatory mediators. Inhibition of IL-17 production appears to be a common point of regulation for RvE1 and RvD1 for resolution of allergic airway responses (Serhan et al., 2000; Haworth et al., 2011).

SUMMARY AND CONCLUSIONS

The recent discovery of resolvins, endogenously generated from the essential fatty acids docosahexaenoic acid and eicosapentaenoic acid, has uncovered molecular and cellular mechanisms for the resolution of acute and adaptive inflammation. These specialized and stereospecific pro-resolving chemical mediators play important roles in limiting allergic airway responses and promoting catabasis of the inflamed lung. In the lung, resolvins are enzymatically generated during cell–cell interactions, often between leukocytes and structural cells. Their interactions with specific receptors establish resolution circuits with cell type specific functional responses. While RvD1 and RvE1 share some cellular targets and pro-resolving actions, there is accumulating evidence for distinct counter-regulatory signaling pathways, including distinct receptors. In response to acute inflammation, these endogenous mediators blunt the inflammatory response by inhibiting aberrant neutrophil trafficking and activation, stimulating efferocytosis of apoptotic neutrophils and promoting anti-angiogenic, anti-fibrotic, and anti-infective actions. In allergic immune responses, resolvins enlist NK cells to facilitate clearance of activated T cells and activate macrophages (in a non-phlogistic manner) for phagocytic removal of allergen deposited in inflamed lung. Rapidly formed during inflammatory responses, these autacoids are also rapidly inactivated by eicosanoid oxidoreductases. In the setting of chronic inflammatory lung disease, airway levels of omega-3 fatty acids and pro-resolving mediators are decreased. With no curative therapy currently available for asthma or several other chronic inflammatory diseases, the development of resolvin stable analogs is leading to exciting new potential therapeutic approaches in acute and adaptive chronic inflammation that emphasize these natural homeostatic pathways.

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Resolution of inflammation in obesity-induced liver disease

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Low-grade inflammation in adipose tissue is recognized as a critical event in the development of obesity-related co-morbidities. This chronic inflammation is powerfully augmented through the infiltration of macrophages, which together with adipocytes, perpetuate a vicious cycle of inflammatory cell recruitment and secretion of free fatty acids and deleterious adipokines that predispose to greater incidence of metabolic complications. In the last decade, many factors have been identified to contribute to mounting unresolved inflammation in obese adipose tissue. Among them, pro-inflammatory lipid mediators (i.e., leukotrienes) derived from the omega-6 polyunsaturated arachidonic acid have been shown to play a prominent role. Of note, the same lipid mediators that initially trigger the inflammatory response also signal its termination by stimulating the formation of anti-inflammatory signals. Resolvins and protectins derived from the omega-3 polyunsaturated docosahexaenoic and eicosapentaenoic acids have emerged as a representative family of this novel class of autacoids with dual anti-inflammatory and pro-resolving properties that act as "stop-signals" of the inflammatory response. This review discusses the participation of these endogenous autacoids in the resolution of adipose tissue inflammation, with a special emphasis in the amelioration of obesity-related metabolic dysfunctions, namely insulin resistance and non-alcoholic fatty liver disease.

Keywords: obesity, omega-6 fatty acids, eicosanoids, omega-3 fatty acids, resolvins, stromal-vascular macrophages, Kupffer cells

RESOLUTION OF INFLAMMATION: CIRCUITS AND CHEMICAL MEDIATORS

Inflammation plays a vital role in host defense against invasive pathogens and tissue and wound repair. Inflammation is part of the innate immune response and is initiated by a cascade of signals in response to an infection or injury that leads to the recruitment of specialized inflammatory cells, particularly neutrophils (PMN), into injured tissue to neutralize and eliminate the injurious stimuli (Barton, 2008; Chen and Nuñez, 2010). The innate immune response not only acts as the first line of defense against an insult, but it also provides the necessary signals to instruct the adaptive immune system for an effective response to deal with the noxious agent. Although inflammation is important in eradication of pathogens, unresolved, chronic inflammation that occurs when the offending agent is not removed or contained is detrimental to the host, resulting in tissue damage, fibrosis, and loss of function (Barton, 2008; Chen and Nuñez, 2010).

Since unresolved inflammation is detrimental to the host, higher organisms have evolved protective mechanisms to ensure resolution of the inflammatory response in a specific time-limited manner (Serhan et al., 2008). Once considered a mere passive process of dilution, resolution is today envisioned as a highly orchestrated process coordinated by a complex regulatory network of cells and mediators. This novel insight offers the possibility to harness resolution factors that clear inflammation and use them to ameliorate the pathologies associated with chronic inflammation. This has the benefit to avoid any unwanted side-effect observed during the long-term therapy with anti-inflammatory drugs such as cyclooxygenase (COX) inhibitors. COX inhibitors, like aspirin (ASA) or ibuprofen, can cause gastrointestinal irritation and renal damage when used in high doses (Wallace and Vong, 2008). Although at first glance selective COX-2 inhibitors looked like to overcome NSAID toxicity on the gastrointestinal tract, COX-2 inhibitors as Vioxx were later withdrawn from the market for their increased risk of cardiovascular thrombotic events (Wallace and Vong, 2008). For this reason, the search for novel targets and the identification of molecular circuits and chemical mediators involved in resolution represent a priority in anti-inflammatory therapy.

Among the molecules that facilitate resolution, lipid mediators derived from the metabolism of essential polyunsaturated fatty acids have attracted most attention. The first recognized family of specialized pro-resolving mediators (SPM) was the lipoxins (LXs). LXs are conjugated trihydroxytetraene-containing eicosanoids generated from endogenous sources of the omega-6 arachidonic acid (Serhan, 2002). A major route of transcellular LX biosynthesis is initiated by 15-lipoxygenase (15-LO) forming 15*S*-hydroxyeicosatetraenoic acid (15*S*-HETE), which is rapidly converted to LXA_4 by 5-LO (**Figure 1**; Serhan et al., 1984). Another major route of transcellular LX biosynthesis is the generation of 15-epi-LXs through a circuit initiated by acetylation of COX-2 by ASA (Clària and Serhan, 1995). In this route, when ASA inhibits

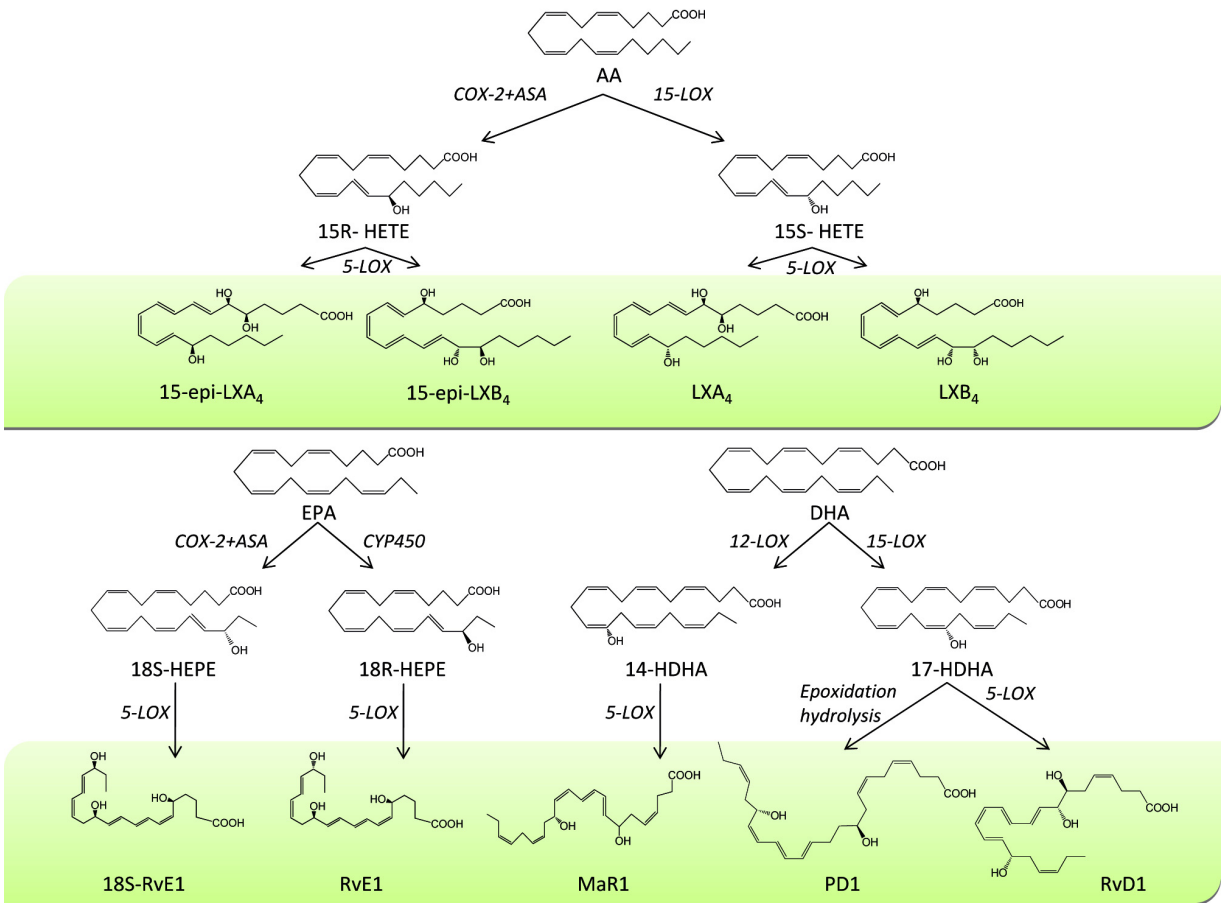

FIGURE 1 | Biosynthesis of specialized pro-resolving mediators (SPM). During the process of resolution of inflammation, the omega-6 fatty acid arachidonic acid (AA) is converted by 15-lipoxygenase (15-LOX) to 15*S*-hydroxyeicosatetraenoic acid (15*S*-HETE), which is rapidly converted to LXA_4 and LXB_4 by 5-LOX. Formation of 15-epi-LXA_4 and 15-epi-LXB_4 from 15*R*-HETE can also occur after acetylation of cyclooxygenase-2 (COX-2) by aspirin (ASA). Similarly, the omega-3 fatty acid eicosapentaenoic acid (EPA) is converted into 18-hydroperoxy-EPE (18-HEPE) by aspirin-treated COX-2 or through cytochrome P450 (CYP450) and subsequently transformed by 5-LOX into 18*S*- or 18*R*-resolvin (Rv) E1. DHA is converted into 17-hydroxy-DHA (17-HDHA) by 15-LOX which subsequently is transformed by 5-LOX into RvD1 and by epoxidation hydrolysis into protectin D1 (PD1), respectively. Finally, DHA is transformed by 12-LOX into 14-hydroxy-DHA (14-HDHA) and by 5-LOX into maresin 1 (MaR1).

prostaglandin (PG) formation in cells bearing a cytokine-induced COX-2, the resulting ASA-acetylated COX-2 converts arachidonic acid into 15*R*-HETE. Subsequently, 15*R*-HETE is transformed by 5-LO of activated neutrophils into 15-epi-LXs, which carry the carbon-15 alcohol in the *R* configuration, instead of the *S* as in the native LXs (**Figure 1**; Clària and Serhan, 1995). These SPM act as "stop-signals" for inflammation and inhibit leukocyte chemotaxis, adhesion to and transmigration across endothelial monolayers in response to LTB_4 (Serhan et al., 2008). LX stable analogs inhibit *in vivo* LTB_4-induced leukocyte rolling, adherence, margination and extravasation and when applied topically to mouse ears they dramatically inhibit leukocyte infiltration and vascular permeability (Serhan et al., 2008).

Resolvins are the second family of SPM with recognized anti-inflammatory and pro-resolving properties. Resolvins are endogenous lipid mediators generated from the omega-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). They were initially identified using a lipidomics-based approach that combined liquid chromatography and tandem mass spectrometry within self-limited inflammatory exudates captured during the "spontaneous resolution" phase of acute inflammation (Serhan et al., 2000, 2002). Resolvins are classified into D- and E-series in accordance with their biosynthetic precursor, either DHA or EPA, respectively. Schematically, resolvin biosynthesis is initiated by 15-LO which transforms endogenous sources of DHA into 17*S*-hydroxy-DHA which is further transformed by leukocyte 5-LO into resolvin (Rv) D1 and RvD2 (**Figure 1**; Hong et al., 2003). Endothelial cells expressing COX-2 treated with aspirin also transform DHA into 17*R*-hydroxy-DHA which is further converted by 5-LO into 17*R*-RvD1 (**Figure 1**; Serhan et al., 2000, 2002). DHA can also be metabolized into a dihydroxy-containing derivative via an intermediate epoxide that opens via hydrolysis and subsequent rearrangements to form protectin (PD) 1 (**Figure 1**; Serhan et al., 2000, 2002; Hong et al., 2003). Similarly, RvE1 biosynthesis is initiated when EPA is converted to 18-hydroperoxy-EPE by aspirin-treated COX-2

or through cytochrome P450 activity (Serhan et al., 2000; Haas-Stapleton et al., 2007). By transcellular biosynthesis, 18-hydroperoxy-EPE is transformed by 5-LO of neighboring leukocytes into RvE1 via a 5(6)epoxide intermediate (**Figure 1**; Serhan et al., 2000, 2002).

RvD1, RvD2, PD1, and RvE1 are potent SPM, which contrary to their metabolic substrates, DHA and EPA, exert their biological actions at the nanomolar range. Indeed, the potency of these SPM is notable with concentrations as low as 10 nM producing a 50% reduction in PMN transmigration. Two receptors (ALX/FPR2 and GPR32) have been shown to transmit RvD1 signals (Krishnamoorthy et al., 2010), whereas a G-protein coupled receptor (ChemR23) signals for RvE1 (Arita et al., 2005a). The full structural elucidation, stereochemical assignment and biological actions for these compounds were first completed in RvE1. RvE1 was readily shown to decrease PMN infiltration and T cell migration, reduce tumor necrosis factor (TNF) α and IFNγ secretion, inhibit chemokine formation, and block interleukin (IL)-1-induced NF-κB activation (Schwab et al., 2007; Bannenberg and Serhan, 2010). RvE1 was also shown to stimulate macrophage phagocytosis of apoptotic PMN and to be a potent counter-regulator of L-selectin expression (Schwab et al., 2007; Dona et al., 2008). RvE1 displayed potent anti-inflammatory actions *in vivo*, protecting mice against experimental periodontitis, colitis, peritonitis, and brain ischemia-reperfusion (Arita et al., 2005b; Bannenberg and Serhan, 2010). A RvE1-initiated resolution program for allergic airway response was identified by Haworth et al. (2008). Similarly, RvD1 and RvD2 were reported to reduce inflammatory pain, block IL-1β transcripts induced by TNFα in microglial cells and function as potent regulators limiting PMN infiltration into inflamed brain, skin, and peritoneum (Hong et al., 2003; Sun et al., 2007). RvD2 in particular has been shown to be a potent endogenous regulator of excessive inflammatory responses in mice with microbial sepsis (Spite et al., 2009). Moreover, PD1 has been reported to exert protective actions in acute models of inflammation by blocking PMN migration and infiltration into the inflammatory site (Serhan et al., 2006). Finally, these SPM expedite the resolution process by paving the way for monocyte migration and their differentiation to phagocytosing macrophages, which remove dead cells (efferocytosis) and then terminate the inflammatory response by promoting macrophage efflux into lymphatics (Schif-Zuck et al., 2011).

RESOLUTION OF ADIPOSE TISSUE INFLAMMATION IN OBESITY

Abdominal obesity and insulin resistance are the predominant underlying risk factors for the metabolic syndrome and related co-morbidities such as type 2 diabetes, dyslipidemia, and non-alcoholic fatty liver disease (Elks and Francis, 2010). A wealth of evidence indicates that metabolic disorders associated with obesity are initiated by the presence of a chronic "low-grade" state of inflammation in the adipose tissue (Ferrante, 2007; Elks and Francis, 2010). This "low-grade" inflammatory state is aggravated by the recruitment of inflammatory cells, mainly macrophages in the adipose tissue (Ferrante, 2007; Elks and Francis, 2010). As a consequence of this unresolved inflammatory response, the production of pro-inflammatory adipokines [i.e., IL-6, TNFα, and monocyte chemotactic protein-1 (MCP-1)] is increased while the secretion of adiponectin, an anti-inflammatory and insulin-sensitizing adipokine, is reduced (**Figure 2A**; Ferrante, 2007; Elks and Francis, 2010). In addition to adipokines, the formation of pro- and anti-inflammatory lipid mediators is also severely deregulated in obesity. Indeed, we have recently demonstrated that the production of SPM (i.e., RvD1 and PD1 and the metabolic precursors 14-HDHA, 17-HDHA, 18-HEPE) is deficient in inflamed obese adipose tissue (Clària et al., 2012). Whether the response to these mediators is also impaired and whether this SPM deficit is a generalized property of obese tissues are open questions that need to be addressed.

Adipose tissue inflammation is also driven by the activation of classical pro-inflammatory pathways such as arachidonate 5-LO. Indeed, over-expression of FLAP is a common finding in adipose tissue of patients and animals with obesity and insulin resistance (Kaaman et al., 2006; Horrillo et al., 2010). Moreover, linkage studies have identified 5-LO as a gene with pleiotropic actions on adipose fat accumulation and pancreatic function (Mehrabian et al., 2008). The ability of adipose tissue to generate 5-LO-derived products has recently been challenged by Horrillo et al. (2010). These authors have demonstrated the presence of all enzymes necessary for the formation of 5-LO products (5-LO, FLAP, LTA_4 hydrolase, and LTC_4 synthase) as well as all receptors involved in leukotriene (LT) signaling (BLT1, BLT2, CysLT1, and CysLT2) in adipose tissue of both lean and obese mice (Horrillo et al., 2010). Importantly, adipose tissue samples from obese mice showed increased formation of 5-LO products, mainly LTB_4 (Horrillo et al., 2010). Similar findings have been reported in visceral adipose tissue from obese Zucker rats (Chakrabarti et al., 2011). An important observation of the study by Horrillo et al. (2010) was that LTB_4 unequivocally triggered an inflammatory response in adipose tissue by inducing the nuclear translocation of p50 and p65 subunits of NF-κB. Secondary to LTB_4-induced NF-κB activation, there was an enhanced release of MCP-1 and IL-6, which directly connect adipose tissue inflammation with insulin resistance and hepatic steatosis (Horrillo et al., 2010). The physiological consequences of these changes in adipose tissue function were corroborated *in vivo* by observing that either pharmacological inhibition of the 5-LO pathway or genetic deletion of *Alox5*, the gene coding for 5-LO, alleviate insulin resistance and hepatic steatosis in obese animals (Horrillo et al., 2010; Martínez-Clemente et al., 2010).

In sharp contrast to the pro-inflammatory actions for the most part of omega-6-derived products, omega-3-derived lipid mediators act as "braking signals" of the persistent vicious cycle leading to unremitting inflammation in obese adipose tissue. Endres et al. (1989) were the first to demonstrate anti-inflammatory properties of the omega-3 fatty acids. Since then, supplementation of omega-3 fatty acids has proven to exert overall benefits in obesity and metabolic syndrome. In a recent series of experiments, González-Périz et al. (2009) have demonstrated that administration of an omega-3-enriched diet to *ob/ob* mice, an experimental model of obesity and fatty liver disease, resulted in increased adiponectin levels and reduced insulin resistance and hepatic steatosis. These changes occurred in parallel with augmented formation of omega-3-derived SPM in

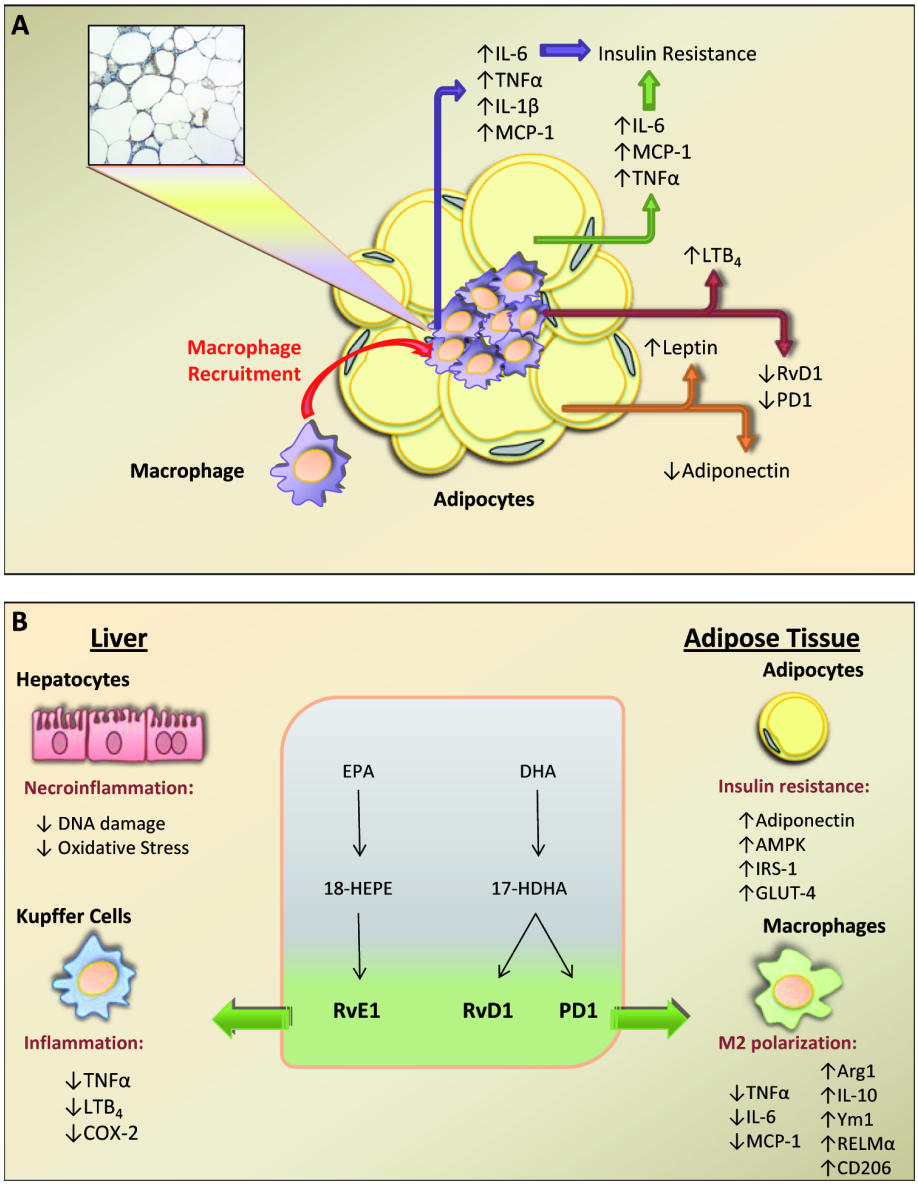

FIGURE 2 | (A) Schematic overview summarizing the cross-talk between macrophages and adipocytes in obese adipose tissue. Obese adipose tissue shows a remarkable infiltration of macrophages which form "crown-like" structures that surround necrotic adipocytes. This recruited macrophages together with hypertrophy and/or hyperplasia of adipocytes produce an aberrant release of pro-inflammatory adipokines [tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-1β, and monocyte chemotactic protein-1 (MCP-1)] that leads to insulin resistance. Unbalanced formation of pro-inflammatory leukotriene (LT) B_4 and leptin accompanied by a deficit in anti-inflammatory mediators [i.e., resolvin (Rv) D1, protectin (PD) 1, and adiponectin] contributes to a state of unresolved inflammation in obese adipose tissue. **(B)** Schematic representation of the protective actions of specialized omega-3-derived mediators on liver and adipose tissue. Eicosapentaenoic acid (EPA) is converted into 18-hydroperoxy-EPE (18-HEPE) and resolvin (Rv) E1, whereas DHA is converted into 17-hydroxy-DHA (17-HDHA) and RvD1 and protectin D1 (PD1). In the liver, these specialized pro-resolving mediators (SPM) protect hepatocytes from DNA damage and oxidative stress and dampen inflammation by inhibiting TNFα, LTB_4, and cyclooxygenase-2 (COX-2) in Kupffer cells. In adipose tissue, SPM exert insulin sensitizing actions by up-regulating adiponectin, AMP-activated protein kinase (AMPK), insulin receptor signaling-1 (IRS-1) and glucose transporter-4 (GLUT-4) in adipocytes and promoting M2 polarization [arginase 1 (Arg1), IL-10, chitinase 3-like 3 (Ym1), resistin-like molecule (RELM)-α, and CD206] while inhibiting M1 markers (TNFα, IL-6, and MCP-1) in macrophages.

adipose tissue, while formation of the omega-6-derived products PGE_2, 5-HETE, and LTB_4 was significantly inhibited (González-Périz et al., 2009). Along these lines, intraperitoneal injection of nanogram doses of RvE1 elicited significant insulin-sensitizing effects by inducing adiponectin, glucose transporter-4 (GLUT-4) and insulin receptor signaling-1 (IRS-1) expression in adipose tissue and conferred significant protection against hepatic steatosis (González-Périz et al., 2009). Similarly, in leptin receptor-deficient (*db/db*) obese and diabetic mice, nanogram doses of RvD1 improved glucose tolerance, decreased fasting blood glucose, and

increased insulin-stimulated Akt phosphorylation while reducing the formation of crown-like structures rich in inflammatory macrophages in adipose tissue (Hellmann et al., 2011). Recently, similar beneficial actions have been described for LXA_4 in an experimental model of age-associated adipose inflammation (Börgeson et al., 2012).

Omega-3-derived mediators can also induce changes in the status of macrophage polarization toward a pro-resolution phenotype. Tissue macrophages are phenotypically heterogeneous and 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). Macrophages are broadly characterized by their activation (polarization) state according to the M1/M2 classification system (Mantovani et al., 2007). In this classification, the M1 designation is reserved for classically activated macrophages following stimulation with IFNγ and LPS, whereas the M2 designation is applied to the alternatively activated macrophages after *in vitro* stimulation with IL-4 and IL-13. M1 macrophages secrete high amounts of TNFα, IL-1β, and IL-6, whereas M2 macrophages dampen pro-inflammatory cytokine levels and promote resolution of inflammation and tissue repair (Gordon and Taylor, 2005). M1/M2 macrophage polarization can be monitored by assessing the expression of selected markers. M1-associated markers include inducible nitric oxide synthase (iNOS) and classical pro-inflammatory mediators such as TNFα, IL-1β, IL-6, and MCP-1. In contrast, established M2 markers include scavenger, mannose (CD206) and galactose (Mgl-1) receptors, arginase 1, IL-10, chitinases Ym1 and Ym2, and resistin-like molecule (RELM)-α (Martínez et al., 2009).

In a recent study, Titos et al. (2011) have demonstrated that RvD1 consistently induced M2 polarization in adipose tissue macrophages. These investigators first noticed that DHA did not modify the total number of macrophages in obese adipose tissue, but markedly reduced the percentage of $CD11b^{high}/F4/80^{high}$ expressing cells in parallel with the emergence of low-expressing CD11b/F4/80 macrophages, suggesting a phenotypic switch in macrophage polarization. Indeed, these investigators further demonstrated that DHA and RvD1 up-regulated a complete panel of M2 markers including IL-10, CD206, RELM-α, and Ym1, and remarkably stimulated arginase 1 expression while promoting non-phlogistic macrophage phagocytosis and attenuating IFNγ/LPS-induced Th1 cytokine secretion (Titos et al., 2011). These results were in agreement with those reported by Hellmann et al. (2011), who showed the ability of RvD1 to improve insulin resistance in obese-diabetic mice, by reducing macrophage $F4/80^{+}CD11c^{+}$ cell accumulation and increasing the percentage of positive F4/80 cells expressing the M2 marker Mgl-1 in adipose tissue. The ability of resolvins to modify macrophage plasticity has also been demonstrated by Schif-Zuck et al. (2011), who reported that administration of RvD1 and RvE1 to peritonitis-affected mice 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. As the majority of macrophages that accumulate in obese adipose tissue are M1 inflammatory type, these findings are a strong argument in favor of the pro-resolution actions of omega-3-derived mediators in obese adipose tissue.

RESOLUTION OF OBESITY-INDUCED STEATOHEPATITIS

Lipids, adipokines, and other soluble factors released by inflamed adipose tissue have a direct impact on other insulin-sensitive tissues, especially on the liver. In fact, both adipose and hepatic tissues have immediate access to a vast network of blood vessels that implicate a direct connection between these two tissues. This connection is exemplified by the observation that the circulating fatty acid pool derived from fat is the primary contributor to hepatic steatosis, the initial stage in non-alcoholic steatohepatitis (Donnelly et al., 2005). In this context, adiponectin represents a paradigmatic example of the direct control of adipokines on liver function. Adiponectin, which is an adipokine with potent anti-inflammatory and insulin-sensitizing properties, is a hepatoprotective adipokine lowering hepatic steatosis and insulin resistance and preventing liver fibrosis (Tilg, 2010). Importantly, adiponectin is able to up-regulate the RvE1 receptor ChemR23 in primary human adipocytes, which expression is seriously compromised in human and rodent fatty liver (Wanninger et al., 2012).

To better appreciate how adipose tissue influences hepatic inflammation and the progression from steatosis to steatohepatitis, it is necessary to fully understand the complex cellular architecture of the liver. The hepatic tissue is arranged in a peculiar fenestrated capillary network known as the hepatic sinusoid (Wisse et al., 1996). The morphological features of the hepatic sinusoid provide a unique environment where each single hepatocyte is in close contact with other hepatocytes as well as with non-parenchymal sinusoidal liver cells, including Kupffer cells, endothelial cells, and hepatic stellate cells (Wisse et al., 1996). In terms of inflammation, Kupffer cells, the liver resident macrophages, play the most relevant role and have been classically considered the major sinusoidal cell type involved in hepatic eicosanoid formation (Decker, 1990). Indeed, Kupffer cells express COX-1, COX-2, and 5-LO and generate relevant amounts of PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2, LTB_4, and $LTC_4/LTD_4/LTE_4$ (Decker, 1990; Titos et al., 2000, 2003). These resident hepatic macrophages are also able to generate LXA_4 from endogenous sources of arachidonic acid or by transcellular biosynthesis from 15*S*-HETE released by nearby 15-LO-containing hepatocytes (Clària and Planagumà, 2005). Unlike LTB_4 and PGE_2, Kupffer cell-derived LXA_4 down-regulates the cytokine–chemokine axis in adjacent hepatocytes (Planagumà et al., 2002).

Liver tissue is also a rich source of omega-3-derived SPM, such as PD1 and its intermediate precursor 17*S*-HDHA (González-Périz et al., 2006). These SPM produced an amelioration of necroinflammatory liver injury, an effect that was associated with a decrease in hepatic COX-2 expression and PGE_2 formation and reduced genotoxic DNA damage and oxidative stress in isolated hepatocytes (González-Périz et al., 2006). More important, these SPM reduced TNFα release in macrophages, recognized as the predominant effector cells involved in the inflammatory cascade leading to hepatocyte damage. 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 its inhibition is linked to lower necroinflammatory liver injury and fibrosis (Titos et al., 2000, 2003, 2005).

SUMMARY

Obesity and the associated metabolic disorders are characterized by the presence of a chronic "low-grade" inflammatory response in insulin sensitive tissues, in particular adipose tissue and liver. The mechanisms explaining this observation are unknown but unremitting inflammation is likely to be the consequence of an impaired resolution. Resolution of inflammation (the so-called, "catabasis") is not a passive process that simply occurs when the stimulus disappears, but it is a highly regulated process that requires the coordinated action of pro-resolution SPM. Among these, in recent years we have witnessed an emergence of a number of SPM carrying both anti-inflammatory and pro-resolution properties, namely LXs, resolvins, and protectins. A schematic representation of the actions of these lipid mediators on adipose tissue and liver cells is shown in **Figure 2B**. In summation, these autacoids enhance inflamed adipose tissue catabasis and provide powerful templates for the design of novel therapies to combat the progression of metabolic complications associated with obesity.

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Resolution of inflammation: therapeutic potential of pro-resolving lipids in type 2 diabetes mellitus and associated renal complications

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The role of inflammation in the pathogenesis of type 2 diabetes mellitus (T2DM) and its associated complications is increasingly recognized. The resolution of inflammation is actively regulated by endogenously produced lipid mediators such as lipoxins, resolvins, protectins, and maresins. Here we review the potential role of these lipid mediators in diabetes-associated pathologies, specifically focusing on adipose inflammation and diabetic kidney disease, i.e., diabetic nephropathy (DN). DN is one of the major complications of T2DM and we propose that pro-resolving lipid mediators may have therapeutic potential in this context. Adipose inflammation is also an important component of T2DM-associated insulin resistance and altered adipokine secretion. Promoting the resolution of adipose inflammation would therefore likely be a beneficial therapeutic approach in T2DM.

Keywords: inflammation, resolution, lipxoins, resolvins, protectins, renal inflammation

INFLAMMATION AND COUNTER-REGULATORY LIPID MEDIATORS

The inflammatory response is necessary for effective host defense, although it must eventually dissipate to ensure tissue homeostasis and avoid pathologic conditions such as abscess formation, scarring, fibrosis, and eventual organ failure (Lawrence and Gilroy, 2007). Indeed, compromised resolution has been proposed as an underlying mechanism in many prevalent chronic diseases such as arthritis, diabetes, and atherosclerosis (Serhan et al., 2008; Maderna and Godson, 2009). It is now recognized that the resolution of inflammation is a dynamically regulated process orchestrated by mediators that play important counter-regulatory roles including cytokines, chemokines, and lipid mediators such as the lipoxins (LXs), resolvins, and protectins (Serhan, 2009). These mediators reduce vascular permeability and inhibit polymorphonuclear cell (PMN) recruitment, while promoting recruitment of monocytes and stimulating efferocytosis (Serhan et al., 2008). It has also been proposed that pro-resolving lipids stimulate lymphatic drainage of leukocytes (Arita et al., 2005b). Interestingly, the signaling pathways initially inducing prostaglandin (PG)E_2 and PGD_2 formation and thus the onset of inflammation, may actively switch the production of lipid mediators from pro-inflammatory to pro-resolving by inducing 5-lipoxygenases (LO) necessary for production of LXs, protectins, and resolvins (Serhan and Savill, 2005). In this way physiological inflammation programs its own resolution and promotes tissue homeostasis (Levy et al., 2001).

Abbreviations: ACE inhibitors, angiotensin-converting-enzyme inhibitors; AIM, antioxidant inflammation modulator; ARBs, angiotensin receptor blockers; ATMs, adipose tissue Mϕs; CKD, chronic kidney disease; CLS, crown like structures; CRP, C-reactive protein; DHA, docosahexaenoic acid; DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; EPA, eicosapentaenoic acid; HUVECs, human umbilical vein endothelial cells; IL, interleukin; LO, lipoxygenase; LXA_4, lipoxin A_4 (S),6(R),15,Trihydroxyeicosa-7E,9E,11Z,13E-tetraenoic acid; M.tb, *Mycobacterium tuberculosis*; maresins, MΦ mediators in resolving inflammation; miRNA, micro RNA; Mϕ, macrophages; PG, prostaglandin; PMN, polymorphonuclear cell; RA, rheumatoid arthritis; RAS, renin–angiotensin system; Rvs, resolvins; T2DM, type 2 diabetes mellitus; UUO, unilateral ureteric obstruction.

LIPOXINS

The LXs are produced endogenously at sites of inflammation as counter-regulatory lipid mediators with anti-inflammatory, pro-resolving, and anti-fibrotic bioactions (Serhan et al., 2008; Maderna and Godson, 2009). LXs are typically generated by transcellular metabolism between neutrophils, platelets, and resident tissue cells, such as epithelial cells (Lefer et al., 1988; Serhan, 2007), through the sequential action of 5-LO and either 12-LO or 15-LO (Serhan, 2005; Parkinson, 2006). LXs limit leukocyte chemotaxis (Lee et al., 1989) and activation of neutrophils and eosinophils (Bandeira-Melo et al., 2000), while stimulating Mϕ efferocytosis of apoptotic cells (Godson et al., 2000; Mitchell et al., 2002; Reville et al., 2006). Lipoxin A_4 (LXA_4) and its positional isomer lipoxin B_4 (LXB_4) are the principal LX species found in mammals. Although the LXB_4 receptor remains to be identified, the LXA_4 receptor FPR2/ALX is expressed on cells of diverse lineage, including fibroblasts (Wu et al., 2006a), renal mesangial cells (McMahon et al., 2002; Mitchell et al., 2004), and epithelial cells (Nascimento-Silva et al., 2007). LXs are protective in several experimental models of disease, e.g., inflammatory bowel diseases (Fiorucci et al., 2004), periodontal disease (Serhan, 2004; Kantarci and Van Dyke, 2005; Kantarci et al., 2006), and cardiovascular

disease (Serhan, 2005). LXs have also been reported to act as vasodilators (von der Weid et al., 2004) and may reprogram Mϕs from a classically activated (M1) phenotype to a spectrum of alternative activation (Mitchell et al., 2002). The bioactions of LXs are summarized in **Table 1**. The impact of LXs in maintaining the exquisite equilibrium between effective host defense and homeostasis is remarkably illustrated by the fact that over production of LXs may compromise host defense to pathogens. In the case of *Mycobacterium tuberculosis (M.tb)*, increased LXA_4 production is associated with decreased TNF-α activity and results in an inadequate inflammatory response (Tobin et al., 2010). Conversely, LXA_4 increases survival rate in *Toxoplasma gondii* infection where a compromised immune response due to diminished LO activity and LX biosynthesis is detrimental (Aliberti, 2005).

LIPOXIN RECEPTORS AND SYNTHETIC LIPOXIN ANALOGS

The principal LXA_4 receptor is FPR2/ALX, which has been identified and cloned in numerous cell types, including monocytes

Table 1 | Lipoxin induced bioactions.

Cell type	Bioactions *in vitro*
LXA_4, LXA_4-analogs and aspirin-triggered lipoxins (ATLs)	
Monocytes	Stimulate chemotaxis and adhesion without causing ROS production (Maddox and Serhan, 1996)
Macrophages	Stimulate efferocytosis while reducing inflammatory cytokine secretion (IFN-γ and IL-6) and increasing pro-resolving cytokine secretion (IL-10) (Mitchell et al., 2002; Schwab et al., 2007)
	Switch Mϕ phenotype from inflammatory to pro-resolving
PMN	Inhibit chemotaxis, adhesion, and transmigration (Chiang et al., 2006).
	Inhibit pro-inflammatory cytokine secretion (Jozsef et al., 2002)
	Inhibit ROS production (Levy et al., 1999; Börgeson et al., 2010)
	Enhance CCR5 expression on apoptotic PMN (Ariel et al., 2006)
	Attenuate P-selectin-mediated PMN–endothelial cell interactions (Papayianni et al., 1996)
DCs	Regulated as monocytes differentiate into DCs (Yang et al., 2001)
	Trigger SOCS-2 expression (Machado et al., 2006)
Eosinophils	Inhibit chemotaxis, IL-5, and eotaxin secretion (Soyombo et al., 1994; Bandeira-Melo et al., 2000; Levy et al., 2002)
Platelet	Inhibit *Porphyromonas gingivalis*-induced aggregation (Börgeson et al., 2010)
T cells	Inhibit anti-CD3 Ab induced TNF-α (Ariel et al., 2003)
NK-cells	Block cytotoxicity (Ramstedt et al., 1985, 1987)
PBMC	Inhibit anti-CD3 Ab induced TNF-α (Ariel et al., 2003)
Endothelium	Inhibit P-selectin mobilization (Scalia et al., 1997)
	Upregulate IL-10 while inhibiting LTD_4 and VEGF stimulated proliferation and angiogenesis (Baker et al., 2009)
Epithelium	Inhibit TNF-α induced IL-8 (Bonnans et al., 2007)
	Inhibit epithelial to mesenchymal transition (Wu et al., 2010)
Fibroblasts	Inhibit proliferation (Wu et al., 2006a)
	Inhibit IL-1β induced IL-6, IL-8, and MMP-3 (Sodin-Semrl et al., 2000)
Mesangial cells	Inhibit inflammatory cytokine production (Wu et al., 2006b), proliferation and cell cycle progression (Badr et al., 1989; Mitchell et al., 2004, 2007; Wu et al., 2005, 2006b) as well as ROS production (Mitchell et al., 2007)
GI epitlelium (enterocytes)	Antagonize TNF-α stimulated neutrophil-enterocyte interactions *in vitro* and attenuate TNF-α chemokine release and colonocyte apoptosis in human intestinal mucosa *ex vivo* (Goh et al., 2001)
	Inhibit TNF-α induced IL-8 (Gewirtz et al., 2002)
Hepatocytes	Reduce PPARα and CINC-1 expression (Planaguma et al., 2002)
Astrocytoma cells	Inhibit IL-1β induced IL-8 and ICAM-1 expression (Decker et al., 2009)
LXB_4 and LXB_4-analogs	
Monocytes	Stimulate monocytes recruitment, chemotaxis and adherence without causing ROS production (Maddox and Serhan, 1996)
	Increase adherence of undifferentiated THP-1 to laminin (Maddox et al., 1998)
PBMC	Inhibit anti-CD3 Ab induced TNF-α (Ariel et al., 2003)
PMN	Inhibit PMN migration across endothelium (HUVEC monolayer; Maddox et al., 1998)
	Attenuate P-selectin-mediated PMN–endothelial cell interactions (Papayianni et al., 1996)
NK cells	Inhibit cytotoxicity (Ramstedt et al., 1985)

and Mϕs (Maddox et al., 1997), T cells (Ariel et al., 2003), synovial fibroblasts (Sodin-Semrl et al., 2000), renal mesangial cells (McMahon et al., 2002), and enterocytes (Gronert et al., 1998). In contrast to conventional GPCRs, which typically show very specific ligand binding, the FPR2/ALX receptor binds pleiotropic ligands, both lipids and small peptides, such as acute phase proteins (Chiang et al., 2000), and may elicit ligand-dependent pro-inflammatory or anti-inflammatory responses (Chiang et al., 2006; Maderna and Godson, 2009). Krishnamoorthy et al. (2010) recently found that LXA_4 also interacts with another G-protein coupled receptor, namely GPR32.

LXA_4 undergoes rapid inactivation *in vivo*, primarily by PG dehydrogenase-mediated oxidation and reduction (Serhan et al., 1995) and efforts have been made to design chemically stable LX analogs. Because the three-dimensional molecular structure of the FPR2/ALX receptor is as of yet unknown, designing LXA_4 analogs is based on experimentally discovered structure/function relationship of LXA_4. The LXA_4 molecule can be considered in three regions; the lower chain, the upper chain, and the tetraene side chain (Duffy and Guiry, 2010). The first generation LXA_4 analogs carry modifications in the lower alkyl chain, to increase metabolic stability and prevent oxidation (Clish et al., 1999). The second generation analogs are collectively referred to as 3-oxa-LXA_4 and were constructed carrying modifications in the upper chain (Petasis et al., 2005), replacing the C_3 methylene group with an oxygen molecule (Guilford and Parkinson, 2005). The third generation LXA_4 analogs are characterized by replacement of the triene structure with a benzene ring (O'Sullivan et al., 2007; Petasis et al., 2008). Importantly, the *o*-[9, 12]-Benzo-15-epi-LXA_4 has been shown to activate the FPR/ALX receptor in a similar manner to native LXA_4, using an engineered β-arrestin system (Sun et al., 2009).

RESOLVINS, PROTECTINS, AND MARSEINS

Resolvins, protectins, and maresins are pro-resolving lipids discovered by Serhan et al. (2000) through sophisticated lipidomic analysis of resolution phase exudates in the murine dorsal air pouch model. Resolvins may be divided into the E series (RvEs) and D series (RvDs), which are generated from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, the most common forms of ω-3 PUFA. Similarly, protectins and maresins are generated from DHA. Like the LXs, resolvins are generated in a transcellular manner by the sequential action of LO. Protectins and maresins on the other hand are generated by single cells, but also through the action of LO. In neutrophils RvE1 has been shown to bind the GPCR LTB_4 receptor BLT1 with a K_d of 45 nM (Arita et al., 2007), whereas in Mϕ and dendritic cells RvE1 bind ChemR23 with a K_d of 11.3 ± 5.4 nM and B_{max} indicating approximately 4,200 binding sites per cell (Arita et al., 2005b; Kohli and Levy, 2009). RvD1 has also been reported to interact both with FPR2/ALX and GPR32 in phagocytes (Krishnamoorthy et al., 2010). As of yet it is not entirely clear which receptor the protectins and maresins act through, although PD1 has a high affinity surface binding site on human PMN and retinal pigment epithelium cells (Bannenberg and Serhan, 2010). Resolvins, protectins, and maresins all display potent anti-inflammatory and pro-resolving effects inhibiting production of pro-inflammatory mediators, regulating neutrophil trafficking and promoting efferocytosis (Schwab et al., 2007; Serhan, 2009). The effects of these lipid mediators are summarized in **Table 2**.

INFLAMMATION AND TYPE 2 DIABETES MELLITUS

Diabetes mellitus (DM) is a serious metabolic disorder of glucose homeostasis reflecting destruction of the β-cells of the pancreas and subsequent lack of insulin production (type 1 DM, T1DM) or decreased target organ sensitivity to insulin and β-cell dysfunction (type 2 DM, T2DM). T2DM is defined as having a fasting plasma glucose ≥7.0 mmol/l and affects over 90% of diabetics, or an estimated 285 million people globally (Cusi, 2010). T2DM imposes significant socioeconomic burdens through its many diabetes-associated complications. These can be divided into microvascular complications [diabetic nephropathy (DN), neuropathy, and retinopathy] and macrovascular complications [atherosclerosis, ischemic heart disease, stroke, and peripheral vascular disease often resulting in amputations] (Wild et al., 2004). Risk factors of T2DM include genetic preposition, ethnicity, high blood pressure, and high cholesterol, but obesity is frequently cited as the primary cause.

The role of inflammation in diabetes is becoming more evident and elevated circulating interleukin (IL)-1β, IL-6, and C-reactive protein (CRP) are predictive of T2DM (Navarro and Mora, 2006; de Luca and Olefsky, 2008; Donath and Shoelson, 2011). These inflammatory markers are primarily derived from the adipose tissue and the liver. The hypothesis that the pathogenesis of T2DM reflects an inflammatory disorder is supported by preclinical studies and clinical trials using anti-inflammatory agents (Donath and Shoelson, 2011). Examples of these include IL-1β receptor blockers, anti-TNF-α and IL-6 therapies, as well as the use of salsalate. We will now briefly discuss current attempts to use anti-inflammatory therapeutics to attenuate the pathology of diabetes.

Interleukin-1β is a key regulator of inflammation both in T1DM and T2DM and has been shown to induce pancreatic β-cell apoptosis and exacerbate the systemic inflammation associated with diabetes, for instance by augmenting adipocyte TNF-α and IL-6 production (Akash et al., 2012). Patients with T2DM display increased IL-1β levels (Boni-Schnetzler et al., 2008), while its naturally occurring IL-1 receptor antagonist (IL-1Ra) is diminished (Maedler et al., 2004). Interest has been directed toward using IL-1Ra as a therapeutic in T2DM. Clinical trials show that the IL-1Ra anakinra improves glycemia and β-cell secretory functions, while attenuating systemic inflammation (Donath and Shoelson, 2011). For instance, anakinra administered over a 13-week period in T2DM patients increased insulin production, while glycosylated hemoglobin, i.e., HbA1c and the inflammatory marker CRP were significantly reduced (Larsen et al., 2007). The limitation with IL-1Ra lies in its short half-life, but successful attempts have been made to increase its stability by fusing IL-1Ra with peptides such as human serum albumin (HLA) or elastin-like polypeptides (ELPs), although these compounds remain to be tested in diabetic models (Akash et al., 2012).

TNF-α is also implicated in the pathogenesis of insulin resistance (IR) and its expression correlates with reduced insulin-stimulated glucose disposal (Kern et al., 2001). TNF-α is elevated

Table 2 | Resolvin, protectin, and maresin induced bioactions.

Cell type	Bioactions *in vitro*
Resolvin E1	
Macrophages	Stimulates efferocytosis while reducing IFN-γ and IL-6 (Schwab et al., 2007)
PMN	Decreases transendothelial and epithelial migration (Campbell et al., 2007)
	Stimulates L-selectin shedding, while reducing CD18 expression and inhibiting PMN rolling *in vivo* (Dona et al., 2008)
	Attenuates BLT1 depended TNF-α and NF-κB activation (Arita et al., 2007)
	Enhances CCR5 expression on apoptotic PMN (Ariel et al., 2006)
Dendritic cells	Inhibits migration (Arita et al., 2005a)
	Reduces IL-12 production from DCs stimulated with pathogen extract (Arita et al., 2005a)
Platelets	Disruptes platelet aggregation (Dona et al., 2008; Fredman et al., 2010)
Resolvin D1	
Microglia cells	Inhibits IL-1β expression (Serhan et al., 2002)
Protectin D1	
PMN	Enhances CCR5 expression on apoptotic PMN (Ariel et al., 2006)
Mϕ	Stimulates efferocytosis while reducing IFN-γ (Schwab et al., 2007)
T cell	Promotes apoptosis, inhibits TNF-α and IFN-γ (Ariel et al., 2005)
Glia cells	Reduces IL-1β-induced NF-κB activation and COX2 expression (Marcheselli et al., 2003), reduces amyloid β-42-induced nerotoxicity, promotes amyloid β-induced apoptosis (Lukiw et al., 2005)
Epithelium	Protects from apoptosis induced by oxidative stress (Mukherjee et al., 2004)
Maresin 1	
Macrophage	Stimulates efferocytosis (Serhan et al., 2009)

both in obese rodents (Uysal et al., 1997) and obese humans (Hotamisligil et al., 1995; Kern et al., 2001) and furthermore decreases upon weight loss (Kern et al., 1995). TNF-$\alpha^{-/-}$ *ob/ob* mice have significantly improved insulin sensitivity (Uysal et al., 1997) and obese mice lacking the TNF-α receptor are protected from high fat diet induced IR (Romanatto et al., 2009). However, in humans TNF-α neutralizing antibodies does not appear to improve insulin sensitivity in obese subjects (Ofei et al., 1996; Rosenvinge et al., 2007). Nevertheless, TNF-α blockers are often used to treat rheumatoid arthritis (RA) and it was recently reported that obese RA patients receiving TNF-α blockers displayed improved fasting glucose and increased circulating adiponectin levels (Stanley et al., 2010), possibly warranting more studies in the field. IL-6 is also an important inflammatory mediator in diabetes and increased levels correlate with IR (Pradhan et al., 2001), although it appears to have a dual role. Whereas IL-6 causes IR in adipocytes (Rotter et al., 2003) and anti-IL-6 therapy over a 6 month period diminished HbA1c in diabetic RA patients (Ogata et al., 2011), the IL-6 derived from skeletal muscle during exercise appears beneficial (Pedersen et al., 2003). The use of anti-IL-6 blockers as an anti-inflammatory therapeutic in diabetes has therefore been debated. Salsalate on the other hand is a very interesting drug in the context of diabetes and has been shown to reduce CRP, FFA, and triglycerides while increasing insulin sensitivity and adiponectin levels (Koska et al., 2009; Goldfine et al., 2010). Salsalate may, however, cause gastric irritation and should be used with caution in pregnancy (Torloni et al., 2006; Chyka et al., 2007). Collectively these studies indicate the potential of using anti-inflammatory therapeutics to attenuate T2DM.

RESOLUTION OF ADIPOSE INFLAMMATION IN TYPE 2 DIABETES MELLITUS

There is a growing appreciation that adipose tissue is not merely an insulating energy store but is actually an endocrine organ regulating appetite, glucose and lipid metabolism, blood pressure, inflammation, and immune function (Kershaw and Flier, 2004). Adipose tissue has been shown to play a particularly important role in the systemic inflammation associated with obesity, IR, and diabetes. Factors such as prolonged obesity or aging cause a state of systemic low-grade inflammation, which induces monocyte recruitment to the adipose tissue. Adipose tissue is a source of pro-inflammatory cytokines and adipose tissue Mϕ (ATM) derived TNF-α, IL6, and IL-1β contribute to adipose IR and exacerbates systemic inflammation (Lumeng et al., 2007b). Promoting resolution of adipose inflammation would likely be a beneficial therapeutic approach, reducing the risk of developing obesity-associated complications, such as IR and T2DM (Donath and Shoelson, 2011).

Given the spectrum of anti-inflammatory and pro-resolution bioactions of LXs and other counter-regulatory lipid mediators, these may provide a potential intervention to attenuate adipose inflammation (Gonzalez-Periz and Claria, 2010). We recently reported a role of LXA_4 in adipose inflammation, culturing adipose explants of aging mice as an *ex vivo* model of adipose inflammation (Börgeson et al., 2012). We confirmed that LXA_4

increased expression of critical components of insulin sensitivity, including the glucose transporter GLUT-4 and IRS-1, consistent with restoring insulin sensitivity in the tissue. Furthermore, LXA_4 decreased IL-6 secretion while increasing production of the pro-resolving IL-10, suggesting that LXA_4 acted in a pro-resolving manner (Börgeson et al., 2012). Indeed, IL-10 inversely correlates with T2DM and has been shown to inhibit IL-6-induced IR, attenuate MCP-1 secretion, and promote GLUT-4 and IRS-1 expression (Lumeng et al., 2007a; Gonzalez-Periz and Claria, 2010). The study also demonstrated that LXA_4 partially rescued MΦ-inhibited adipose glucose uptake *in vitro* (Börgeson et al., 2012). Inflammatory MΦs are a key component of augmented adipose IR (Lumeng et al., 2007b; Cusi, 2010; Spencer et al., 2010). Importantly, LXA_4-mediated reversal of insulin desensitization correlated with restored adipose Akt activation, which is necessary for translocation of the glucose sensitizing GLUT-4 receptor from the cytosol to the plasma membrane (Börgeson et al., 2012). Interestingly, RvD1 also increased insulin-stimulated pAkt in adipose tissue of obese *db/db* mice (Hellmann et al., 2011). Furthermore, LXA_4 inhibited MΦ TNF-α production, which is an important cytokine previously demonstrated to inhibit adipose glucose uptake *in vitro* (Gao et al., 2003). LXA_4 also inhibited MCP-1 secretion, though the importance of MCP-1 in adipose inflammation has been debated (Chen et al., 2005; Inouye et al., 2007). The reduction of inflammatory cytokines may suggest that LXA_4 promoted restoration of insulin sensitivity by altering MΦ phenotype toward resolution. Finally, LXA_4 also appeared to have a direct impact on adipocytes as it rescued TNF-α-induced desensitization to insulin-stimulated Akt activation, which also correlated with increased GLUT-4 translocation.

The beneficial effects of ω-3 PUFA, RvE1, and PD1 have also been shown in *ob/ob* mice (Gonzalez-Periz et al., 2009). Both ω-3 PUFA enriched diet and intraperitoneal injections of RvE1 increased expression of genes involved in glucose transport (GLUT-4) and insulin signaling (IRS-1), as well as genes involved in insulin sensitivity (PPARγ). Similar to ω-3 PUFA, RvE1 increased adiponectin levels, as did PD1 when incubated with adipose explants from *ob/ob* mice (Gonzalez-Periz et al., 2009). Additional studies show that RvD1 decrease accumulation of ATMs and improve insulin sensitivity while reducing fasting blood glucose in *db/db* diabetic mice (Hellmann et al., 2011). Interestingly, the total number of ATMs remained unaltered with RvD1 treatment, but the ratio of M2:M1 increased. The number of adipose crown like structures (CLS) in obese animals was also reduced by 50–60% (Hellmann et al., 2011) and RvD1 significantly increased circulating adiponectin and adipose phosphorylation of AMPK. The study also reports diminished IL-6 secretion (Hellmann et al., 2011), which has previously been shown to suppress adiponectin in 3T3-L1 adipocytes (Fasshauer et al., 2003) and may explain the restored adiponectin levels, which in turn have been shown to increase insulin sensitivity (Kristiansen and Mandrup-Poulsen, 2005; Kadowaki et al., 2006).

INFLAMMATION AND DIABETIC NEPHROPATHY

Diabetic nephropathy presents a particularly important problem as it develops in 25–40% of diabetic patients and is the major cause of end-stage kidney disease (Ritz et al., 1999). DN is a type of chronic kidney disease (CKD) rising in prevalence in concert with chronic DM in susceptible individuals. In addition to being the leading cause of renal failure, T2DM is also an independent risk factor in the development of cardiovascular disease (Syed and Khan, 2011). DN reflects the convergence of inflammatory, metabolic, and hemodynamic factors. Inflammation causes glomerulosclerosis, tubular atrophy, damage to renal vasculature, and fibrosis (Ferenbach et al., 2007). Renal matrix accumulation arises in response to paracrine and autocrine mediators produced by resident and infiltrating cells, such as mesangial cells and Mϕs.

Promoting inflammatory resolution is likely an attractive approach when trying to prevent renal fibrosis and CKD (Börgeson and Godson, 2010). The mechanisms by which resolution of renal inflammation occurs naturally and how they are subverted in disease are only beginning to be understood. Important components include efferocytosis of apoptotic cells and a change of the cytokine milieu from pro-inflammatory to anti-inflammatory/pro-resolving (Ferenbach et al., 2007; Börgeson and Godson, 2010). Biphasic regulation of renal inflammation and NF-κB also appears important, where the first peak mediated through p65/p50 heterodimers induces inflammation through pro-inflammatory mediators such as MCP-1 and RANTES. The second peak on the other hand (p50/p50 homodimers) promotes resolution by downregulating MCP-1/CCL1, RANTES/CCL5, and TNF-α (Panzer et al., 2009), while inducing expression of pro-resolving IL-10 (Cao et al., 2006). Similarly, to other pathologies it also appears that the phenotype of Mϕs is important in CKD (Wada et al., 2004; Sung et al., 2007). Whereas M1 Mϕs are detrimental, the M2a and perhaps even more so the M2c phenotype is beneficial (Wang and Harris, 2011).

Mϕs play an important role in DN as previously reported by Tesch (2008, 2010). Mϕs increase production of ROS, pro-inflammatory cytokines, and pro-fibrotic growth factors that contribute to the formation of myo-fibroblasts. Mϕs also appear to directly activate fibroblasts to a pro-fibrotic (myo-fibroblast) phenotype through secretion of galectin-3 (Henderson et al., 2008). Inhibition of Mϕ recruitment has been suggested to attenuate disease in several models of renal fibrosis with varying efficacy (Wada et al., 2004; Sung et al., 2007). For instance, MCP-$1^{-/-}$ mice are protected against renal injury in a model of T1DM (Chow et al., 2006) and furthermore urinary levels of MCP-1 are predictive of renal injury in humans and have been proposed as a diagnostic marker of progressive diabetic kidney disease (Tesch, 2008). There is a growing appreciation that the plasticity of Mϕs is an important factor in disease progression (Duffield, 2011; Wang and Harris, 2011) and that Mϕs also contribute to the resolution of renal inflammation. For instance, Mϕ efferocytosis of apoptotic cells is coupled to the generation of anti-inflammatory mediators such as IL-10 (Ricardo et al., 2008). To this effect, re-programming Mϕs *ex vivo* toward a M2 phenotype (IL-4/IL-13 stimulation) provides protection in models of renal disease, whereas the M1 phenotype (LPS stimulation) is detrimental (Wang et al., 2007). Additional research suggests that M2a and M2c phenotypes are both renoprotective, but that the latter appears to be the more effective (Wang and Harris, 2011).

EXPERIMENTAL THERAPEUTICS AND DN

Diabetic nephropathy is a chronic disease and current therapeutics primarily focus on glycemic and blood-pressure control through drugs targeting the renin–angiotensin system (RAS), such as angiotensin-converting-enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs). However, these treatment regimes only slow the progression of the disease, but do not halt or reverse it. Furthermore, prolonged use of RAS inhibitors may induce hyperkalemia, reduction in systemic blood pressure and decreased renal blood flow. Therefore, there is a profound need for novel therapeutic strategies in this field and the search is ongoing (Decleves and Sharma, 2010; Shepler et al., 2012). Examples of experimental therapeutics that show potential include bardoxolone methyl, which in clinical trials increases estimated glomerular filtration rate (eGFR) and creatinine clearance, while inhibiting inflammation in diabetic patients with stage 3b-4 CKD (Pergola et al., 2011a,b; Thomas and Cooper, 2011). Pirfenidone is an oral anti-fibrotic and anti-inflammatory agent which shows therapeutic potential in DN, although it was initially developed for treatment of idiopathic pulmonary fibrosis. In a randomized, double blind study pirfenidone increased eGFR and decreased markers of inflammation (TNF, INF-γ, and IL-1; Sharma et al., 2011) and has also demonstrated anti-fibrotic potential in both *in vitro* (Hewitson et al., 2001) and *in vivo* (RamachandraRao et al., 2009; Takakuta et al., 2010) models of renal disease. Vitamin D analogs, e.g., paricalcitol, may also be renoprotective agents through negatively regulating the RAS system and attenuation of renal fibrosis in rodent unilateral ureteric obstruction (UUO) models inhibiting accumulation of ECM as well as TGF-β1 and MCP-1 gene expression signaling (Li and Batuman, 2009; Li, 2010). Vitamin D analogs have also been suggested to prevent podocyte injury by promoting expression of slit diaphragm proteins (Li, 2011) and shows promising potential in emerging clinical trials reducing proteinuria in CKD patients (Li, 2010).

As inflammation is a common denominator in CKD and a hallmark of DN, pro-resolving therapeutics may have potential benefit. We recently reported that LXs are protective in CKD, as pre-treatment with LXA_4 and benzo-LXA_4 modulates inflammation and fibrosis in early UUO-induced injury (Börgeson et al., 2011a). UUO is an established model of progressive tubulointerstitial fibrosis and inflammation, relevant to CKD of diverse etiologies, including DN. UUO induces marked Mϕ infiltration, tubular cell death, fibroblast activation, and possible phenotypic transition of resident renal cells characteristic of progressive renal fibrosis (Higgins et al., 2007; Chevalier et al., 2009). Benzo-LXA_4 and LXA_4 attenuated UUO-induced fibrotic responses such as collagen accumulation by inhibiting collagen-1α2 gene expression, expression of collagen chaperone HSP47 and TGF-β1 signaling pathways (Börgeson et al., 2011a). Interestingly, RvD1 has also been demonstrated to attenuate collagen deposition in a murine model of renal ischemia reperfusion (Duffield et al., 2006). Specifically, LXs inhibited UUO-induced TGF-β1 canonical (Smad2) and non-canonical (Akt, Erk, and p38MAPK) signaling pathways, translating to reduced pro-fibrotic signaling (Börgeson et al., 2011a). Although LXA_4 did not alter the expression of TGF-β1, it did inhibit expression of MMP2 and CTGF. This is indeed noteworthy since MMP2 activates latent TGF-β1 and is a major driver of TGF-β1-mediated fibrosis. The LXA_4 mediated reduction of CTGF, both at mRNA and protein levels, would likely result in reduced fibrotic responses. The anti-fibrotic effect of LXs has been demonstrated in several *in vitro* systems, inhibiting proliferation and cell cycle progression in mesangial cells (Börgeson and Godson, 2010). Recent data also demonstrate protection by RvE and RvD in murine UUO (Qu et al., 2012). LXs also appeared to shift Mϕ phenotype and displayed significant pro-resolving actions in UUO-induced CKD. Whereas the total number of Mϕ and MCP-1 remained unaltered, LX treated animals displayed decreased pro-inflammatory IFN-γ and TNF-α cytokines and increased pro-resolving IL-10 levels (Börgeson et al., 2011a). Indeed, it appeared that the LXs induced a shift the Mϕ phenotype toward an early stage M2c reparative phenotype, based on the high IL-10 expression induced by benzo-LXA_4, although TGF-β1 remained unaffected (Börgeson et al., 2011a).

Micro RNA (miRNA) may also prove an important therapeutic target in DN, as they have demonstrated importance in CKD pathogenesis (Kato et al., 2007; Wang et al., 2008; Long et al., 2010). We recently reported that whereas TGF-β1 downregulates expression of the miRNA let-7c in renal epithelia, LXA_4 enhances let-7c expression, and attenuates TGF-β1 fibrotic responses as let-7c targets expression of the TGF-βR1 (Brennan et al., in revision). Importantly, LXs inhibit ROS production (Börgeson and Godson, 2010; Börgeson et al., 2011b; Wu et al., 2012), which may be analogous to the antioxidant effect of bardoxolone methyl (Rojas-Rivera et al., 2012). Indeed, bardoxolone methyl is an antioxidant inflammation modulator (AIM) compound, targeting Nrf2 which is a master regulator of the antioxidant response. Interestingly, LXA_4 has been shown to inhibit LPS-mediated ROS production and to downregulate Nrf2 protein levels in human umbilical vein endothelial cells (HUVECs; Pang et al., 2011).

CONCLUSION

Increasing evidence supports the role of chronic inflammation in the pathogenesis of T2DM and associated complications such as DN. Pro-resolving mediators, such as LXs, resolvins, and protectins, attenuate diabetes-related pathologies, including kidney disease and adipose inflammation. Thus promoting the resolution of inflammation through use of these lipids may provide a novel therapeutic strategy in the fight against diabetes-related pathologies.

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Omega-3 fatty acid-derived resolvins and protectins in inflammation resolution and leukocyte functions: targeting novel lipid mediator pathways in mitigation of acute kidney injury

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Inflammation, in conjunction with leukocytes, plays a key role in most acute kidney injury (AKI). Non-resolving renal inflammation leads to chronic fibrosis and renal failure. Resolvin D series (RvDs) and E series (RvEs), protectins, and maresins (MaRs) are endogenous omega-3 fatty acid-derived lipid mediators (LMs) that potently promote inflammation resolution by shortening neutrophil life span and promoting macrophage (Mf) non-phelogistic phagocytosis of apoptotic cells and the subsequent exit of Mfs from inflammatory tissue. 14*S*,21*R*-dihydroxy docosahexaenoic acid (14*S*,21*R*-diHDHA), a Mf-produced autacrine, reprograms Mfs to rescue vascular endothelia. RvD1, RvE1, or 14*S*,21*R*-diHDHA also switches Mfs to the phenotype that produces pro-resolving interleukin-10. RvDs or protectin/neuroprotectin D1 (PD1/NPD1) inhibits neutrophil infiltration into injured kidneys, blocks toll-like receptor -mediated inflammatory activation of Mfs and mitigates renal functions. RvDs also repress renal interstitial fibrosis, and PD1 promotes renoprotective heme-oxygenase-1 expression. These findings provide novel approaches for targeting inflammation resolution and LMs or modulation of LM-associated pathways for developing better clinical treatments for AKI.

Keywords: resolvins, protectins/neuroprotectins, maresins, 14*S*,21*R*-diHDHA, inflammation-resolution, kidney-injury, fibrosis, leukocytes

ACUTE KIDNEY INJURY: AN INFLAMMATORY DISEASE

ACUTE KIDNEY INJURY: AN UNMET MEDICAL CHALLENGE

Acute kidney injury (AKI), formerly known as "acute renal failure," causes a decline of kidney function (Bonventre and Yang, 2011). AKI occurs in many conditions, and AKI mortality is quite significant (Bonventre and Yang, 2011). Patients with AKI have a high chance of developing chronic or end-stage renal disease if they survive. Pharmacologic treatment and renal replacement therapy are only preventive or supportive and have not reduced AKI mortality (Negi and Shigematsu, 2012). The current treatment for AKI is still only preventive or supportive (Bonventre and Yang, 2011). Kidney ischemia/reperfusion injury (KIR) is a common cause of AKI (Bonventre and Yang, 2011).

INFLAMMATION, LEUKOCYTES, AND INFLAMMATION RESOLUTION: CRUCIAL TO ACUTE KIDNEY INJURY AND CHRONIC FIBROSIS

Inflammation plays a critical role in pathogenesis and recovery of AKI (Bonventre and Yang, 2011). AKI is characterized by infiltration and activation of leukocytes neutrophils, macrophages (Mfs), dendritic cells (DCs), and lymphocytes as well as damage (apoptosis and necrosis) of vascular endothelia and tubular epithelia (**Figure 1**). The activated leukocytes produce reactive oxidative species (ROS) and inflammatory factors, both of which damage the surrounding tissue. Mfs and DCs participate in both the innate and adaptive immune responses. Mfs infiltrated into kidneys during the first 48 h after KIR are mainly inflammatory M1 type that injures the tissue, whereas non-inflammatory M2 Mfs predominate later and are correlated with kidney repair (Lee et al., 2011). Regulatory T-cells are protective in AKI (Ko et al., 2010). B-cell deficiency confers protection from KIR injury (Burne-Taney et al., 2003). This type of injury also stimulates expression of adhesion molecules by vascular endothelia, such as ICAM-1 and VCAM-1, promoting leukocyte accumulation around injured sites. The injury-enhanced interaction of endothelia and leukocytes produces inflammatory cytokines, prostaglandins, leukotrienes, and complements, compromising endothelial junctions due to swelling and loss of glycocalyx and actin cytoskeleton. AKI inflammation goes into a positive feedback amplification as more blood leukocytes infiltrate through the vascular endothelial barrier into other renal tissue and become activated until inflammation resolution dominates over the inflammation (**Figure 1**) (Borgeson and Godson, 2010; Bonventre and Yang, 2011). Tubular epithelia, mesangium, and pericytes also produce inflammatory factors after injury or interaction with leukocytes, such as TNF-α, IL-8, IL-6, and IL-1β, leading escalated kidney inflammation and damage (**Figure 1**) (Bonventre and Yang, 2011).

While leukocyte-led inflammation causes tissue injury; a natural force for inflammation resolution is gaining ground both in parallel and in series (Serhan et al., 2002; Kieran and Rabb, 2004;

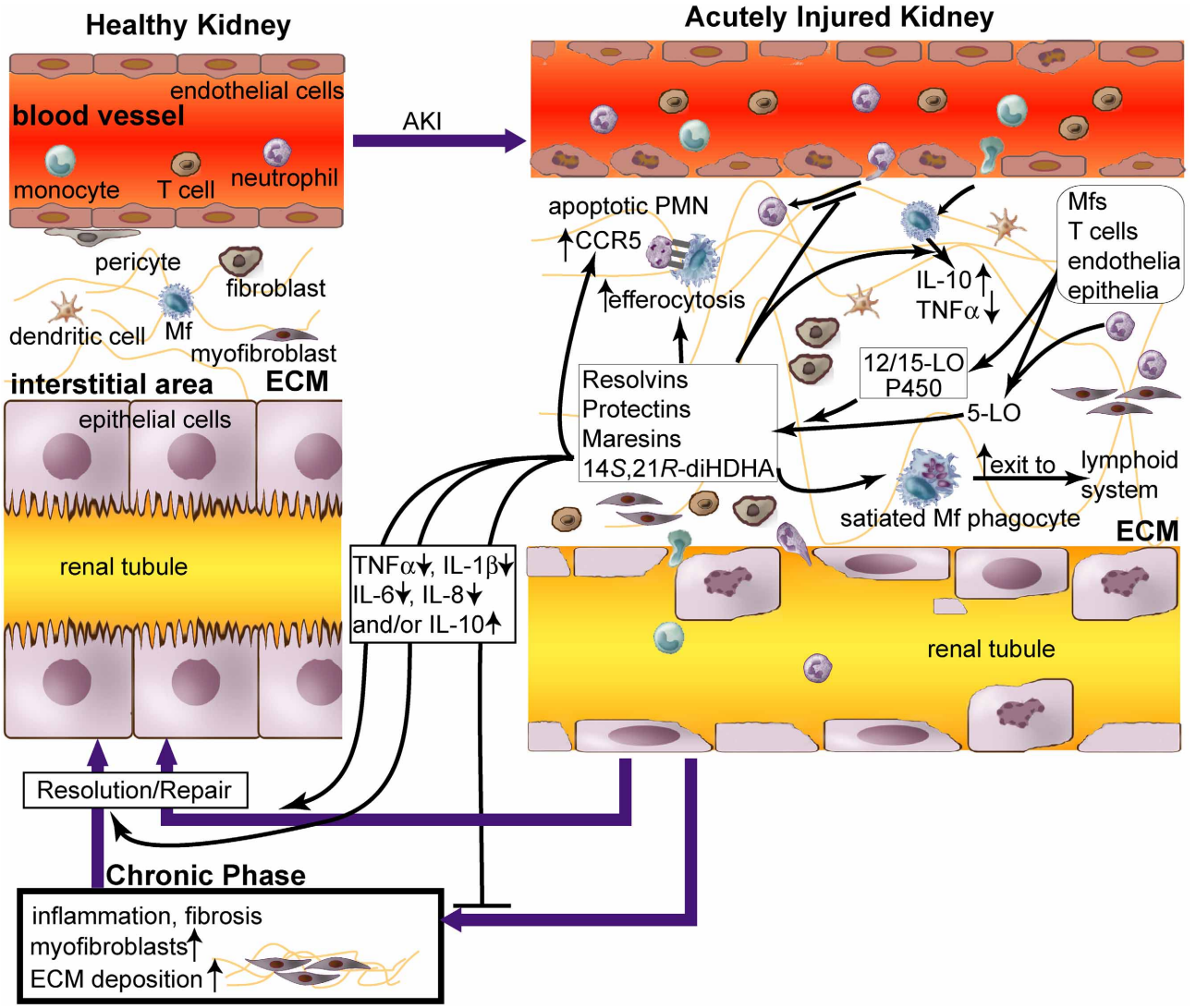

FIGURE 1 | Role of lipid mediators, resolvins and protectins in inflammation resolution in acute renal injury (AKI). AKI causes (1) damage of renal vascular endothelium; (2) diapedesis of neutrophils and monocytes through endothelium; (3) accumulation of neutrophils, macrophages (Mfs), dendritic cells (DCs), T cells, fibroblasts, and myofibroblasts in renal tissue; and (4) apoptosis and necrosis of tubular epithelium. Impaired inflammation resolution that occurs during AKI leads to chronic fibrosis [i.e., excessive extracellular matrix (ECM, as light-green lines) produced by myofibroblasts and other cells in the kidneys] and ultimately chronic renal failure (Bonventre and Yang, 2011; Serhan and Petasis, 2011). Although leukocytes cause tissue damage when activated to inflammatory phenotypes by AKI in the initial phase, they could promote inflammation resolution and tissue repair when modulated by pro-resolving lipid mediators, including resolvins and protectins. Resolvins, protectins, maresins, and 14*S*,21*R*-diHDHA are produced by 12/15-lipoxygenase (LO), p450, and/or 5-LO, in trans-cellular or intracellular biosynthetic systems of leukocytes or leukocytes plus endothelia/epiothelia. These lipid mediators act as paracrines and autacrines of leukocytes to promote resolution of AKI-initiated inflammation and fibrosis and rescue of kidney functions. They also inhibit accumulation of neutrophils and Mfs in kidneys during acute inflammation in AKI (Duffield et al., 2006; Tian et al., 2012), promote Mf non-phlogistic efferocytosis of apoptotic neutrophils (Hong et al., 2008), enhance apoptotic leukocyte expression of CCR5 that scavenges critical inflammatory chemokines, and accelerate phagocyte exit from the inflammatory site via the lymphatics (Ariel et al., 2005; Ariel and Serhan, 2007). These lipid mediators increase the level of pro-resolving, anti-fibrotic IL-10 and reduce the levels of inflammatory cytokines TNFα, IL-1β, IL-6, and/or IL-8 in Mfs or renal tissue. Thus these lipid mediators act as effectors for inflammation resolution and injury repair, which restores AKI-damaged kidneys to homeostasis. Administration of these lipid mediators promotes resolution of inflammation and injury in AKI.

Kluth, 2007; Serhan and Petasis, 2011). Certain macrophage phenotypes promote inflammation resolution in AKI (Alikhan et al., 2011; Lee et al., 2011). Particularly, pro-resolving lipid mediators (LMs) are produced by leukocytes or interaction of leukocytes, endothelium, and epithelium (Hong et al., 2003; Serhan and Petasis, 2011). These mediators trigger signaling that reduces production of inflammatory factors, enhances non-phlogistic efferocytosis, and promotes the switch of inflammatory leukocytes to pro-resolution reparative phenotypes (**Figure 1**, **Table 1**) (Hong et al., 2008).

Table 1 | Selected characteristics of n3-PUFAs-derived lipid mediators.

Lipid mediator	Pre-cursor	Enzyme(s) for biosynthesis	Receptor(s)	Activate signaling	Deactivate signaling	Inhibiting inflammatory molecule expression	Promoting pro-resolving cytokine expression
RvD1	DHA	5-LO + (12/15-LO or 15-LO) (a–c)	FPR2/ALXR GPR32 (l)			IL-8 (p) MIP-1β (p) RANTES (p) IL-6 (p) VCAM-1 (p) TNFα (q) IL-1β (q)	IL-10 (q)
RvE1	EPA	5-LO + (12/15-LO or 15-LO) (c–e)	CMKLR1/ ChemR23 BLT1 (e)	PI3K Akt ERK1/2 (m)	NFκB (e)	IL-8 (p) VCAM-1 (p) MIP-1β (p) RANTES (p) TNFα (p) VCAM-1 (p) IL-1β (q)	IL-10 (q)
PD1/NPD1	DHA	12/15-LO or 15-LO (b, c, f)		PI3K Akt mTOR/p70S6K (n)	NFκB (o)	COX2 (r)	
14*S*,21*R*-diHDHA	DHA	(12/15-LO or 12-LO) + P450 (g–k)		PI3K Akt p38-MAPK (h–k)			IL-10 (i)

Notes: (a) (Serhan et al., 2002); (b) (Hong et al., 2003); (c) (Serhan and Petasis, 2011); (d) (Serhan et al., 2000); (e) (Arita et al., 2005a); (f) (Serhan et al., 2006); (g) (Lu et al., 2010); (h) (Tian et al., 2011a); (i) (Tian et al., 2011b); (j) (Tian et al., 2010); (k) (Tian et al., 2012); (l) (Spite et al., 2009); (m) (Ohira et al., 2010); (n) (Faghiri and Bazan, 2010); (o) (Marcheselli et al., 2003); (p) (Tian et al., 2009); (q) (Schif-Zuck et al., 2011); (r) (Mukherjee et al., 2004). BLT1, leukotriene B4 receptor; CMKLR1/ChemR23, chemokine-like receptor 1/chemerin receptor 23; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; FPR2, formyl peptide receptor 2; GPR32, G protein-coupled receptor 32; MAPK, mitogen-activated protein kinases; MIP-1β, macrophage inflammatory protein 1β; mTOR/p70S6K, mammalian target of rapamycin/p70 ribosomal S6 kinase; PI3K, phosphatidylinositol 3-kinase; RANTES, regulated upon activation normal T cell expressed and presumably secreted.

Renal chronic fibrosis is the formation of excessive fibrous connective tissue in kidneys due to excessive accumulation in the extracellular matrix in response to chronic inflammation or repeated injury. Although appropriate local and transient renal fibrosis is needed for repair in the early phase of AKI, chronic fibrosis is a major detrimental feature in the later phases (Borgeson and Godson, 2010). Chronic fibrosis can eventually lead to end-stage renal failure. Leukocytes play a crucial role in renal fibrosis during AKI (Borgeson and Godson, 2010) (**Figure 1**). Lipoxin (LX) A_4, protectin/neuroprotectin D1 (PD1/NPD1), or resolvin D1 (RvD1) suppresses chronic fibrosis in KIR-injured kidneys (Godson et al., 2000; Duffield et al., 2006; Borgeson and Godson, 2010; Borgeson et al., 2011). Mfs interact with fibroblasts and pericytes, the key cells that can transdifferentiate into fibrosis-forming myofibroblasts (**Figure 1**) (Duffield, 2010). Mfs under inflammatory activation produce pro-fibrotic factors—such as TGF-β1, IL-13, and platelet-derived growth factor (PDGF) (Ko et al., 2008)—and also contribute to renal fibrosis (Young et al., 1995). Moreover, Mf depletion reduces renal fibrosis, but Mfs also can produce anti-fibrotic factors, such as IL-10, specific matrix metalloproteinases, and Endo180, in addition to phogocytose fibrotic extracellular matrix, apoptotic myeofibroblasts, and tissue debris (Vernon et al., 2010). Adoptive transfer of Mfs into mice at the chronic inflammation phase ameliorates chronic renal fibrosis (Nishida et al., 2005). This demonstrates that certain Mf phenotypes contribute to the prevention or resolution of chronic renal fibrosis.

In the following sections, we will present a concise review on omega-3 polyunsaturated fatty acids (n3-PUFA)-derived LMs that promote the resolution of inflammation and chronic fibrosis as well as repair in AKI.

SPECIALIZED ANTI-INFLAMMATORY, PRO-RESOLVING LIPID MEDIATORS DERIVED FROM n3-PUFAs: RESOLVINS, PROTECTINS, AND MARESINS (FIGURE 1, TABLE 1)

CHEMICAL STRUCTURES AND FORMATION *In vivo* AND *In vitro*

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the major n3-PUFAs present in fish oils, have beneficial effects that could prove helpful in preventing and/or treating inflammatory diseases (Kelley et al., 1999; Simopoulos, 2002). As such, the molecular and cellular mechanisms behind these beneficial effects are of significant interest and have been explored (Bazan et al., 1984; Serhan et al., 2000, 2002; Hong et al., 2003; Marcheselli et al., 2003; Serhan, 2011). The structures and bioactivities

of several families of novel n3-PUFAs-derived LMs that are both anti-inflammatory and pro-resolving have been discovered (Serhan, 2011). Some of these compounds were termed resolvins since they are formed in the resolution phase of inflammation and potently promote resolution (Serhan et al., 2002). Another DHA-derived LM, 10,17S-docosatriene, was discovered (Serhan, 2011). It was termed neuroprotectin D1 if generated in neural tissue for its protection in neurons, glial cells, and brain stroke; or protectin D1 for other tissue (Bazan, 2005; Serhan, 2011).

Resolvin D series (RvDs) are derived from DHA. During inflammation, endogenous DHA is converted to 17*S* hydroxyl-containing RvDs (RvD1–RvD6) and docosa-conjugated triene-containing PD1/NPD1 via 15-lipoxygenase (LO) (15*S*-lipoxygenation)-initiated biochemical pathways (Serhan et al., 2002; Hong et al., 2003; Marcheselli et al., 2003) or to 14*S* hydroxyl-containing maresins (MaRs) via 12-LO (12*S*-lipoxygenation)-initiated biochemical pathways. 5-LO catalyzes sequentially with 15-LO or 12/15-LO, generating RvDs (Hong et al., 2003) and some MaRs (Serhan et al., 2009). PD1, in isolated human cells and murine cells, was found to be 10*R*,17*S*-dihydroxy-docosa-4*Z*, 7*Z*, 11*E*, 13*E*, 15*Z*, 19*Z*-hexaenoic acid (Serhan et al., 2006). RvD1, RvD2, PD1/NPD1, and/or their biosynthetical pathway marker 17*S*-hydroxyl DHA (17*S*-HDHA) have been found in blood (Hong et al., 2003), ischemia-injured brains, retinal pigment epithelial cells, and/or AKI kidneys (Marcheselli et al., 2003; Mukherjee et al., 2004; Duffield et al., 2006; Bazan et al., 2011), demonstrating the existence of these compounds and/or pathways in injured tissue or cells. The interaction of endothelial cells and leukocytes promotes their biosynthesis (Tian et al., 2009) (**Figure 1**). RvE1 and 17*R*-hydroxyl epimers of RvDs and PD1, on the other hand, are generated through 15*R*-lipoxygenation pathways catalyzed by aspirin-acetylated cyclocygenase-2 (COX-2) or cytochrome P450 (in contrast to the typical 15-LO-catalyzed 15*S*-lipoxygenation of arachidonic acid) (Serhan et al., 2000, 2006). They were found in exudates, blood, and brains of humans and animals treated with aspirin (Serhan, 2011; Bazan et al., 2012). This provides new molecular insights for aspirin-based anti-inflammatory medication besides inhibiting COXs to produce inflammatory prostaglandins and thromboxins.

Recently we found several additional new pro-healing LMs: 14*S*,21*R*-dihydroxy-docosa-4*Z*, 7*Z*, 10*Z*, 12*E*, 16*Z*, 19*Z*-hexaenoic acid (14*S*,21*R*-diHDHA) and its epimers (Lu et al., 2010; Tian et al., 2011a,b). 14*S*,21*R*-diHDHA, as a positional isomer of maresin-1 (Serhan et al., 2009), is generated from DHA in Mfs, neutrophils, and cutaneous wounds. 12-LO and P450 catalyze sequentially to convert DHA to 14*S*,21*R*-diHDHA and 14*S*,21*S*-diHDHA through the intermediacy of 14*S*-HDHA (formed via 12*S*-lipoxygenation from DHA) (Lu et al., 2010; Tian et al., 2011a).

BIOACTIONS

Resolvins, protectins, and MaRs recapitulate beneficial bioactions of DHA or EPA with several order-of-magnitudes higher potency (in nanomolar and picomolar range) compared to their precursors (DHA or EPA) (Serhan and Petasis, 2011). These LMs have potent anti-inflammatory and pro-resolving effects, since they inhibit inflammatory factor expression and neutrophil infiltration, and since they promote non-phlogistic Mf phagocytosis of apoptotic cells (Serhan and Petasis, 2011). Such actions have been revealed in many *in vivo* models of inflammatory diseases, as well as *in vitro* experiments on diverse types of cells critical to these diseases. These actions include dermal inflammation, peritonitis, periodontitis, colitis and intestinal inflammation, asthma and airway inflammation, cystic fibrosis, acute lung or kidney injury, glomerulonephritis, and brain stroke (Marcheselli et al., 2003; Serhan and Petasis, 2011). RvE1 and its analogs are currently undergoing clinic trials for diseases of the eye, lung, kidney, skin, and intestines (Serhan and Petasis, 2011). Bazan et al. discovered that PD1/NPD1 resolves inflammation in brain and eye (Marcheselli et al., 2003; Mukherjee et al., 2004; Lukiw et al., 2005). PD1 or LXA_4 blocks inflammatory cytokine secretion from human T-cells and enhances CCR5 expression on apoptotic PMN (**Figure 1**), which accelerates clearance of inflammatory CCR5 ligands (Ariel et al., 2003, 2005). PD1 also promotes T-cell apoptosis (Ariel et al., 2005), as well as reduces the neutrophil lifespan in peritonitis (Bannenberg et al., 2005) and neutrophil-survival signaling for IL-1β (Hong et al., 2003). RvE1 promotes phagocytosis-induced neutrophil apoptosis and resolution of pulmonary inflammation (El Kebir et al., 2012). Several comprehensive reviews on these mediators are already available (Borgeson and Godson, 2010; Serhan and Petasis, 2011; Bazan, 2012).

14*S*,21-diHDHA and 14*R*,21-diHDHA promote or restore wound healing (Lu et al., 2010) impaired by alcohol intoxication (Tian et al., 2010) or diabetes (Tian et al., 2011a). Also, 14*S*,21*R*-diHDHA enhances VEGF release, vascularization, and migration of endothelial cells in diabetic mice. It also remedies angiogenic and pro-healing functions of mesenchymal stem cells (MSCs) and Mfs attenuated by diabetes, including their production of VEGF and/or IL-10 (Tian et al., 2011b). 14*S*,21*R*-diHDHA reduces hyperglycemia-induced ROS generation by inflammatorily-activated Mfs (Tian et al., 2011b). Thus, 14*S*,21*R*-diHDHA is a specific pro-resolving LM that may promote the protection or repair of the renal endothelium and epithelium during AKI.

RECEPTORS (TABLE 1)

Two G-protein-coupled receptors have been identified for RvE1: (**1**) BLT1 in neutrophils; and (**2**) CMKLR1/ChemR23 in Mfs and DCs (Arita et al., 2005a,b). RvD1 has also been reported to interact with both FPR2 or LXA_4 receptor (ALXR) and GPR32 in phagocytes (Spite et al., 2009). ALXR is expressed in neutrophils (Fiore et al., 1994) and monocytes (Maddox et al., 1997), and it activates T-cells (Ariel et al., 2003), intestinal or bronchial epithelial cells (Bonnans et al., 2003; Kucharzik et al., 2003), and renal mesangial cells (McMahon et al., 2000; Maderna and Godson, 2009), implying ALXR existence in renal podocytes and tubular epithelium. PD1/NPD1 stereoselectively and specifically binds with retinal pigment epithelial cells and neutrophils, suggesting specific receptors for NPD1 in both the immune and visual systems (Marcheselli et al., 2010). However, the exact NPD1 receptor(s) needs to be identified. The receptors of other resolvins, protectins, and marsins are likely to exist based on their structure-activity association, but have not been discovered yet.

CELL SIGNALING (TABLE 1)

Through CMKLR1 or BLT1 receptors, RvE1 represses the activation of NFκB (Arita et al., 2005a, 2007), a crucial regulator of innate immune responses in kidneys (Mulay et al., 2012). NPD1 or RvE1-CMKLR1 interactions activate PI3K and Akt, which involves mTOR signaling; RvE1 also activates ERK1/2 (Faghiri and Bazan, 2010; Ohira et al., 2010). 14*S*,21*R*-diHDHA activates PI3K, Akt, and P38-MAPK, but not ERK1/2 (Tian et al., 2010, 2011a, 2012). PI3K-Akt signaling regulates cell survival, and activation of MAPK pathways is essential in wound healing and associated angiogenesis (Tian et al., 2010, 2011a). These signaling systems are relevant to AKI (Borgeson and Godson, 2010; Tian et al., 2012).

METABOLIC DEACTIVATION

RvD1 is converted by eicosanoid oxidoreductases (EORs) to 17-oxo-RvD1 and 8-oxo-RvD1. The former is an inactivation metabolite, while the latter is still effective in suppressing neutrophil infiltration (Sun et al., 2007). RvE1 is metabolized to 12-oxo-RvE, 18-oxo-RvE1, 10,11-dihydroxy RvE, 19-hydroxy RvE1, 20-hydroxy RvE1 in tissue or cells, of which the first four metabolites are inactive partially or completely in inflammation resolution, and thus are representative for RvE1 metabolic deactivation (Arita et al., 2006; Hong et al., 2008). Human neutrophils convert PD1 to its omega-22 hydroxy product (Serhan and Petasis, 2011). The metabolic deactivation of resolvins could be excessively up-regulated in pathological conditions, resulting in their deficiency, or diminishing the pharmacological efficacy of administered resolvins. Molecular engineering has been used to overcome this problem; for example, A *p*-fluorophenoxyl added to RvE1 ω-terminal blocks the critical metabolic inactivation of RvE1 without attenuating the anti-inflammatory pro-resolving activities (Arita et al., 2005a; Hong et al., 2008).

RESOLVIN D SERIES AND PROTECTIN D1 RESOLVE INFLAMMATION AND MITIGATE AKI (FIGURE 1, TABLE 1)

Based on the findings that DHA-derived RvDs and PD1 promote inflammation resolution (Serhan, 2011) and DHA supplementation reduces KIR injury in dogs and rats (Neumayer et al., 1992; Kielar et al., 2003), Duffield and colleagues studied the treatment of murine KIR with RvDs and PD1. The study showed that administration of RvDs (RvD1:RvD2:RvD3 = 1:2:1), RvD1, or PD1 attenuates functional and morphological kidney injury, reduces accumulation of inflammatory neutrophils and Mfs, and suppresses TLR-mediated activation of Mfs. TLR signaling in Mfs and lymphocytes is involved in sustained chronic inflammation (Foell et al., 2007; Kato et al., 2008). RvDs treatment until 72 h after ischemia inhibits renal interstitial chronic fibrosis (Duffield et al., 2006). Interstitial chronic fibrosis and persistent leukocyte infiltration (chronic inflammation), resulting from AKI, leads to scarring and chronic renal failure (Morgera et al., 2002; Duffield and Bonventre, 2004). RvDs and PD1 likely have additional cellular sites of action in the kidney, e.g., on the endothelium and vascular tone, interstitial fibroblasts, mesangial cells, pericytes, DCs, and T-cells because of their pro-resolving and anti-fibrotic ability (Duffield et al., 2006). They may modulate the actions of monocytes/Mfs and neutrophils in the kidney. Hassan and Gronert found that PD1 amplified renoprotective heme-oxygenase-1 (HO-1) expression in ischemia-injured and non-injured kidneys, while PD1 inhibited neutrophil infiltration in murine KIR (Hassan and Gronert, 2009). These results support their notion that the interaction of the 12/15-LO and HO-1 systems provides a positive feedback loop that amplifies anti-inflammatory, pro-resolving signals.

Godson and colleagues found that arachidonic acid-derived LXs are pro-resolving in several types of renal injury; LXs play a reparative role in glomerulonephritis, and reduce proteinuria, glomerular inflammation, and mesangial cell proliferation (Kieran et al., 2004; Wu et al., 2006; Borgeson and Godson, 2010). LXs are protective against murine KIR, where a LX-stable analogue gives functional and morphological protection and attenuates inflammatory cytokine responses (Leonard et al., 2002). LXs also up-regulate genes of tight-junction proteins claudin 1, 3, and 7, which likely reduce inflammatory leukocyte infiltration; Moreover, they found that LXs attenuate renal chronic fibrosis and related gene expression in mesangial cells (Borgeson and Godson, 2010). LXA_4 analog or RvE1 remarkably prolong renal allograft survival in mice, which is consistent with LXA_4 inhibition of calcineurin activity and inflammatory cytokine release by human neutrophils. Also, RvE1 counter-regulates leukocytes partially via increased LXA_4 biosynthesis (Levy et al., 2011). Since AKI is the major complication of renal allograft transplantation (Bellomo et al., 2012), these results further demonstrate the effectiveness of LXA_4 or RvE1 in reducing AKI. LX actions converge with the pro-resolving characteristics of RvD1, as LXA_4 and RvD1 both activate the same G-protein coupled receptors ALXR/FPR2 and GPR32.

14*S*,21*R*-diHDHA PROMOTES MESENCHYMAL STEM CELLS IN RESOLUTION OF INFLAMMATION AND PREVENTION OF AKI

MSCs have shown potential to resolve inflammation and repair injury in renal failure (Togel et al., 2005). MSCs treated with 14*S*,21*R*-diHDHA more efficiently inhibit KIR-induced elevation of serum creatinine levels and reduce renal tubular cell death, as well as infiltration of neutrophils, Mfs, and DCs to renal tissue. Conditioned media from 14*S*,21*R*-diHDHA-treated MSCs reduce the generation of TNF-α and ROS by Mfs under KIR conditions. Infusion of 14*S*,21*R*-diHDHA-treated MSCs more efficiently reduce KIR-renal damage compared to untreated MSCs. Treated MSCs are resistant to apoptosis *in vivo* (when transplanted under capsules of AKI-injured kidneys) and *in vitro* (when cultured under simulated KIR conditions). This enhancement of MSC viability involves PI3K-Akt signaling. Additionally, treatment of MSCs with 14*S*,21*R*-diHDHA promotes secretion of renotrophic hepatocyte growth factor and insulin growth factor-1. In brief, 14*S*,21*R*-diHDHA promotes MSC amelioration of AKI (Tian et al., 2012).

RESOLVINS, PROTECTINS, AND MARESINS ACT ON LEUKOCYTES RELATED TO FIBROSIS IN AKI

Although the mechanisms that resolvins and PD1 use to reduce renal chronic fibrosis in AKI (Duffield et al., 2006) remain to be further delineated, the following findings provide hints for future

research on this subject. PD1, RvD1, or RvE1 switches Mfs to pro-resolving phenotypes, including CD11b^{low} Mfs, which are more capable in efferocytosis and emigration to lymphoid organs for inflammation resolution (**Figure 1**) (Schwab et al., 2007; Schif-Zuck et al., 2011; Ariel and Serhan, 2012). RvD1, RvE1, or 14*S*,21*R*-diHDHA induces Mfs to produce more anti-fibrotic IL-10 (Schif-Zuck et al., 2011; Tian et al., 2011b). These pro-resolving LMs, acting in concert in AKI, not only inhibit inflammation, but also shift the macrophage roles from pro-inflammatory (M1) or pro-fibrotic phenotypes to phenotypes that promote resolution as well as anti-fibrotic, regulatory functions (**Figure 1**, **Table 1**) (Duffield et al., 2006; Serhan and Petasis, 2011; Ariel and Serhan, 2012).

CONCLUDING REMARKS AND PERSPECTIVES

The discoveries of n3-PUFA-derived resolvins, protectins, and MaRs in the last two decades have provided unconventional knowledge and opened new frontiers for understanding the mechanisms involved in inflammation resolution. These LMs are produced endogenously by enzymes in leukocytes and tissue and act as paracrines and autacrines of leukocytes. Experiments have already shown that selected LMs promote resolution of AKI-caused inflammation and chronic fibrosis and rescue kidney function. LMs inhibit recruitment of neutrophils and monocytes to kidneys during acute inflammation, and they likely switch Mfs and T-cells toward anti-inflammatory pro-resolving phenotypes in AKI, as observed in other inflammatory conditions (**Figure 1**, **Table 1**). Mechanisms behind the actions of these LMs and their regulatory roles on leukocytes provide the basis for developing leukocyte-related modalities for efficient AKI treatment. These LMs or their mimics may be of therapeutic importance for treating AKI. More studies need to be conducted to further delineate the kinetic process for these LMs in reprogramming the phenotypes of leukocytes, which regulate the resolution of renal inflammation and chronic fibrosis and recover renal functions in AKI. Additional up-stream or down-stream signaling pathways involved should also be studied, as they may yield novel mechanistic targets and insights for AKI treatment.

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Targeting cytosolic proliferating cell nuclear antigen in neutrophil-dominated inflammation

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New therapeutic approaches that can accelerate neutrophil apoptosis under inflammatory conditions to enhance the resolution of inflammation are now under study. Neutrophils are deprived of proliferative capacity and have a tightly controlled lifespan to avoid their persistence at the site of injury. We have recently described that the proliferating cell nuclear antigen (PCNA), a nuclear factor involved in DNA replication and repair of proliferating cells is a key regulator of neutrophil survival. The nuclear-to-cytoplasmic relocalization occurred during granulocytic differentiation and is dependent on a nuclear export sequence thus strongly suggesting that PCNA has physiologic cytoplasmic functions. In this review, we will try to put into perspective the physiologic relevance of PCNA in neutrophils. We will discuss key issues such as molecular structure, post-translational modifications, based on our knowledge of nuclear PCNA, assuming that similar principles governing its function are conserved between nuclear and cytosolic PCNA. The example of cystic fibrosis that features one of the most intense neutrophil-dominated pulmonary inflammation will be discussed. We believe that through an intimate comprehension of the cytosolic PCNA scaffold based on nuclear PCNA knowledge, novel pathways regulating neutrophil survival can be unraveled and innovative agents can be developed to dampen inflammation where it proves detrimental.

Keywords: inflammation, neutrophil, apoptosis, PCNA, cystic fibrosis

INTRODUCTION

In the acute phase of inflammatory diseases, neutrophils are rapidly recruited to sites of injury or infection where they engulf and kill invading microorganisms (Witko-Sarsat et al., 2000). Moreover, recent studies have underscored an unsuspected neutrophil plasticity that can influence and shape the immune response (Mantovani et al., 2011). Neutrophil apoptosis, the process of programmed cell death that prevents the release of neutrophil histotoxic contents, should be tightly regulated (Kennedy and DeLeo, 2009; Fox et al., 2010) to limit the destructive capacity of neutrophil products to surrounding tissues (Nathan, 2006). The subsequent recognition and phagocytosis of apoptotic neutrophils by macrophages is central to the successful resolution of an inflammatory response. However, it has been reported that neutrophils can phagocytosed apoptotic cells and might participate in the clearance of apoptotic neutrophils at the site of inflammation (Esmann et al., 2010). It is increasingly apparent that the dying neutrophil itself exerts anti-inflammatory effects through modulation of surrounding cell responses (Kobayashi et al., 2002), particularly macrophage inflammatory cytokine release (Ariel and Serhan, 2012). In several inflammatory diseases including arthritis (Wright et al., 2010), vasculitis (Abdgawad et al., 2012), or cystic fibrosis (CF; McKeon et al., 2008; Moriceau et al., 2009, 2010), neutrophil apoptosis was delayed, thus potentiating the deleterious inflammatory response. Recent studies have highlighted the complexity of neutrophil death mechanisms and uncovered the involvement of novel pathways (Geering and Simon, 2011). Neutrophil survival induced for instance by cytokine such as G-CSF involves a complex gene pattern as evidence by gene array studies (Drewniak et al., 2009). As an example, we have identified the proliferating cell nuclear antigen (PCNA) as a key element controlling neutrophil survival. In neutrophils, that are non-proliferating cells, PCNA localization was strictly cytosolic and correlated with the grade of their viability (Witko-Sarsat et al., 2010).

A SOPHISTICATED REGULATION OF APOPTOSIS IS REQUIRED TO CONTROL NEUTROPHIL ACTIVATION

Like other cells, a neutrophil possesses both pro-survival and death pathways, the balance of which determines its fate (Witko-Sarsat et al., 2011). Several studies have unraveled the standard cascade of events, which classically include mitochondrial outer membrane permeabilization (MOMP) followed by release of cytochrome c (that is very weak in neutrophils) and other pro-apoptotic proteins into the cytosol, caspase activation, DNA fragmentation, chromatin condensation, loss of membrane asymmetry, formation of

apoptotic bodies (Geering and Simon, 2011; Kepp et al., 2011) and, finally, generation of "eat me signals" that stimulate the uptake of apoptotic cells by phagocytes (Paidassi et al., 2009). In neutrophils, the apoptotic machinery presents specific features that render these cells peculiar and very interesting to study as a model in which apoptosis control is cell cycle-independent because they cannot proliferate (Witko-Sarsat et al., 2011). Hence, a complete cell cycle arrest was observed in band cells and segmented neutrophils from bone marrow and in circulating mature neutrophils (Theilgaard-Monch et al., 2005). Expression patterns of apoptosis genes studied by microarray indicated that death control occurred by the p53 pathway in promyelocytes and by death receptor pathways in bone marrow neutrophils. Neutrophil apoptosis is inhibited by a continuous expression of the short lifespan Bcl-2 homolog myeloid cell leukemia-1 (Mcl-1; Thomas et al., 2010). It has been clearly shown that neutrophil survival was regulated by the inducible expression of the short-lived Mcl-1 (Moulding et al., 2001). In that respect, Mcl-1 can be considered as a potential target to modulate neutrophil's fate (Milot and Filep, 2011). However, the molecular mechanisms controlling this "spontaneous or constitutive" apoptosis still remain obscure: it is not clear whether neutrophil apoptosis occurred because of the lack of external surviving signals or because of its "internal clock". It has been reported that deactivation of phosphatidylinositol 3,4,5-triphosphate/Akt signaling mediates neutrophil spontaneous death (Zhu et al., 2006). Accordingly, neutrophils depleted of Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a phosphatase that negatively regulates Akt activity, live much longer than wild-type neutrophils. Some surprising insights into neutrophil survival mechanisms came when cyclin-dependent kinases (CDK) happened to play a key role in the regulation of neutrophil survival (Rossi et al., 2006). Notably, CDK are implicated in the regulation of the cell cycle and constitute targets for anti-cancer therapies (Malumbres et al., 2009). Inhibition of CDK by roscovitine can trigger neutrophil apoptosis by interfering with the phosphorylation of RNA polymerase II and with neutrophil transcriptional capacities thereby inducing neutrophil apoptosis (Leitch et al., 2012).

PCNA: A NOVEL PLAY FOR THIS FASCINATING ACTOR THAT ESCAPES FROM NUCLEUS TO MEDIATE NEUTROPHIL SURVIVAL

An unanticipated finding came with our discovery that PCNA, an ancestral nuclear protein involved in DNA replication, was present in resting neutrophil cytosol and was degraded upon apoptosis. In fact, PCNA happened to be an actor of neutrophil survival (Witko-Sarsat et al., 2010) but it remains to be investigated whether it could regulate "the neutrophil internal clock". Historically, PCNA was described as an antigen for autoimmune disease in systemic lupus erythematosus patients, detected only in the proliferating cells (Mahler et al., 2012). The tight association of PCNA with cancer transformation resulted in the use of PCNA as a diagnostic and prognostic cell cycle marker in tumors (Stoimenov and Helleday, 2009).

With the aim to start understanding the molecular mechanisms whereby PCNA exerts its anti-apoptotic activities and how the cytosolic PCNA scaffold is regulated, we will next discuss key issues based on our knowledge of nuclear PCNA, assuming that similar principles governing its function are conserved between nuclear and cytosolic PCNA.

A UNIQUE BUT CONSERVED TRIDIMENSIONAL STRUCTURE

Proliferating cell nuclear antigen is a ubiquitous protein that has a unique ring-shaped structure (Krishna et al., 1994) and a highly conserved amino acid sequence (Prosperi, 2006). PCNA has been identified in all eukaryotes from unicellular organisms to humans. Striking is the similarity in molecular structure between yeast and human that share 35% amino acid sequences identity but have highly superimposable three-dimensional structure (Stoimenov and Helleday, 2009). Trimeric PCNA exhibits sixfold symmetry as a result of having two globular domains in each monomer (**Figure 1**). The importance of PCNA in DNA replication is tightly linked with its ring-shaped structure that allows to slide freely on duplex DNA (Kelman, 1997). Remarkably, PCNA mutants that cannot form trimers failed to stimulate the polymerase Pol δ (Jonsson et al., 1995). Deletion of the PCNA gene in the yeast showed that PCNA was an essential protein required for DNA replication and knocking out the PCNA gene in mice was lethal (Kelman, 1997).

PCNA: THE SECOND TO NONE IN THE COORDINATION OF COMPLEX BIOLOGICAL PROCESSES

One key issue is that PCNA has no known enzymatic activity but can interact with a diverse array of proteins and cellular factors to regulate their activities: PCNA has been named the "professional recruiting agent" or more elegantly the "dancer with multiple partners" (Maga and Hubscher, 2003). PCNA-interacting proteins can be classified into two groups (Moldovan et al., 2007): the first consists of enzymes involved in nucleic acid metabolism, including DNA replication [replication factor C (RFC), DNA polymerase δ and ε, FEN-1, DNA ligase I] and repair (MutL homolog 1, MutS homolog 2, Uracil-DNA glycosylase 2) that are localized for the majority exclusively in nucleus. In contrast, the second group consists of cell cycle regulatory proteins (p21/waf1/Cip1, p57, CDK2, growth arrest and DNA damage (Gadd45), and the myeloid-differentiation primary-response (MyD118), Mcl-1) that localized both within the nuclei or the cytoplasmic compartment depending on the cell type. Except for Mcl-1, the major anti-apoptotic Bcl-2 homolog expressed in neutrophils (Thomas et al., 2010), these latter PCNA partners have not been studied in the neutrophil survival context. We have previously shown that, in mature neutrophils, PCNA was constitutively associated with procaspase-3, procaspase-8, procaspase-9, and procaspase-10, presumably sequestering them within the cytosol to prevent their activation. In line with this notion, recombinant PCNA was shown to interfere with in vitro procaspase 9 activation (Witko-Sarsat et al., 2010), thus strongly suggesting that PCNA association with procaspases represents a way to block their activation.

p21/waf1 DESTABILIZED THE PCNA SCAFFOLD AND TRIGGERED NEUTROPHIL APOPTOSIS

p21/waf1 is a well characterized PCNA partner that has been identified in a protein complex containing PCNA, cyclin, and CDK

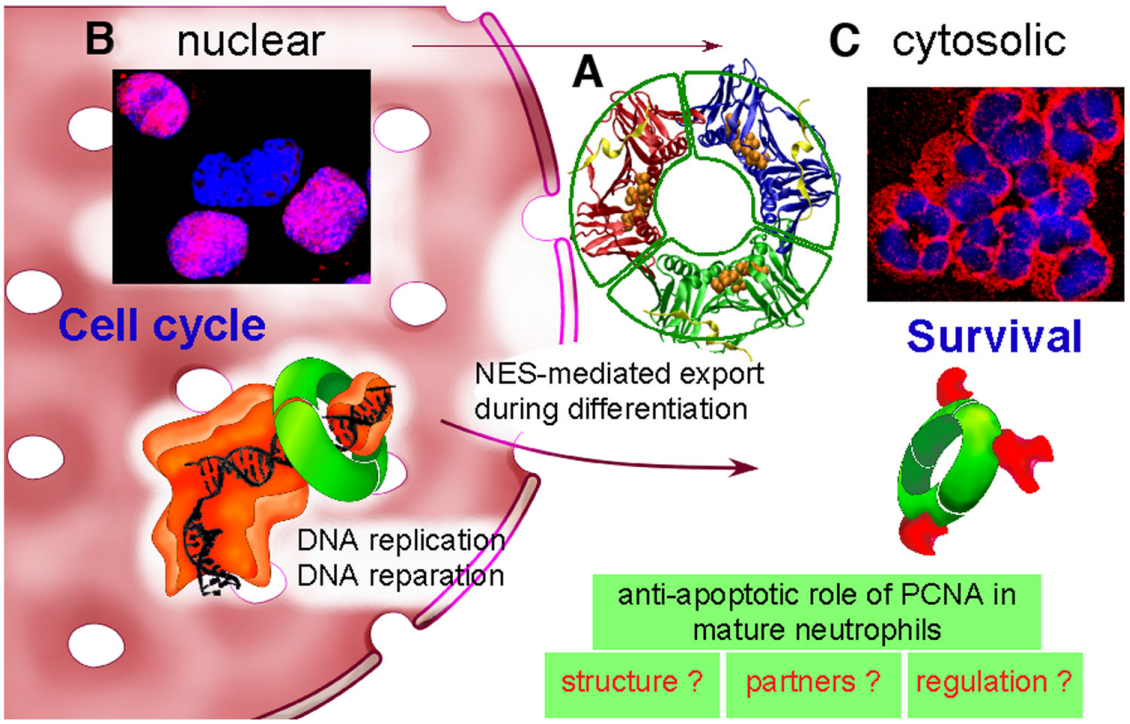

FIGURE 1 | Dual life of PCNA: from nucleus to cytosol. (A) PCNA is a scaffold protein, which acts as a trimer (represented here in red–blue–green) as shown by its molecular tridimensional structure (PDB ID: 1VYM, PyMOL Molecular Graphics System). PCNA forms a ring-shaped complex, which can encircle the DNA and orchestrate DNA replication and reparation. PCNA monomers have two similar globular domains, linked by a long flexible loop called the interdomain-connecting loop that binds numerous PCNA partners including p21/waf1. A synthetic peptide from the sequence of p21/waf1 (represented here in yellow) is bound to this interdomain-connecting loop. The nuclear export sequence (NES) is composed of a central helix (represented here with orange balls) localized in the inner face of the PCNA trimer. **(B)** In proliferating cells such as the myeloid CD34$^+$ precursor cells, PCNA is nuclear as shown by PCNA immunolabeling (in red) and DNA staining with Hoechst (in blue). The purple color results from the colocalization between PCNA and DNA within nucleus. PCNA is a trimeric ring (represented in green), which exerts its activity during DNA replication by braceleting DNA. PCNA has an important role in DNA synthesis because it is part of the polymerase Pol δ holoenzyme (represented in orange). **(C)** In contrast, in neutrophils, PCNA is localized exclusively within the cytosol as shown by the red color of the cytoplasm and the blue nuclei with its typical polylobular form. During granulocytic differentiation, there is a nuclear-to-cytosol relocalization that involves a NES that we have recently characterized (Bouayad et al., 2012). In neutrophils, under pro-survival conditions, cytosolic PCNA is associated with multiple partners, including procaspases-3, -8, -9, and -10 to prevent their activation, and is stabilized by the treatment of neutrophils with G-CSF (Witko-Sarsat et al., 2010). On the opposite, death signals, either directly or indirectly can target PCNA, leading to a disruption of the PCNA scaffold and thus triggering apoptosis (Witko-Sarsat et al., 2011). Further studies are required to characterize PCNA structure, to identify its partners and to understand how cytosolic PCNA can regulate neutrophil survival.

(Xiong et al., 1992; Waga et al., 1994). p21/waf1 is a p53-responsive gene but p21 expression can also be p53-independent (Biggs and Kraft, 1995). The p21 has two different inhibitory effects on the entry of the cell into S-phase. One is to inhibit the kinase activity of CDK and the other is to inhibit DNA replication via an interaction with PCNA (Goubin and Ducommun, 1995). Based on previous studies, synthetic peptides such as the carboxy-p21 peptide (residues 141–160 on the p21/waf1 sequence) carrying the consensus sequence for binding to the PCNA interdomain-connecting loop (**Figure 1**) was shown to act as an effective competitor for PCNA partners and to interfere with its functions (Warbrick, 2000). Remarkably, this carboxy-p21 triggered neutrophil apoptosis and concomitant PCNA degradation, in addition to impair the capacity of G-CSF to prolong neutrophil survival in vitro (Witko-Sarsat et al., 2010). Thus, the observation that the carboxy-p21 triggered neutrophil apoptosis by disturbing the PCNA scaffold clearly showed to us that PCNA is pivotal in maintaining neutrophil survival. Whether p21/waf1 expression controls the PCNA scaffold in neutrophils has not been investigated yet. The expression of p21 has been shown to be downregulated during granulocytic differentiation (Yaroslavskiy et al., 1999) and its expression in mature neutrophils is low under resting conditions (Klausen et al., 2004). Surprisingly, p21 mRNA has been shown to be strongly upregulated *in vivo* in human neutrophils isolated from bronchoalveolar lavages following LPS intratracheal instillation (Coldren et al., 2006). Whether p21–PCNA interaction could play a role in neutrophil survival will require further investigations.

CYTOSOL AS A PHYSIOLOGIC PLAYGROUND FOR PCNA ACTION

The peculiar exclusive cytoplasmic localization was a feature of mature neutrophils as, for instance, PCNA was detectable exclusively in the nucleus of CD34$^+$ cells or in myeloblasts isolated from human bone marrow aspirates (Witko-Sarsat et al., 2010). More recently we have provided evidence of an active PCNA nuclear export that involved the chromosome region maintenance

1 (CRM1) exportin (Turner et al., 2012). Accordingly, leptomycin B, an inhibitor of the CRM1 exportin inhibited this PCNA relocalization during granulocytic differentiation of human primary $CD34^+$ cells or in promyelocytic cell lines (Bouayad et al., 2012). Using enhanced green fluorescent protein fusion constructs, we demonstrated that PCNA relocalization involved a nuclear export signal (NES) located from I11 to I23 in the PCNA sequence. However, this NES, located at the inner face of the PCNA trimer (**Figure 1**) was not functional in wild-type PCNA, but instead, was fully active and leptomycin B-sensitive in the monomeric PCNAY114A mutant. We also provided evidence that nuclear-to-cytoplasmic relocalization that occurred physiologically during myeloid differentiation was essential for PCNA anti-apoptotic activity in mature neutrophils. It is noteworthy that the PCNA NES was extremely conserved between species (Bouayad et al., 2012) thus suggesting that this CRM1-dependent export of PCNA was part of the physiologic PCNA trafficking presumably occurring in cells other than neutrophils, thus uncovering a novel aspect of PCNA functions. Notably, the presence of PCNA has been recently described in the cytosol of cancer cells (Naryzhny and Lee, 2010).

POST-TRANSLATIONAL MODIFICATIONS OF CYTOSOLIC PCNA: A KEY IN NEUTROPHIL SURVIVAL?

Another level of complexity in the ballet of PCNA partners within nucleus, is the multiplicity of PCNA post-translational modifications that modulate specific protein interactions (Moldovan et al., 2007). In fact, phosphorylation (although controversial), ubiquitination, sumoylation, and acetylation that have been described for nuclear PCNA offer a great deal of options to modulate PCNA activities (Naryzhny and Lee, 2004). In neutrophils, we have observed that PCNA was ubiquitinated and was degraded via the proteasome during apoptosis (Witko-Sarsat et al., 2010). The levels of PCNA were found to time-dependently decrease in neutrophils undergoing apoptosis regardless of whether the triggering signaling cascade passed through the extrinsic (death receptors) or the intrinsic pathway (mitochondria). Since proteasome inhibitors reversed such a PCNA diminution, we concluded that a proteasome-mediated PCNA degradation, triggered along both the death receptor and mitochondrial apoptotic cascades, was responsible for apoptosis-induced PCNA degradation.

MODULATING PCNA SCAFFOLD IN NEUTROPHIL-DRIVEN INFLAMMATION: THE MODEL OF CYSTIC FIBROSIS

THE PROMINENT ROLE OF NEUTROPHIL IN CYSTIC FIBROSIS AIRWAY INFLAMMATION

Cystic fibrosis which is a lethal autosomal recessive disorder caused by mutation of the CF transmembrane regulator (CFTR) gene, is characterized by an intense neutrophil-dominated airway inflammation (Cantin, 1995) and a chronic bacterial colonization with *Pseudomonas aeruginosa*. Plugging in small airways contributes to the morbidity and mortality in CF (Burgel et al., 2007), leading to respiratory failure and the need for lung transplantation (Burgel and Nadel, 2008). The prognosis is tightly linked with the severity of the inflammatory process. Hence, the extraordinary numbers of neutrophils accumulating within airways of CF patients has led to the hypothesis of an innate immunity failure (Bals et al., 1999). Today, the current treatment involves antibiotherapy and mucolytic drugs but therapeutic intervention in CF remains a challenge (Pier, 2012). Anti-inflammatory drugs for CF lung disease appear to have some beneficial effects on disease progression. These agents include oral corticosteroids and ibuprofen, as well as azithromycin, which, in addition to its antimicrobial effects, also possess anti-inflammatory properties. Inhaled corticosteroids, antioxidants, nutritional supplements, and protease inhibitors have a limited impact on the disease. Adverse effects limit therapy with oral corticosteroids and ibuprofen (Narasimhan and Cohen, 2011). Hence, the lack of promising candidate emphasizes the need for fresh approaches in the management of airway inflammation in CF, for instance by targeting neutrophil apoptosis in combination with antibiotherapies.

Previous studies on CF patient's neutrophils indicated functional disturbances in bacterial phagocytosis, killing, and other effector functions (Downey et al., 2009). Because of the extreme heterogeneity of CF patients in terms of infectious status (Witko-Sarsat et al., 1999), the comparisons of experiments and results are difficult. Indeed, we have previously shown that neutrophils from CF parents who were heterozygous for CFTR mutation, had also disturbed neutrophil functions thus suggesting the possibility of an innate neutrophil defect in CF (Witko-Sarsat et al., 1996; Moriceau et al., 2010). Accordingly, a recent study has provided evidence that the absence of the CFTR from myeloid-derived cells slows the resolution of inflammation (Bonfield et al., 2012). Gene-expression patterns of neutrophils from clinically stable and healthy controls have shown dramatic differences (Adib-Conquy et al., 2008). This was consistent with a perturbed "inflammatory program" in CF neutrophils (Hayes et al., 2011), which remains to be investigated (Tirouvanziam et al., 2008). It should also be mentioned that perpetuation of inflammation in the CF airway may also be amplified by defective macrophage clearance mechanisms. It has been shown that persistence of infection in CF was partly due to ineffective uptake and killing of pathogens due to a defective macrophage innate response (Wright et al., 2009). Notably, a defect in apoptotic cell clearance has also been reported in CF (Vandivier et al., 2002).

DYSREGULATED NEUTROPHIL APOPTOSIS: A POTENTIAL TARGET FOR THERAPEUTIC INTERVENTION

Given the number of neutrophils in CF airways (Danel et al., 1996; **Figure 2A**), late neutrophil apoptosis could have devastating consequences. This neutrophil-dominated airway inflammation typical of the CF condition is representative of a chronic but active inflammatory process with neutrophil persistence suggesting a defect in apoptotic neutrophil clearance by macrophages (**Figure 2B**). Indeed, we (Moriceau et al., 2010) and others (McKeon et al., 2008) reported that neutrophils from CF patients undergo delayed apoptosis and have decreased levels of the pro-apoptotic protein Bax (Dibbert et al., 1999), thereby slowing their removal by macrophages and potentiating airway inflammation. In an attempt to modulate the delayed apoptosis in neutrophils from CF patients, roscovitine was used at 10 μM to restore normal apoptosis levels for CF PMN (Moriceau et al., 2010).

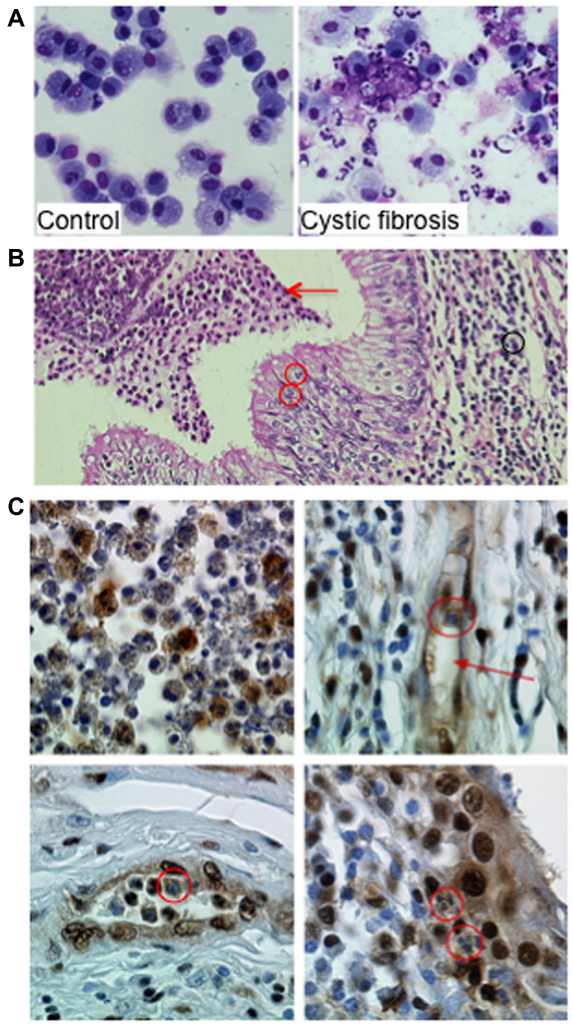

FIGURE 2 | Proliferating cell nuclear antigen (PCNA) expression in neutrophils within airways from CF patients. (A) Neutrophils and macrophages from bronchoalveolar lavages (BAL) from a control donor and from a patient with CF. The control BAL was composed mainly of alveolar macrophages. In contrast, in BAL from CF 95% of the cells are neutrophils either viable or apoptotic. Few macrophages and lymphocytes are present as well as cell debris and mucus (Giemsa stain ×630 original magnification). **(B)** Neutrophil-dominated inflammation in lung explants from a CF patient. Acute inflammation within the lung in CF patient with characteristic histologic features: (1) inflammatory infiltrate with neutrophils (black circle and arrow) and lymphocytes in the lamina propria, (2) transepithelial neutrophil migration (red circles), and (3) accumulation of degenerating and apoptotic neutrophils in the lumen (red arrow; Hematoxylin–Eosin–Safran staining ×200 original magnification). Lung explant specimens from CF patients were obtained at transplantation (Hôpital Européen Georges Pompidou). **(C)** Immunoperoxidase labeling of PCNA on paraffin sections of lung explants from a CF patient. Labeling was performed using a rabbit polyclonal anti-PCNA antibody (Ab5, diluted 1:100, Calbiochem) and immunoperoxidase detection (Dako) as previously described (Moriceau et al., 2009; ×630 original magnification). The airway lumen contents neutrophils expressing PCNA (upper left panel). Neutrophils within vessels (red arrow, upper right panel, red circle on the lower left panel) expressed high amounts of PCNA as did neutrophils found in the epithelium (red circle on lower right panel). This strong PCNA expression in neutrophil cytosol contrasts with the lack of labeling observed in cells present in the lamina propria, including fibroblasts and lymphocytes. Similar observations were made in lung explants from four CF patients.

Whether CDK-mediated survival pathway could crosstalk with the PCNA scaffold is currently unknown and would require more investigations.

We recently identified coronin-1A (Grogan et al., 1997) as a cytosolic protein overexpressed in CF neutrophils, a finding that was consistent with its anti-apoptotic function (Moriceau et al., 2009). Coronin-1 expression investigated by immunohistochemistry of pulmonary tissues obtained from CF patients during transplantation clearly showed a strong coronin-1A expression in neutrophils at the site of inflammation (Moriceau et al., 2009). Similar immunohistochemistry labeling of PCNA in neutrophils within the airway lumen showed a great variation in their PCNA contents reflecting their different apoptosis rates (**Figure 2C**). In contrast, the cytoplasm of neutrophils present within the mucosa, lamina propria and vessels were strongly labeled thus indicating their survival state (**Figure 2C**). These observations suggest that PCNA was highly expressed in neutrophils infiltrating the lung of CF patients and might play a role in neutrophil survival at the site of inflammation. Whether PCNA could be associated to coronin-1A is currently unknown but should require more investigation. Investigation of cytosolic PCNA within neutrophils at sites of inflammation (for instance in vasculitis or rheumatoid arthritis) should also be explored.

Strategies aiming at potentiating neutrophil apoptosis by targeting the PCNA scaffold in CF have to be carefully investigated and could be combined with other anti-inflammatory or anti-infectious therapeutic strategies to achieve a maximum efficacy in term of dampening neutrophil-driven inflammation. It might be possible to adjust this type of therapy to avoid any risk of neutropenia-induced infection.

CONCLUSION

Highlighting peculiar pathways used by neutrophils to control their survival (Geering and Simon, 2011) are of pivotal importance for the development of novel anti-inflammatory strategies (Duffin et al., 2010). Even in the absence of proliferation, PCNA seems to have a conservative function for preserving neutrophil's life. This particular cytosolic PCNA localization strongly suggests that this could be harnessed for therapeutic purposes when neutrophils would be out of control such as in sustained inflammation. This will be our main challenge to exploit all the data on nuclear PCNA gathered during more than five decades of work, and try to be creative to understand how the enigmatic PCNA scaffold participates to neutrophil survival.

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Myeloid nuclear differentiation antigen, neutrophil apoptosis and sepsis

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Sepsis and septic shock are characterized by prolonged inflammation and delayed resolution, which are associated with suppression of neutrophil apoptosis. The role of the intrinsic apoptotic pathway and intracellular factors in regulation of neutrophil apoptosis remain incompletely understood. We previously reported that the nuclear factor MNDA (myeloid nuclear differentiation antigen) is fundamental to execution of the constitutive neutrophil death program. During neutrophil apoptosis MNDA is cleaved by caspases and relocated to the cytoplasm. However, when challenged with known mediators of sepsis, human neutrophils of healthy donors or neutrophils from patients with sepsis exhibited impaired MNDA relocation/cleavage parallel with myeloid cell leukemia-1 (MCL-1) accumulation and suppression of apoptosis. MNDA knockdown in a model cell line indicated that upon induction of apoptosis, MNDA promotes proteasomal degradation of MCL-1, thereby aggravating mitochondrial dysfunction. Thus, MNDA is central to a novel nucleus-mitochondrion circuit that promotes progression of apoptosis. Disruption of this circuit contributes to neutrophil longevity, thereby identifying MNDA as a potential therapeutic target in sepsis and other inflammatory pathologies.

Keywords: MNDA, sepsis, neutrophils, MCL-1, mitochondria, internal apoptosis pathway, inflammation

INTRODUCTION

Different types of hematopoietic cells participate in the inflammatory response to microbial infection. Among them, circulating neutrophils are rapidly recruited into infected or injured tissues. They are the first line of defense against pathogens and are key regulators of the initial response to microbial infection. Effective removal of neutrophils from inflamed tissues is critical for timely resolution of inflammation. However, because of the disruption of neutrophil programmed cell death in inflammatory-related conditions, including sepsis, neutrophils persist in tissues and blood and portend poor prognosis. Here, we will discuss the recent discovery of a novel nuclear to mitochondrion circuit that is involved in the control of neutrophil apoptosis and disrupted during sepsis.

SEPSIS

Sepsis and septic shock (hereafter commonly referred as sepsis) are portent major medical challenges that result from a harmful host response to infection. Sepsis has a high prevalence and morbidity. At the beginning of this century, Angus et al. (2001) reported that, at the time, 9.3% of all cases of death in the USA was caused by sepsis. The incidence of sepsis was then evaluated as 3 cases per 1000 people, and 2.26 cases per 100 hospital-discharged patients. The mortality was estimated at 26.6% of all sepsis cases but, this percentage was significantly higher with elderly patients. The prevalence of this disease is increasing year after year despite advances in critical care. It is now considered to be the 10th leading cause of mortality in the United States (Melamed and Sorvillo, 2009).

Sepsis results from an inappropriate host response to infection. The initial stage of sepsis is usually considered to result from an exaggerated or dysregulated inflammatory response to infection (Pene et al., 2012). As sepsis persists, a shift toward immunosuppression is observed (Hotchkiss and Nicholson, 2006), concomitant with occurrence of organ failure and secondary infection. The severity of sepsis is frequently evaluated by various scoring systems, including the APACHE II (Acute Physiology and Chronic Health Evaluation II; Knaus et al., 1985) or SAPS II (Simplified Acute Physiology Score II; Le Gall et al., 1993) within the first 24 h of hospitalization. The score is based on measurements of vital parameters such as blood pressure, heart rate, respiratory rate, temperature, neutrophil count, etc. Intriguingly, both high and low blood neutrophil counts (neutrophilia and neutropenia, respectively) portend poor prognosis. Despite extensive efforts, specific molecular markers for identifying patients with high risk for sepsis or its more severe form, septic shock, have not been identified. Molecular markers with limited accuracy and specificity have been proposed for defining the stages of the disease. For instance, the prototypic acute-phase reactant C-reactive protein can be used as a marker of systemic inflammation during sepsis, whereas high-levels of procalcitonin are detectable at early stage of bacterial infection (Aalto et al., 2004).

NEUTROPHILS AND SEPSIS

Neutrophils are the first line of defense against pathogens. They generate different proteolytic enzymes as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) to destroy invading microorganisms following phagocytosis, or

extracellulary by neutrophil extracellular trap (NET) formation (Papayannopoulos et al., 2010; Metzler et al., 2011).

Neutrophils have the shortest life span among leukocytes and undergo constitutive programmed cell death (apoptosis). This process is essential for regulation of neutrophil homeostasis. Constitutive apoptosis renders neutrophils unresponsive to extracellular stimuli and allows their recognition and removal by macrophages (Savill et al., 2002; Gilroy et al., 2004). This process is critical for termination of the inflammatory response and tissue repair. Following discharging their function, at the inflammatory locus, extravasated neutrophils are though to predominantly undergo apoptosis. However, signals from the inflammatory milieu can either accelerate or suppress the cell death program, thereby affecting the fate of neutrophils (Gilroy et al., 2004). Suppressed neutrophil apoptosis is often detected in patients with inflammatory pathologies, including sepsis and septic shock and portends poor prognosis (Keel et al., 1997; Matute-Bello et al., 1997; Taneja et al., 2004). Exposure of neutrophils to inflammatory mediators such as GM-CSF, IL-8 or to bacterial constituents results in delayed apoptosis (El Kebir and Filep, 2010; Geering and Simon, 2011). Preserving neutrophil activities at the sites of infection may be required for complete elimination of invading pathogens, but could also aggravate injury to the host, resulting in persistent tissue damage. Therefore, the regulation of neutrophil apoptosis is critical to control the balance between their antimicrobial effectiveness and potential deleterious effect on tissues.

Signaling pathways promoting survival of neutrophils during sepsis are converging to control expression and degradation of key factors influencing the programmed cell death. In mature neutrophils, the anti-apoptotic protein myeloid cell leukemia-1 (MCL-1) and the pro-apoptotic protein Bcl2-associated X (BAX) are critical for the regulation of mitochondrial transmembrane potential ($\Delta\Psi_m$), and hence, activation of effector caspases (El Kebir and Filep, 2010; Geering and Simon, 2011; Milot and Filep, 2011). Since the control of mitochondrial transmembrane potential is central to the intrinsic apoptotic pathway, these discoveries placed forth the intrinsic apoptotic pathway in regulation of neutrophil apoptosis.

INTRINSIC APOPTOSIS PATHWAY AND MCL-1 IN NEUTROPHILS

MCL-1 is an anti-apoptotic factor of the Bcl-2 family. MCL-1 accumulation protects against formation of the BAK-BAX heterodimer on the external mitochondrial membrane and subsequent release of cytochrome *c* along with other molecules influencing apoptosis like SMAC/Diablo, endonuclease G, and AIF (apoptosis-inducing factor), from the mitochondrial inner membrane. Hence, MCL-1 protects $\Delta\Psi_m$ and thus regulates the internal apoptotic pathway.

Unlike other members of the Bcl-2 family, MCL-1 protein has a short half-life and its levels of expression change substantially as neutrophils age and upon exposure of neutrophils to inflammatory mediators (Moulding et al., 2001; Craig, 2002). Indeed, MCL-1 protein expression inversely correlates with the degree of neutrophil apoptosis in both experimental models and clinical settings. Rapid loss in MCL-1 corresponds to development of apoptosis and MCL-1 knockdown results in dramatic decreases in the neutrophil lifespan (Moulding et al., 1998; Dzhagalov et al., 2007). Modification in *Mcl-1* transcription accounts for most variation of MCL-1 expression observed upon stress conditions (Dong et al., 2011). At the transcription level, *Mcl-1* is regulated by different transcription factors including MYC, NF-κB (RelA/p65), STAT5, and HIF-1α (Akgul et al., 2000; Negrotto et al., 2006; Beverly and Varmus, 2009; Thomas et al., 2010). RNA processing and protein accumulation/turnover are also important for regulation of MCL-1 expression (Bae et al., 2000). The turnover of MCL-1 results primarily from the proteasome activity (Zhong et al., 2005). MULE/Arf-BP1, an E3 ubiquitin ligase, ubiquitinates MCL-1 and subsequently enhances its proteasomal degradation (Zhong et al., 2005). This activity can be counterbalanced by the activity of the deubiquitinase USP9X which was demonstrated to deubiquitinate and thereby, to stabilize MCL-1 (Schwickart et al., 2010). However, surprisingly little is known about regulation of MCL-1. We have identified myeloid nuclear differentiation antigen (MNDA) as a regulator of the proteasomal degradation of MCL-1 (Fotouhi-Ardakani et al., 2010 and see below).

ROLE OF MITOCHONDRIA IN NEUTROPHIL APOPTOSIS

In neutrophils, mitochondria have an atypical function and their role seems to be restricted to apoptosis (van Raam and Kuijpers, 2009). This view has been nourished by the observation that neutrophils rely on glycolysis for energy formation and even for a long time mitochondria could not be detected in these cells. The electron transport chain is inefficient to transport electrons from complexes III to IV in neutrophils (van Raam et al., 2008). However, it is not to say that it exerts no activity in neutrophils since, inhibitors of the mitochondrial respiratory chain complex I can modulate the severity of lung injury evoked by LPS (Zmijewski et al., 2009). Enhanced production of H_2O_2 by neutrophils results in inhibition of IκB-α degradation hence preventing the activation of NF-κB, a key regulator of inflammatory gene expression in neutrophils (Zmijewski et al., 2008). Thus, the mitochondrial respiratory chain appears to be only partially active in neutrophils.

MNDA: A KEY COMPONENT OF A NOVEL NUCLEUS TO MITOCHONDRION CIRCUIT

Different factors exerting their activity in the nucleus have been reported to participate in and influence the internal apoptosis pathway. While some nuclear proteins including E2F1, STAT3, HIF-1α, and NF-κB are well known to regulate expression of genes encoding pro- or anti-apoptotic factors, other nuclear proteins like MNDA, p53, p21/WAF1, proliferating cell nuclear antigen (PCNA), nur77, SHP, and possibly p73, have been reported or proposed to act as nuclear signals (transducers) to influence the intrinsic apoptotic pathway upon relocation or specific cytoplasmic accumulation (Chipuk et al., 2003; Dumont et al., 2003; Mihara et al., 2003; Wang, 2005; Fotouhi-Ardakani et al., 2010; Witko-Sarsat et al., 2010; Milot and Filep, 2011). Some of these factors have been reported to directly affect pro- or anti-apoptotic factors and hence, apoptosis. MNDA is one of them.

Myeloid nuclear differentiation antigen is a human hematopoietic specific factor of the HIN-200 family. This family of factors is composed of the functionally related proteins IFI16, AIM2, IFIX,

and MNDA (Choubey and Panchanathan, 2008). MNDA localizes predominantly to the nucleus and is expressed mainly in myeloid cells. It has been suggested that MNDA may function as a master regulator of monocytic and granulocytic lineages (Novershtern et al., 2011). Recently, MNDA has been proposed to be a transcription factor (Suzuki et al., 2012). Like other members of the HIN-200 family, MNDA contains a pyrin/PAAD/DAPIN domain that mediates binding between proteins involved in apoptotic and inflammatory signaling pathways (Fairbrother et al., 2001). It also contains a HIN-200 domain, which is thought to promote protein–protein (Dawson and Trapani, 1996; Choubey and Panchanathan, 2008) and protein–DNA interactions (Jin et al., 2012). *MNDA* gene regulation is influenced by interferons (Choubey and Panchanathan, 2008). MNDA was initially proposed to regulate myeloid cell differentiation as well as development of sporadic myelodysplastic syndrome (Briggs et al., 2006).

The potential implication of MNDA in regulation of apoptosis in myeloid cells and in inflammation has been directly assessed in neutrophil granulocytes (Fotouhi-Ardakani et al., 2010). In bone marrow-derived and mature neutrophils, MNDA is predominantly located in the nucleus. In neutrophils undergoing apoptosis, MNDA is cleaved by caspases, presumably caspase-3, and relocated to the cytoplasm. However, the cleavage of MNDA is likely not required for its cytoplasmic accumulation since the full-length MNDA could also be detected in the cytoplasm. Culture of human neutrophils with inflammatory mediators, like bacterial constituents and platelet-activating factor, promotes their survival and indicates a clear correlation between the degree of neutrophil apoptosis and MNDA cleavage as well as cytoplasmic accumulation. These findings suggest that MNDA could participate in regulation of apoptosis in neutrophils.

A causal relationship between MNDA and apoptosis has been established in a model cell line, the promyelocytic leukemia cell line HL-60, which expresses endogenous MNDA (Duhl et al., 1989; Savli et al., 2002). We created two MNDA-deficient HL-60 cell lines by the stable genomic integration of vectors encoding specific small hairpin RNA (shRNA). In these engineered model cell lines, knockdown of MNDA partially protected HL-60 cells against genotoxic stress-induced apoptosis, markedly attenuated activation of caspase-3, but not caspase-8, and prevented mitochondrial dysfunction (Fotouhi-Ardakani et al., 2010). These observations identify MNDA as a modulator of the intrinsic (mitochondrial) pathway of apoptosis.

The importance of the anti-apoptotic factor MCL-1 in control of $\Delta\Psi_m$ and neutrophil apoptosis (Moulding et al., 1998; Dzhagalov et al., 2007) led us to interrogate whether MNDA could influence the internal pathway of apoptosis via MCL-1. Interestingly, we found that: (i) MNDA co-immunoprecipitates with MCL-1; and (ii) after induction of apoptosis, MCL-1 accumulation was greatly enhanced in MNDA-deficient HL-60 cells compared to MNDA proficient HL-60 cells (Fotouhi-Ardakani et al., 2010). Similar results were obtained in the presence of the protein synthesis inhibitor cycloheximide, suggesting that MNDA influences the turnover of MCL-1 protein. Since MCL-1 turnover is mainly regulated by proteasomal degradation (Zhong et al., 2005), we blocked the proteasome activity with MG132 and found that under such condition, MNDA failed to affect MCL-1 accumulation. These findings confirm that the rapid fall in MCL-1 expression is due to proteasomal degradation and indicate that, when present, MNDA promotes proteasomal degradation of MCL-1. By contrast, MNDA knockdown slowed down MCL-1 turnover and rendered HL-60 cells resistant to genotoxic stress-induced apoptosis, indicating that MNDA regulation of MCL-1 degradation is required for the execution of the constitutive cell death program. Collectively these findings indicate that cytoplasmic accumulation of MNDA is not merely a consequence, but rather an important mechanism promoting apoptosis in HL-60 cells and likely, in mature human neutrophils (**Figure 1**).

It is not known whether co-immunoprecipitation of MNDA and MCL-1 resulted from direct protein–protein interaction or which region(s) of MNDA is(are) required for this association. However, the MNDA PAAD/DAPIN/Pyrin domain, which is common to different proteins involved in apoptosis and inflammation, and/or the HIN-200 domain that mediates protein–protein interactions (Asefa et al., 2004) could be critical for the MNDA interaction with MCL-1. Indeed, the PAAD/DAPIN/Pyrin domain was shown to promote self-association of MNDA (Xie et al., 1997), and might also mediate association with other proteins. For instance, IFI16, which contains a PAAD/DAPIN/Pyrin domain, interacts with p53, thereby modulating senescence and apoptosis (Song et al., 2008). In mice, members of the HIN-200 family were shown to promote inflammation through interacting with NF-κB (Min et al., 1996). These results demonstrate that a member of the HIN-200 family or a protein with the PAAD/DAPIN/Pyrin domain co-immunoprecipitates with an anti-apoptotic protein of the Bcl-2 family to regulate apoptosis. It remains to be investigated whether this mechanism is common to all MNDA expressing cells including hematopoietic progenitors (Briggs et al., 2006).

ROLE FOR MNDA DURING SEPSIS

It is well established that neutrophils isolated from the peripheral blood of healthy volunteers undergo apoptosis when cultured for 24–48 h *in vitro*. By contrast, under the same conditions of culture, neutrophils of patients with sepsis exhibit markedly prolonged survival due to suppressed apoptosis (Keel et al., 1997; Matute-Bello et al., 1997; Fotouhi-Ardakani et al., 2010; Paunel-Gorgulu et al., 2012). The enhanced neutrophil longevity is associated with preserved $\Delta\Psi_m$ and inversely correlates with cytoplasmic accumulation of MNDA (Fotouhi-Ardakani et al., 2010). As predicted from the comprehensive study on MNDA in model cell lines (see above), during neutrophil apoptosis MNDA is relocated from the nucleus to the cytoplasm whereby it directly interacts with MCL-1 and promotes its proteasomal degradation (**Figure 1**). Although the signaling pathways involved in these events have not been elucidated, MNDA remains sequestered in the nucleus of neutrophils of patients in sepsis (Fotouhi-Ardakani et al., 2010). Consistently, culture of neutrophils from healthy volunteers with LPS, bacterial DNA, or platelet-activating factor partially replicated the abnormalities seen in the clinical samples, including the sequestration of MNDA to the nucleus (Fotouhi-Ardakani et al., 2010). Most interestingly, similar results were obtained when neutrophils of healthy donors were cultured in presence of serum from sepsis patient (Fotouhi-Ardakani et al., 2010). These findings suggest that neutrophils integrate yet unidentified cues

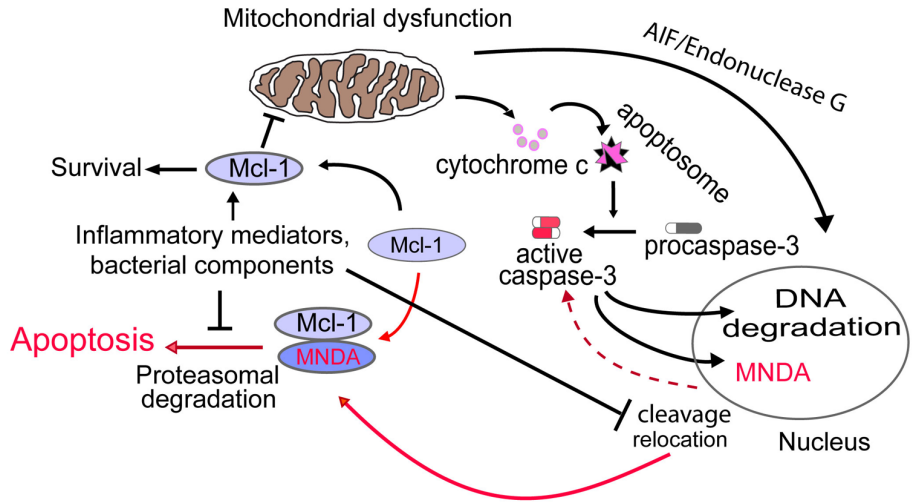

FIGURE 1 | Proposed model for MNDA regulation of neutrophil apoptosis. Cytoplasmic relocation and cleavage of MNDA results in aggravation of mitochondrial dysfunction through promotion of proteasomal degradation of MCL-1. Activation of this novel nucleus-mitochondrion circuit would then accelerate execution of the apoptotic death program. Conversely, prevention of MNDA relocation and cleavage would prolong neutrophil survival by retarding apoptosis. The mechanisms by which bacterial components and/or inflammatory modulators could negatively influence MNDA relocation and cleavage and hence, interfere with this nuclear-mitochondrial circuit remains to be defined. Broken line indicates yet undefined mechanism.

from the inflammatory milieu, which would prevent the cytoplasmic relocation and/or accumulation of MNDA, events that favor neutrophil apoptosis. Although our pilot clinical study was not powered to assess outcome, we noted that patients who had died exhibited markedly suppressed neutrophil apoptosis with minimal or complete absence of MNDA translocation and/or cleavage in neutrophils. Suppressed apoptosis in circulating neutrophils may contribute to neutrophilia, which predicts a poor prognosis, whereas delayed apoptosis in emigrated or trapped neutrophils contributes to aggravation of tissue injury, in particular damage to the airways (Matute-Bello et al., 1997; Hotchkiss and Nicholson, 2006). Apoptotic neutrophils sequester cytokines during endotoxin shock in mice (Ren et al., 2008) and thus may contribute to resolution of sepsis. Conversely, failure of neutrophils to undergo timely apoptosis would likely impair this pro-resolution effect. Clearly, additional studies are required to assess the precise role of MNDA in facilitating resolution of inflammation.

In conclusion, cytoplasmic accumulation of MNDA plays an important role in the progression of apoptosis. This represents a novel mechanism whereby MNDA, which predominantly localizes to the nucleus, regulate MCL-1 degradation and consequently mitochondrial function following its accumulation in the cytoplasm. The investigation of MNDA in neutrophils demonstrates that prevention of cytoplasmic MNDA accumulation likely contributes to suppressed apoptosis of neutrophils in patients with sepsis. Therefore, targeting MNDA may have a therapeutic potential for the treatment of sepsis and other inflammatory disorders.

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New insights for C5a and C5a receptors in sepsis

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The complement system plays a central role in inflammation and immunity. Among the complement activation products, C5a is one of the most potent inflammatory peptides with a broad spectrum of functions. There is strong evidence for complement activation including elevated plasma level of C5a in humans and animals with sepsis. C5a exerts its effects through the C5a receptors. Of the two receptors that bind C5a, the C5aR (CD88) is known to mediate signaling activity, whereas the function of another C5a binding receptor, C5L2, remains largely unknown. Here, we review the critical role of C5a in sepsis and summarize evidence indicating that both C5aR and C5L2 act as regulating receptors for C5a during sepsis.

Keywords: sepsis, C5a, receptor, inflammation, complement

INTRODUCTION

The complement system is composed of more than 30 heat-labile plasma proteins (Guo and Ward, 2005). Although complement activation plays a key role in innate immune defenses against invading bacteria, over-activation of complements leads to many inflammatory diseases including sepsis (Huber-Lang et al., 2001a,b, 2002a,b; Laudes et al., 2002b; Guo et al., 2004; Guo and Ward, 2005; Rittirsch et al., 2008). The complement system acts as an enzymatic cascade through a variety of protein-protein interactions, and complement activation occurs after a variety of different stimuli. Three well-known pathways are involved in complement activation: classical pathway, mannose-binding lectin (MBL) pathway, and alternative pathway (Guo et al., 2004; Guo and Ward, 2005) (**Figure 1**). The classical pathway can be activated by direct association of C1q with the microbial pathogen surfaces. It can also be initiated by binding of C1q to antigen-antibody complexes during an adaptive immune response. The MBL pathway is trigged by binding of MBL to carbohydrate structures containing mannose present on bacterium or virus surfaces. The alternative pathway is activated by binding of spontaneously activated complement C3 protein (C3b fragment) to pathogen surfaces. All the three pathways result in a series of enzymatic cleavage reactions, leading to formation of C3 convertase, at which the three pathways converge (Guo and Ward, 2005). C3 convertase can lead to the formation of C3a, C3b, C5a, C5b, C6, C7, C8, and C9, among which C5b, C6, C7, C8, and C9 form a membrane attack complex (C5b-9), which is used by host to lyse gram-negative bacteria. Coagulation pathway was recently suggested as a novel pathway of complement activation acting-independently of the formation of canonical C3/C5 convertases (Huber-Lang et al., 2006) (**Figure 1**). In this pathway, thrombin functions as a C5 convertase in the absence of C3, leading to the production of C5a and formation of C5b-9 (Huber-Lang et al., 2006). Moreover, in multiple trauma patients, factor VII-activating protease (FASP), which was activated by circulating nucleosomes released from necrotic cells, interacted with complement proteins in plasma, and cleaved C3 and C5 to produce C3a and C5a (Kanse et al., 2012). However, the mechanistic basis underlying the interaction between coagulation pathway and complement pathway remains poorly understood.

Sepsis represents a spectrum of clinical symptoms characterized by the inability of host to regulate the inflammatory response (Riedemann et al., 2003b). In the United States, it affects at least 600,000 persons per year, leading to around 250,000 annual deaths (Ward, 2010; Bosmann et al., 2011b). The systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, septic shock, and multiorgan failure (MOF) are currently used to characterize the progressive stages of this very complex and therapeutically challenging disorder of the immune and inflammatory systems (Hoesel et al., 2006). Bacterial infections can progress to sepsis, but detection of bacteremia is not a prerequisite for making the clinical diagnosis of sepsis. Sepsis can stimulate complement activation in both humans and animals, resulting in increased levels of C3a, C4a, and C5a in plasma (Bengtson and Heideman, 1988; Smedegard et al., 1989; de Boer et al., 1993; Nakae et al., 1994). It has been demonstrated that classical, MBL, and alternative pathways all participate in complement system activation, and play important roles in sepsis (Celik et al., 2001; Windbichler et al., 2004; Dahlke et al., 2011). Importantly, a recent study using CLP-induced sepsis model in mice lacking

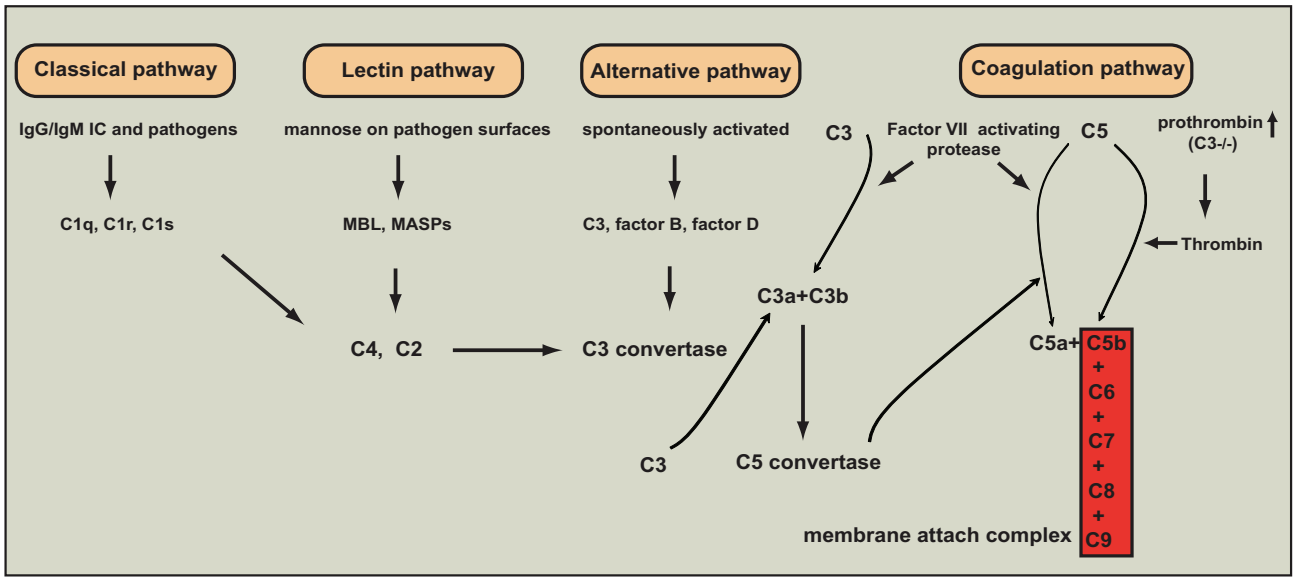

FIGURE 1 | The various pathways of complement activation. Four major pathways are involved in complement activation: classical, lectin, alternative, and coagulation pathways.

either the alternative ($fD^{-/-}$) or classical ($C1q^{-/-}$) complement activation pathway provides clear evidence that the classical pathway and the alternative pathway exert distinctly different contribution to the innate host response during sepsis by showing that the classical pathway is important for clearing bacteria in the early development of sepsis, whereas the alternative pathway may play a more important role for the later phase of development (Dahlke et al., 2011). During sepsis, over-activation of complement system causes multiple organ damage and compromised immune responses (Guo et al., 2003). Among complement system, C5a is the most powerful inflammatory mediator, which can lead to adverse systemic consequences by a broad spectrum of mechanisms in sepsis (Ward, 2004; Guo and Ward, 2005). C5a exerts its effect through its receptors: C5aR and C5L2. The roles of C5a signaling in inflammatory injury associated with sepsis are becoming defined. Here, we review the recent data for the critical roles of C5a, C5aR, and C5L2 during sepsis.

ROLE OF C5a IN SEPSIS

Human C5a is composed of 74 amino acids, which is a glycosylated peptide. NMR spectroscopy demonstrated that C5a contains four helices, which are connected by loops. The helical structures are cross-linked by disulfide bonds, which make the molecule quite stable in the presence of oxidative stress (Ward, 2010). It has been well established that C5a production could be due to plasma complement activation pathways. In addition, studies indicated that C5a could also be generated through cleavage of C5 by phagocytic cell-derived serine proteases that have C5 convertase activity (Huber-Lang et al., 2002c). These cells include alveolar macrophages and neutrophils (Huber-Lang et al., 2002c). Interestingly, a recent study shows that M-ficolin, a pattern-recognition molecule which activates the complement system in a manner similar to MBL pathway, was released by phagocytes during bacterial sepsis, and its cord blood level was positively related to circulating phagocytes and early-onset sepsis in neonates (Schlapbach et al., 2012).

The roles of C5a in sepsis have been investigated in subhuman primate model of sepsis-induced by intravenous injection of *Escherichia coli* (*E. coli*) into monkeys. In this model, C5a neutralizing antibody reduced several septic parameters (Stevens et al., 1986; Hangen et al., 1987). As a result, all septic animals treated with anti-C5a antibody survived, and did not developed severe lung edema and decreased oxygenation (Stevens et al., 1986; Hangen et al., 1987). In contrast, 75% of animals treated with control IgG died with decreased oxygenation, increased extravascular lung water, and profound hypotension (Stevens et al., 1986; Hangen et al., 1987).

The molecular mechanisms underlying the harmful effects of excessive C5a on innate immune functions during sepsis are being defined. C5a inhibited phagocytic activity of normal blood neutrophil in a dose-dependent manner (Huber-Lang et al., 2002b). Furthermore, blood neutrophils from septic rats showed defect in phagocytosis (Huber-Lang et al., 2002b). In contrast, neutrophils from cecal ligation and puncture (CLP) rats treated with antibody to C5a preserved the phagocytic activity. C5a treatments also led to suppression of $p47^{phox}$ phosphorylation, and its subsequent translocation to the cell membrane and assemble of NADPH oxidase, which resulted in inhibition of respiratory burst in neutrophils (Huber-Lang et al., 2002b). C5a-induced defects in phagocytosis and NADPH oxidase assembly caused defective bactericidal activity of neutrophils, leading to increased bacterial counts (Huber-Lang et al., 2002b). In CLP-induced sepsis model, 50% of rats receiving anti-C5a antibody treatment survived during a 10-day survival study, while the survival rate

was only 9.5% in the septic group treated with normal IgG (Czermak et al., 1999). The improved survival was linked to reduced bacterial colony forming-units (CFU) in blood, spleen, and liver, and improved H_2O_2-generating ability of neutrophils by C5a blockade (Czermak et al., 1999).

Complement activation occurs during sepsis in human, leading to the generation of anaphylatoxins including C3a, C4a, and C5a (Nakae et al., 1996). Appearance of high levels of anaphylatoxins was correlated with MOF that is a key factor resulting in death, and lower anaphylatoxin levels could only be identified in surviving septic patients but not non-surviving persons (Bengtson and Heideman, 1986; Nakae et al., 1996). In addition, *in vitro* experiment demonstrated that neutrophils in patients surviving from sepsis-induced MOF had defect in chemotactic response to C5a, which might be related with inability of C5a to bind to neutrophils (Solomkin et al., 1981; Goya et al., 1994). In experimental sepsis, C5a blockade attenuated the parameters of MOF, and maintained normal chemotactic function of neutrophils (Huber-Lang et al., 2001a; Flierl et al., 2006). Importantly, C5a blockade given at 12 h after the initiation of sepsis has protective effects against detrimental influence of septic shock (Huber-Lang et al., 2001b). However, it remains to be determined whether, in human beings with sepsis, there may be a similar "time window" during which anti-C5a treatment can be an effective method to improve survival.

C5a REGULATION OF INFLAMMATORY MEDIATORS

C5a promotes proinflammatory mediators' production in many cell types (**Table 1**). For example, C5a stimulated the synthesis and release of cytokines such as TNF-α, IL-1β, and IL-6 by human peripheral blood mononuclear cells (Schindler et al., 1990; Scholz et al., 1990). In addition, C5a promoted generation of IL-8, IL-1β, and RANTES at mRNA level in human umbilical cord endothelial cells (HUVEC) (Monsinjon et al., 2003). A recent study found that IL-17F production in mouse peritoneal macrophages was significantly induced by LPS at both mRNA and protein levels (Bosmann et al., 2011a). Interestingly, C5a amplified LPS-stimulated IL-17F generation by enhancing Akt phosphorlation in a MyD88-dependent manner (Bosmann et al., 2011a). C5a can also exert *in vivo* immunoregulatory functions (**Table 2**). For example, plasma level of IL-17F was dramatically elevated in both LPS- and CLP-induced septic mice, which correlated with C5a concentration (Bosmann et al., 2011a). Furthermore, IL-17F level was greatly decreased in septic mice receiving C5a blocking antibody, suggesting that IL-17F production was positively regulated by C5a during sepsis. C5a can also synergistically induce the production of cytokines and chemokines with LPS in various cells. These include IL-1 and TNF from mouse peritoneal macrophages and human monocytes (Cavaillon et al., 1990), IL-8 from human neutrophils (Strieter et al., 1992), and TNF-α, macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant-1 (CINC), and IL-1β from rat alveolar epithelial cells (Riedemann et al., 2002c). Similarly, exposure of mouse dermal microvascular endothelial cells to LPS or IL-6, followed by exposure to C5a, resulted in a synergistic effect on the generation of MIP-2 and monocyte chemoattractant protein-1 (MCP-1) (Laudes et al., 2002a). Our recent study demonstrated that C5a increased IgG immune complex-stimulated TNF-α, MIP-2, and MIP-1α expression by enhancing phosphorylation of both p38 and p44/42 MAPKs in a Fcγ receptor-dependent manner (Yan et al., 2012). C5a also plays a pivotal role in lymphocyte inflammatory responses. For example, C5a modulated IL-22 and IL-17 expressions by human CD4+ T cells (Gerard et al., 2005). Moreover, C5a-induced a robust Th1 polarization, while inhibited Th2 response in trinitrobenzene sulfonic acid-induced model of colitis, which contributed to the exacerbation of intestinal damage (Chen et al., 2011). The role of C5a in innate lymphocyte activation during *E. coli*-induced sepsis was recently reported (Fusakio et al., 2011). In this study, using $C5aR^+/C5aR^-$ mixed bone marrow chimeras, the cognate C5a/C5aR interaction on NKT cells was identified as a critical factor for NKT cell activation and the recruitment during sepsis. Furthermore, there is a synergistic interaction between C5a/C5aR and TLRs, which enhances the production of TNF-α and IFN-γ from NKT and NK cells in co-cultures with dendritic cells (DC) (Fusakio et al., 2011). DC are bridges linking innate and adaptive immunity, their functions are affected by C5a. When cultured with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), DCs from C5-deficient mice secreted much less IL-12 in comparison with those from C5-sufficient animals (Moulton et al., 2007). Furthermore, C5-deficient DCs fully restored the IL-12 generating capacity when incubated with BCG in presence of C5a (Moulton et al., 2007), suggesting that C5a may contribute to the generation of acquired immune responses in mice by modulating Th1 response.

On the other hand, C5a can also limit the pro-inflammatory mediators' production. For example, in an experimental allergic model, C5a suppressed DC-derived IL-23 production, which led to inhibition of Th17 cell differentiation and proliferation, and limited the severe airway hyper-responsiveness (Lajoie et al., 2010). C5a can also suppress many other pro-inflammatory mediators' expression. For example, Mycobacterium tuberculosis (MTB)-infected macrophages from C5-deficient mice showed enhanced growth of MTB coinciding with a reduced secretion of both cytokines (TNF-α, IL-1β, IL-6, and IL-12) and chemokines (KC, MIP-2, and MIP-1α) (Jagannath et al., 2000). Both LPS and IFN-γ-induced IL-12 expression were markedly suppressed by C5a in human monocytes (Wittmann et al., 1999). IL-6 expression was significantly reduced by C5a in HUVECs (Monsinjon et al., 2003). Moreover, C5a significantly suppressed LPS-induced TNF-α expression by increasing the expression of cytosolic IκBα, an inhibitor of NF-κB activation, in neutrophils (Riedemann et al., 2003a). Interestingly, a recent study showed that C5a exhibited anti-inflammatory effect during endotoxic shock by suppressing IL-17A and IL-23 production from CD11b(+)F4/80(+) macrophages (Bosmann et al., 2012). Mechanistically, endotoxin-induced generation of C5a resulted in activation of the PI3-K-Akt and MEK1/2-ERK1/2 pathways, leading to IL-10 production, followed by suppression of IL-17A and IL-23 expressions (Bosmann et al., 2012).

Complement system is activated at early time during sepsis, causing C5a production, which may play a central role in generation of "inflammatory cytokine storm." During sepsis, there is an increase of both pro-inflammatory mediators in blood including IL-6, TNF-α, IL-1β, IL-8, and IFN-γ, and

Table 1 | C5a regulation of inflammatory mediators.

Cell type	Clinical condition associated with *in vitro* model of choice	Stimulus	Signaling effectors involved	Immune reactions	References
Neutrophil	Sepis	C5a		Reduced phagocytic activity, respiratory burst, bactericidal activity, and chemotactic response	Solomkin et al., 1981; Goya et al., 1994; Huber-Lang et al., 2002b
			PI3-K, Akt, ERK1/2, PKC, and Bcl-XL	Reduced apoptosis	Perianayagam et al., 2002; Suvorova et al., 2008
				Increased IL-6 production	Riedemann et al., 2004
		C5a + LPS	IκBα	Reduced TNF-alpha production	Riedemann et al., 2003a
			p38 and p44/42 MAPKs	Increased IL-6 production	Riedemann et al., 2004
			PI3-K	Increased IL-8 and IL-1beta production	Wrann et al., 2007
		C5a + LPS		Increased IL-6 generation in the presence of anti-C5L2 antibody treatment	Scola et al., 2009
Monocyte	Sepis	C5a		Increased TNF-alpha, IL-1beta, and IL-6 production	Schindler et al., 1990; Scholz et al., 1990
		C5a + LPS		Elevated IL-1, TNF, and IL-8 generation	Cavaillon et al., 1990; Strieter et al., 1992
		C5a + LPS/IFN-gamma		Reduced IL-12 expression	Wittmann et al., 1999
Human umbilical cord endothelial cell	Sepis	C5a		Increased IL-8, IL-1beta, RANTES, and tissue factor expressions Reduced IL-6	Ikeda et al., 1997; Monsinjon et al., 2003
Macrophage	Sepis	C5a + LPS	MyD88 and Akt	Enhanced IL-17F generation	Bosmann et al., 2011a
				Enhanced IL-1, and TNF generation	Cavaillon et al., 1990
			PI3-K, Akt, MEK1/2 and ERK1/2	Reduced IL-17A, and IL-23 expressions that are C5aR-but not C5L2-dependent while increased IL-10 production	Bosmann et al., 2012
	IgG immune complex-associated diseases	C5a + IgG IC	p38 and p44/42 MAPKs	Amplified expressions of MIP-2, MIP-1alpha, TNF-alpha	Yan et al., 2012
Macrophage lack of C5	Tuberculosis	Mycobacterium tuberculosis (MTB)		Enhanced growth of MTB Reduced secretion of TNF-alpha, IL-1beta, IL-6, IL-12, KC, MIP-2, and MIP-1alpha	Jagannath et al., 2000
Alveolar epithelial cell		C5a + LPS		Increased expressions of TNF-alpha, MIP-2, CINC, and IL-1beta	Riedemann et al., 2002c

(Continued)

Table 1 | Continued

Cell type	Clinical condition associated with *in vitro* model of choice	Stimulus	Signaling effectors involved	Immune reactions	References
Dermal microvascular endothelial cells	Sepis	C5a + LPS/IL-6		Increased MIP-2, and MCP-1production	Laudes et al., 2002a
CD4 + T cell	Age-related macular degeneration	C5a		Enhanced IL-22, and IL-17 generation	Gerard et al., 2005
NKT and NK	Sepis	C5a + ligands for TLRs		Increased expressions of TNF-alpha, and IFN-gamma	Fusakio et al., 2011
C5 deficient dendritic cell	Tuberculosis	*Mycobacterium bovis* Bacillus Calmette-Guerin (BCG)		Reduced IL-12 expression	Moulton et al., 2007
Adrenal medulla cell	Sepis	C5a	Caspase	Increased apoptosis	Flierl et al., 2008a
γ^{d}T cell	Sepis	C5a		Enhanced C5aR, and IL-17 expressions	Haviland et al., 1995
Thymocytes from septic rats	Sepis	C5a	Caspase-3, -6, 9, cytochrome c, and Bcl-X L	Enhanced apoptosis	Guo et al., 2000

anti-inflammatory factors such as IL-10, IL-13, IL-4, and TGF-β (Wolkow, 1998; Titheradge, 1999; Le Tulzo et al., 2002; Guo et al., 2004; Flierl et al., 2008b). Sepsis-induced imbalance between pro-inflammatory and anti-inflammatory responses leads to apoptosis, immunosuppression, and multiple organ dysfunction (Guo et al., 2004). Neutrophils are generally regarded as driving force for acute inflammation. The role of C5a in sepsis is best studied by its effects on neutrophil inflammatory responses. For example, a recent study demonstrated that elevated serum IL-6 level during CLP-induced sepsis was due to increased level of C5a (Riedemann et al., 2004). Importantly, neutrophil depletion resulted in a more than 50% decrease of IL-6 level, suggesting that neutrophils are the major contributor of C5a-regulated IL-6 production during sepsis (Riedemann et al., 2004). In another study, anti-C5a monoclonal antibody led to an over 75% decrease in serum IL-6 bioactivity in septic pigs receiving intravenous injection of *E. coli* when compared with control group (Hopken et al., 1996). *In vitro*, either LPS or C5a significantly induced IL-6 expression in neutrophils (Riedemann et al., 2004). Importantly, C5a enhanced LPS-stimulated IL-6 generation by rapidly inducing phosphorylation of p38 and p44/42 MAPKs (Riedemann et al., 2004). In human neutrophils, C5a significantly boosted TLR-4-dependent generation of IL-1β and IL-8, which was controlled in an inhibitory fashion by the PI3K pathway (Wrann et al., 2007). Furthermore, PI3K signaling pathway exerts an overall protective role during the onset of sepsis in rodents by limiting C5a-mediated effects on neutrophil cytokine generation, and promoting oxidative burst and phagocytosis (Wrann et al., 2007). Thus, these studies suggest a leading role of C5a in the imbalance of inflammatory network during sepsis. However, whether other cell populations such as monocytes and NKT cells are responsible for the cytokine storm *in vivo* during sepsis and how the complex interactions between these cells contribute to the acute inflammatory processes in sepsis remains a puzzle.

C5a REGULATION OF COAGULATION PATHWAYS DURING SEPSIS

During sepsis, blood monocytes, tissue macrophage, and endothelial cells serve as sensors of invading microorganisms by using pattern recognition receptors. The interactions between the host receptors and the conserved structures of pathogens lead to activation of inflammatory and coagulation pathways. It is well known that coagulation cascade is activated in septic patients. There are two pathways involved in blood coagulation: extrinsic and intrinsic pathways. Extrinsic pathway is responsible for initiation of blood clotting, and intrinsic pathway is the initiator of blood coagulation amplification (Aird, 2003). During sepsis, elevated expression of tissue factor (TF) was found on the surfaces of tissue macrophages and circulating monocytes, which led to initiation of extrinsic clotting cascade, thrombin production, and fibrin formation (Aird, 2003). At the same time, sepsis suppresses natural anti-coagulant responses, which results in increased thrombin production, fibrin formation and consumption of clotting factors, and decreased protein C in blood (Aird, 2003). Injection of exogenous protein C inhibited initiation of coagulation pathway, reduced organ dysfunction, and improved survival rate in a sepsis model performed in baboon, while *in vivo*

Table 2 | *In vivo* immunoregulatory properties of the C5a/C5aR system.

Model	Treatment	Outcomes	References
E. coli-induced sepsis	C5a neutralizing antibody	Increased survival rate, decreased lung edema and oxygenation	Stevens et al., 1986; Hangen et al., 1987
	anti-C5a antibody	Decreased IL-6 level in serum	Hopken et al., 1996
	C5aR knockout	Attenuation of NK and NKT cell activation Reduced TNF-alpha and IFN-gamma release by NK and NKT cells Impaired recruitment of NK and NKT cells to the site of infection Increased survival rate	Fusakio et al., 2011
LPS-induced endotoxic shock	C5a neutralizing antibody	Attenuated septic parameters	Smedegard et al., 1989
	C5aR knockout	Increased circulating IL-23 and IL-17A level Increased resistance to endotoxic shock	Van Epps et al., 1990; Bosmann et al., 2012
	C5L2 knockout	Increased serum IL-1beta while decreased survival rate	Han et al., 2011
CLP-induced sepsis	anti-C5a antibody	Reduced bacterial colony forming-units while improved respiratory burst Reduced IL-17F level in serum Attenuated coagulant paremeters Reduced apoptosis of adrenal medulla cell Ameliorated septic encephalopathy Restoration of neutrophil to spontaneous apoptosis Reduced inflammatory mediators' production by cardiomyocytes while attenuation of cardiac dysfunction Restoration of C5aR content on neutrophils	Czermak et al., 1999; Laudes et al., 2002b; Guo et al., 2003, 2006; Flierl et al., 2008a, 2009; Atefi et al., 2011; Bosmann et al., 2011a
	C5aR knockout	Decreased plasma levels of IL-1beta, IL-6, MIP-2, and MIP-1alpha while increased survival rate	Rittirsch et al., 2008
	C5aR antagonist	Improved survival	Huber-Lang et al., 2002a
	C5aR antibody	Reduced IL-6 and TNF-alpha production in serum, and bacterial burden while improved survival	Zahedi et al., 2000
	anti-C5L2 antibody	Increased serum IL-6 level	
	C5L2 knockout	Decreased serum levels of IL-1beta, MIP-2, MIP-1alpha, and HMGB1 while improved survival Increased pro-inflammatory mediators' production from cardiomyocytes	Rittirsch et al., 2008; Atefi et al., 2011
House dust mite-induced allergic asthma	C5/C5aR knockout	Increased IL-23 production by dendritic cells and Th17 cell differentiation and proliferation Enhanced airway hyperresponsiveness	Lajoie et al., 2010
	C5L2 knockout	Attenuated asthmatic phenotye	Johswich et al., 2006
IgG IC-induced acute lung injury	C5L2 knockout	Reduced lung inflammation	Gerard et al., 2005

blockade of protein C activation by using anti-protein C antibody worsened *E. coli*-induced septic shock (Taylor et al., 1987). However, due to risk of serious bleeding in 35% patients receiving rhAPC (recombinant human activated protein C), the FDA and European Medicines Agency (EMEA) have recently withdrawn their support and recommends not using the product, and the manufacturer has withdrawn the product from the market (Kylat and Ohlsson, 2012).

A number of evidences indicate the involvement of C5a in coagulation pathway. The recombinant human C5a stimulated TF expression in a dose-dependent fashion in HUVECs (Ikeda et al., 1997). In addition, C5a-induced TF production in human leukocytes (Muhlfelder et al., 1979). In the CLP-induced sepsis model, C5a neutralizing antibody ameliorated coagulation/fibrinolytic protein changes in rats, thus preventing dissemination of intravascular coagulation (Laudes et al., 2002b). In septic rats receiving anti-C5a antibody, coagulant parameters were greatly attenuated (Laudes et al., 2002b). Additionally, C5a markedly induced IL-8 generation in HUVECs (Monsinjon et al., 2003), which could in turn induce the fibrin deposition and

promote thrombogenesis as well as proliferation and structural reorganization of endothelial cell (Guo et al., 2004). Therefore, the involvement of C5a in activation of coagulation pathways seems to be mediated by up-regulated expression of IL-8 in human beings, and C5a neutralizing antibody treatment may be an effective approach to prevent coagulation-induced organ damage during sepsis. The coagulation system also has profound effects on the complement activation. It has been shown that thrombin is capable of generating C5a in the absence of C3 (Huber-Lang et al., 2006). A most recent study provided novel insights into the complex interaction between the coagulation/fibrinolysis cascades and the complement system *in vitro* and *ex vivo* (Amara et al., 2010). This study established multiple links between various factors of the coagulation and fibrinolysis cascades and the central complement components C3 and C5 by demonstrating that thrombin, human coagulation factors (F) XIa, Xa, and IXa, and plasmin were all found to effectively cleave C3 and C5 (Amara et al., 2010). Thus, it is possible that C5a pathway and coagulation/fibrinolysis cascades during sepsis can regulate each other by positive-feedback mechanisms.

ROLE OF C5a IN CELL APOPTOSIS DURING SEPSIS

Immunosuppression occurs in humans and rodents during sepsis, which is due to reduced number of T and B lymphocytes in lymphoid tissues and in circulation (Guo et al., 2000; Riedemann et al., 2002a; Hotchkiss and Nicholson, 2006; Ward, 2008). Apoptosis appears to be the predominant factor that is responsible for lymphoid cell loss and the associated pathogenesis during sepsis (Song et al., 2000). It has been reported that early lymphocyte apoptosis in blood stimulated by sepsis in human being was associated with low survival rate (Le Tulzo et al., 2002). Apoptosis can be induced via both the extrinsic (TNF-α, Fas ligand) and intrinsic pathways (mitochondrial) during sepsis (Ward, 2010), and prevention of lymphoid cell apoptosis could markedly attenuate parameters of sepsis and improve survival (Hotchkiss et al., 2000; Oberholzer et al., 2001). *In vitro* experiments demonstrated that when exposed to C5a, thymocytes from septic rats showed increased apoptotic rate, which was attributable to the increased caspase-3, -6, and -9 activities (Riedemann et al., 2002a). However, C5a exposure alone could not stimulate normal thymocyte apoptosis (Guo et al., 2004), suggesting that other factors such as TNF-α and Fas ligand-induced by sepsis were indispensible for C5-indued apoptotic death of thymocytes. Furthermore, *in vivo* experimental data showed that thymocyte apoptosis was induced in a time-dependent fashion during sepsis, leading to around 50% loss of thymus weight 24 h after onset of sepsis (Guo et al., 2000). Thymocyte apoptosis was due to elevated ratio of apoptotic accelerators to anti-apoptotic proteins, because the activities of caspase-3, -6, -9 and cytochrome c-level in cytosol were significantly increased 12 h after CLP induction of sepsis, while Bcl-X_L content was greatly reduced (Guo et al., 2000). Importantly, C5a neutralizing antibody treatment maintained caspase-3, -6, and -9 activities at basal levels, prevented increase of ctyosolic cytochrome c concentration and decrease of Bcl-X_L level (Guo et al., 2000). These studies indicated that intrinsic pathway participated in sepsis-induced thymocyte apoptosis, which could be intervened by C5a blockade (**Figure 2**).

C5a can also contribute to apoptosis of other cell types (**Figure 2**). Recent study showed that C5a treatment caused significant apoptosis of adrenal medulla cells (PC12), leading to impaired generation of catecholamines in a dose- and time-dependent manner (Flierl et al., 2008a). *In vivo*, apoptosis of adrenal medulla cells was markedly increased after CLP-induced sepsis, which was greatly reversed by C5a blockade (Flierl et al., 2008a). Furthermore, pan-caspase inhibitor treatment prevented C5a-induced PC12 cell apoptosis during sepsis (Flierl et al., 2008a), suggesting that elevated caspase activities are critical for C5a-induced adrenal medulla cell apoptosis. Septic encephalopathy secondary to a breakdown of the blood-brain barrier (BBB) is a known complication of sepsis. Using CLP-induced sepsis model, a recent study demonstrated that the neutralization of C5a greatly ameliorated pathophysiological changes associated with septic encephalopathy (Flierl et al., 2009). Furthermore, C5a/C5aR signaling was also linked to increased caspase 3 activity and apoptosis in mouse brain endothelial cells (Jacob et al., 2011).

While C5a stimulated apoptosis of several cell types during sepsis, it provides anti-apoptotic signals to neutrophils (**Figure 2**). *In vitro* experiments showed that C5a inhibited spontaneous human neutrophil apoptosis by activating PI3-K/Akt signaling pathway (Perianayagam et al., 2002). In addition, C5a stimulation could lead to activation of ERK1/2 (Suvorova et al., 2008) and protein kinase C (PKC) (Simon, 2003). Both ERK1/2 and PKC can provide neutrophils with anti-apoptotic signals (Simon, 2003). Thus, C5a might be involved in delayed neutrophil apoptosis through multiple signaling pathways. It is noteworthy that C5a plays a key role in generation of inflammatory mediators such as IL-1β, IL-6, and IL-8 in humans (Strieter et al., 1992; Hopken et al., 1996), all of which can stimulate anti-apoptotic signals in neutrophils (Simon, 2003). We have previously observed that neutrophils from septic rats showed delayed spontaneous apoptosis when compared with those from normal animals (Guo et al., 2006). In contrast to normal serum, septic sera treatment led to significant resistance of neutrophils isolated from normal rats to apoptotic death, which was due to activation of both Akt and ERK1/2 (Guo et al., 2006). In sharp contrast, septic sera from rats receiving anti-C5a antibody restored the sensitivity of neutrophils to spontaneous apoptosis (Guo et al., 2006). C5a-induecd resistance of neutrophils to apoptosis was due to enhanced phosphorylation of Akt and ERK1/2, and increased expression of X-linked inhibitor of apoptosis and Bcl-X_L(Guo et al., 2006). These studies together suggest that the distinct effects of C5a on apoptosis in various cell types may induce different pathophysiology in sepsis. Increased apoptotic death of lymphocytes and adrenal medulla cells led to immunosuppression during sepsis, while decreased apoptotic rate caused release of more toxic cellular products from activated neutrophils (**Figure 1**). Together, these events may result in delayed pathogen elimination, normal tissue damages, and finally MOF.

EFFECT OF C5a ON CARDIAC DYSFUNCTION DURING SEPSIS

Defect in cardiac function is often induced in septic patients and has been referred to as "cardiomyopathy of sepsis." "Septic cardiomyopathy" has been characterized by *in vitro* defective cardiomyocyte (CM) function. During sepsis, left ventricular

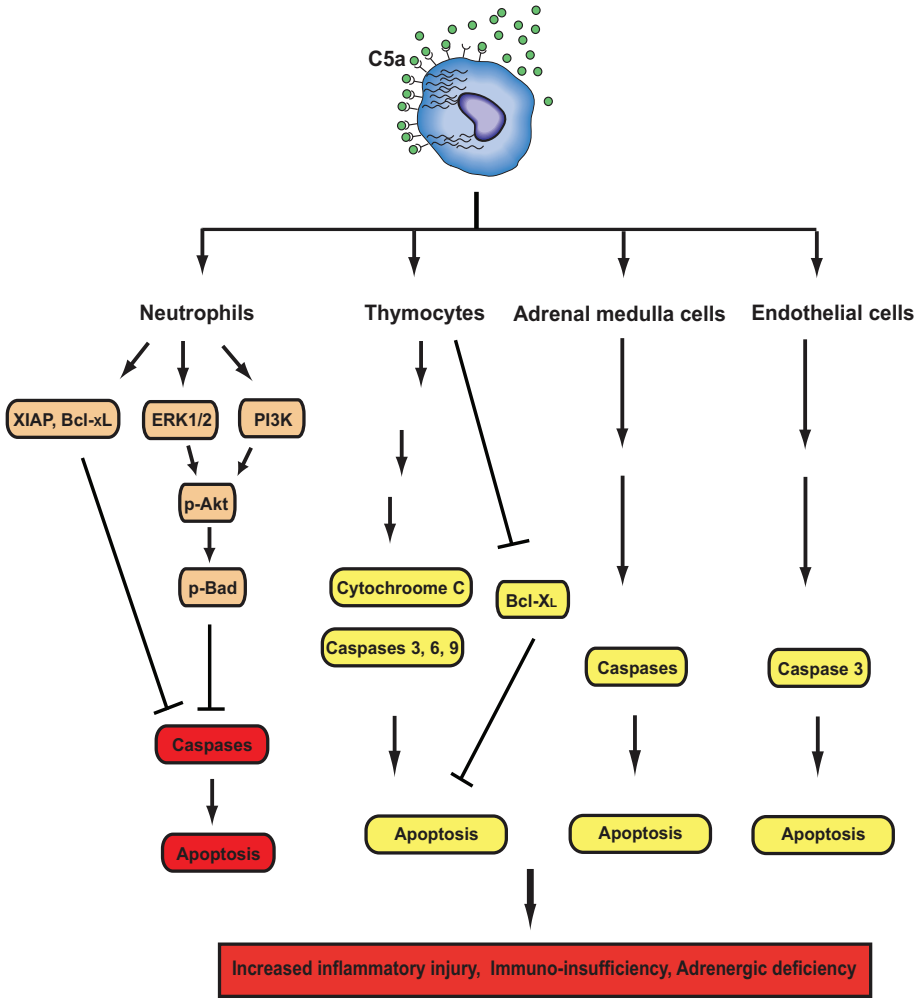

FIGURE 2 | Effects of C5a signals on apoptosis during sepsis. C5a have both anti- and pro-apoptotic activities depending on cell types. In neutrophils, C5a activates PI3K and ERK1/2 pathways, leading to phosphorylation of Akt and subsequent phosphorylation of Bad. Phosphorylated Bad inhibits cytochrome C release from mitochondria to prevent the formation of the apoptosome, thereby inhibiting neutrophil apoptosis. C5a together with LPS induces XIAP production, which can inhibit the formation of the apoptosome. Sepsis enhances Bcl-xL expression and reduces Bim expression. C5a and LPS can also enhance Bcl-xL expression. All of these events are in favor of maintaining the integrity of mitochondria and preventing neutrophil apoptosis. In thymocyes, adrenal medulla cells, and endothelial cells, C5a can induce apoptosis by enhancing caspase activities or inhibiting Bcl-xL expression.

pressures were greatly reduced, and CMs isolated from septic rats exhibited defective contractility and relaxation (Niederbichler et al., 2006). Importantly, when incubated with C5a, CMs isolated from both sham and CLP animals developed defective contractility and relaxation (Niederbichler et al., 2006). These defects were attenuated in septic rodents receiving anti-C5a antibody treatment, indicating that C5a might play a central role in cardiac dysfunction during sepsis. "Cardiosuppressive cytokines," the definition of which is based on their ability to disrupt normal contractile function of normal CMs, have been described in patients with sepsis, and include IL-6, TNF-α, and IL-1β (Cain et al., 1999; Joulin et al., 2007; Ward, 2010). Furthermore, a recent study showed that polymicrobial sepsis greatly induced generation of inflammatory mediators in hearts, and CMs isolated from septic rodents spontaneously secreted cytokines and chemokines (IL-6, TNF-α, IL-1β, MIP-1α, MIP-2, MCP-1, KC, and IL-10) in a time-dependent manner (Atefi et al., 2011). In contrast, CMs obtained from septic rodents receiving neutralizing antibody to C5a produced significant less amount of the inflammatory mediators. Thus, C5a production during sepsis resulted in increased expressions of cytokines and chemokines in CMs, leading to cardiac dysfunction (Atefi et al., 2011) (**Figure 3**). The role of IL-10 in CM function during sepsis is unclear. IL-10 is considered to have anti-inflammatory effect and may be protective of septic heart by antagonizing other inflammatory mediators' effects, which represents a negative feedback mechanism regulated by C5a (**Figure 3**). In line with this hypothesis, a recent study shows that IL-10 prevents TNF-α induced cardiomyocyte apoptosis (Dhingra et al., 2011).

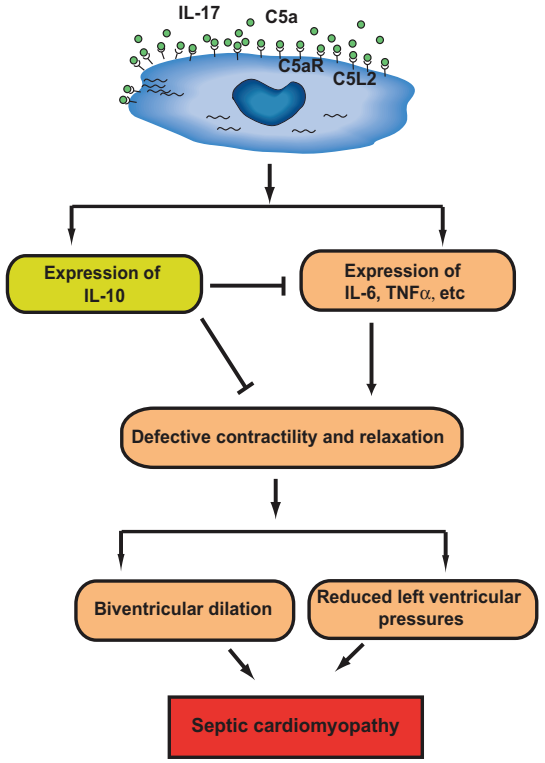

FIGURE 3 | Role of C5a signaling in cardiac dysfunction in sepsis. In cecal ligation and puncture (CLP)-induced sepsis, expression of C5aR, but not C5L2, on CMs is increased. C5a increased production of inflammatory mediators (IL-6, TNF-α, etc.,) and anti-inflammatory mediator (IL-10) from CMs in a C5aR-, C5L2-, and IL-17-dependent mechanism. The resulting cytokine storm impaired CM contractility leading to "septic myocardiopathy." C5a-dependent IL-10 may function as a negative feedback regulator of CM function during sepsis.

NEUTROPHILS AND COLLATERAL TISSUE DAMAGE IN SEPSIS

During CLP-induced sepsis, multiple organ failure occurs. When compared with control, CLP mice displayed higher level of plasma urea level, which indicated that the filtrating function of the kidneys was impaired (Dahlke et al., 2011). In addition, renal vascular permeability was significantly induced by septic shock, which was demonstrated by increased extravascular Evans blue leak (Benjamim et al., 2005). Liver cell damage and abnormal liver function were also induced by CLP as proved by elevated GOT/AST level—an indicator of liver cell injury, and bilirubin level, which suggested impairment of normal liver function (Dahlke et al., 2011). Moreover, CLP leads to pulmonary dysfunction. Histological assay showed that CLP-induced lung structural change such as alveolar swelling and inflammatory cell accumulation (Dahlke et al., 2011). Impairment of other organs, such as thymus, adrenal medulla, and heart, were also observed during septic shock (Riedemann et al., 2002a; Niederbichler et al., 2006; Flierl et al., 2008a). CLP-induced collateral tissue damages might be due to bacterial accumulation in lungs, kidneys, livers, and spleens (Riedemann et al., 2002b; Scott et al., 2003; Dahlke et al., 2011). However, whether CLP could induce bacterial burden in heart is still an open question. During sepsis-induced systemic inflammation, neutrophil influx into lungs and livers were elevated, as reflected by increased MPO activity in the corresponding organs (Scott et al., 2003; Dahlke et al., 2011). Transmigration of neutrophil from vascular vessels into collateral tissues is indispensible for bacterial clearance; however, excessive neutrophil accumulation could lead to tissue damages. It has been demonstrated that during CLP-induced sepsis, rat receiving anti-C5 antibody showed decreased bacterial load in spleen and liver compared with those receiving control IgG (Buras et al., 2004). In addition, anti-C5 treatment attenuated lung injury by reduced neutrophil influx (Buras et al., 2004), indicating that CLP might stimulate inappropriate neutrophil accumulation in tissues. Cardiac dysfunction-induced by CLP could be also alleviated by blocking C5a signaling (Niederbichler et al., 2006). Moreover, CLP-induced bacterial influx into lungs, kidneys, and livers could be reduced by disruption of C5aR (Riedemann et al., 2002b). Furthermore, disruption of C5aR could prevent thymocytes and adrenal medulla cells from apoptotic death (Riedemann et al., 2002a; Flierl et al., 2008a). However, the effect of C5aR on neutrophil accumulation in different organs and tissue (kidney, liver, lung and spleen) damages remains largely unknown. In addition, the role of C5L2 in bacterial dissemination, tissue accumulation of neutrophils, and organ damages is still enigmatic, though C5L2 deficient mice were resistant to CLP-induced systematic inflammatory reactions and subsequent death (Rittirsch et al., 2008).

EXPRESSION AND FUNCTION OF C5aR IN SEPSIS

C5a can bind two receptors on the cells: C5aR and C5L2. C5aR (CD88) is a G-protein-coupled receptor with seven transmembrane segments. C5aR has a molecular weight of 45 kDa, and binds to C5a with high affinity, to a lesser extent, to C5a des Arg. The expression and function of C5aR in neutrophils during sepsis have been studied. After CLP in rats, C5aR content on neutrophils gradually decreased, reached the nadir at 24 h after onset of sepsis, and progressively increased thereafter (Guo et al., 2003). Mechanistically, the dynamic change of C5aR on neutrophil surface during sepsis might be due to internalization, followed by reconstitution (Guo et al., 2003). The result was consistent with the previous study that the association of C5a with C5aR caused rapid internalization of the ligand-receptor complex in neutrophils, followed by recycling of C5aR to the cell surface (Van Epps et al., 1990; Naik et al., 1997; Gilbert et al., 2001). Importantly, intravenous administration of neutralizing antibody to C5a markedly prevented decrease of C5aR content on neutrophils (Guo et al., 2003), suggesting that sepsis-induced rapid internalization of C5aR was likely caused by systemic appearance of C5a. Except for C5a-induced internalization, C5aR expression could also be regulated at transcription level by other inflammatory mediators generated during sepsis. For instance, C5aR mRNA expression was greatly reduced in monocytes and monocyte-derived dendritic cells by Th2 cytokine IL-4 (Soruri et al., 2003), which was significantly up-regulated during sepsis (Song et al., 2000). Surface content of C5aR on neutrophils might play an important role in their function. The lowest level of C5aR

content on neutrophils 24 h after onset of CLP was accompanied by defective oxidative burst [decreased production of reactive oxygen species (ROS), especially H_2O_2] (Guo et al., 2003), which might be important for bacterial killing ability of neutrophils. Furthermore, the gradually increased expression of C5aR on neutrophils after 24 h CLP was correlated with elevated oxidative burst activity (Guo et al., 2003). Therefore, it seems that low level of C5aR on neutrophils might lead to reduced ROS production and followed high bacterial burden 24 h after CLP. However, the exact relationship between C5aR level and production of reactive nitrogen species (RNS) that may be more important for bactericidal activity is still unknown. On the other hand, there is no direct evidence demonstrating that reduced oxidative burst activity of neutrophils was due to decreased C5aR on surfaces; hence, use of C5aR knockout neutrophils is necessary to examine its influence on ROS and RNS expressions during CLP-induced sepsis.

The role of C5aR in sepsis was recently determined by gene knockout approach. In mid-grade CLP, 31% of wild type mice survived, whereas 80% of C5aR-deficient mice survived in a 7-days survival study, indicating the contribution of C5aR to harmful outcome of CLP-induced sepsis (Rittirsch et al., 2008). Furthermore, plasma levels of IL-1β, IL-6, MIP-2, and MIP-1α were obviously down-regulated in C5aR knockout mice when compared with wild type littermates (Rittirsch et al., 2008), suggesting that the harmful effects of C5aR during sepsis might result from C5a-mediated cytokine storm. Unfortunately, although C5aR blockade treatment resulted in lower bacterial burden in various organs, the influence of C5aR on bacterial counts was not investigated in C5aR-knockout mice. In line with this result, disruption of the C5a receptor gene significantly increases resistance to acute Gram-negative bacteremia, and endotoxic shock following an intravenous infusion of purified *E. coli* endotoxin (Hollmann et al., 2008). The role of C5aR in sepsis was also investigated by using a C5aR antagonist, C5aRa. C5aRa is a cyclic peptide to compete with C5a for binding to C5aR. During sepsis, C5aRa treatment blocked chemotactic responses of neutrophils to C5a, and prevented C5a/C5aR-induced paralysis of innate immunity, which led to improved survival in a 9-days survival study (Huber-Lang et al., 2002a). These studies further indicate C5aR as a potential therapeutic target in sepsis.

Originally, C5aR was thought to be exclusively expressed in myeloid cells such as macrophages, monocytes, neutrophils, basophils, and eosinophils (Solomkin et al., 1981; Gerard et al., 1989; Kurimoto et al., 1989; Werfel et al., 1992; Bosmann et al., 2012). There were now growing evidences that C5aR is expressed on a variety of non-myeloid cells. These include bronchial and alveolar epithelial cells, smooth muscle cells, Kupffer cells, endothelial cells, astrocytes, kidney tubular epithelial cells, and other parenchymal cells of solid organs such as lung, kidney, liver, and heart (Strunk et al., 1988; Gasque et al., 1995; Haviland et al., 1995; Lacy et al., 1995; Wetsel, 1995; Schieferdecker et al., 1997; Fayyazi et al., 2000; Zahedi et al., 2000; Drouin et al., 2001; Riedemann et al., 2002c; Sun et al., 2009). During the onset of experimental sepsis in rodents, up-regulated expression of C5aR was found in whole organs including lung, thymus, kidney, liver, and heart (Riedemann et al., 2002b) (Riedemann et al., 2003c), though CLP-induced C5aR level on neutrophils was reduced (Guo et al., 2003). Because lower C5aR level was accompanied by defective respiratory burst in neutrophils (Guo et al., 2003), disruption of C5aR function in other cell types except for neutrophils might contribute to improved survival rate during CLP-induced sepsis. Functionally, mice receiving blocking antibody to C5aR immediate after onset of CLP showed dramatically improved survival in a 7-days survival study (Riedemann et al., 2002b). Furthermore, anti-C5aR treatment led to a significant reduction of serum levels of IL-6 and TNF-α, and bacterial counts in a variety of organs (lung, liver, and kidney) when compared with normal IgG injection (Riedemann et al., 2002b). Using CLP-induced sepsis model in mice, IL-6 blockade was shown to have protective effects on sepsis, which are linked to reduced C5a receptor expression in lung, liver, kidney, and heart (Riedemann et al., 2003c). In another study, C5aR expression was markedly elevated on bronchial epithelial cells in LPS-induced systemic inflammation model (Drouin et al., 2001). However, the pathogenic role of C5aR signaling pathway in these organs during sepsis remains poorly understood.

C5aR was constitutively expressed in γδT cells and its expression was further enhanced in mice undergoing sepsis at both transcription and translation level (Han et al., 2011). *In vitro*, C5aR expression was elevated in γδT cells treated with C5a (Han et al., 2011), and incubation of γδT cells with C5a stimulated IL-17 expression (Han et al., 2011), implying the involvement of C5a/C5aR signaling in the release of inflammatory mediators from γδT cells during sepsis. Interestingly, our previous data showed that IL-17 released from γδT cells during experimental sepsis contributed to high concentrations of pro-inflammatory mediators and bacteremia, leading to a low survival rate (Flierl et al., 2008b).

C5aR expression in other cells and organs plays an important role in apoptosis during sepsis. C5aR expression was increased in thymocytes as early as 3 h after CLP, and peaked at 12 h (Riedemann et al., 2002a). The increased C5aR expression was accompanied by the elevated binding of C5a to the receptor on cell surfaces, leading to apoptosis-mediated loss of lymphoid cells (Riedemann et al., 2002a). Therefore, C5aR may be a possible therapeutic target to control unexpected apoptotic loss of lymphoid cells at the early stage of sepsis, preventing lethal immunosuppression. Clinically, catecholamines are frequently used last-resort drugs to prevent cardiovascular dysfunctions during severe sepsis. However, the mechanisms regulating their production during sepsis remain largely unknown. Recently, it was found that blockade of both C5aR and C5L2 abolished adrenomedullary apoptosis *in vivo* during sepsis, further suggesting that C5aR and C5L2 may be promising targets with implications on future complement-blocking strategies in the clinical setting of sepsis (Flierl et al., 2008a). C5aR in heart may also play a critical role in the development of reversible cardiac dysfunction commonly occurred during sepsis. A recent study demonstrated that C5aR mRNA level in hearts rose almost 3-fold as early as 6 h after CLP (Atefi et al., 2011). Furthermore, CMs isolated from C5aR- or C5L2-knockout rodents undergoing sepsis secreted low level of inflammatory mediators, which was comparable to sham group (Atefi et al., 2011).

C5aR was expressed in splenic NK and NKT cells (Fusakio et al., 2011). NK and NKT cells from C5aR knockout mice infected with *E. coli* expressed less CD69 (the marker of NK and NKT cell activation) when compared with their wild type counterparts, suggesting that C5aR signaling regulates the activation of NK and NKT cells (Fusakio et al., 2011). Furthermore, C5aR deficiency resulted in a reduced release of IFN-γ and TNF-α by NKT and NK cells and in an impaired recruitment of NKT and NK cells to the site of infection (Fusakio et al., 2011). Importantly, the absence of C5aR, NKT, and NK cells, but not of C5L2, led to significantly increased survival from sepsis, which was associated with reduced IFN-γ and TNF-α serum levels (Fusakio et al., 2011). These results together indicate that C5aR activation may represent a novel pathway driving detrimental effects of NKT and NK cells during sepsis. In addition, C5a and Toll-like receptor (TLR) acted synergistically to stimulate TNF-α and IFN-γ expressions in NK and NKT cells (Fusakio et al., 2011). Interestingly, the cognate antigen-mediated NKT cell activation was inhibited by C5a, suggesting that C5a might play a dual role in NKT cell activation (Fusakio et al., 2011).

ROLE OF C5L2 IN SEPSIS

C5L2 is the newly identified C5a receptor, which has a molecular weight similar to C5aR. C5L2 belongs to a subfamily of C3a, C5a, and fMLP receptors, and like C5aR, it is expressed in various types of cell such as granulocytes and dendritic cells (Ohno et al., 2000). While C5L2 binds to C5a and C5a des Arg with high affinity, the interaction between C5L2 and other ligands such as C3a and C3a des Arg, is still a matter of controversy (Gerard et al., 2005; Kalant et al., 2005; Johswich et al., 2006; Chen et al., 2007; Scola et al., 2009). Unlike C5aR, C5L2 is uncoupled from G-proteins due to the replacement of arginine by leucine in the DRY region of the third intracellular loop, and the association of C5L2 with C5a induces no intracellular calcium influx (Okinaga et al., 2003; Scola et al., 2009). C5L2 was thus proposed to function as a recycling decoy receptor to remove active complement fragments from the extracellular environment (Scola et al., 2009). The majority of C5L2 are located in cytosol in the "resting" PMN, which was in striking contrast to C5aR that mainly appears to be on cell surfaces (Johswich et al., 2006; Scola et al., 2009). C5L2 can play both anti-inflammatory and pro-inflammatory roles. For example, C5L2 could protect mice from IgG immune complex-induced acute lung injury and inflammation (Gerard et al., 2005). Conversely, in a mouse model of OVA- or house dust mite-induced allergic asthma, C5L2 deficiency led to a attenuated asthmatic phenotype with the decreased airway hyper-responsiveness (AHR) and Th2 cytokine expression, and reduced airway accumulation of lymphocytes and eosinophils numbers as well as serum IgE level. Therefore, C5L2 may play opposite roles in distinct diseases (Zhang et al., 2010).

The functional role of C5L2 in sepsis remains poorly understood. In CLP-induced sepsis, C5L2 expression in neutrophils was increased, and C5L2 on cell surfaces did not undergo internalization as C5aR (Gao et al., 2005), suggesting the expression of C5aR and C5L2 are regulated by different mechanisms during sepsis. C5L2 expression was significantly increased in lung and liver in septic mice (Gao et al., 2005). Importantly, anti-C5L2 antibody-treated mice showed increased serum IL-6 level during CLP-induced sepsis (Gao et al., 2005). Furthermore, *in vitro* study using blood neutrophils showed that IL-6 expression-induced by LPS and C5a was further amplified by anti-C5L2 antibody treatment (Gao et al., 2005), indicating that C5L2 negatively regulated IL-6 generation. In line with these results, a recent study shows that TLR activation enhances C5a-induced pro-inflammatory responses in peripheral blood mononuclear cell (PBMC) and whole blood by negatively modulating the C5L2 (Raby et al., 2011). These data support the hypothesis that C5L2 could act as a "decoy" receptor to dampen inflammatory response during CLP-induced sepsis. Contrary to these speculations, C5L2 was shown to be a functional receptor rather than merely a decoy receptor (Rittirsch et al., 2008). In a mid-grade CLP model, 31% of wild type mice survived, whereas all C5L2 knockout mice survived in a 7-days survival study, suggesting a critical role of C5L2 in the harmful outcome of sepsis (Rittirsch et al., 2008). The effect of C5L2 during sepsis was linked to its regulation of both inflammatory cytokines (IL-1β, MIP-2, and MIP-1α) and plasma high mobility globulin β1 (HMGB1) in the blood (Rittirsch et al., 2008). These data suggest that C5L2 is a positive regulator of sepsis. In contrast to the finding in CLP model, C5L2-deficient mice showed increased susceptibility to lethal effects of LPS injection compared with control littermates (Chen et al., 2007). Furthermore, LPS-injected mutant mice showed higher IL-1β serum levels, indicating that the increased susceptibility was associated with elevation of some inflammatory cytokines (Chen et al., 2007). These results suggest that C5L2 plays a key role in the regulatory mechanism that protects against LPS-induced shock responses. Interestingly, C5L2 seems to have a functional role in heart during sepsis. Cardiomyocyte (CMs) isolated from wild mice undergoing sepsis produced high levels of IL-6, TNF-α, IL-1β, MIP-1α, MIP-2, MCP-1, and KC, while CMs from C5L2-deficient mice secreted significant low level of the inflammatory mediators (Atefi et al., 2011). These data suggest that C5aR and C5L2 contribute synergistically to the harmful consequences in heart during sepsis.

CONCLUSIONS

Sepsis in human beings results in a high death rate. The therapeutic options remain limited and controversial. Following the recent updated review that no evidence suggests APC should be used for treating patients with severe sepsis or septic shock (Marti-Carvajal et al., 2012) and withdraw of Xigris [a recombinant human activated protein C (rhAPC)] from market in 2011, the search for "silver bullet" for the treatment of sepsis will continue. In septic human beings, there is abundant evidence for complement activation and C5a production. Interception of C5a or its receptors in the CLP model greatly improves survival in septic rodent. Mechanically, these observations are mainly linked to the recovery of blood neutrophil function during sepsis. Thus, anti-C5a strategy holds great promise for the treatment of sepsis. Eculizumab (trade name Soliris), a recombinant humanized monoclonal antibody that inhibits C5 cleavage by the C5 convertase via binding to C5 was recently approved for atypical hemolytic-uremic syndrome (aHUS), a disease that causes abnormal blood clots to form in the kidneys (2011). This will encourage

the development of effective humanized monoclonal antibody targeting C5a or its receptors.

On the other hand, the molecular signaling whereby C5a/C5aRs regulates neutrophil function at different stages of sepsis remains poorly understood. Furthermore, although both C5aR and C5L2 are expressed in various other cell types and organs, their potential role in organ function during sepsis are not known. Importantly, many anti-C5a antibodies also bind C5, thus preventing the formation of the terminal complement complex C5b-9 which is important for controlling bacterial infection. Clearly, the anti-C5a strategy remains to be carefully evaluated in future clinical research and trials. Interestingly, a recent study shows that resolvin 2 (RvD2), a new member of lipid mediators enzymatically generated within resolution networks that possess unique and specific functions to orchestrate catabasis, potently reduced C5a-mediated neutrophil-endothelial interactions to reduce microbial peritonitis (Spite et al., 2009). Furthermore, RvD2 significantly inhibited C5a-stimulated extracellular superoxide generation (Spite et al., 2009). In CLP-induced sepsis, RvD2 sharply decreased the excessive cytokine production, neutrophil recruitment, bacterial burden while increasing peritoneal mononuclear cells and macrophage phagocytosis (Spite et al., 2009). These pro-resolving actions together translate to increased survival from CLP-induced sepsis (Spite et al., 2009). It is tempting to speculate that C5a/C5aRs signaling pathway may be a major target of resolvins. Understanding how the mechanisms by which activation of C5a/C5aR/C5L2 regulate cell and organ function including inflammatory responses and apoptosis is no doubt a fruitful field for future progress in prevention and treatment of sepsis.

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Allopurinol reduces antigen-specific and polyclonal activation of human T cells

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Allopurinol is the most popular commercially available xanthine oxidase inhibitor and it is widely used for treatment of symptomatic hyperuricaemia, or gout. Although, several anti-inflammatory actions of allopurinol have been demonstrated *in vivo* and *in vitro*, there have been few studies on the action of allopurinol on T cells. In the current study, we have assessed the effect of allopurinol on antigen-specific and mitogen-driven activation and cytokine production in human T cells. Allopurinol markedly decreased the frequency of IFN-γ and IL-2-producing T cells, either after polyclonal or antigen-specific stimulation with Herpes Simplex virus 1, Influenza (Flu) virus, tetanus toxoid and *Trypanosoma cruzi*-derived antigens. Allopurinol attenuated CD69 upregulation after CD3 and CD28 engagement and significantly reduced the levels of spontaneous and mitogen-induced intracellular reactive oxygen species in T cells. The diminished T cell activation and cytokine production in the presence of allopurinol support a direct action of allopurinol on human T cells, offering a potential pharmacological tool for the management of cell-mediated inflammatory diseases.

Keywords: allopurinol, T cells, xanthine oxidase, Th1 cytokines, anti-inflammatory

INTRODUCTION

Allopurinol is the most popular commercially available xanthine oxidase inhibitor. This molecule and its *in vivo* active metabolite oxypurinol act as hypoxanthine analogs that irreversibly inhibit xanthine oxidase leading to an inhibition of the catalytic reaction that generates uric acid from hypoxanthine and xanthine, with the concomitant production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Elion, 1988). Since its approval by the Food and Drug Administration in 1966, allopurinol has been used for treatment of symptomatic hyperuricaemia, or gout (Pacher et al., 2006). However, allopurinol presents a wide range of other potential therapeutic applications such as chronic kidney disease, ischemic reperfusion injury, ischemic heart disease, hypertension, chronic heart failure (Pacher et al., 2006), vascular endothelial dysfunction (George and Struthers, 2009), lens-induced uveitis (Augustin et al., 1994, 1996) and chemotherapy of parasitic infections such as leishmaniasis and Chagas disease (Apt et al., 2005; Harzallah et al., 2010). It was also demonstrated that allopurinol reduces rejection in renal transplant recipients when combined with azathioprine/cyclosporine/prednisolone regimens (Chocair et al., 1993).

Several anti-inflammatory actions of allopurinol have been demonstrated *in vivo* and *in vitro*, probably related with its dose-dependent free radical scavenging ability (Namazi, 2004). Allopurinol decreases the production of Tumor Necrosis Factor-α by human mononuclear cells (Oláh et al., 1994), down-regulates the expression of Intercellular Adhesion Molecule 1 (ICAM-1 or CD54) and $P2X_7$ purinergic receptor on human monocytes/macrophages (Mizuno et al., 2004), decreases antigen-specific B cell responses in Ovalbumin-immunized BALB/c mice (Kato et al., 2000) and blocks the induction of Monocyte Chemotactic Factor-1 and Interleukin-6 production in rat vascular smooth muscle cells (Lee et al., 2005). However, the action of allopurinol on T cells is less known. In this regard, allopurinol has been reported to significantly suppress phytohaemagglutin-induced lymphocyte blastogenesis (Kurashige et al., 1985). As allopurinol is a widely prescribed drug with immunomodulatory action and has been proposed as a good candidate for treatment of inflammatory-mediated diseases in which T cells are involved (Grus et al., 2003; Govani and Higgins, 2010), it would be of interest to clarify any putative capacity of allopurinol to modulate T cell activation. Herein, we show that allopurinol diminishes activation as well as cytokine and intracellular reactive oxygen species production by T cells following antigen specific or mitogen-driven stimulation of human peripheral blood mononuclear cells (PBMC).

Abbreviations: 7-AAD, 7-aminoactinomycin D; AL, allopurinol; DCF, 2′ 7′-dichlorofluorescein; DCFH-DA, 2′ 7′-dichlorofluorescin di-acetate; DV, drug vehicle; HSV-1, herpes simplex virus-1; iROS, intracellular reactive oxygen species; PBMC, peripheral blood mononuclear cells; PMA, Phorbol 12-myristate 13-acetate; *T. cruzi*, *Trypanosoma cruzi*.

MATERIALS AND METHODS

SELECTION OF STUDY POPULATION

Healthy adult volunteers ($n = 21$) aged 27–53 years and asymptomatic chronically *Trypanosoma cruzi*-infected adult subjects ($n = 7$) aged 38–52 years living in urban areas of Buenos Aires, Argentina, were recruited at the Hospital Interzonal General de Agudos "Eva Peron" Buenos Aires, Argentina. Subjects with hypertension, ischemic heart disease, cancer, HIV infection, syphilis, diabetes, arthritis, or serious allergies were excluded from the present study. This protocol was approved by the Institutional Review Board of the Hospital Interzonal General de Agudos "Eva Peron." Signed informed consents were obtained from all individuals before inclusion in the study, and are stored by the authors.

COLLECTION OF HUMAN PBMC

Blood samples were obtained by venipuncture and PBMC were isolated by density gradient centrifugation on Ficoll-hypaque (Amershan, Sweden) and were cryopreserved for later analysis.

DRUGS AND ANTIGENS

Allopurinol was purchased from USP/BP, Italy. Whole viral particles from Herpes Simplex virus 1 (HSV-1), strain F, were kindly provided by Dr. Carlos A Pujol (Laboratorio de Virología, Departamento de Química Biológica, Universidad de Buenos Aires, Argentina) and obtained as described elsewhere (Matsuhiro et al., 2005). Briefly, HSV-1 strain F was originally obtained from the American Type Culture Collection (Rockville, USA) and propagated in Vero (African green monkey kidney) cells grown in Eagle's minimum essential medium (EMEM, Sigma, USA) supplemented with 1.5% calf serum in 150 cm^2 cell culture flasks (BD Falcon, USA). Supernatant from HSV-1-infected Vero cell cultures was titrated by plaque formation and used as antigenic stimulation. Supernatant from non-infected Vero cell cultures did not induce T cell responses in ELISPOT assays with PBMC. Peptides derived from Influenza (Flu) virus with high binding affinity for the common class I HLA-supertypes A01, A02, A03, B27, and B35 were synthesized at the University of Georgia Molecular Genetics Instrumentation Facility (Athens, USA). A commercial vaccine (Tetanol Pur, ELEA, Novartis, Germany) was used as an antigen source for tetanus toxoid. An amastigote lysate preparation derived from the Brazil strain of *Trypanosoma cruzi (T. cruzi)* was obtained as previously described (Laucella et al., 2004).

DETERMINATION OF ALLOPURINOL CYTOTOXICITY

Three $\times 10^6$ PBMC were incubated in 24-well plates in complete RPMI 1640 10% fetal calf serum in the presence or absence of allopurinol in a concentration range from 25 to 300 μg/ml or drug vehicle (10 mM NaOH) during 2, 24 or 48 h at 37°C. The frequency of total viable and nonviable $CD8^+$, $CD4^+$, and $CD14^+$ cells were determined by staining with 1 μg/ml 7-Amino-actinomycin D (7-AAD) in combination with anti-human CD8 (APC), anti-human CD4 (PE) and anti-human CD14 (FITC) antibodies (Becton Dickinson, USA) for 30 min at 4°C. Cells were then washed, fixed with 2% paraformaldehyde and acquired in a FacsCalibur flow cytometer (Becton Dickinson, USA). Analysis was performed with FlowJo software (Tree Star, USA). At least 5×10^5 events were collected per sample.

IFN-γ AND INTERLEUKIN 2 ENZYME-LINKED IMMUNOSORBENT SPOT (ELISPOT) ASSAYS

The number of antigen-specific Interferon-gamma (IFN-γ)-secreting or interleukin-2 (IL-2)-secreting T cells for the different stimuli assessed was determined by *ex vivo* ELISPOT using commercial kits (ELISPOT Human IFN-γ and IL-2 Sets; Becton Dickinson, USA), as described by the manufacturer. Cryopreserved PBMC were seeded in triplicate wells, at a concentration of 4×10^5 cells/well, and were stimulated with HSV-1 (at a multiplicity of infection of 10 plaque forming units/cell), Flu-derived peptide pool (1 μg/ml/peptide), tetanus toxoid (1/20 dilution) or *T. cruzi* lysate (10 μg/ml) in the presence of drug vehicle alone (10 mM NaOH) or allopurinol (300 μg/ml in 10 mM NaOH) for 18–20 h. Stimulation of PBMC with 20 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma, USA) plus 500 ng/ml Ionomycin (Sigma, USA) in media was used as positive control of cytokine secretion, while PBMC incubated in media with the addition of allopurinol or drug vehicle served to determine the basal levels of spot-forming cells. For set-up of experimental conditions, 4×10^5 PBMC were stimulated with 100 ng/ml of anti-CD3 in combination with 100 ng/ml of anti-CD28 antibodies (Becton Dickinson, USA) in the presence of drug vehicle alone or different allopurinol concentrations for 18–20 h. Spots were counted and analyzed by Analyzer and ImmunoSpot software (version 6.5; CTL, USA). Responses were considered positive if the number of spot-forming cells in the presence of antigen was at least twice the number of spots in the presence of media, and the number of spots in the latter was less than 10 per 4×10^5 cells. The number of specific IFN-γ and IL-2-secreting T cells was calculated by subtracting the value of the wells containing media alone from the antigen/mitogen-stimulated spot count. Only subjects that presented positive antigen-specific responses were included in these assays.

CD69 SURFACE EXPRESSION

Cryopreserved PBMC were incubated over night at 37°C in 24-well plates in complete RPMI 1640 10% fetal calf serum at a density of 10^6 cells/ml. Cells were then stimulated with 1 μg/ml of anti-CD3 in combination with 1 μg/ml of anti-CD28 antibodies (Becton Dickinson, USA) in the presence of drug vehicle alone or 300 μg/ml allopurinol during 5 h. PBMC cultured with complete RPMI in the presence of drug vehicle alone or 300 μg/ml allopurinol served as unstimulated control cells. Then, the cells were washed and stained with anti-human CD4 (PerCP), anti-human CD8 (APC) and anti-human CD69 (PE) (Becton Dickinson, USA) for 30 min at 4°C. At least 5×10^5 events were collected per sample in a FacsCalibur flow cytometer (Becton Dickinson, USA). Analysis was performed with FlowJo software (Tree Star, USA).

INTRACELLULAR REACTIVE OXYGEN SPECIES (iROS) DETERMINATION

iROS levels were determined as described previously (Yano et al., 1998). Briefly, cryopreserved PBMC were thawed and 1×10^6 PBMC were stained with anti-human CD3 APC (Becton

Dickinson, USA) in PBS for 15 min at room temperature. Cells were then washed and further incubated with 100 nM 2′ 7′-dichlorofluorescin di-acetate (DCFH-DA, Sigma, USA) for 15 min at 37°C and shaking, with or without 300 μg/ml allopurinol in PBS. In this case, allopurinol was directly dissolved into PBS to avoid the use of drug vehicle. Thereafter, 100 nM PMA or PBS alone was added to the culture for additional 1hr at 37°C under shaking. Cells were acquired in a FacsCalibur flow cytometer (Becton Dickinson, USA) and analyzed with FlowJo software (Tree Star, USA). At least 2×10^5 events were collected per sample. The intracellular hydrolyzed and oxidized form, 2′ 7′-dichlorofluorescein (DCF), was detected at the FL-1 channel. DCF mean fluorescence intensity represents a measure of iROS.

STATISTICAL ANALYSIS

The Kruskal-Wallis nonparametric test was used to compare the percentages of surface markers expression and 7-AAD incorporation in PBMC treated with different concentrations of allopurinol or drug vehicle. The Wilcoxon signed rank test was applied to compare the frequencies and sizes of spot-forming cells, CD69 expression and iROS production between untreated and allopurinol-treated PBMC. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

In vitro TREATMENT WITH ALLOPURINOL EXERTS NO CYTOTOXICITY AND HAS NO INFLUENCE ON CD4, CD8, AND CD14 EXPRESSION

In order to rule-out any possible cytotoxic effect of allopurinol on human PBMC, cell viability was evaluated by staining with 7-AAD, a nucleic acid fluorescent dye that penetrates the cell membrane of nonviable cells and allows the quantification of dying or dead cells by flow cytometry. Allopurinol did not affect either cell viability (**Figures 1A–D**) or the constitutive expression of CD4, CD8, and CD14, regardless the dose (**Figures 1E–G**) in a 48 h assay. Neither significant difference in cell viability or in the expression of surface markers was recorded after 2 or 24 h-incubation in the presence of allopurinol (data not shown).

ALLOPURINOL DECREASES POLYCLONAL PRODUCTION OF IFN-γ AND IL-2 BY HUMAN PBMC

We have evaluated the effect of allopurinol on the ability of human T cells to secrete IFN-γ and IL-2 after stimulation with different pathogen-specific antigens. Considering that previous studies have shown that treatment of human PBMC with allopurinol in a range of 25–100 μg/ml impairs several monocyte functions (Mizuno et al., 2004), experimental conditions for cytokine secretion by the ELISPOT technique were set-up by stimulation of PBMC with anti CD3/CD28 antibodies in the presence of 25–300 μg/ml allopurinol. Although decreases in both IFN-γ (**Figures 2A–C**) and IL-2 (**Figures 2E–G**) production were already observed at 100 μg/ml allopurinol, these decreases became significant with 300 μg/ml allopurinol. Thus, the latter concentration was chosen for further studies. Drug vehicle alone did not exert any effect on cytokine production (**Figures 2D** and **H**).

In vitro TREATMENT WITH ALLOPURINOL ATTENUATES ACTIVATION-DRIVEN CD69 EXPRESSION IN HUMAN T CELLS

CD69 is a cell surface molecule upregulated early after T cell activation (Hara et al., 1986; Cosulich et al., 1987; Risso et al., 1989). In this study, we also evaluated the effect of allopurinol on early events of human T cell activation by measuring alterations in CD69 expression. As shown in **Figure 3**, allopurinol attenuates CD69 upregulation on $CD4^+$ (**Figure 3A** and top panels **Figure 3C**) and $CD8^+$ (**Figure 3B** and bottom panels **Figure 3C**) T cells after anti-CD3/CD28 stimulation. The mean fold increase in CD69 expression after anti-CD3/CD28 stimulation was 38% and 30% lower in the presence of allopurinol for $CD4^+$ and $CD8^+$ T cells, respectively, compared with drug vehicle alone. Conversely, neither allopurinol nor the drug vehicle altered the expression of CD69 on unstimulated T cells after the 5 h-incubation period (**Figures 3A–C**).

In vitro TREATMENT WITH ALLOPURINOL REDUCES iROS PRODUCTION IN HUMAN T CELLS

T cells increase the production of ROS early after activation and their effector function is strongly regulated by ROS, through distinct T cell receptor (TCR) pathways (Devadas et al., 2002; Williams and Kwon, 2004; Yan and Banerjee, 2010). Taking into account that allopurinol is a potent scavenger of ROS (Moorhouse et al., 1987) and presents antioxidant activities *in vivo* (Augustin et al., 1994), we hypothesized that allopurinol action on T cell activation could be explained by its scavenging action during early activation events of T cells. We therefore studied the allopurinol action on iROS production in human T cells by measuring DCF mean fluorescence intensity in response to PMA, a known stimulus for iROS production in human T cells (Yano et al., 1998). Allopurinol significantly reduced either the levels of spontaneous (**Figures 4A** and **B**) or PMA-induced (**Figures 4A** and **C**) iROS in total T cells.

ALLOPURINOL DECREASES ANTIGEN-SPECIFIC IFN-γ AND IL-2-PRODUCING T CELL RESPONSES IN HUMAN PBMC

The effect of allopurinol on antigen-specific cytokine production by T cells was also evaluated. To achieve this aim, T cell responses specific for pathogen-derived antigens representative of different infection models, including a chronic viral infection (HSV-1), a solved viral infection (Flu) and a parasitic chronic infection (*T. cruzi*) were measured ex vivo by IFN- γ and IL-2 ELISPOT assays. T cell responses specific for Tetanus toxoid were also analyzed in a group of vaccinated subjects. Allopurinol markedly decreased the frequency of IFN-γ-producing T cells independently of the antigen used as stimuli, including full viral particles from HSV-1, a Flu-derived peptide pool with high binding affinity for common class I HLA-supertypes, tetanus toxoid and a *T. cruzi* lysate preparation (**Figures 5A–D**). Likewise, IL-2 production was also diminished in the presence of allopurinol upon stimulation with *T. cruzi* antigens (**Figure 5E**). The mean spot size of IFN-γ was significantly decreased in the presence of allopurinol, indicating not only a reduction in the frequency of IFN-γ-producing T cells, but also in cytokine production at a single cell level (**Figures 6A** and **B**). As seen after polyclonal stimulation, drug vehicle alone did not have any effect on cytokine

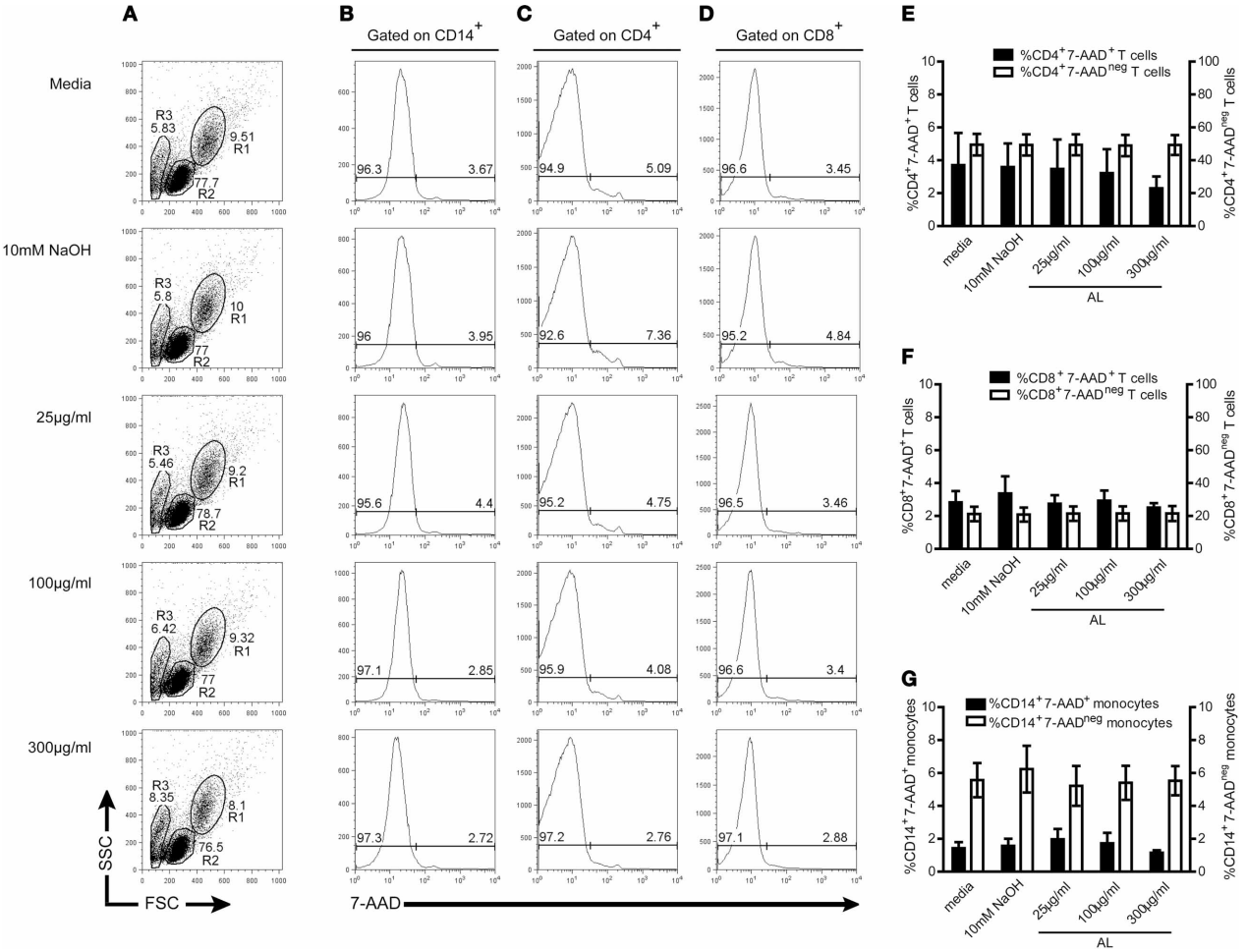

FIGURE 1 | *In vitro* treatment with allopurinol exerts no cytotoxicity and has no influence on CD4, CD8, and CD14 constitutive expression. PBMC from healthy donors were isolated by density gradient centrifugation on Ficoll-hypaque. PBMC were incubated in the presence or absence of allopurinol (25–300 μg/ml) or drug vehicle (10 mM NaOH) during 48 h and analyzed by four color flow cytometry. The frequency of total non-viable and viable $CD14^+$, $CD4^+$, and $CD8^+$ cells were determined by staining with 1 μg/ml 7-aminoactinomycin D (7-AAD) in combination with CD14 (FITC), CD4 (PE), and CD8 (APC) antibodies. **(A–D)** Representative gate selection and analysis for monocytes and T cells. Monocytes (R1) and lymphocytes (R2) were gated by forward and side light scatter **(A)**, including an area generally enriched in death cells (R3). $CD14^+$, $CD4^+$, or $CD8^+$ cells were selected, and from these populations 7-AAD histograms were made to determine the percentages of 7-AAD^+ (on the right) and 7-AAD^- (on the left) cells on total $CD14^+$ **(B)**, $CD4^+$ **(C)** or $CD8^+$ **(D)** T cells. Mean percentages ± SEM of $CD4^+7\text{-}AAD^+$ **(E)**, $CD8^+7\text{-}AAD^+$ **(F)** or $CD14^+7\text{-}AAD^+$ **(G)**, (left Y axis, black bars) and $CD4^+7\text{-}AAD^-$ **(E)**, $CD8^+7\text{-}AAD^-$ **(F)** or $CD14^+7\text{-}AAD^-$ **(G)**, (right Y axis, white bars) from 5 samples assessed. $P > 0.05$ among different allopurinol concentrations, drug vehicle, and media alone, as determined by Kruskal–Wallis test. AL, allopurinol.

production after antigen-specific stimulation (**Figures 5A–E** and **Figures 6A** and **B**).

DISCUSSION

Xanthine Oxidase inhibitors have emerged as therapeutic tools for inflammatory-mediated diseases, including those in which T cells are involved (Grus et al., 2003; Pacher et al., 2006; Govani and Higgins, 2010; Ng et al., 2011; Sliem and Nasr, 2011). In the present study, the effect of allopurinol on late (i.e., cytokine production) as well as on early (i.e., CD69 expression and iROS production) events of human T cell activation was investigated. We show, for the first time, that allopurinol decreases class I and class II-restricted antigen-specific and polyclonal IFN-γ and IL-2 production, in addition to the expression of activation markers by human T cells.

Antigen-specific activation of T cells requires recognition of peptide-MHC complexes by the TCR on antigen presenting cells, costimulation and the expression of adhesion molecules. The recognition of antigen by the TCR initiates transcriptional activation of particular genes that mediate T cell responses. We found that allopurinol decreased IFN-γ production by T cells specific for different pathogens probably due to a direct effect of the drug on T cells as well as on antigen-presenting cells. This view is supported by other findings showing that allopurinol downregulates the expression of ICAM-1 on monocytes (Mizuno et al., 2004) and modulates protein kinase C activity

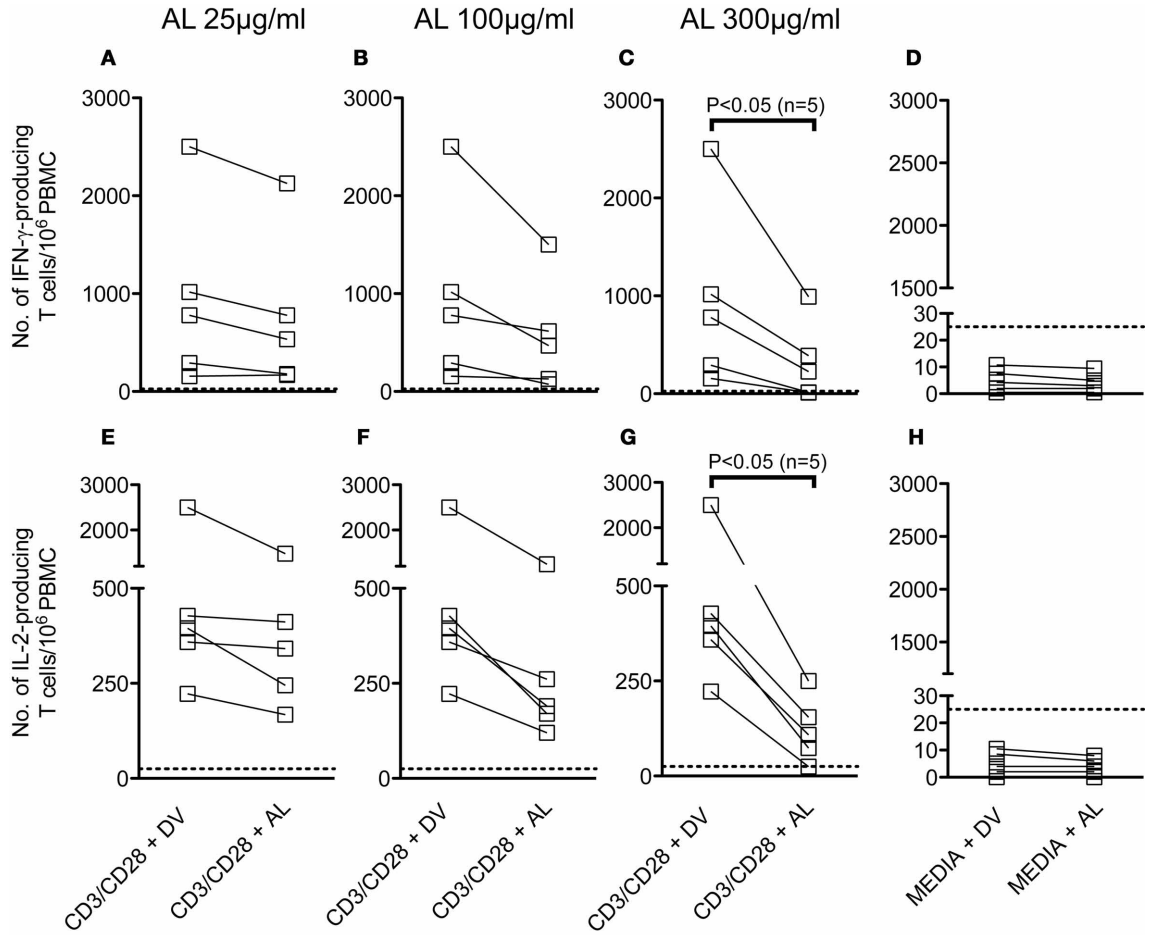

FIGURE 2 | Effect of different concentrations of allopurinol on polyclonal activation-driven IFN-γ and IL-2 production by human T cells. PBMC from healthy donors were stimulated with anti-CD3 (100 ng/ml) plus anti-CD28 (100 ng/ml) antibodies, or remained with media alone, in the presence of different concentrations of allopurinol (+ AL) or drug vehicle (+ DV), for 18–20 h and the number of IFN-γ-secreting **(A–D)** or IL-2-secreting **(E–G)** T cells were determined by *ex vivo* ELISPOT assays. **(D** and **H)** The samples were cultured in media with the addition of AL or DV. Each line represents an individual subject. Panels **A–C** and **E–G** show the net number of spot-forming cells (spot-forming cells in media were subtracted). Dotted lines represent the threshold for positive IFN-γ or IL-2 ELISPOT responses, as defined in Materials and Methods. Comparisons between T cell responses in the absence or presence of allopurinol upon stimulation were performed by Wilcoxon signed rank test.

(Kang et al., 2006), one of the main enzymes involved in biochemical pathways inducing T cell responses. IFN-γ secretion was also reduced in the presence of allopurinol after polyclonal stimulation of T cells with anti-CD3/CD28 that mimics signals generated by the TCR complex, bypassing the need for T cell-antigen presenting cell interaction, supporting that allopurinol might exert a direct action on T cells. Moreover, we showed that IL-2 production, a cytokine that drives T cell activation, is reduced in the presence of allopurinol. The lower amounts of antigen-specific cytokine production, as evidenced by the smaller spot size and the attenuation of CD69 upregulation upon activation with CD3/CD28, further support the specific effect of allopurinol on T cell activation.

Diverse evidences have shown that the redox state can influence T cell function *in vitro* and *in vivo* (Griffiths et al., 2011). Cross-linking of the TCR and the co-stimulatory molecule CD28 in human T cells results in enhanced iROS production that is needed for NF-κB and IL-2 expression (Los et al., 1995) and is consistent with an important role for ROS in the immediate early events during activation (Yan and Banerjee, 2010). Lipid metabolism, mitochondria, and/or NADPH oxidases have been claimed to be the source of iROS during T cell activation (Williams and Kwon, 2004). Although xanthine oxidase also mediates ROS generation, this enzyme is not expressed by human immune cells (Dröge, 2002). Xanthine oxidase is distributed in the liver, gut, lung, kidney, heart, and brain, accounting for only a minor proportion of total ROS production under normal conditions (Dröge, 2002). Thus, the modulation of T cell function observed herein would be accounted for the ROS scavenging action of allopurinol but not for an inhibition in ROS production mediated by the xanthine oxidase enzyme. Thus, allopurinol would deprive T cells from iROS which are required during the early activation of the NF-kB complex (Los et al., 1995) and PKC (Kang et al., 2006), thus promoting a

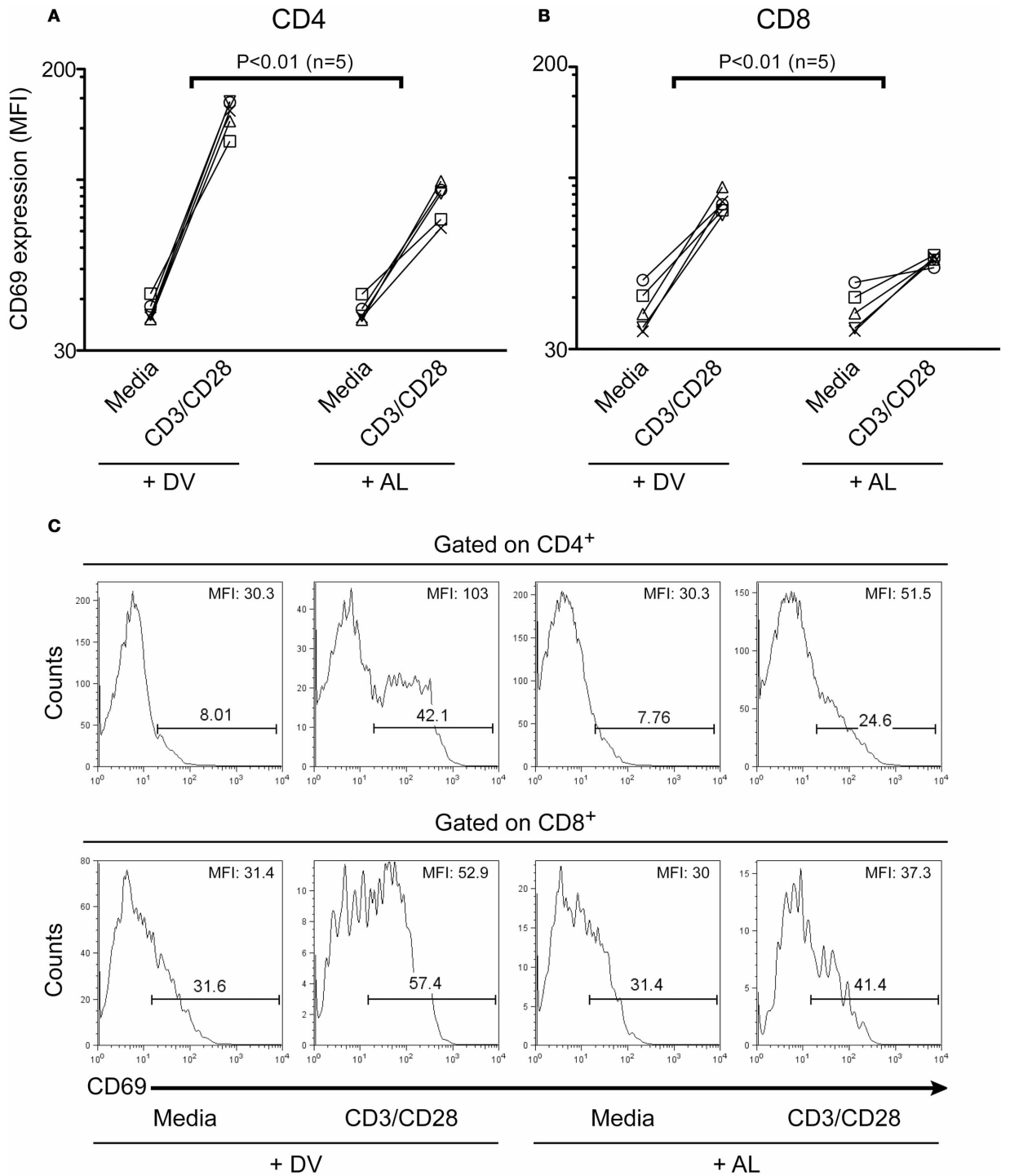

FIGURE 3 | Allopurinol attenuates activation-driven CD69 upregulation in CD4$^+$ and CD8$^+$ T cells. PBMC were incubated for 5 h with media or with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, in the presence of allopurinol (+ AL, 300 μg/ml) or drug vehicle alone (+ DV). Cells were then stained with anti-CD4 (PerCP), anti-CD8 (APC) and anti-CD69 (PE) and analyzed with four color flow cytometry. Lymphocytes were gated by forward and side light scatter. From this population single color CD4 or CD8 staining histogram was made, and the expression of CD69 was analyzed. Each line shows the mean fluorescence intensity (MFI) of CD69 expression on CD4$^+$ **(A)** or CD8$^+$ **(B)** T cells before and following stimulation with anti-CD3/CD28 antibodies, in the presence of drug vehicle (+ DV) or allopurinol (+ AL), for each subject evaluated. Comparisons between the differences in net CD69 expression (subtracting CD69 MFI in media) with and without allopurinol were performed by Wilcoxon signed rank test. Representative histogram plots of CD69 expression on CD4$^+$ (**C**, top panels) and CD8$^+$ (**C**, bottom panels) T cells with the indicated treatment. The numbers over the bars indicate the percentages of CD69$^+$ cells and the numbers in the top right corner indicate the MFI of CD69 expression on CD4$^+$ (top) and CD8$^+$ (bottom) T cells.

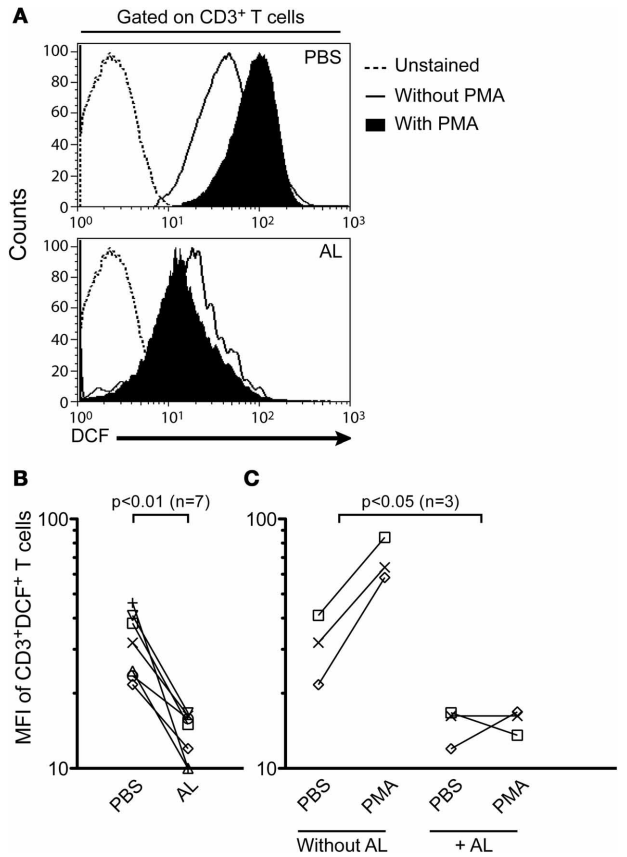

FIGURE 4 | Allopurinol attenuates spontaneous and mitogen-activated iROS production in human T cells. PBMC were stained with anti-CD3 PE and incubated at 37°C for 1 h with DCFH-DA and PBS alone or with PMA (100 nM) in the presence (+ AL, 300 μg/ml) or absence of allopurinol. The oxidized form, DCF represents a measure of iROS. Lymphocytes were gated by forward and side light scatter. From this population single color CD3 staining histogram was made, and the expression of DCF in the gated population was analyzed. **(A)** Representative iROS expression on $CD3^+$ T cells in the absence (upper panel) or presence (bottom panel) of allopurinol. **(B)** Cumulative data of spontaneous iROS production from 7 subjects. Each line represents spontaneous iROS levels for each patient in the absence (PBS) or in the presence (AL) of allopurinol. **(C)** Cumulative data of induced iROS production. Each line show iROS release for each subject prior and following stimulation with PMA in the presence (+ AL) or absence of allopurinol. Comparisons were performed by Wilcoxon signed rank test. MFI: mean fluorescence intensity.

hyporesponsive state of T cells. In agreement with this notion, allopurinol has been recently shown to reduce NF-κB pathway activation, pro-inflammatory cytokines production, and oxidative stress in different *in vivo* models of inflammation (Correa-Costa et al., 2011; Aldaba-Muruato et al., 2012; Demirel et al., 2012).

Both T cells and dendritic cells elevate the intracellular oxidation status upon antigen-specific interaction, and bidirectional dendritic cell-T cell communication can be blocked by interfering with redox regulation pathways (Matsue et al., 2003). Moreover, alteration in redox status of T cells and dendritic cells has been recently proposed as a putative mechanism of regulatory T cells action (Yan et al., 2010). Although, we cannot rule out the possibility that the effect of allopurinol on antigen-specific T cell responses is due to, at least partially, an impairment in antigen presentation by the action of allopurinol on antigen-presenting cells, the reduction of iROS production by PMA-stimulated T cells in the presence of allopurinol for short periods of time further supports the idea of a direct action of allopurinol on T cell activation. Future studies regarding the effect of allopurinol on the expression of costimulatory molecules, as well as studies at the transcriptional level on T cells, will help us to further clarify these ideas.

$CD4^+$ IFN-γ-producing T_{h1} cells have long been associated with the pathogenesis of many organ-specific autoimmune diseases (Dardalhon et al., 2008). Herein, we report that these responses might be inhibited by allopurinol, *in vitro*. A beneficial effect of allopurinol on cell-mediated diseases is also supported by an improvement of the response to thiopurine treatment by the addition of allopurinol in individuals with inflammatory bowel disease, a T_{h1}-polarized disease (Gardiner et al., 2011; Smith et al., 2012). Inflammation has also been pointed out as a potential target for therapy of chronic heart failure (Celis et al., 2008), that has been strongly correlated with T cell activation and altered T_{h1}/T_{h2} balance (Cheng et al., 2009). iROS production by phagocytic leukocytes is also increased in heart failure patients (Castro et al., 2003) and it is a major cause of endothelial dysfunction as iROS can act both, as triggers or amplifiers of the inflammatory response (Deschamps and Spinale, 2006; Castro et al., 2008). Several studies have demonstrated that treatment with xanthine oxidase inhibitors of patients suffering from heart failure resulted in reduced oxidative stress and improved endothelial function (Landmesser et al., 2002; Castro et al., 2005; Hare et al., 2008). Our results showed that allopurinol decreases iROS production by T cells which might have important therapeutic implications since down-regulation of inflammatory T cells is the treatment of choice for many inflammatory diseases. In agreement with this notion, the use of a different immunomodulating agent, Pentoxifylline, improved the clinical status of patients with idiopathic-dilated and ischemic cardiomyopathy (Barnett and Touchon, 1990; Skudicky et al., 2000; Sliwa et al., 2002, 2004).

Allopurinol is rapidly absorbed *in vivo*, reaching peak plasma concentrations within 30–60 min, following oral administration (Pea, 2005). Allopurinol has relatively short half-life in plasma (2–3 h) because it is rapidly metabolized *in vivo* and converted almost completely into to the oxidized metabolite, oxypurinol, which has the same therapeutic pattern but a much longer elimination half-life (14–30 h) than the parent compound (Pea, 2005). Allopurinol is negligibly bound to plasma proteins, and gets spread to different tissues, including vascular tissue, liver, intestine, and heart (Murrell and Rapeport, 1986). Therefore, the maximum possible concentration of allopurinol *in vivo* is hard to estimate, making it difficult to predict the *in vivo* physiological relevance of drug concentrations applied, *in vitro*, in the present study. Moreover, it has been demonstrated that allopurinol can be differentially metabolized *in vitro* and *in vivo* (Kramer and Feldman, 1977).

Determination of plasma concentrations of oxypurinol highly differs depending on the population studied, the dose and

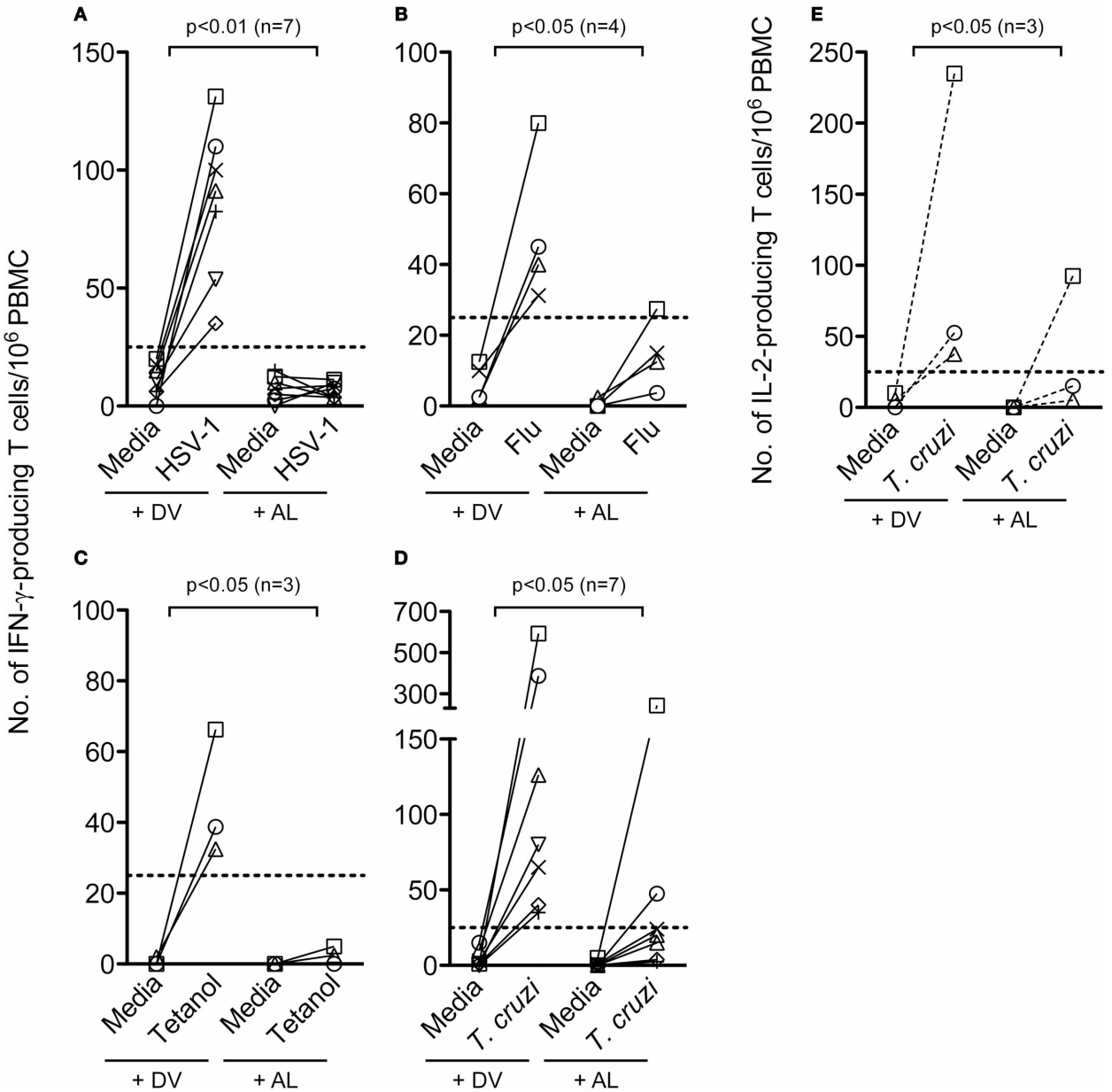

FIGURE 5 | Allopurinol attenuates antigen-specific activation-driven IFN-γ and IL-2 production by human T cells. PBMC from healthy donors were stimulated with full viral particles from HSV-1 **(A)**, a Flu-derived peptide pool **(B)**, tetanus toxoid **(C)**, or cultured in the absence of antigen stimulation (media), in the presence of allopurinol (+ AL, 300 μg/ml) or drug vehicle alone (+ DV) for 18–20 h, and the number of IFN-γ-secreting or IL-2-secreting **(E)** T cells were determined by *ex vivo* ELISPOT assays. PBMC from a group of chronically *T. cruzi*-infected subjects were stimulated with a *T. cruzi*-derived amastigote lysate **(D** and **E)**. Each line represents an individual subject. Dotted lines represent the threshold for positive T cell responses, as defined in Materials and Methods. Comparisons between T cell responses in the absence or presence of allopurinol upon stimulation were performed by Wilcoxon signed rank test.

the route of administration, the length of treatment and the time-points after drug intake chosen for measurements (Rodnan et al., 1975; Breithaupt and Tittel, 1982; Berlinger et al., 1985; Murrell and Rapeport, 1986; Emmerson et al., 1987; Day et al., 1988a,b; Graham et al., 1996; Turnheim et al., 1999; Guerra et al., 2001; Kaya et al., 2006; Panomvana et al., 2008; Stocker et al., 2008; Van Dijk et al., 2008; Torrance et al., 2009; Stamp et al., 2011). For instance, Van Dijk et al. demonstrated that allopurinol reaches a mean maximal plasma concentration of 41.90 μg/ml within minutes after a dose of 15 mg/kg body weight administered i.v. in pregnant sows (Van Dijk et al., 2008). In a survey studying 50 adult patients receiving variable daily doses of allopurinol, with 83% of the patients taking 300 mg/day, during 6 ± 7.5 years on average, a wide range of plasma oxypurinol concentration (i.e., from 2.8 to 55.8 μg/ml) was observed (Day et al., 1988b). Differences in the levels of

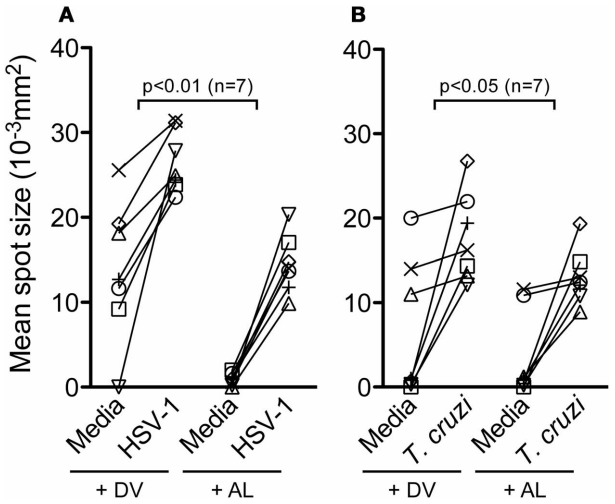

FIGURE 6 | Allopurinol decreases IFN-γ secretion at a single cell level. IFN-γ ELISPOT assays were performed by stimulation, or not (media), of PBMC from healthy donors with full viral particles from HSV-1 **(A)**, or PBMC from a group of chronically *T. cruzi*-infected subjects with a *T. cruzi*-derived amastigote lysate **(B)**, in the presence of allopurinol (+ AL, 300 μg/ml) or drug vehicle (+ DV), for 18–20 h. Mean spot sizes represent an estimate of IFN-γ production per cell. Each line represents an individual subject. Comparisons between T cell responses in the absence or presence of allopurinol upon stimulation were performed by Wilcoxon signed rank test.

serum oxypurinol from patient to patient taking the same dose of allopurinol may result from differences in absorption, rate of metabolism, xanthine oxidase levels, the amount of drug bound to xanthine oxidase in the tissues and/or renal function. Even diet and exercise can significantly alter the pharmacokinetics of oxypurinol (Berlinger et al., 1985; Kaya et al., 2006).

In the present work, we observed that a single dose of 100 μg/ml allopurinol decreased T cell production of cytokines within hours after *in vitro* culture, but reaching a statistical significant reduction upon treatment with 300 μg/ml of allopurinol. As oxypurinol accumulates and reaches a steady-state level during long-term administration (Murrell and Rapeport, 1986), the effect of allopurinol observed within hours after *in vitro* culture is probably only comparable to the effect observed in subjects receiving long-term treatment with allopurinol. In this regard, allopurinol is generally well tolerated, allowing long-term treatments and administration of high doses (Momeni and Aminjavaheri, 1995; Momeni et al., 2002).

Allopurinol, administered at 600 mg/day during 3 months, constitutes a second line drug for the treatment of chronic Chagas disease, caused by *Trypanosoma cruzi* infection. Allopurinol was also shown to be safe and effective in the treatment of Chagas disease reactivation after heart transplantation (Almeida et al., 1996; Bestetti and Theodoropoulos, 2009). In a recent pilot study of a sequential combined treatment with allopurinol and benznidazole in the chronic phase of *Trypanosoma cruzi* infection (Pérez-Mazliah et al., 2012), we have observed that the frequency of peripheral naïve $CD4^+$ and $CD8^+$ T cells, that are generally diminished in this phase of the infection (Albareda et al., 2006, 2009), is improved along with a decrease in the frequency of peripheral $CD4^+$ and $CD8^+$ T cells expressing the activation marker HLA-DR after completion of allopurinol administration. These changes were sustained following the consecutive treatment with benznidazole, supporting the immunomodulating activity of allopurinol, *in vivo*, on human T cells.

Allopurinol hypersensitivity syndromes, like the Stevens-Johnson syndrome, is an infrequent but life-threatening adverse effect that affects about 0.4% patients receiving allopurinol therapy (Arellano and Sacristán, 1993; Pluim et al., 1998) and whose mechanisms remain unclear. Three potential factors involved in allopurinol hypersensitivity syndromes are the genetic background, dose accumulation, and immunological responses to the drug. However, a relationship between the appearance of allopurinol hypersensitivity syndromes and the use of high doses has not been demonstrated (George et al., 2006). Risk factors for development of allopurinol hypersensitivity syndromes include aging, renal impairment, diuretic use, and some ethnics groups (Chinese descent) (Lee et al., 2008). In the same pilot study mentioned above (Pérez-Mazliah et al., 2012), the use of allopurinol in doses of 600 mg/day for 90 consecutive days was very well tolerated and we did not observe any case of severe adverse reaction. This observation is in agreement with previous studies (Gallerano et al., 1990; Momeni and Aminjavaheri, 1995; Apt et al., 1998; Momeni et al., 2002; Apt et al., 2003, 2005), in which doses even higher than 1 g/day of allopurinol during months were administered without registering high incidence of adverse effects.

The immunomodulatory action of allopurinol described in the present work in combination with previous observations could raise the idea that allopurinol would favor the occurrence of opportunistic infections. We have not observed a higher incidence of opportunistic infections in chronically *Trypanosoma cruzi*-infected patients under treatment with doses of 600 mg per day of allopurinol during 3 months (Pérez-Mazliah et al., 2012). This is again in accordance with previous observations, even in long-term and high dose treatments with allopurinol (Gallerano et al., 1990; Momeni and Aminjavaheri, 1995; Apt et al., 1998, 2003, 2005; Momeni et al., 2002). Thus, the immunomodulatory effect of allopurinol does not seem to generate the level of immunosuppression required to favor opportunistic infections *in vivo*, at least in the context of the studies cited herein.

Nonetheless, due to its low frequent but potentially severe side effects as well as its immunomodulatory action, it is highly recommended a close follow-up of patients receiving allopurinol throughout the entire duration of the treatment. Particular attention should be paid to those populations at high risk of developing side effects and those presenting alterations in normal immune system function. In summary, this study raise evidence in line with an immunomodulatory action of allopurinol on human T cells, offering a potential pharmacological tool for the management of cell-mediated inflammatory diseases.

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