



Pro-Resolving Maresins in Inflammation: New Pathway and Mechanisms With Oral Pathogens

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**Pro-resolving Maresins in inflammation:
New pathway and mechanisms with oral pathogens**

A Thesis Presented by

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to

The Faculty of Medicine

In partial fulfillment of the requirements

for the degree of

Doctor of Medical Sciences

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ABSTRACT

Objectives

The aim of this thesis was to study the biosynthesis of Maresin 1 (MaR1) in human macrophage and to investigate the potential defect of this pathway in patients with localized aggressive periodontitis as well as the role of MaR1 in regulating phagocyte functions and leukocyte-platelet aggregation using LAP as a disease model.

Methods

Human recombinant enzyme (12-lipoxygenase and soluble epoxide hydrolyse) and synthetic intermediates were used to investigate the maresin pathway by LC/MS/MS-based lipid mediator (LM) metabololipidomics. Human macrophage 12-LOX sequence was crosschecked with platelet-type 12-LOX. Enzyme expression levels in human myeloid mononuclear lineage were determined with flow cytometry. Peripheral blood was collected from LAP patients and age/gender-matched healthy controls (HC). Neutrophils and monocytes were isolated, and macrophages were obtained from monocytes with GM-CSF incubation. LAP and HC macrophage 12-LOX expression levels were monitored by flow cytometry and endogenous maresin metabolite levels were investigated using LM metabololipidomics. Periodontal pathogens including *Porphyromonas gingivalis* (*P. g*) and *Aggregatibacter actinomycetemcomitans* (*A. a*) were incubated with neutrophils and macrophages to investigate phagocyte functions. Phagocytosis and intracellular anti-microbial reactive oxygen species (ROS) production

were determined using fluorescence plate reader. Bacterial killing by macrophages and in whole blood was evaluated by assessing residual colony forming units following incubation with bacteria.

Results

We demonstrated that human macrophage 12-LOX is identical with platelet-type 12-LOX and is upregulated from monocyte to different mononuclear lineage. 12-LOX converts DHA to 14*S*-HpDHA, which is a key intermediate in MaR1 biosynthesis. In addition, soluble epoxide hydrolyze (sEH) is responsible for the further production of novel anti-inflammatory and pro-resolving Maresin 2 (MaR2; 13, 14*S*, di-HDHA). Since 12-LOX is the key-initiating enzyme, investigation begin with macrophages from LAP patients, which have lower 12-LOX expression (~30% lower) and reduced MaR1 (87.8±50 vs. 239.1±32 pg/ 10⁶ cells) levels compared to macrophages from healthy controls (HC). Functionally, LAP macrophages gave impaired phagocytosis (~40% lower) and killing of periodontal pathogens including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. In addition, neutrophils from LAP patients also displayed diminished kinetics (~30% slower) and reduced maximal phagocytosis (~20% lower) of these pathogens. Exogenous MaR1 rescued the impairment with LAP phagocytes enhancing phagocytosis (31-65% increase, 1nM), intracellular anti-microbial reactive oxygen species production (26-71% increase, 1nM) and bacterial killing of these periodontal pathogens *in vitro*. Additionally, MaR1 retained biological action and enhanced bacterial killing in the whole blood *ex vivo* from HC and LAP patients (22-37%

reduction of bacterial titers, 10nM). MaR1 counter-regulated leukocyte-platelet aggregation against two stimulus (platelet activating factor (PAF) and *P. gingivalis*) and regulated the expression levels of surface adhesion molecules (CD18 and P-selectin). Patients with LAP had elevated leukocyte-platelet aggregation (40% higher compared to HC) in the whole blood that could be reduced by MaR1 in a dose-dependent manner (40-60% reduction of the excess aggregates, 1-100nM).

Conclusions

These results indicate that 12-LOX is the key enzyme in maresin biosynthetic pathway. LAP macrophages, had lower 12-LOX expression levels and dysregulated maresin biosynthesis. Reduced phagocyte functions were established with both LAP macrophages and neutrophils; exogenous MaR1 rescued this impairment with LAP patients and also enhanced the functions of HC phagocytes. Additionally, MaR1 enhanced bacterial killing and reduced elevated leukocyte-platelet aggregates in LAP whole blood. Together these results suggest that therapeutics targeting maresin pathway may have clinical application treating LAP and oral diseases associated with infection and uncontrolled inflammation.

Grant support

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RESEARCH BACKGROUND

Resolution of inflammation is an active process

It is now known that the resolution of inflammation is an active process orchestrated by specialized pro-resolving lipid mediators (SPMs) (Serhan, 2011; Serhan, 2014). In the past, it was thought that the resolution phase of inflammation is a passive process as described in the *Taber's Cyclopedic Medical Dictionary* (Taber, 1970) regarding the definition of **Resolution**: *1. Decomposition; absorption or breaking down of the products of inflammation. 2. Cessation of inflammation without suppuration and the return to normal.* It was widely believed that the whole inflammatory process was driven by pro-inflammatory signals such as leukotrienes (LT), prostaglandins (PG), and cytokines. With the structure elucidation of molecules within self-resolving inflammatory exudate, it was uncovered that pro-resolving agonists exist and a temporal class-switch occurred from classic pro-inflammatory lipid mediators to SPMs including lipoxins (LX), resolvins (Rv), protectins (PD), and maresins (MaR) during the resolution of inflammation (Levy et al., 2001; Serhan, 2014) (Fig. 1). These SPMs are pro-resolving signals for counter-regulating inflammation and agonists for homeostasis. Acute inflammation is protective, yet if not resolved, may result in chronic inflammation, fibrosis, tissue damage, and loss of organ function (Serhan et al., 2008; Nathan and Ding, 2010). Thus, uncontrolled inflammation is an unifying factor in the pathogenesis of several chronic inflammatory diseases including cardiovascular, lung, joint (Nathan and Ding, 2010), and periodontal

diseases (Van Dyke and Serhan, 2003). Thus, dysregulated biosynthesis of pro-resolving lipid mediators may underline the pathogenesis of uncontrolled chronic inflammatory diseases (Serhan, 2007; Serhan 2014) and its therapeutic potential is of great interest to the public.

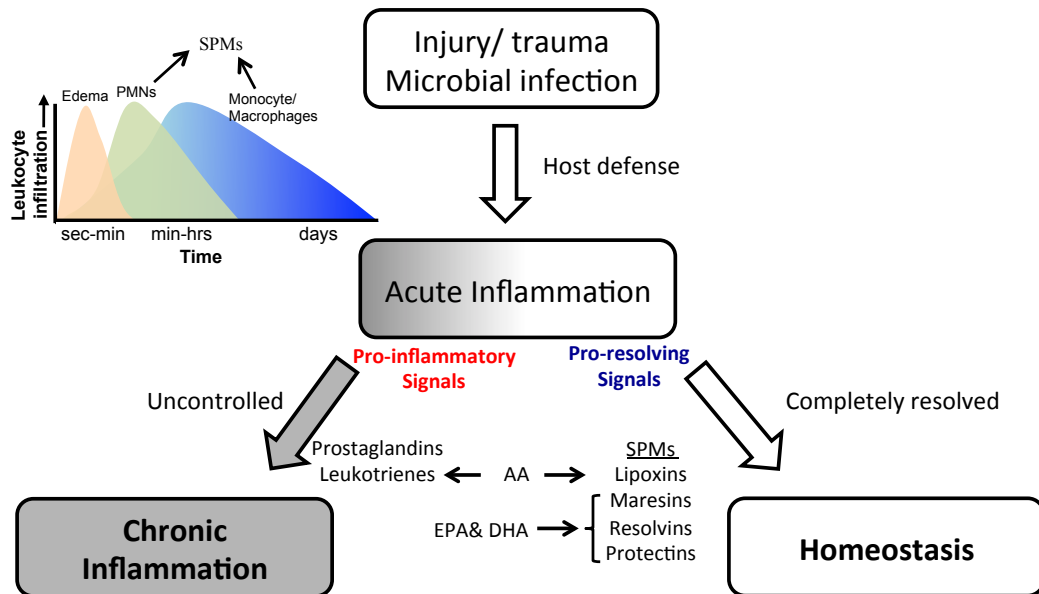


Figure 1. Pro-inflammatory and pro-resolving signals in inflammation. Following injury or infection, acute inflammation is protective as part of the host defense response. If acute inflammation is not controlled or unresolved, it will turn to chronic inflammation and diseases. The ideal outcome of acute inflammation is complete resolution, during which there is a lipid mediator class-switching from pro-inflammatory mediators to specialized pro-resolving mediators (SPMs) as pro-resolving signals. These mediators may be converted from arachidonic acid (AA), docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) to lipoxins, maresins, resolvins, and protectins. (Figure adapted from Serhan, 2011 FASEB J)

Endogenous agonists for resolution of inflammation:

The emergence and functional roles of specialized pro-resolving lipid mediators

In the late 1980s, Serhan, Samuelsson and colleagues reported the identification of lipoxins (LX) along with its structures and actions (Samuelsson et al., 1987). LXs were considered the first family of pro-resolving lipid mediators originated from arachidonic acid (AA). Before that time, despite “natural” resolution of inflammation was considered passive, “resolvent mollificants” that control the inflammation were documented in several chapters of the *Canon of Medicine* (Avicenna, 1999). However, what constitutes a “resolvent” was unknown at that time, nor was its chemical form. In recent years, novel families of SPMs derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were identified and their chemical structures and biological functions elucidated (Schwab et al., 2007; Spite et al., 2009; Serhan et al., 2009). These SPMs are endogenous agonists for resolution and possess anti-inflammatory and pro-resolving bioactions as illustrated in Fig. 2 (Serhan et al., 2008). These mechanisms mediated by SPMs include: limiting neutrophilic infiltration; stimulating non-phlogistic recruitment of monocytes (which means without eliciting any inflammatory response); activating macrophage phagocytosis of microorganisms and apoptotic cells; and increasing the exit of phagocytes via the lymphatics (Serhan et al., 2002; Schwab et al., 2007; Campbell et al., 2007). In addition to these generalized actions, SPMs exert specialized actions towards specific cell types in various systems (Serhan, 2014).

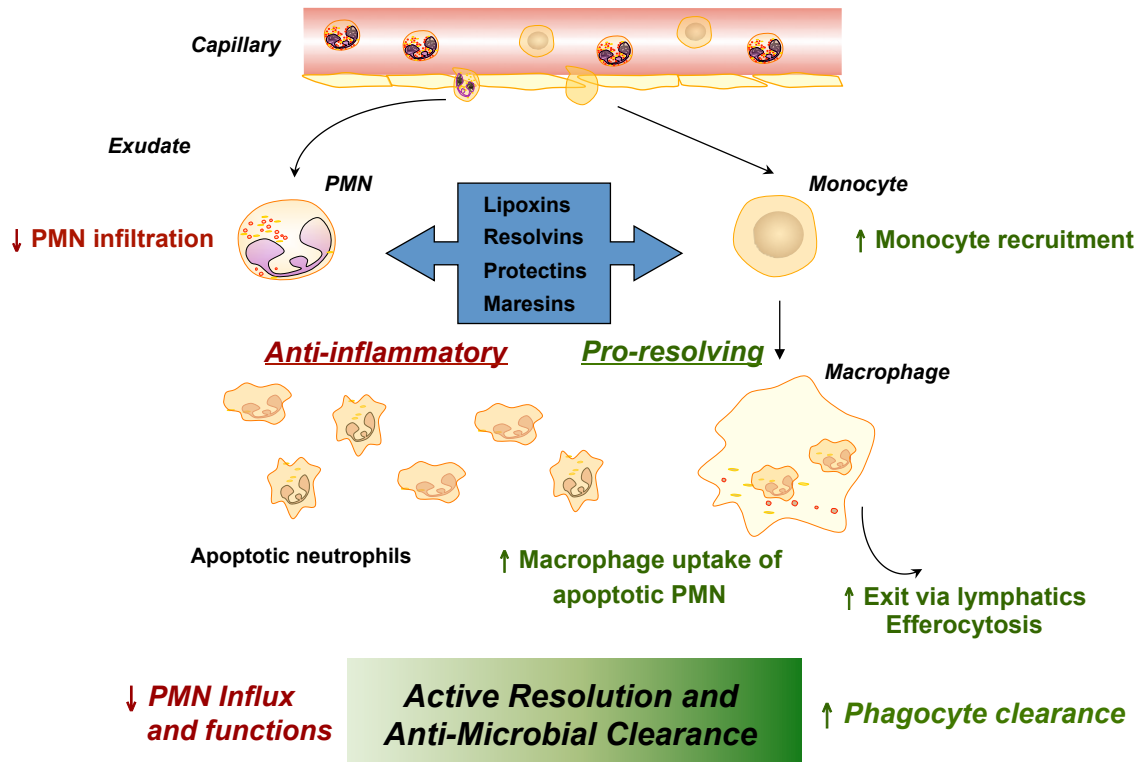


Figure 2. SPMs are anti-inflammatory and pro-resolving. The general actions of SPMs are anti-inflammatory and pro-resolving in counter-regulating inflammation. They limit neutrophilic infiltration as anti-inflammatory action; recruiting non-inflammatory macrophages to enhance uptake of apoptotic neutrophils and efferocytosis for clearance as pro-resolving actions. These programmed functions make SPMs potent agonists for resolution in inflammation (Figure adapted from Serhan, Chiang, and Van Dyke, 2008 Nat Rev Immunol).

The biosynthesis, structures, and specialized functions of SPMs:

From mediator class-switching to leads for resolution physiology

SPMs are produced not only during lipid mediator class-switching but also were identified in human tissues implying its potential role in maintaining homeostasis (Serhan, 2014; Colas et al., 2014). Recently, the complete stereochemistry of various SPMs were elucidated as shown in Fig. 3 and total organic synthesis was achieved. We may be able to further study their specialized actions for homeostasis physiology.

However, we need to appreciate and extend our current knowledge with the major players in acute inflammation (neutrophils and macrophages) and its interaction with SPMs during resolution to envision our future directions. When neutrophils are first recruited to the site of acute inflammation, they play the role of initiating the inflammation giving a pro-inflammatory phenotype. By utilizing either cyclooxygenase (COX) or lipoxygenase (LOX), neutrophils can rapidly biosynthesize pro-inflammatory mediators (eg. PGs and LTs) from AA within minutes to elicit inflammatory responses. Recent results, however, indicate that as inflammation progresses, the neutrophils in confined exudates stop producing chemo-attractants (such as LTB₄) and within hours begin to convert arachidonic acid to lipoxins, thereby acting as pro-resolving phenotype to actively terminate inflammation and promote resolution (Levy et al., 2002; Serhan et al., 2005). Pro-inflammatory PGE₂ also activates the production of lipoxins which may indicate that our immune system starts to tune in SPMs during the initial phase of inflammation to control its progress (Serhan et al., 2005). Macrophages also play an important role in the

class-switching process and resolution. Different phenotypes and resolution-phase macrophages were also recently described and involved in homeostasis (Mosser et al., 2008; Bystrom et al., 2008). The macrophage functions involved in homeostasis including pro-resolving actions, wound healing, neovascularization and further regenerative potentials (Bystrom et al., 2008). Thus, the chemical mediators produced by macrophage during resolution phase of inflammation are of wide interest. Recently, Maresin 1 (MaR1) was identified converting from DHA by macrophages (Serhan et al., 2009). In this thesis, we will focus on the biosynthesis and novel actions of MaR1.

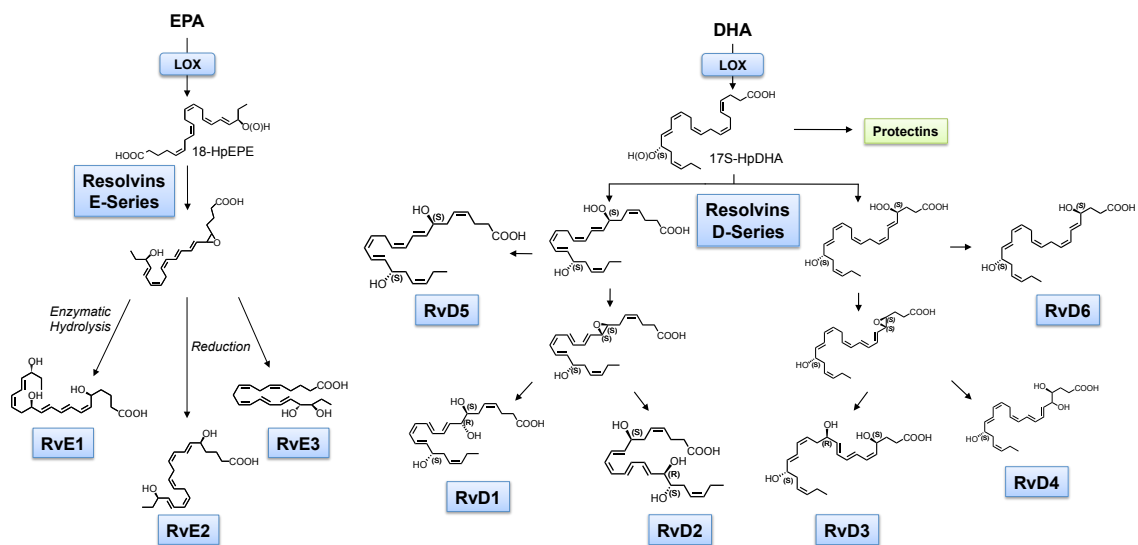


Figure 3. Structures and diverse pathways of Resolvin E and D series. Complete stereochemistry of several SPM families was established and total organic synthesis achieved. We gradually understand the biosynthesis of each pathway in human and their specialized role in resolution physiology. (Figure adapted from Serhan, 2014 Nat Rev)

Maresin: Macrophage mediators in resolving inflammation

Maresin 1 (MaR1) was first identified in self-resolving inflammatory exudate and murine macrophages (Serhan et al., 2009). It was later discovered that human myeloid monocyte derived lineage also produce MaR1 (Dalli et al., 2012; Serhan et al., 2012), and that MaR1 is present in the synovial fluid of patients with rheumatoid arthritis and human serum, demonstrating its endogenous nature in human (Giera et al., 2012; Colas et al., 2014). MaR1 exerts potent anti-inflammatory and pro-resolving actions: including the reduction of neutrophil recruitment and enhancement of phagocytosis (Serhan et al., 2009) and is organ protective in lung inflammation (Abdulnour et al., 2014). In addition to its pro-resolving actions, MaR1 also promotes tissue regeneration and relieves neurological pain (Serhan et al., 2012).

The biosynthetic pathway of maresins is of interest for investigation since its epoxide intermediate (13*S*, 14*S*-epoxy-maresin) proved to be crucial for shifting macrophage phenotype from M1 to M2 and inhibit the enzyme that produces LTB₄, namely the LTA₄ hydrolase (LTA₄H) (Dalli et al., 2013) (Fig. 4). MaR1 is converted from DHA via lipoxygenation, epoxidation and enzymatic hydrolysis. (Fig. 4) It was first shown that MaR1 was converted from DHA by 12/15-lipoxygenase (12/15-LOX) in the mouse (Serhan et al., 2009). Although human macrophages produce maresins (Dalli et al., 2012), the enzyme responsible for its production has not been identified in human. For aim 1 of this thesis, the human macrophage maresin biosynthetic pathway was examined

and a potential defect of this pathway in localized aggressive periodontitis patients was scrutinized with novel actions of MaR1 being investigated for aim 2 and 3.

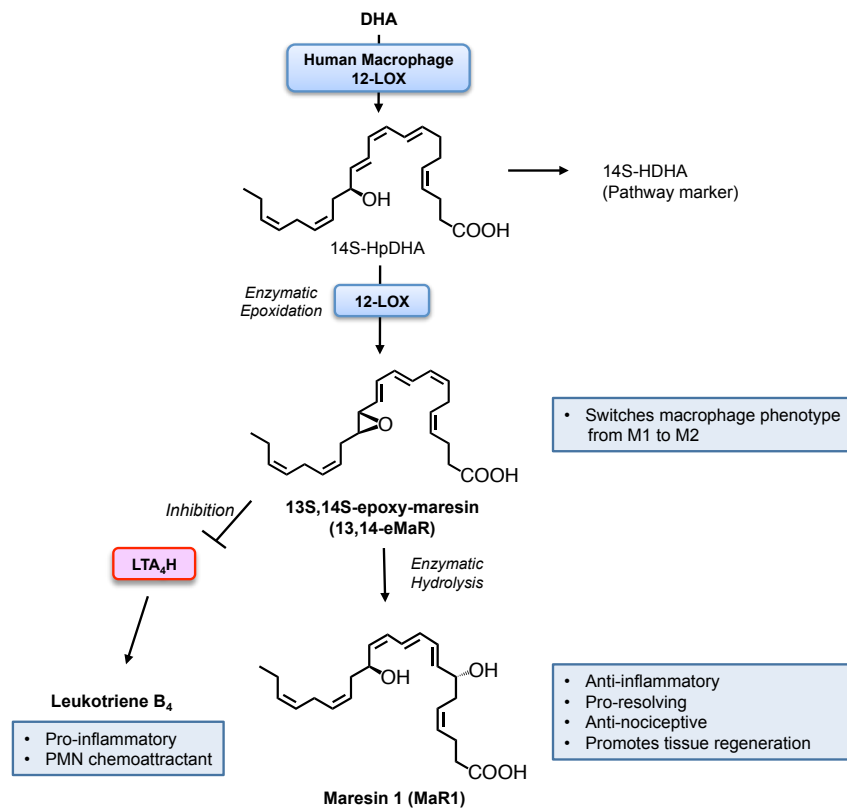


Figure 4. The biosynthesis of Maresin 1 and bioactive intermediate epoxy-maresin. DHA is converted to 14S-hydroperoxy-docosahexanoic acid (14S-HpDHA) intermediate by human macrophage 12-LOX. 14S-HpDHA can be hydrolyzed to 14S-HDHA or goes through 12-LOX-mediated epoxidation to 13S, 14S-epoxy- docosahexanoic acid (13,14-eMaR) and further converted to MaR1 by human macrophage. 13,14-eMaR inhibits the enzyme leukotriene A4 hydrolysis and thus reduces the production of pro-inflammatory LTB₄. (Figure adapted from Dalli et al., 2013 FASEB J)

HYPOTHESIS AND SPECIFIC AIMS

Central hypothesis of the thesis

“There is a defect in the leukocyte maresin biosynthetic pathway in localized aggressive periodontitis (LAP) patients and reduced maresin production contributes to the abnormal leukocyte functions and excessive platelet leukocyte aggregation in this disease.”

This hypothesis was addressed by the following specific aims (Fig. 5):

Aim 1 examined in human myeloid mononuclear lineage

novel biosynthesis mechanisms of maresins

Aim 2 investigated if there is a dysregulation of maresin biosynthesis in LAP patients and if

it is associated with functional defect of the leukocytes

Aim 3 assessed whether Maresin 1 (MaR1) could regulate leukocyte-platelet aggregation

Results from these proposed specific aims provided new insights into the role of maresin and its biosynthetic pathway in the pathogenesis of localized aggressive periodontitis (LAP). The therapeutic potential of maresin was tested and new therapeutic approach targeting this pro-resolving maresin biosynthesis may be developed. A potential link between oral inflammation and systemic inflammation through the excessive formation of platelet-leukocyte aggregates in the peripheral blood in LAP patient was also examined if regulated by MaR1.

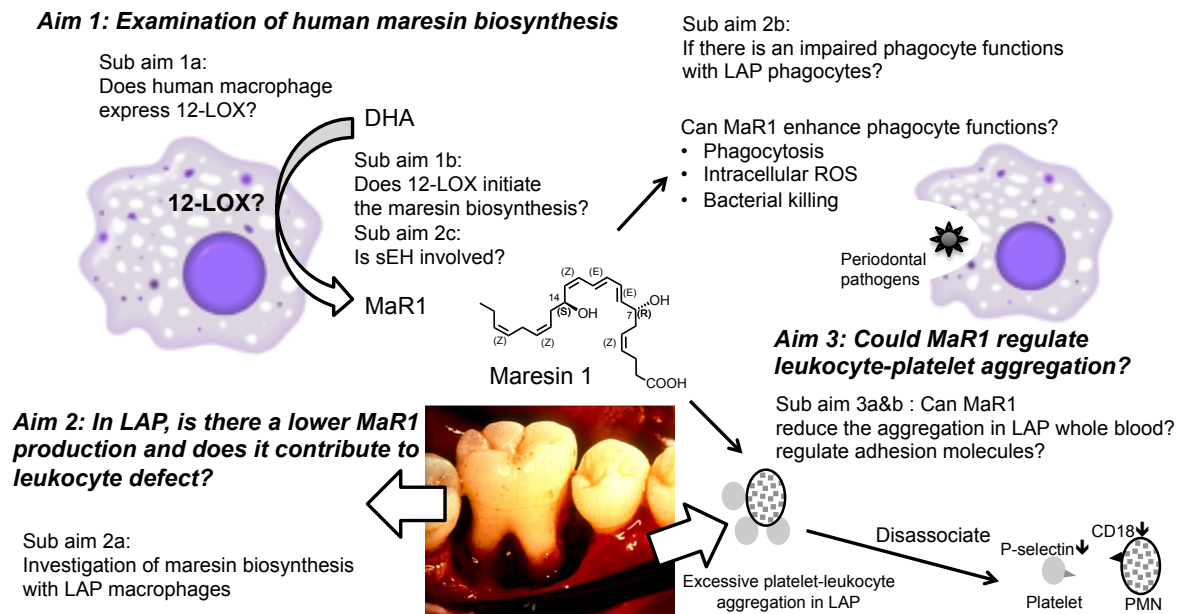


Figure 5. Scheme of specific aims: MaR1 biosynthesis and its new mechanism of actions with LAP leukocytes and oral pathogens. Aim 1 examined the maresin biosynthesis in human macrophage; aim 2 investigated the potential defect of this pathway in LAP patients and the role of MaR1 in regulating phagocyte functions; aim 3 assessed whether MaR1 regulate leukocyte-platelet aggregation in human whole blood.

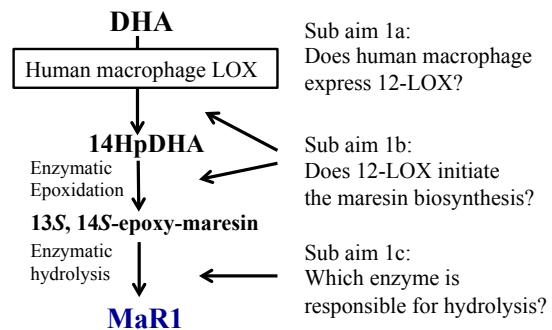
RESEARCH STRATEGY:

SIGNIFICANCE, INNOVATION, APPROACH

Aim 1 examined in human myeloid mononuclear lineage novel biosynthesis mechanisms of maresins

Rationale and significance: MaR1 was first identified from co-incubation of murine resident macrophages with DHA and 14HpDHA (Serhan et al., 2009) and later from human macrophage endogenous biosynthesis (Dalli et al., 2012; Serhan et al., 2012). Given MaR1's potent pro-

resolving, antinociceptive and regenerative potentials, its endogenous biosynthesis is of great interest for therapeutic investigation. It was found that 12/15-LOX deficient mice have a significant reduction of 14HDHA production and for the wild type mouse by adding lipoxygenase inhibitor esculetin with the exudate diminished its production (Dalli et al., 2012). However, little is known about what type of lipoxygenase in human macrophages is responsible for the biosynthesis of 14HpDHA and maresin-epoxide intermediate and whether other bioactive maresins could be produced. 12-LOX, the proposed key enzyme for the initiation of maresin metabolome is tested. Sub aim 1a questioned whether human myeloid mononuclear lineage express 12-LOX. And sub aim 1b examined the major product of 12-LOX from DHA in human macrophage to see if it is the key enzyme as proposed for MaR1 production.



Approaches: Human recombinant enzyme (12-LOX, soluble epoxide hydrolyse) and synthetic intermediates (14-HDHA, 13,14-diHDHA) were used to confirm the products and stereochemistry of the pathway by LC/MS/MS-based LM metabololipidomics. Identification of 12-LOX in human myeloid mononuclear lineage with flow cytometry (Fig. 6) and examination of the mRNA levels were conducted. Amplification of cDHA from M0 macrophage was also done to investigate the sequence of the enzyme and crosscheck with human platelet-type 12LOX.

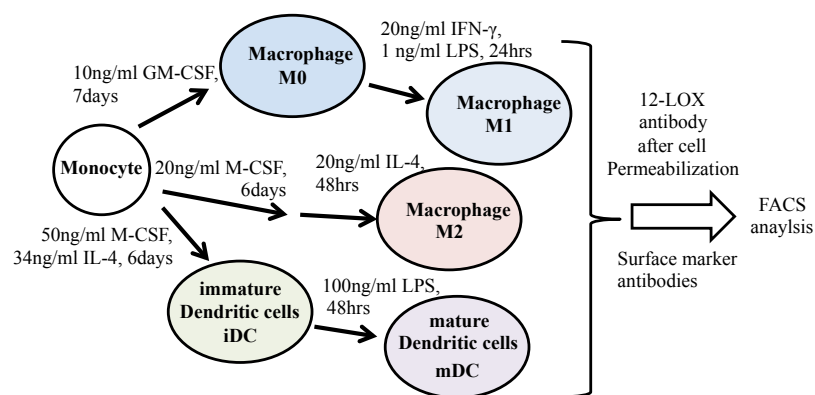


Figure 6. Examination of 12-LOX enzyme expression in human myeloid mononuclear lineage. Differentiation of human peripheral monocytes to macrophages and dendritic cells according to established protocols (Bellora et al., 2010; Bender et al., 1996). Different phenotypes of macrophage and dendritic cells were then subject to surface markers verification and permeabilized for determination of 12-LOX enzyme expression by flow cytometry.

Aim 2 investigated if there is a dysregulation of maresin biosynthesis in LAP patients and if it is associated with functional defect of the leukocytes

Rationale and significance: Since the level of 14HDHA is significantly lower in LAP patient's activated peripheral blood compared to healthy control (Fredman et al., 2011), it is likely that there is a perturbation in the biosynthetic pathway. Therefore, LAP was chosen as a model disease to study the biosynthesis of MaR1. We hypothesized that there is a reduced levels of MaR1 in LAP patients, and the next question was the functional impact of MaR1 to these phagocytes (neutrophils and macrophages). Earlier it was discovered that LAP neutrophils display defective functions in phagocytosis of *Staphylococcus aureus* (Cainciola et al., 1977; Van Dyke et al., 1986). Recently, macrophages from LAP patients were also found to display impaired phagocytosis of zymosan (Fredman et al., 2011). It is unknown whether these phagocytes also give reduced phagocytosis of periodontal pathogens, which may be more clinically relevant. In addition, other SPMs have shown their actions in infection, including RvE1 has been shown to alter the biofilm in the rabbit periodontitis model and both *A.actinomycetemcomitans*. and *P.gingivalis* dropped to undetectable level after treatment (Hasturk et al., 2007). It has also been shown that RvD1 and RvD5 enhance the bacterial killing of *E. coli* and thus lower the antibiotic requirements (Chiang et al., 2012).

Approaches: Peripheral blood was collected from LAP patients and age/gender-matched healthy controls (HC) from Forsyth Institute. Neutrophils and monocytes were isolated, and monocytes were differentiated into macrophages in the presence of GM-CSF for 7 days. Endogenous lipid mediators (LM) were investigated using LM metabololipidomics. Periodontal pathogens

including *Porphyromonas gingivalis* (*P.g.*) and *Aggregatibacter actinomycetemcomitans* (*A.a*) were incubated with neutrophils and macrophages to investigate phagocyte functions. Phagocytosis and intracellular anti-microbial reactive oxygen species (ROS) production were determined using fluorescence plate reader. Bacterial killing by macrophages and in whole blood was evaluated by assessing residual colony forming units following incubation with bacteria. 12-lipoxygenase (LOX) expression was measured by flow cytometry (Fig. 7).

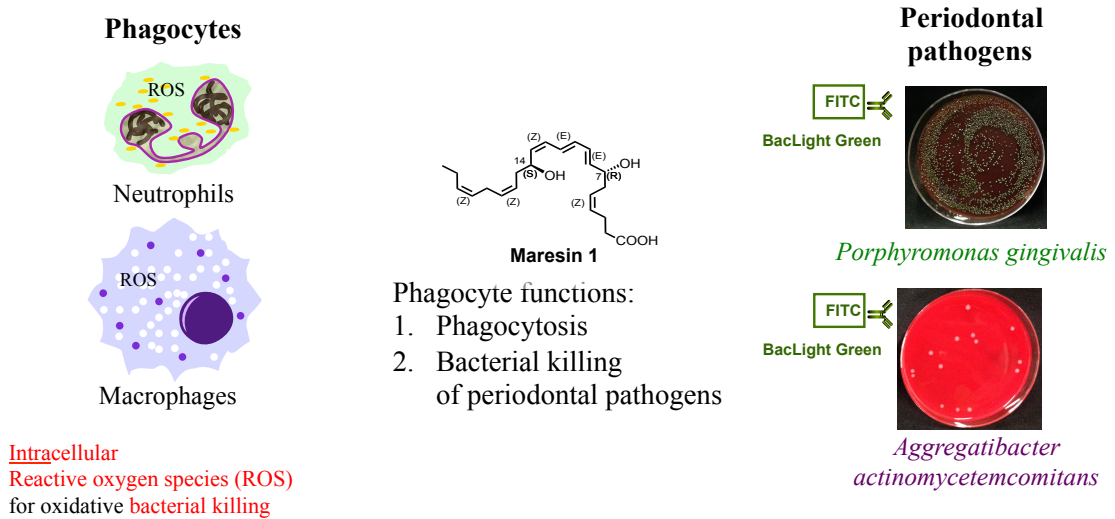


Figure 7. Investigation the actions of MaR1 on phagocyte functions. Two phagocytes (neutrophils and macrophages) was prepared from two different subjects (LAP patients and healthy donors) and incubated with two periodontal pathogens (*P. gingivalis* and *A. actinomycetemcomitans*) to test the action of MaR1 on phagocyte functions including phagocytosis, intracellular ROS production, and killing of periodontal pathogens.

Aim 3 assessed whether MaR1 could regulate leukocyte-platelet aggregation

Rationale and significance: The circulating leukocyte-platelet aggregates in LAP were found to be approximately two-folds higher than the matched healthy control (Fredman et al., 2011). Since this may be one of the mechanisms that links the oral and systemic inflammation as it has been associated with several systemic diseases and proposed as an early markers for acute myocardial infarction (AMI) (Forum et al., 2001) and type 2 diabetes mellitus (DM) (Patko et al., 2012). Within the SPMs family, LXA₄ was shown to reduce PMN aggregates (Fiore et al., 1995) and along with RvE1 and RvE2, they all reduce CD18 expression on neutrophil and monocytes (Filep et al., 1999, Dona et al., 2008, Oh et al., 2012). It was reasonable to predict that SPMs and specifically MaR1 may regulate the leukocyte-platelet aggregation via reducing the expression of the adhesion molecules. The significance of this aim was to apply MaR1 in treating the excessive leukocyte-platelet aggregates in the LAP whole blood *ex vivo*. This whole blood assay could also be used to compare in parallel with the potential therapeutic potency of enhancing bacterial killing in the whole blood in aim 2. The successful results together would demonstrate the therapeutic potential of MaR1 in human whole blood that could be extended to intercepting the impact of oral infection to systemic inflammatory diseases.

Approaches: Healthy human whole blood was collected and treated with MaR1 before adding agonists for aggregation including platelet activating factor (PAF) and *P. gingivalis*. The levels of leukocyte (neutrophil and monocyte) – platelet aggregation was determined using flow cytometry. The expression levels of surface adhesion molecules including CD18 and P-selectin were also monitored. Peripheral blood from LAP patients was collected and treated with MaR1 with or without the addition of *P. gingivalis*.

CHAPTER 1

The biosynthesis of maresins in human macrophage

Introduction

Maresins biosynthesis in macrophages involves an initial addition of oxygen molecule of DHA at a 14 position (Serhan et al., 2009), followed by epoxidation of the 14 hydroperoxy-intermediate that is subsequently converted to 13*S*,14*S*-epoxy-maresin (Dalli et al., 2013). Evidence for 13*S*,14*S*-epoxy-maresin was recently established from both human macrophages (Serhan et al., 2009) and recombinant human 12-LOX incubation (Dalli et al., 2013) using alcohol trapping. The complete stereochemistry of this epoxide intermediate was determined, 13*S*,14*S*-epoxy-docosa-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid, by total organic synthesis as well as its biological actions were demonstrated (Dalli et al., 2013).

Given the potent pro-resolving actions of MaR1 (Serhan et al., 2009& 2012), it remained of interest to establish the role of human 12-LOX in macrophages in maresin biosynthesis, and whether 13*S*, 14*S*- epoxy-maresin intermediate is converted to additional bioactive mediators by human macrophages. Here, we characterized the human macrophage 12-LOX and its role in maresin biosynthesis, and identified a new bioactive macrophage mediator (13*R*,14*S*- dihydroxy-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid) coined Maresin 2 (MaR2) that displays anti-inflammatory and pro-resolving actions.

Materials and methods

Materials used in this study:

RPMI 1640 and DPBS (with or without calcium and magnesium) were from Lonza (Hopkinton, MA, USA). Ficoll-Histopaque 1077-1, lipopolysaccharide (LPS) and zymosan A were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant granulocyte-monocyte colony stimulating factor (GM-CSF), macrophage colony stimulating factor, interferon γ (IFN- γ) and interleukin-4 were purchased from R&D System (Minneapolis, MN, USA). Fetal calf sera, ampicillin and Bac-to-Bac Baculovirus expression system were from Invitrogen (Grand Island, NY, USA). High capacity cDNA reverse transcription kit was from Applied Biosystems (Grand Island, NY, USA). Phusion PCR kit and cloning enzymes were from New England BioLabs (Ipswich, MA, USA). All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Bio-scale Mini Macro-prep High Q columns were from BioRad (Hercules, CA, USA). Sephacryl S-100 HR resin was from GE Life Sciences (Pittsburgh, PA, USA). FACS analysis antibodies against the surface marks were from BioLegend (San Diego, CA, USA). FITC rat anti-mouse Ly6G (clone IAS), purified rat anti-mouse CD16/32 (mouse BD Fc block) were purchased from BD Bioscience (San Jose, CA), and PE rat anti-mouse F4/80 (clone BM8) and PerCPCy5.5 rat anti-mouse CD11b (clone Mac-1) were purchased from eBioscience (San Diego, CA). Human 12-lipoxygenase monoclonal antibody was from Novus Biologicals (Littleton, CO, USA). Human soluble epoxide hydrolase poly-clonal antibody, soluble epoxide hydrolase, AA, DHA, d₃-5S-

HETE and d₄-LTB₄ purchased were from Cayman Chemicals (Ann Arbor, MI, USA). Human soluble epoxide hydrolase shRNA constructs were from OriGene (Rockville, MD, USA). C18 SPE columns were from Waters (Milford, MA, USA). Eclipse Plus C18 column was from Agilent (Santa Clara, CA, USA). All liquid chromatography solvents were from Fisher Scientific (Pittsburgh, PA, USA).

Human macrophage and dendritic cell preparation

Human peripheral blood mononuclear cells (PBMC) from whole blood (purchased from Children's Hospital Blood Bank, Boston, MA, de-identified from healthy donors) were isolated by density gradient centrifugation using Histopaque-1077. Monocytes (MC) were obtained by adhesion purification, and were cultured in RPMI with 10 ng/mL human recombinant GM-CSF at 37°C for 7 days to obtain macrophages (M0). M1 macrophages were obtained by stimulating M0 macrophages with 20 ng/ml IFN- γ and 1 ng/ml LPS for 24 h. M2 macrophages were obtained by incubating monocytes with 20 ng/ml macrophage colony stimulating factor for 6 days and stimulating with 20 ng/ml IL-4 for 48 h (Bellora et al., 2010). Immature dendritic cells (iDC) were obtained by incubating isolated MCs with 50 ng/ml GM-CSF and 34 ng/ml IL-4 for 7 days, and mature dendritic cells (mDC) were stimulated by 100 ng/ml LPS for 24 h (Bender et al., 1996). Phenotypic lineage of monocyte-derived cells was confirmed by flow cytometry staining with fluorescent-conjugated antibodies, including CD163, CD54 and CD80 (Bellora et al., 2010).

Quantitative real-time PCR (qPCR)

Total RNA was isolated from cultured cells using High Pure miRNA Isolation Kit (Roche), and cDNA was synthesized with High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Real-time PCR was performed using the SYBR-green (Fredman et al., 2012). GAPDH was employed as internal control. Expression was measured on an ABI Prism cycler (Applied Biosystems, Foster City, CA) and data were analyzed using the $\Delta\Delta C_t$ method. The forward and reverse primers used were as follows: 12-LOX (59-GATGATCTACCTCCAAATATG-39 and 59-CTGGCCCCAGAAGATCTGATC-39) and GAPDH (59-AG-CCACATCGCTCAGACAC-39 and 59-GCCCAATACGACCA- AATCC-39).

Cloning of human macrophage 12-lipoxygenase

12-LOX cDNA was synthesized with high capacity cDNA reverse transcription kit (Applied Biosystems) and a 12-LOX specific RT primer from M0 macrophage total mRNA. The primer sequence is 59-AGA AAG TTT ACT GCT CCC CTG G- 39. Two pairs of primers were designed to amplify the upstream and downstream regions. The two regions cover the whole coding area of 12-LOX cDNA and had 200 bp overlap. The upstream primer sequences were: 59-CTC CCC TCG CCT AAG CTG CTG-39; 59-CTT GAA GAT GGG GTG CAG TCC-39. The downstream primer sequences are: 59-ATT

CAG CCT CCC AAC CCC AGC TCT-39; 59-GGT TTA ACT GGG GGA GGA AAT
AGA GCC T-39. PCRs were performed with Phusion PCR kit (New England BioLabs)
following manufacturer's instruction to amplify the upstream and downstream regions.
Human 12-LOX full-length cDNA coding 663 amino acid residues was obtained by using
the obtained upstream-downstream PCR products as template, and performing a second
round of PCR (SOEing PCR). NdeI and BamHI restriction sites were included in front of
start codon and after stop codon, respectively. 12-LOX full-length cDNA was inserted
into pET20b vector and sequenced.

Expression of human macrophage 12-LOX protein in sf9 cells

12-LOX protein was obtained with Bac-to-Bac Baculovirus Expression System
(Invitrogen) and the manufacturer's instructions. In brief, 12-LOX cDNA was sub-cloned
into pFastBac vector. pFastBac-12-LOX plasmid was transformed into DH10Bac E. coli,
and was recombined with bacmid. The fragment with 12-LOX was donated into bacmid.
The bacmid harboring 12-LOX was isolated and transfected into insect sf9 cells. After 5
days, the first generation virus (P1) was harvested from the media. 0.5 to 1 ml of P1 was
used to infect 10 ml of sf9 cells to obtain the second generation of virus (P2). 125 ml of
sf9 cells with density of 26106 cells/ml in suspension culture were infected with 10 ml P2
at 27°C for 72 h. Cells were stored at -80°C after centrifugation at 2000 g for 10 min.

Protein purification

Sf9 cells were suspended in 50 mM Tris-HCl (pH 8) buffer supplemented with protease inhibitor cocktail (Sigma), and disrupted by sonication (Branson, 20 s pulse for 5 times). Soluble fraction was isolated by centrifugation at 15000 g for 30 min for column chromatography. 12-LOX protein was purified to homogeneity by ion exchange and size exclusion column chromatography consecutively using Bio-scale Mini Macro-prep High Q (5 ml) and Sephacryl S-100 HR (80 ml) columns at a flow rate of 1.0 and 0.35 ml/min, respectively. For Q column, a linear gradient between 0 and 1 M NaCl in 50 mM Tris-HCl (pH 8) was used to elute all proteins in 20 column volumes. The fractions with 12-LOX activity (see next section) were collected, pooled, concentrated and loaded onto Sephacryl S-100 HR column with 50 mM Tris-HCl (pH 8). All procedures were conducted on ice or at 4°C. Protein purity was determined to be about 90% by SDS-PAGE. Small aliquots of concentrated human recombinant 12-LOX were flash frozen in liquid nitrogen and stored at -80°C until use.

Kinetic assays

Assays were performed using a Cary 60 UV-Vis spectrophotometer. Either AA or DHA from 1 μ M to 50 μ M was used as substrate [S], and 12-LOX concentration [E] was fixed at 100 nM. Assays were conducted either in calcium-depleted buffer (50 mM Tris, pH 8.0, 1 mM EGTA, 2 mM MgCl₂, 0.03% Tween 20) or in calcium-supplemented buffer (50 mM Tris, pH 8.0, 1 mM EGTA, 2 mM MgCl₂, 3 mM CaCl₂, 0.03% Tween 20). Conversion of AA to HpETEs or DHA to HpDHA was monitored by UV absorbance at

235–236 nm. At each given substrate concentration, initial rate (V_0) was obtained using extinction coefficient $\epsilon_{236} \approx 23,000 \text{ M}^{-1} \text{ cm}^{-1}$. V_0 and substrate concentration [S] were fit into Michaelis-Menten equation: $V_0 = [E] \times k_{\text{cat}} \times [S] / (K_M + [S])$ to determine K_M and K_{cat} .

Mouse peritonitis

All animal procedures were approved by the Standing Committee on Animals of Harvard Medical School (Protocol 02570) and performed in accordance with institutional guidelines. Male FVB mice (6–8 weeks old, weighing 22–25 g) were purchased from Charles River Laboratories. Mice were housed 4 mice per cage in specific pathogen-free facilities in a humidity (45–55%) and temperature (23–25°C) controlled environment with a 12 h light-dark cycle. Mouse peritonitis was carried out as in (Bannenberg et al., 2005) and efforts were made to minimize suffering (e.g. if an increase in pain or stress behavior was noted, the mice were euthanized according to Protocol #02570). Briefly, mice were anesthetized by inhaled isoflurane according to Protocol 02570 and randomly assigned to be administered intravenously with 1 ng 13*R*,14*S*-diHDHA, MaR1, or vehicle (saline) immediately prior to the administration of 0.1 mg zymosan (Sampaio et al., 2010). At 4 h, mice were euthanized with overdose of isoflurane followed by cervical dislocation and peritoneal lavages collected. PMN numbers were assessed by light microscopy and flow cytometry. PMNs were identified as CD11b⁺ and Ly6G⁺ cells.

Human macrophage phagocytosis

Human M0 macrophages were plated onto 96-well plates (5×10^4 cells/well) and phagocytosis was carried out after 24 h. Cells were incubated with either vehicle (PBS containing 0.1% ethanol), MaR1 as in (Serhan et al., 2009 & 2012) or 13R,14S-diHDHA at indicated concentrations (15 min, 37°C). All incubations were conducted in PBS containing calcium and magnesium (PBS⁺⁺). Subsequently either FITC-labeled zymosan (5×10^5 particles/well) or fluorescent labeled apoptotic PMN (1.5×10^5 cells/well) were added to each well and cells incubated for a further 60 min at 37°C, pH 7.45. The cells were washed 3 times with PBS⁺⁺ and extracellular fluorescence was quenched using Trypan Blue (1:15 diluted). Phagocytosis was assessed using a SpectraMax M3 plate reader (Molecular Devices).

Human recombinant enzyme incubations

To identify the chirality of 12-LOX product(s), DHA (5 μ M) was mixed with the isolated human macrophage 12-LOX (0.2 μ M) in 100 μ L 20mM Tris (pH8), 100mM KCl at 37°C for 10 min. In select incubations, DHA (10 μ M) was either incubated with human 12-LOX or with 12-LOX plus sEH (0.28 μ M) in the same Tris buffer as above at 37°C, pH 8.0 for 10 min. All incubations were stopped by 2 volumes of methanol, and 500 pg d₈-5S-HETE and d₄-LTB₄ were added into each sample before extraction as internal standards (Dalli et al., 2012).

Sample extractions and LC-MS/MS

All samples from recombinant enzyme incubations were subjected to liquid-liquid extractions with 6 volumes of diethyl ether. The samples from cell incubations were extracted with SPE columns (Oh et al., 2011). LC-MS/MS analyses were conducted as in (Dalli et al., 2013). In the chiral chromatography separation, Chiralpak AD-RH column was used with methanol/water/acetic acid of 95:5:0.01 (v/v/v) (Oh 2011). The transition ion pairs were: 14-hydroxy- 4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid (14-HDHA) (343>205) and 13,14-diHDHA (359>221). 500 pg d₈-5S-HETE and d₄-LTB₄ were used as internal standards to determine extraction recoveries.

Western blotting

The procedure was modified from (Ohira et al., 2003). In brief, cell lysis was mixed with equal volume of tricine sampling buffer (BioRad), and mixtures were immediately boiled at 95°C for 4 min. The samples were loaded to 10% SDS-PAGE, and was transferred to nitrocellulose membranes after gel separation. Membranes were probed by primary and secondary antibodies consecutively. Primary antibodies were diluted as instructions: 1:500 for anti- human 12-LOX (Novus Biologicals); 1:200 for anti-human sEH (Cayman); 1:200 for anti b-actin (Santa Cruz). Blots were developed by enhanced chemiluminescence (Pierce).

Statistics

All the results were expressed as means \pm standard error. One- way ANOVA was carried out to determine difference between groups. $p < 0.05$ was considered significant.

Results

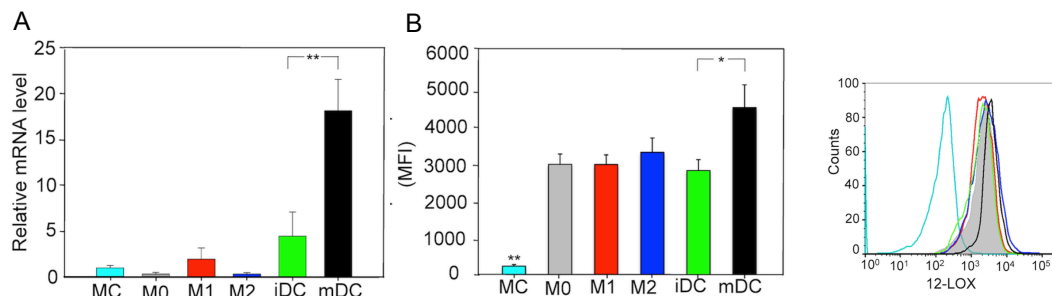


Figure 1. Human macrophages and dendritic cells express human 12-LOX. (A) Expression levels of 12-LOX mRNA in human monocytes (MC), M0, M1, M2 macrophages, immature dendritic cells (iDC) and mature dendritic cells (mDC) were assessed by quantitative PCR. (B) 12-LOX protein expression levels were assessed by flow cytometry. In the left panel, a representative histogram shows 12-LOX present in each cell type. In the right panel, results are mean fluorescent intensity (MFI) of 12-LOX protein levels for each cell type, expressed as mean \pm SEM of 4 separate healthy donors, * $P < 0.05$, ** $P < 0.01$. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

Human monocyte-derived lineage express 12-LOX

We first examined 12-LOX expression levels in human monocytes (MC), macrophages (M0, M1 and M2) and dendritic cells (DC). The cells were prepared in accordance with published methods (Bellora et al., 2010, Bender et al., 1996). Quantitative PCR results using the human platelet 12-LOX sequences as primers (see methods) gave rather low 12- LOX mRNA levels in MC, M0, M1 and M2 cells as well as iDCs (immature dendritic cells) (Fig. 1A). In contrast, the 12-LOX mRNA level in mDC (mature dendritic cells) was significantly higher, or about 3.5 fold compared to iDC. Also, flow-cytometry assessment using fluorescence-labeled antibody against human 12- LOX demonstrated about 10 fold increase in protein expressions in M0, M1 M2 and iDC, and 19 fold increase in mDC, compared to MC (Fig. 1B), indicating that expression of human 12-LOX protein is enhanced with differentiation of MC to macrophages and dendritic cells. Cell lineage was determined by flow cytometry using distinct phenotypic markers (CD80 for M1, CD163 for M2, CD54 for DC, Fig. S1) (Bellora et al., 2010).

We amplified 24-1214 and 1005–2167 bp fragments of 12- LOX from human macrophages (M0) cDNA (Fig. S2). The two fragments covered the entire coding area, and had about 200 bp overlapping. We numbered nucleotides in the present report on the basis of the platelet cDNA sequence (Izumi et al., 1990). We obtained the full length of human macrophage 12-LOX cDNA by SOEing PCR with the 24–1024 and 1005–2167 fragments as template, and the sequencing results showed that M0 12-LOX cDNA

sequence was in accordance with that reported earlier for human platelet 12-LOX (Izumi et al., 1990). Also, western blotting for 12-LOX revealed 75 kD size bands in all lineage cells, which is consistent with the size of human platelet 12-LOX (Fig. S2).

Human macrophage recombinant 12-LOX converts DHA and AA with essentially equivalent catalytic efficiency

We next examined and compared the catalytic efficiencies of AA and DHA with human macrophage recombinant 12-LOX. AA or DHA were incubated with 0.1 μM human recombinant macrophage 12-LOX in the presence or absence of CaCl_2 . The initial rate at each substrate concentration was plotted and fit to Michaelis-Menten equation using non-linear regression to determine k_{cat} (maximal turnover rate per enzyme) and K_M (concentration of substrate with initial rate reaching half of maximal rate, Fig. 2).

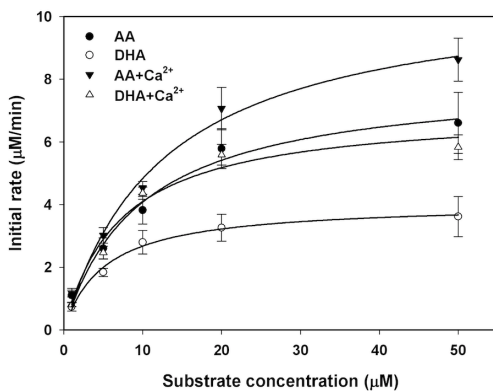


Figure 2. Human 12-LOX converts AA and DHA with essentially equivalent efficiency.

Increasing concentrations of AA or DHA were mixed with 12-LOX (0.1 μM , pH 8.0, R.T.) in the presence or absence of CaCl_2 (2 mM). Initial rates were monitored and plotted versus substrate at indicated concentrations. Each point represents mean \pm SEM, $n = 3$. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

Table 1. Parameters of Michaelis-Menten kinetics.

	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \text{mM}^{-1}$)
AA	9.7±2.1	80.3±6.0	8.3
DHA	5.1±0.7	40.4±1.6	7.9
AA+Ca ²⁺	12.6±2.2	109.4±7.1	8.7
DHA+Ca ²⁺	7.1±2.0	70.2±6.1	9.9

*Initial rate at each substrate concentration was fit into Michaelis-Menten equation to determine K_M and k_{cat} . Results are mean ± SEM.

The calculated k_{cat} for DHA was 2 fold lower than AA; however, the calculated K_M for DHA was about 2 fold lower than that of AA, suggesting that the maximal turnover of DHA by 12-LOX is slower, while affinity of DHA to 12-LOX is higher when compared to AA. The resulting catalytic efficiencies, indicated by k_{cat}/K_M , were essentially equivalent between DHA and AA. Also, addition of CaCl_2 appeared to increase both k_{cat} and K_M , yet lead to no significant increase in catalytic efficiency (k_{cat}/K_M) (Table 1).

***Stereo and positional selective production of 14S-HpDHA
by human macrophage 12-LOX***

Recombinant human macrophage 12-LOX was expressed with baculovirus-insect system, and was purified to apparent homogeneity as described in methods. DHA was incubated with human macrophage 12-LOX. After reduction of reaction mixture using NaBH_4 , the hydroxyl-containing products were separated using chiral high performance liquid

chromatography and identified using lipid mediator metabololipidomics. We found that in these incubations the peak at retention time of ~6 min accounted for ~98% peak area and matched the retention time of 14S-HDHA (Fig. 3A). The MS-MS spectrum for the product beneath this peak matched that of 14-HDHA (Fig. 3B). These results demonstrate that human macrophage 12-LOX inserted oxygen at the C-14 position of DHA predominantly in the S conformation.

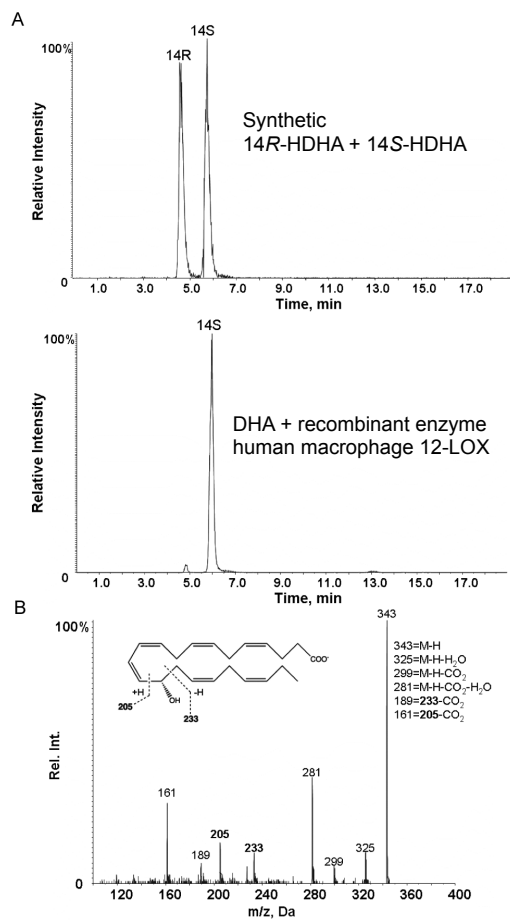


Figure 3. Human macrophage 12-LOX produces 14S-HpDHA. (A) Chiral chromatography of synthetic 14R-HDHA and 14S-HDHA (upper panel). DHA (5 μ M) was incubated with human macrophage 12- LOX (0.2 μ M, 10 min, 37°C, pH 8). Products were extracted (see methods for details) and subject to chiral high performance liquid chromatography-tandem mass spectrometry (m/z, 343>205) (lower panel). (B) MS-MS spectrum of 14S-HDHA from human macrophage 12- LOX incubations (lower panel in A). (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

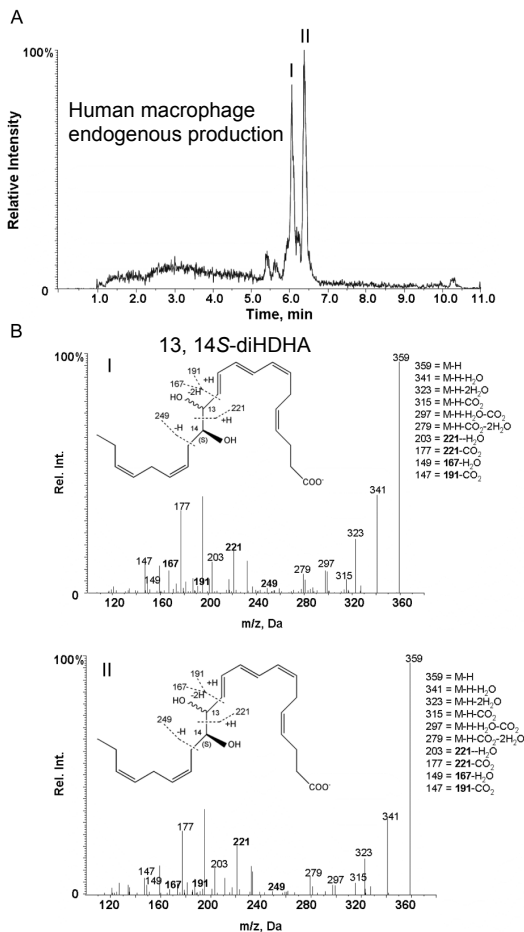


Figure 4. Human macrophage endogenous production of 13R,14S-diHDHA. (A) MRM chromatography for ion pair 359>221 (m/z). Human macrophages were incubated with opsonized zymosan (DPBS^{+/+}, pH 7.45, 15 min, 37°C). Incubations were stopped with 2 volumes of ice-cold methanol, and products were assessed by LM metabololipidomics. (B) MS-MS spectra. *Inset*, diagnostic ions used for identification and corresponding possessed structure fragments. Results are representative of n = 3. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

Identification of endogenous 13,14S-diHDHA from human macrophages

We next investigated whether the 13S,14S-epoxy-maresin, a biosynthetic product of human macrophage 12-LOX (Dalli et al., 2013), was converted by human macrophage to novel products. Using lipid mediator metabololipidomics, we identified 2 endogenous products from macrophages with the fragmentation spectrum for the product under peak I matching 13,14S-diHDHA with the following ions assigned: m/z 359=M-H, m/z 341=M-H-H₂O, m/z 323 = M-H-2H₂O, m/z 315 = M-H-CO₂, m/z 297 = M-H- H₂O-CO₂, m/z 279

= M-H-2H₂O-CO₂, m/z 203 = 221-H₂O, m/z 177=221-CO₂, m/z 149=167-H₂O, and m/z 147=191-CO₂ (Fig. 4). Peak II with retention time of 6.5 min displays the essentially identical fragmentation to peak I, and thus suggests that the product beneath peak II was a stereoisomer of peak I.

Cell-free biosynthesis of 13,14S-HDHA

sEH is expressed in cells of the monocyte lineage (Seidegard et al., 1984, Draper et al., 1999). sEH converts leukotriene A₄ to 5*S*,6*R*-diHETE (Haeggstrom et al., 1986) and epoxyeicosa-trienoic acids to vicinal diols (Chacos et al., 1983). Therefore, we next investigated whether this enzyme was involved in the biosynthesis of 13,14*S*- diHDHA. Co-incubation of both human macrophage 12-LOX and sEH with DHA gave 13,14*S*- diHDHA (peak II, ~0.1% conversion) in comparison to scant formation of this diol product (~0.005% conversion) when only DHA and 12-LOX were present in the incubations (Fig. 5A). The MS-MS fragmentation of 13,14*S*-diHDHA (peak II, Fig. 5B) using this recombinant enzyme system matched those obtained from macrophages (Fig. 4). In the incubations with 12-LOX and DHA, we also identified a double oxygenation product (7*S*, 14*S*-diHDHA; peak I, Fig. 5B), whereas, MaR1 was not identified in these coincubations. These results suggest that 13,14-epoxy-maresin (Dalli et al., 2013) produced from DHA via 12-LOX is substrate of sEH producing 13,14*S*-diHDHA.

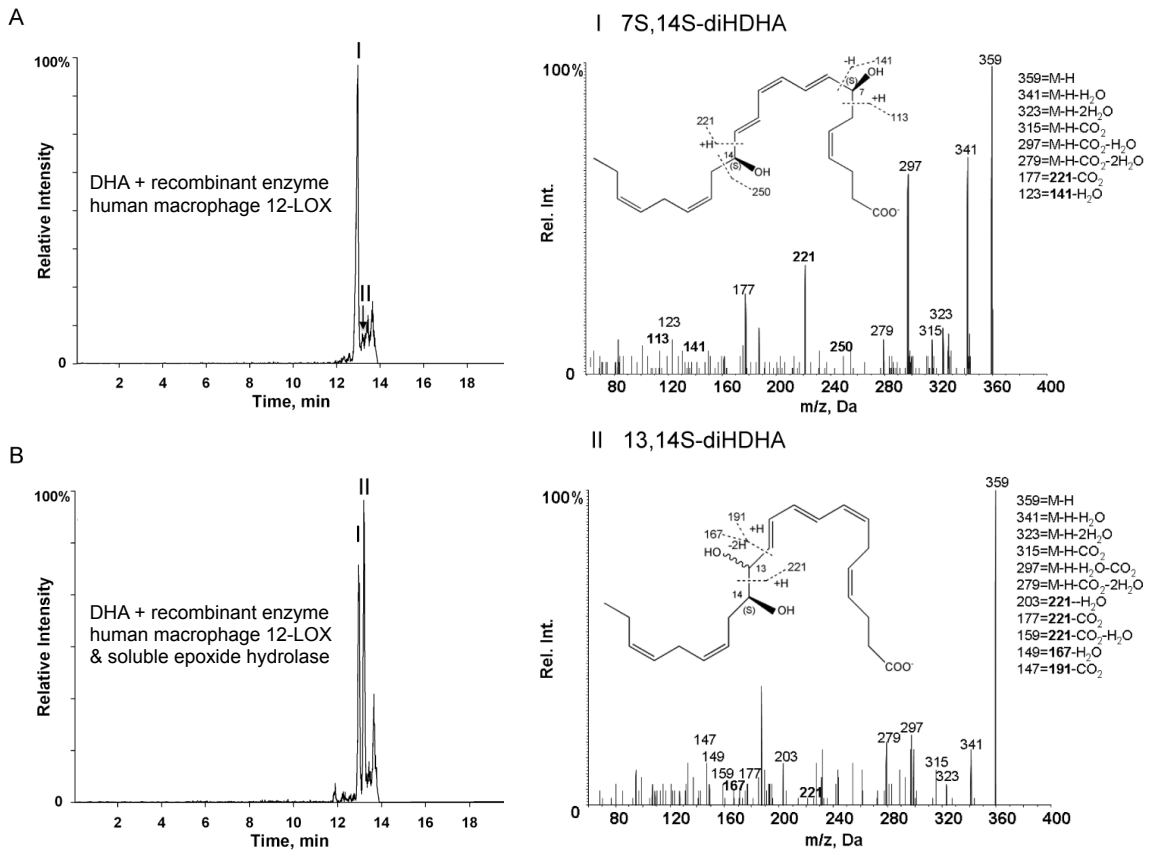


Figure 5. Biosynthesis of 13R,14S-diHDHA in coin incubations with human recombinant 12-LOX and sEH. (A) MRM chromatography for ion pair 359>221. DHA (10 μ M) was incubated with 12-LOX (0.2 μ M) in the absence or presence of 2 U sEH (20 mM Tris, pH 8.0, 100 mM KCl, 37°C, 10 min). (B) MS/MS spectra employed in the identification of 13, 14S-diHDHA (peak II) and 7S, 14S-dihydroxy- 4Z,8E,10Z,12E,16Z,19Z-docosahexaenoic acid (7S,14S-diHDHA) (peak I). Results are representative of n = 3. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

13,14S-diHDHA displays anti-inflammatory and pro- resolving bioactions

We next assessed the potential anti-inflammatory actions of the novel macrophage product isolated by investigating its ability to regulate leukocyte response *in vivo*. Mouse peritonitis was initiated by *i.p.* injection of zymosan (0.1 mg/mouse) (Sampaio 2010). Peritoneal lavages were collected at 4 h and PMN were enumerated by light microscopy and flow cytometry. Systematic administration of 1 ng/mouse of the 13*R*,14*S*-diHDHA prior to initiating peritonitis reduced PMN infiltration into the peritoneum by ~40%; similar values were obtained for MaR1 (Fig. 6A). In addition, 13*R*,14*S*- diHDHA also enhanced human macrophage phagocytosis of zymosan by 90% at a concentration as low as 10 pM, compared to 60% maximal enhancement by MaR1 at 10 nM (Fig. 6B). However, with human apoptotic PMN and macrophages, the MaR1 proved more potent than 13*R*,14*S*-diHDHA at enhancing efferocytosis.

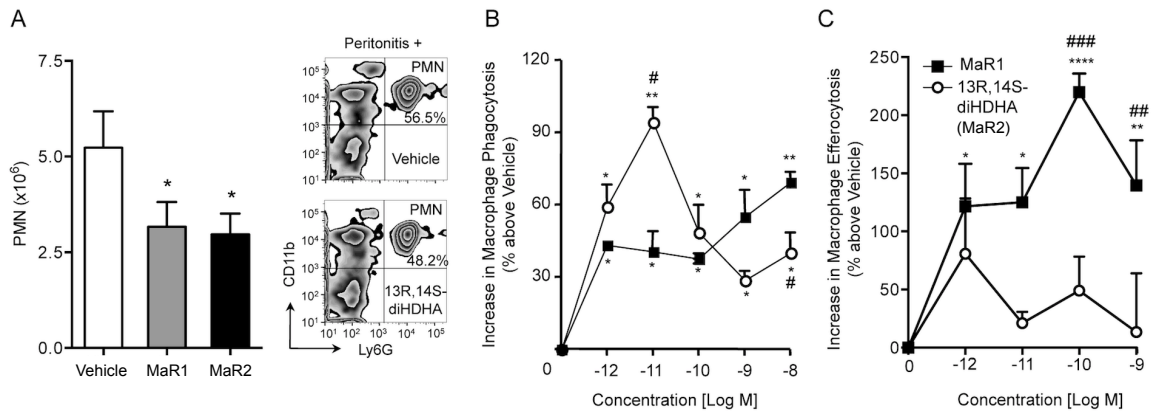


Figure 6. MaR1 and MaR2 (13R,14S-diHDHA) display potent anti-inflammatory and proresolving actions: direct comparison. (A) Mouse peritonitis: exudate PMN numbers *in vivo*. Male mice (6–8 weeks) were administered *i.v.* MaR1, 13R,14S-diHDHA (1 ng/mouse each) or vehicle prior to *i.p.* administration of zymosan (0.1 mg/mouse). Peritoneal exudates were collected, and PMNs enumerated using both light microscopy and flow cytometry. Results are mean \pm SEM. n = 3 mice per treatment from three separate experiments (*P<0.05 vs. vehicle). Enhanced phagocytosis of (B) opsonized zymosan or (C) apoptotic PMN. Human macrophages were seeded in 96-well plates (5×10^4 cells/well) and incubated with vehicle (PBS containing 0.1% ethanol), MaR1 or 13R,14S-diHDHA (PBS^{+/+}, pH 7.45, 37°C, 15 min). (B) FITC-labeled zymosan (5×10^5 particles/well) or (C) fluorescently labeled apoptotic PMN (1.5×10^5 cells/well) were added and cells incubated for an additional 60 min (pH 7.45, 37°C). Non-phagocytosed zymosan or apoptotic PMN were washed, extracellular fluorescence quenched and phagocytosis quantified. Results are mean \pm SEM. n = 3 separate human macrophage preparations (*P<0.05, **P<0.01, ****P<0.0001 vs. vehicle; #P<0.05, ###P<0.001 vs. MaR1). (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

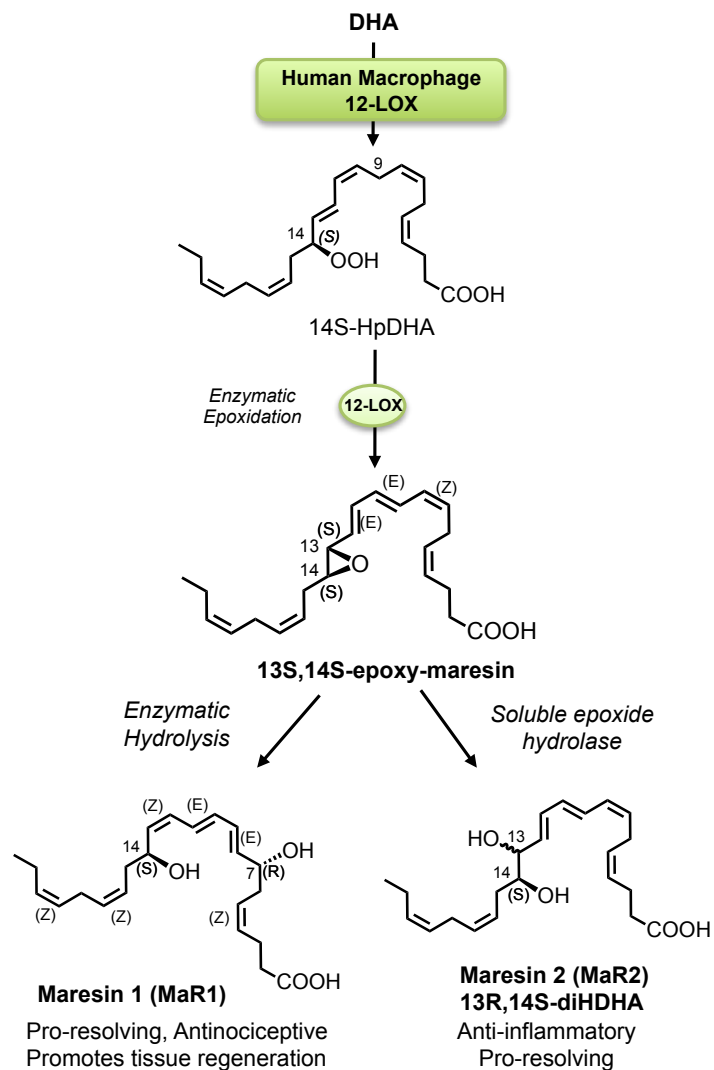


Figure 7. Proposed biosynthetic scheme for MaR1 and MaR2. Human macrophage 12-LOX converts DHA to the 13*S*,14*S*-epoxy-maresin intermediate, and via soluble epoxide hydrolase this intermediate is converted to MaR2. See text for details and stereochemical assignments. (Figure adapted from Dalli et al. 2013 FASEB J, and Deng, Wang et al., 2014 PLoS ONE)

Discussion

In the present study, we identified and cloned the human macrophage 12-LOX involved in the biosynthesis and bioactive maresin metabolome, and found a new member of the maresin family produced from DHA. The human macrophage 12-LOX converted both AA and DHA with essentially equivalent efficiency to produce the hydroperoxy products, respectively, that were predominantly in the carbon 14 with S configuration (98% S). A new 13*R*,14*S*-diHDHA was identified from human macrophages that displayed potent bioactions. Production of 13*R*,14*S*-diHDHA involved oxygenation at C-14 followed by 12-LOX-catalyzed epoxidation and subsequent hydrolysis via sEH. The proposed biosynthetic schemes of MaR1 and 13*R*,14*S*-diHDHA are summarized in Fig. 7. Given the potent anti-inflammatory and pro-resolving actions of 13*R*,14*S*-diHDHA and its biosynthesis from 13*S*,14*S*- epoxy-maresin, we coined this mediator as MaR2.

Maresins are biosynthesized by human macrophages 12-LOX from DHA. MaR1 is the first member of this family to be identified (Serhan et al., 2009). In addition to its anti-inflammatory, pro-resolving (Serhan et al., 2009), tissue regenerative and anti-nociceptive actions (Serhan et al., 2012), MaR1 was recently found to dampen the pro-inflammatory response to organic dust in bronchial epithelial cells (Nordgren et al., 2013), and attenuates mouse colitis (Marcon et al., 2013). MaR1 was also identified in human synovial fluid from rheumatoid arthritis patients (Giera et al., 2012). Another related 12-LOX-derived product, 14*S*, 21*R*-diHDHA, was shown to enhance wound healing and

rescues mesenchymal stem cell function in diabetes and renal ischemia/reperfusion injury (Tian et al., 2012; Lu et al., 2010; Takahashi et al., 1993). In the present manuscript, we identified a novel member of the maresin family, namely 13*R*,14*S*-diHDHA, coined MaR2, that is produced by the human 12-LOX and sEH, which are present in mononuclear cells and macrophages (Seidegard et al., 1984; Draper et al., 1999). MaR2 exhibited similar potency to MaR1 in limiting PMN infiltration, but had an apparent optimal concentration 2–3 log orders lower than MaR1 in enhancing human macrophage phagocytosis of zymosan (Fig. 6). MaR2 also enhanced human macrophage uptake of apoptotic PMN, but was less potent than MaR1. These are the key defining bioactions of a pro-resolving mediator (Tabas et al., 2013; Serhan et al., 2011).

Also, we confirmed that the activities of DHA oxygenation and epoxidation recently identified (Serhan et al., 2009; Dalli et al., 2013) in human macrophages originated from 12-LOX. The nucleotide sequence of the human macrophage 12-LOX was in accordance with human platelet type (Izumi et al., 1990); see Figure S2. Human epidermis also has been shown to express the same platelet type of 12-LOX (Takahashi et al., 1993). Human 12-LOX oxygenates AA at C-12 with S chirality (Hamberg et al., 1974). In the present report, we found that human macrophage 12-LOX oxygenated DHA at the C-14 with a predominant S chirality (Fig. 3). The 14*S*-HDHA profiling obtained here was consistent with that from primary macrophages and DHA (Serhan et al., 2009). The two-carbon position shift in DHA compared to AA is consistent with the tail-first substrate access

(Sloane et al., 1991). In addition, human macrophage 12-LOX displayed an epoxide synthase activity converting 14- hydroperoxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid (14- HpDHA) to 13*S*,14*S*-epoxy-maresin, as demonstrated by methanol trapping (Dalli et al., 2013). This product is a central intermediate in maresin biosynthesis and production of both MaR1 and MaR2 (Fig. 7). 12-LOX also biosynthesizes lipoxins from leukotriene A4 (LTA₄) (Serhan et al., 1990; Romano et al., 1993) and this enzyme is susceptible to suicide inhibition by epoxides, such as with LTA₄ (Romano et al., 1993) or 13*S*,14*S*-epoxy-maresin (Dalli et al., 2013). Interestingly 13*S*,14*S*-epoxy-maresin only inhibits conversion of AA by 12-LOX, while conversion of DHA appeared unaltered (Dalli et al., 2013), thereby suggesting that 13*S*,14*S*-epoxy-maresin exerts a feed- forward action on the maresin pathway and hence in resolution of inflammation.

Macrophages play an indispensable role in resolution of inflammation and re-establishment of homeostasis. M1 macrophages, stimulated by IFN- γ LPS and GM-CSF, resist to microbial invasion and enhance inflammatory response (Mantovani et al., 2005). In contrast, M2 macrophages, differentiated on exposure to IL-4, IL-13 and/ or other cytokines as well as hormones, produce decreased level of pro-inflammatory cytokines and promote resolution (Mantovani et al., 2005; Gordon et al., 2010), whereas DCs play a key role in the transition between innate and adaptive immunity (Steinman et al., 2012). Here, human macrophage 12-LOX initiates biosynthesis of maresins, and more importantly, is responsible for the production of 13*S*,14*S*-epoxy-maresin. Of note, 12-

LOX mRNA expression levels remain unchanged during differentiation of human monocytes to macrophages, and in macrophages, 12-LOX mRNA is not regulated by overnight stimulations with LPS, multiple cytokines or hypoxia (Wuest et al., 2012). In agreement with this, in the present investigation, we did not find significant difference of 12-LOX mRNA levels in human monocytes, and M0, M1 and M2 macrophages. However, 12- LOX mRNA levels in mDC were significantly increased after LPS stimulation, when compared with iDC (Fig. 1). Assessment of 12- LOX protein expression in M0, M1 and M2 macrophages demonstrated significantly increasing 12- LOX protein levels when compared to monocytes (Fig. 1), suggesting that translational or post-translational regulation mechanism also plays a role in establishing 12-LOX protein level in these cells. Of interest, we also found that mDC possess the highest 12-LOX protein level compared to other monocyte-derived lineages examined herein, suggesting that mDC may be a notable source of maresins that can exert pro-resolving actions during resolution of inflammation.

Given the potent actions of maresins in resolution and the role of human macrophage 12-LOX in maresin biosynthesis, we assessed kinetics of conversion for DHA by 12-LOX, finding that 12-LOX catalyzes AA and DHA with equivalent efficiency (Table 1). Enzymatic turnover approaches the maximal rate with incubation of either 50 μ M AA or DHA. This is less than the reported critical micelle concentration for either DHA or AA (Serth et al., 1991). Hence, increased dietary levels of DHA are likely to switch to

maresin production and enhance the resolution phase on the infiltrating macrophages or DC at local microenvironments in lymphoid tissues and elsewhere in humans.

Conclusions

Results obtained in the present studies with recombinant enzymatic co-incubations demonstrated that biosynthesis of 13*R*,14*S*-diHDHA involves a sEH subsequent to 12-LOX (Fig. 5). Mammalian sEH enzymes catalyze hydrolysis of a broad category of epoxides, including epoxyeicosatrienoic acids (Zeldin et al., 1993), LTA₄ (Haeggstrom et al., 1986) and even hepxilins (Cronin et al., 1993). Our finding further demonstrated that 13*S*,14*S*- epoxy-maresin is a new substrate for sEH by coincubation of 12- LOX and sEH with DHA, producing a potent new product (Fig. 5). Since LTA₄ is converted by sEH to 5*S*,6*R*-diHETE with double bond geometry 7*E*,9*E*,11*Z* (Haeggstrom et al., 1988). Based on this mechanism, we tentatively assigned the complete stereochemistry of MaR2 as 13*R*,14*S*-dihydroxy-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid (see Figure 7). Taken together, we identified and cloned a human macrophage 12-LOX and established its expression in monocyte lineage. The enzyme matched the human platelet enzyme cloned earlier (Izumi et al., 1990; Chen et al., 1993; Sheppard et al., 1992). We also identified a novel pro-resolving product from macrophage, MaR2, and characterized its biosynthesis involving 12- LOX and sEH as well as its bioactions. The present findings provide additional evidence for the macrophage pro-resolving metabolome and their potential in regulating inflammation and stimulating resolution.

Chapter 2

The biosynthesis of MaR1 in patients with localized aggressive periodontitis and its regulation on phagocyte functions

Introduction

Localized aggressive periodontitis (LAP) is a clinically distinct form of rapidly progressing periodontal disease caused by abnormal leukocyte-mediated tissue destruction with infection and uncontrolled inflammation (Kantarci et al., 2003). The sites with periodontal pockets in LAP patients harbor higher levels of periodontal pathogens including *Porphyromonas gingivalis* (Lopez et al., 1996) and *Aggregatibacter actinomycetemcomitans* (Slots, 1976; Mandell et al., 1981). In addition, activated peripheral blood from LAP patients exhibits imbalanced lipid mediator profile with higher pro-inflammatory mediators levels including leukotriene B₄ (5*S*, 12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid, LTB₄) and lower pro-resolving mediators levels including lipoxin A₄ (5*S*,6*R*,15*S*-trihydroxyicosa-7*E*,9*E*,11*Z*,13*E*-tetraenoic acid, LXA₄). Of note, 14-hydroxy-docosahexanoic acid (14*S*-hydroxy-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-DHA, 14-HDHA) levels, as the Maresin 1 biosynthetic pathway marker, are reduced in activated peripheral blood from LAP patients compared to those from healthy control donors (Fredman et al., 2011). Therefore, LAP may serve as a model disease to investigate the biosynthesis and action of Maresin 1.

Maresin 1 (MaR1) was first identified in macrophages as a potent pro-resolving lipid mediator (Serhan et al., 2009) and recently in human serum (Colas et al., 2014). Total organic synthesis has been achieved and the complete stereochemistry of bioactive MaR1 is established as *7R, 14S*- dihydroxydocosa-*4Z,8E,10E,12Z,16Z,19Z*-hexaenoic acid (Serhan et al., 2012). In addition to its pro-resolving actions, MaR1 also promotes tissue regeneration, relieves inflammatory neuropathic pain (Serhan et al., 2012) and shifts macrophage phenotype towards M2 (Dalli et al., 2013). In chapter one, we reported that human macrophage 12- lipoxygenase (12-LOX) is identical to platelet-type 12-LOX and established its role as the key initiating enzyme in MaR1 biosynthetic pathway (Dalli et al., 2013; Deng, Wang et al., 2014).

Given that 14-HDHA levels were reduced in LAP patients, the aim of this study was to investigate the expression of 12-LOX within the MaR1 biosynthetic pathway as well as MaR1 levels in LAP patients and its role in regulating phagocyte functions in killing periodontal pathogens.

Materials and methods

Human samples and peripheral blood cell counts

Human samples were obtained following informed consent approved by the Forsyth Institute Review Board (FIRB) (protocol number 11-05). Venous blood (~60ml, 25 units/ml heparin) was collected from patients with a diagnosis of localized aggressive periodontitis (LAP, n = 13, 4 subjects made two visits for independent experiments) according the American Academy of Periodontology (AAP) guideline (Armitage, 1999) and age and gender -matched healthy volunteers (HC, n = 17) with no signs of periodontal disease. All blood donors were otherwise healthy, non-smokers and had denied taking any non-steroidal anti-inflammatory drugs (NSAIDs) for at least two weeks prior to the experiment. Clinically, patients usually have familial aggregation and present with severe, early-onset bone loss around first molars and incisor teeth (Armitage, 1999, Gronert et al., 2004). Most of the LAP patients recruited in this study were the same cohort characterized by a hyper-responsive neutrophil phenotype (elevated LTB₄ and IL-8 induced superoxide generation) (Gronert et al., 2004). The patients were diagnosed by a licensed periodontist (H.H.) in the Center for Clinical and Translational Research at the Forsyth Institute. A group of randomly selected LAP patients with ethnicity-, age-, and gender-matched HC paired donors were submitted for complete blood count (CBC) report (Quest Lab, Boston). In addition, macrophages were prepared from peripheral blood monocytes donated from healthy human volunteers at the Children's Hospital, Boston for independent experiments including the extended dose response of MaR1 in enhancing macrophage phagocytosis and intracellular reactive oxygen species (ROS) generation.

Human neutrophil and monocyte isolation and macrophage culture

Neutrophils and PBMCs were separated from human whole blood by density-gradient Ficoll-Histopaque (Histopaque 1077& 1119, Sigma-Aldrich). Neutrophils were isolated after isotonic lysis of red blood cell contaminants followed by two washes in phosphate-buffered saline (Sigma-Aldrich). Monocytes were obtained after adhesion purification and were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (v/v) (Life Technologies) [For lipid mediator metabololipidomics, monocytes were cultured in phenol red-free RPMI 1640 medium supplemented with 10% human serum (Lonza) as in (Serhan et al., 2012)] and 20 ng/ml human recombinant GM-CSF (R&D Systems) at 37°C for 7 days to be differentiated into macrophages.

Oral bacteria preparation

Porphyromonas gingivalis (A7436, *P. gingivalis*; *P.g*) was cultured on 2% trypticase soy agar (TSA) supplemented with 2.6% Brain Heart Infusion Agar (BD BBL), 1% (w/v) yeast extract (BD BACTO), 5% defibrinated sheep RBC (Northeast Laboratory Services), 5µg/ml of hemin, and 0.5µg/ml vitamin K (Sigma-Aldrich). *Aggregatibacter actinomycetemcomitans* (ATCC Y4, *A. actinomycetemcomitans*; *A.a*), *Fusobacterium nucleatum subsp. nucleatum* (ATCC 25586, *F. nucleatum*; *F.n*), and *Streptococcus intermedius* (ATCC 27335, *S. intermedius*; *S.i*) were grown on TSA Plates (BD Diagnostic System). All cultures were placed in an anaerobic chamber (85% N₂, 10% CO₂, 5% H₂ at 37°C). Colonies were transferred from the plate to respective broth media- *P. gingivalis* to Wilkin's broth (OXOID) and the others into Brain Heart Infusion broth (BD BBL) to grow for 4 days. Bacterial titers were determined at 600nm using a

spectrometer (SmartSpec 3000, Bio-Rad) and adjusted to OD=1.0 (approximates 10^9 CFU/ ml) prior to the experiments.

Expression of 12 Lipoygenase (12-LOX) in human macrophages

Macrophages from LAP patients and HC were gently harvested and permeabilized (Cytotfix/Cytoperm; BD Biosciences). Intracellular staining was carried out using rabbit anti- human 12-LOX Ab (Novus) and Alexa-Fluor conjugated anti-rabbit Ab (Jackson) as used in (Deng et al., 2014). Expression level of 12-LOX was monitored by flow cytometry (FACSCanto II, BD Biosciences) and analyzed with FlowJo (Tree Star).

LC-MS/MS-based lipid mediator (LM) metabololipidomics

All samples for liquid chromatography (LC)-tandem mass spectroscopy (MS/MS) analysis were extracted as in (Colas et al., 2014). Briefly, the macrophage incubations were stopped by adding two volumes of ice-cold methanol with deuterium-labeled internal standards d_8 -5S-HETE, d_4 -LTB₄, d_5 -LXA₄, d_5 -RvD2 and d_4 -PGE₂ (500 pg each) to facilitate quantification and sample recovery. Supernatant was collected after protein precipitation and centrifuged before loaded onto C18 column. LMs were eluted with methyl formate, taken to dryness and resuspended in methanol/water (50:50 vol/vol), prior to injection into the LC-MS-MS system, QTrap 6500 (ABSciex) [For details, please see (Colas et al., 2014)].

MaResin 1 preparation

MaR1 was prepared by total organic synthesis as described in (Serhan et al., 2012) as part of the NIH Program Project (P01- GM095467, CNS).

Macrophage and neutrophil phagocytosis in vitro

GM-CSF-differentiated human macrophages (5×10^4 cells/ well) or neutrophils (10^5 cells/ well) were adhered onto 96-well plates and incubated with the vehicle (PBS containing calcium and magnesium, PBS^{+/+}, Sigma-Aldrich) or indicated concentration of MaR1 for 15 min, followed by incubation with fluorescent-labeled bacteria (BacLight Green, Molecular Probe) (1:50 ratio) or Fluorescent Microspheres (FluoSpheres, Molecular Probe) (1:10 ratio) for indicated time. Plates were gently washed 3 times with PBS^{+/+}, extracellular fluorescence was quenched by Trypan Blue (1:15 diluted), and phagocytosis was determined by measuring total fluorescence (Ex 493/Em525 nm) using a SpectraMax M3 fluorescent plate reader (Molecular Devices). For direct comparison of macrophage phagocytosis between LAP patients and HC, macrophages (2×10^4 cells/well) were used followed by the same conditions as described above and subjected to flow cytometry.

Macrophage and neutrophil intracellular reactive oxygen species generation

Similar to phagocytosis experiment, cells were pre-incubated with fluorescent intracellular ROS indicator carboxy-H₂DCF-DA (Reactive Oxygen Species Detection reagents; Invitrogen) for 30 min and washed. The cells were pre-incubated with vehicles (PBS^{+/+}) or indicated concentration of MaR1 for 15 min prior to addition of bacteria (1:50

ratio) for indicated time. Intracellular ROS generation was determined using a SpectraMax M3 fluorescent plate reader.

Transmission Electron Microscopy

10^6 neutrophils and 5×10^4 macrophages from LAP patients and HC were incubated with *P. gingivalis* (1:50 ratio) for 1 hour and 3 hours respectively; cells were fixed with Formaldehyde-Glutaraldehyde-Picric Acid (FGP) and submitted to Electron Microscopy Core Facility (Harvard Medical School) for further processing and analysis.

Bacterial killing with macrophages and in human whole blood

Macrophages (96-well plate, 5×10^4 / well) and whole blood (90 μ l) from HC and LAP patients were incubated with indicated concentration of MaR1 for 15min followed by addition of bacteria (1:50 and 10^7 CFU/ ml respectively) for 60 min. Supernatant or whole blood was gently mixed, diluted with PBS, and plated onto respective agar plates to determine bacterial titers. Photos were taken after 7 days and colony forming units were counted using ImageJ (NIH).

Statistical analysis

Results are expressed as mean \pm SEM. Statistical analysis was performed using a Student t test (two independent groups) and one-way ANOVA (multiple groups). A p value \leq 0.05 was considered to be significant.

Results

Macrophages from LAP patients have lower 12-lipoxygenase expression and endogenous MaR1 levels

Since 12-LOX is the key enzyme in the biosynthesis of MaR1 (16, 17) (Fig. 1A), we first investigated the expression of human 12-LOX in macrophages from LAP patients. LAP macrophages gave significantly lower 12-LOX expression (~30%) when compared to healthy control subjects (HC) (Fig. 1B). We next identified MaR1 and its biosynthetic pathway marker 14-HDHA in LAP and HC macrophage with GM-CSF incubations using LC-MS-MS based LM-metabololipidomics. Representative multiple reaction monitoring (MRM) chromatograms and MS/MS spectrum for identification of MaR1 and 14-HDHA were shown in Fig. 1C and D. We next quantified these mediators and found that 14-HDHA (625 ± 251 vs. 935 ± 217 pg/ 10^6 cells; ~30% reduction) and MaR1 (87.8 ± 50 vs. 239.1 ± 32 pg/ 10^6 cells; ~65% reduction) levels were significantly lower in LAP macrophages compared to HC (Fig. 1E). Taken together, these findings suggested that MaR1 biosynthesis was dysregulated in LAP macrophages.

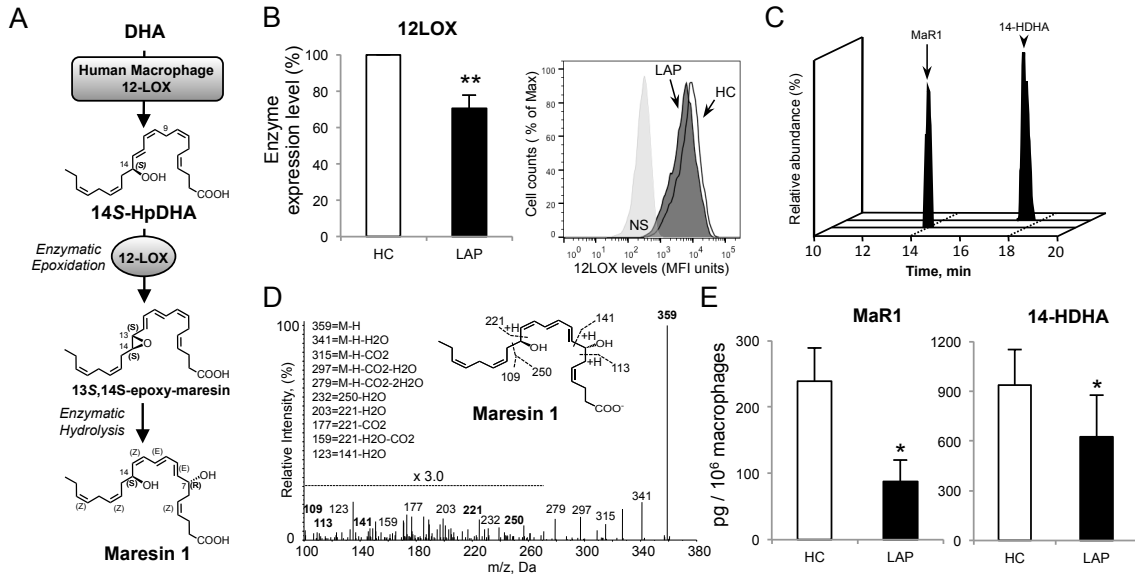


FIGURE 1. Lower 12-LOX expression and MaR1 levels in macrophages from LAP patients. **(A)** Maresin 1 biosynthetic pathway. **(B)** 12-LOX expression was determined using flow cytometry in human macrophages from LAP patients (black bar) and matched healthy controls (HC, open bar) (see methods for details). (Left) Results are expressed as percentage normalized to HC; mean \pm SEM, n=7 paired donors, **p<0.01 LAP vs. HC. (Right) representative histograms. **(C-E)** 14-HDHA and MaR1 levels in human macrophage with GM-CSF incubations determined using LC-MS-MS based LM-metabololipidomics (see methods for details). **(C)** Multiple reaction monitoring (MRM) chromatograms (MaR1 m/z 359/177, 14-HDHA m/z 343/205) and **(D)** Representative MS/MS spectrum used for the identification of MaR1. **(E)** 14-HDHA and MaR1 levels in human macrophage incubations. Results are expressed as mean \pm SEM, n=6 paired donors, *p<0.05, LAP vs. HC.

MaR1 rescued impaired phagocytosis of P. gingivalis and A. actinomycetemcomitans by macrophages from LAP patients.

Since MaR1 enhances macrophage phagocytosis of zymosan and apoptotic PMN (13, 15), we questioned whether MaR1 could enhance phagocytosis of periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*. Indeed, MaR1 dose-dependently (10pM-100nM) enhanced (12-33% above vehicle) phagocytosis of both bacteria by macrophages from healthy donors (Fig. 2A). Because macrophages from LAP patients had lower levels of MaR1, we next investigated their ability to phagocytize these periodontal pathogens. Macrophages from LAP patients gave reduced phagocytosis of *P. gingivalis* (~38% less) and *A. actinomycetemcomitans* (~40% less) compared to HC after 1 hour (Fig. 2B). When MaR1 (0.1-10nM) was incubated with macrophage from LAP patients it gave a statistically significant increase in phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* (~37% and ~65% respectively at 1nM) to comparable levels to those obtained with macrophages from HC (Fig. 2C). These results demonstrated that MaR1 enhanced macrophage phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* rescuing impaired phagocytosis in LAP macrophages.

Given the impaired phagocytosis and killing ability of macrophages from LAP patients, samples were submitted for Transmission Electron Microscopy (TEM) inspection. No obvious structural difference was found under TEM between LAP and HC macrophages. Representative TEM images of LAP macrophages after incubation with *P. gingivalis* were shown (Fig. S4).

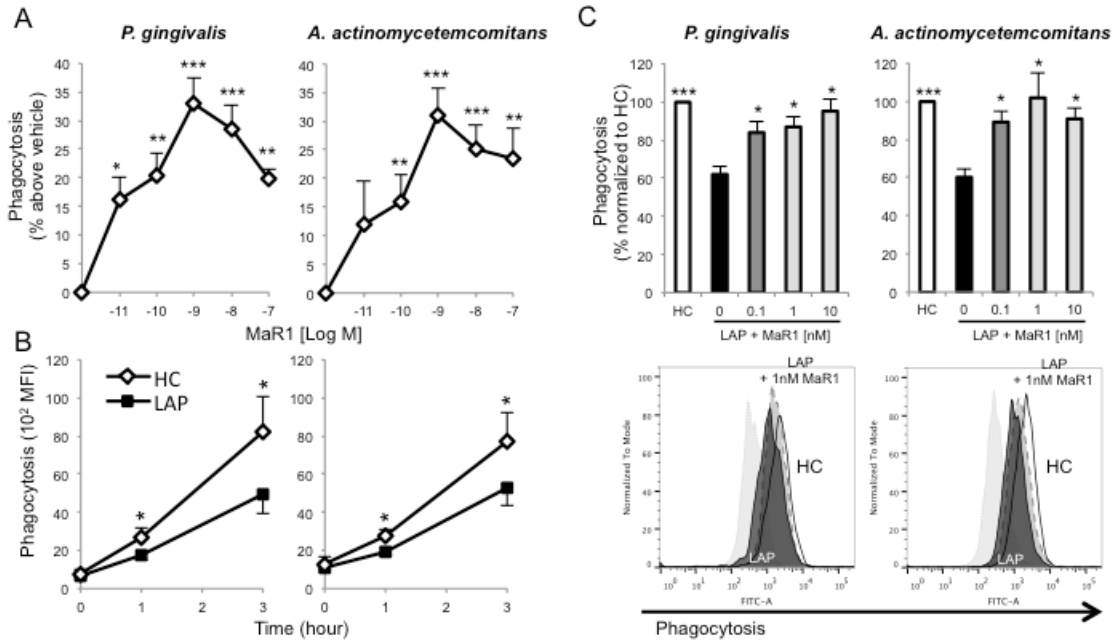


FIGURE 2. MaR1 enhanced phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* and rescued the impairment with LAP macrophages. (A) Macrophages from healthy donors (5×10^4 cells/ well) were incubated with vehicle (PBS) or MaR1 (10pM-100nM) for 15 min prior to addition of fluorescent-labeled bacteria (M ϕ : bacteria, 1:50) for 60 min. Phagocytosis was assessed using fluorescence plate reader. Results are expressed as percent increase above vehicle; mean \pm SEM, n=6-7 healthy donors, *p<0.05, **p<0.01, ***p<0.001 vs. vehicle. (B, C) Macrophages obtained from LAP patients and matched HC (2×10^4 cells/ well) were incubated with the fluorescence-labeled bacteria (1:50) for the indicated time intervals and phagocytosis was assessed using flow cytometry. (B) Results are expressed as mean fluorescence intensity (MFI); mean \pm SEM, n=4-6 paired donors, *p<0.05, HC vs. LAP. (C) Macrophages were incubated with vehicle (PBS) or MaR1 (0.1-10nM) for 15 min followed by incubation with the bacteria for 60 min. Results are expressed as percentage normalized to HC; mean \pm SEM, n=3-6 paired donors (n=6 for vehicle only and n=3-4 for MaR1 does-response with LAP macrophages), *p<0.05, ***p<0.001 vs. LAP plus vehicle only. *Insets* show representative histograms.

MaR1 enhanced intracellular anti-microbial ROS generation and rescued impaired killing of P. gingivalis and A. actinomycetemcomitans by LAP macrophages.

Since MaR1 enhanced macrophage uptake of these pathogens, we next questioned whether MaR1 also enhanced intracellular ROS production for killing following engulfment of the bacteria (Majno and Joris, 2004). To address this question, macrophages from HC and LAP patients were incubated with fluorescent intracellular ROS indicator carboxy-H₂DCF-DA followed by *P. gingivalis* and *A. actinomycetemcomitans* with or without the presence of MaR1 (see methods). Intracellular ROS levels were similar in macrophages from HC and LAP patients without bacteria (Figure 3A *Inset*). Addition of bacteria resulted in ~20-30% increase in intracellular ROS, and MaR1 (1nM) further enhanced intracellular ROS production with both HC and LAP macrophages with greater increases in incubations with *A. actinomycetemcomitans* (33% increase above vehicle) than with *P. gingivalis* (Fig. 3A and for full dose-response, see Fig. S3). These results indicated that MaR1 enhanced macrophage intracellular ROS production when incubated with oral pathogens.

Next, we determined if macrophages from LAP patients have impaired bacterial killing. Live bacteria were incubated with macrophages from HC and LAP patients and the supernatants collected and cultured to determine bacterial titers. Supernatants from LAP macrophages gave higher bacterial titers with both *P. gingivalis* (26% higher) and *A. actinomycetemcomitans* (14% higher) compared to macrophages from HC (Fig. 3B). Incubation of macrophages from HC and LAP patients with MaR1 gave a reduction in bacterial titers with both of *P. gingivalis* and *A. actinomycetemcomitans* (~39% reduction). These results demonstrated that MaR1 enhanced macrophage intracellular

ROS production and rescued the impaired killing of *P. gingivalis* and *A. actinomycetemcomitans* with LAP macrophages.

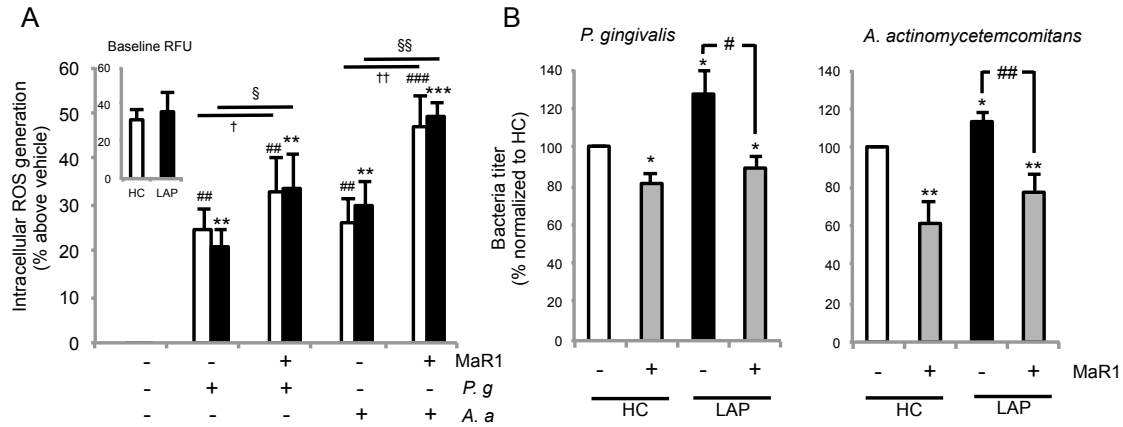


FIGURE 3. MaR1 enhanced intracellular anti-microbial ROS generation and rescued impaired killing of *P. gingivalis* and *A. actinomycetemcomitans* by LAP macrophages. **(A)** Macrophages (2×10^4 cells/ well) were pre-incubated with carboxy- H_2DCF -DA (5 μ M) for 30 min before incubation with vehicle (PBS) or MaR1 (1nM) for 15 min followed by addition of bacteria (1:50) for 60 min. Intracellular ROS generation was assessed using a fluorescence plate reader. Results are expressed as percent increase above vehicle; mean \pm SEM, n=5 paired donors, $^{##}p < 0.01$ vs. HC plus vehicle, $^{**}p < 0.01$ vs. LAP plus vehicle; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. HC in the absence of MaR1, $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ vs. LAP in the absence of MaR1. *Inset* is the relative fluorescence unit (RFU) in the absence of bacteria **(B)** Macrophages from HC and LAP patients (5×10^4 cells/ well) were incubated with vehicle or MaR1 (1nM) for 15 min followed by addition of bacteria (1:50) for 60 min. Serial dilutions of the incubations were plated onto agar plates for 7 days. Colony forming units (CFU) were counted using Image J. Results are expressed as percentage normalized to HC; mean \pm SEM, n=6 paired donors, $^*p < 0.05$, $^{**}p < 0.01$ vs. HC plus vehicle. $^{\#}p < 0.05$, $^{##}p < 0.01$ vs. LAP plus vehicle.

Neutrophils from LAP patients exhibit differential impairment in phagocytosis

We next investigated whether neutrophils from LAP patients also display impaired bacterial clearance and whether this defect is specific to certain bacteria. Incubation of LAP neutrophils with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *S. intermedius* and latex particles gave impaired phagocytosis with a slower kinetics of phagocytosis from 0-2h and also reduced maximal phagocytosis (Fig 4A). We next determined if LAP neutrophils exhibited different levels of impairment to these oral bacteria. Comparison of the kinetics of bacterial phagocytosis demonstrated that LAP neutrophils gave a 51% reduction in rate of phagocytosis of *F. nucleatum*, 32% reduction with *P. gingivalis*, 31% with *A. actinomycetemcomitans*, 23% with *S. intermedius* compared with HC neutrophils (Fig. 4B). Similar results were also obtained when we assessed the extent of phagocytosis at 60 min (Fig. 4C). We also found reduced maximal phagocytosis with LAP neutrophils giving a 46% reduction in *F. nucleatum* and ~20% reduction in the extent of phagocytosis for other bacteria assessed when compared to values obtained with HC neutrophils (Fig. 4C). These results indicated that LAP neutrophils displayed a general impairment in phagocytosis of oral bacteria tested here, yet giving different levels of reduction with different bacteria compared to HC.

Neutrophils from HC and LAP patients were also submitted for TEM examination. Representative TEM images of neutrophils during phagocytosis of *P. gingivalis* were presented (Fig. 5C). No significant structural difference was noted between the neutrophils from LAP patients and HC under TEM (Fig. S5).

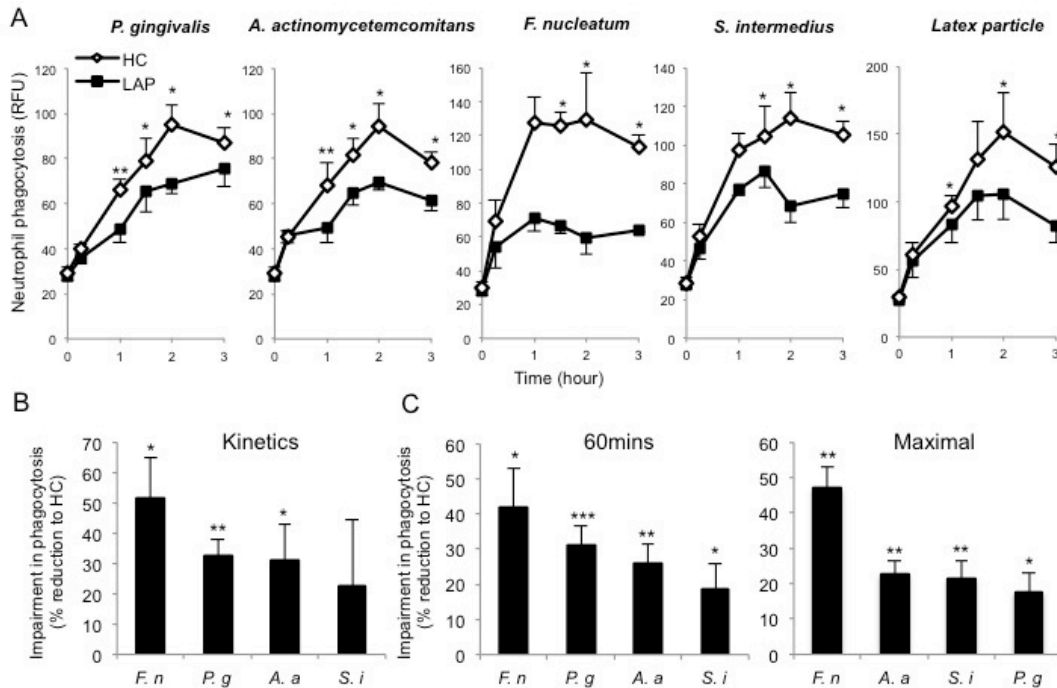


FIGURE 4. Neutrophils from LAP patients gave impaired phagocytosis of oral bacteria. **(A-C)** Isolated human PMN (10^5 cells/ well) were incubated with fluorescent -labeled *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *S. intermedius* (1:50), or latex particles (1:10) for indicated time interval. Phagocytosis was determined using fluorescence plate reader. **(A)** Results are expressed as relative fluorescence unit (RFU); mean \pm SEM, n=3-8 paired donors (n=8 for *P. gingivalis* and *A. actinomycetemcomitans* at 1 hour time point and the rest n=3-4). *p<0.05, **p<0.01, HC vs. LAP. **(B)** The rate of phagocytosis (from 0h to maximal phagocytosis) was calculated for each experiments and represented as the kinetics for each bacteria; Results are expressed as percentage of reduction compared to HC; mean \pm SEM, n=3-4 paired donors, *p<0.05, **p<0.01, HC vs. LAP **(C)** The extent of phagocytosis after 1 hour and the maximal values were obtained; Results are expressed as percentage reduction compared to HC; mean \pm SEM, n=3-8 paired donors, *p<0.05, **p<0.01, ***p<0.001, LAP vs. HC.

MaR1 rescued impaired LAP neutrophil phagocytosis of P. gingivalis and A. actinomycetemcomitans and enhanced intracellular ROS production

Next we questioned whether MaR1 could act on neutrophils and rescue the phagocytic defect with LAP patients. Incubation of neutrophils with MaR1 dose-dependently enhanced neutrophil phagocytosis of *P. gingivalis* (~42% increase above vehicle at 1nM) and *A. actinomycetemcomitans* (~46% increase above vehicle at 1nM) with both LAP and HC neutrophils (Fig. 5A) and rescued the defect with LAP neutrophils above 1pM concentrations. We next assessed whether MaR1 also regulated intracellular ROS production with neutrophils. Incubation of HC and LAP neutrophils with MaR1 gave dose dependent increase in intracellular ROS levels with both *P. gingivalis* and *A. actinomycetemcomitans* (Fig. 5B). These results demonstrated that LAP neutrophils displayed reduced phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* that was rectified by MaR1 to levels seen in neutrophils from HC and MaR1 also enhanced intracellular ROS production in both HC and LAP neutrophils.

Given the impaired phagocytosis and killing ability of macrophages and neutrophils from LAP patients were also submitted for TEM examination. Representative TEM images of neutrophils during phagocytosis of *P. gingivalis* were presented (Fig. 5C). No significant structural difference was noted for the macrophages and neutrophils from LAP patients compared to HC under TEM (Supplemental Fig. 3, 4).

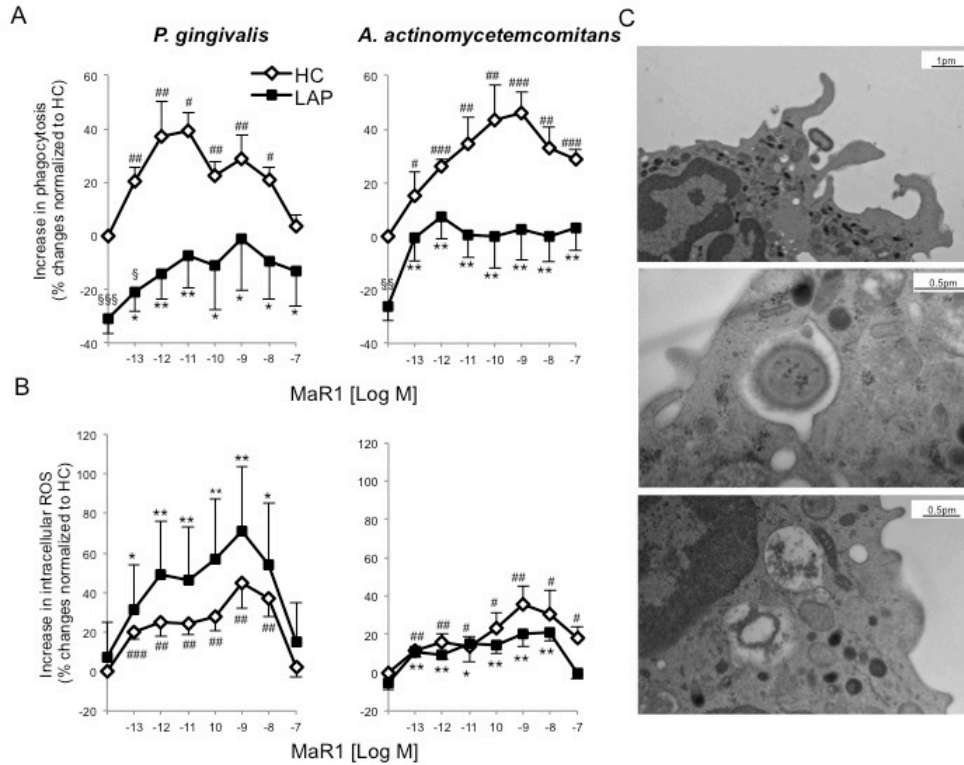


FIGURE 5. MaR1 rescued LAP neutrophil impaired phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* and enhanced intracellular ROS production. (A) Isolated human PMN (10^5 cells/ well) were incubated with MaR1 (0.1pM-100nM) or vehicle (PBS) alone for 15 min prior to addition of fluorescent-labeled bacteria (1:50) for 60 min. Phagocytosis was determined using a fluorescence plate reader. Results are expressed as percent changes normalized to HC; mean \pm SEM, n=5-6. #p<0.05, ##p<0.01, ###p<0.001 HC vs. vehicle; *p<0.05, **p<0.01 LAP vs. vehicle, §p<0.05, §§p<0.01, §§§p<0.001 LAP vs. HC plus vehicle only (B) Isolated human PMNs (10^5 cells/ well) from LAP were incubated with carboxy-H₂DCF-DA for 30 min. Vehicle or MaR1 (0.1pM-100nM) was then added to PMN for 15 min prior to addition of the bacteria (1:50) for 60 min. The intracellular ROS production was quantified using a fluorescence plate reader. Results are expressed as percent changes normalized to HC; mean \pm SEM, n=7, HC #p<0.05, ##p<0.01, ###p<0.001, LAP *p<0.05, **p<0.01; bacteria plus MaR1 vs. bacteria alone. (C) Representative TEM images of neutrophils during phagocytosis of *P. gingivalis*.

LAP whole blood had elevated leukocytes with inverse relationship with bacterial titers

LAP had elevated white blood cell counts including significantly higher neutrophils (4288±296.6 vs. 2231±807.6 cells/ μ l) and monocytes (386.8±29.9 vs. 215.3±48.6 cells/ μ l) compared with ethnicity, age and gender-matched HC (Fig. 6A, Supplemental Table 1.) Incubation of the bacteria with the whole blood gave bacterial titers (both *P. gingivalis* and *A. actinomycetemcomitans*) that had an inverse relationship with the white blood cell counts in the peripheral blood from LAP patients (Fig. 6B); while the relationship was weak in HC whole blood (data not shown). These results indicated that localized oral inflammation may have systemic impact on elevating peripheral leukocytes and the higher numbers of leukocytes gave lower bacterial titers in LAP whole blood.

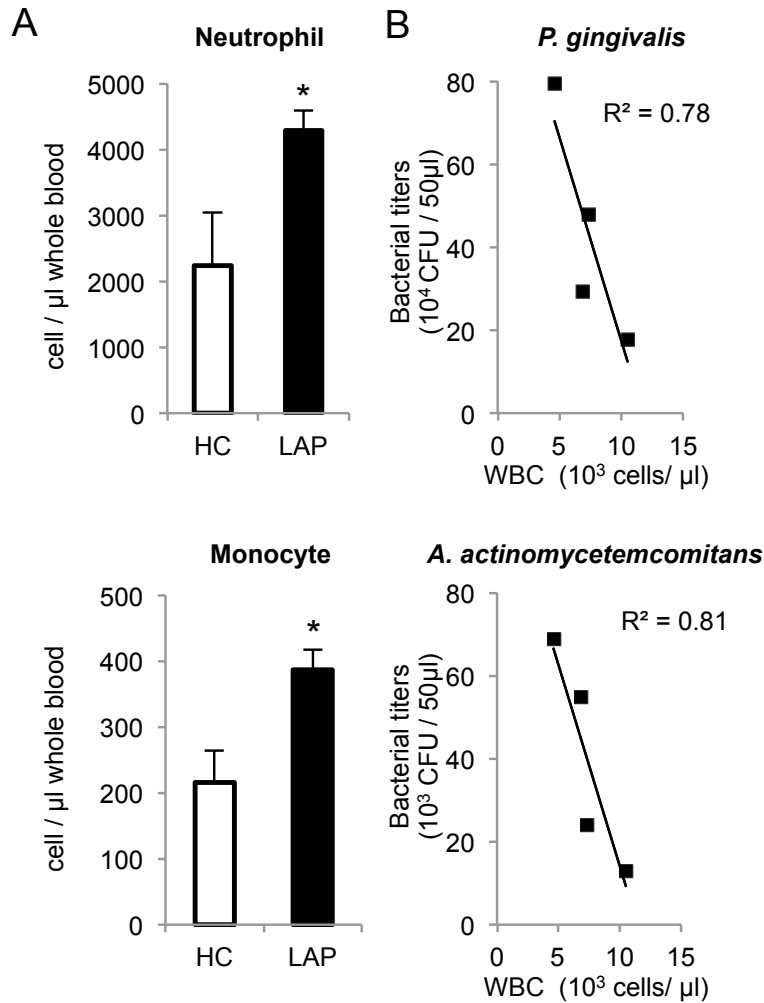


FIGURE 6. LAP whole blood had elevated leukocytes with inverse relationship with bacterial titers. **(A, B)** Peripheral whole blood was collected from LAP patient and ethnicity-age-gender matched HC **(A)** Absolute neutrophil and monocyte counts were obtained from CBC reports and results are expressed as mean \pm SEM, n=4, *p<0.05, HC vs. LAP. **(B)** LAP whole blood were incubated with bacteria (10^7 CFU/ ml) for 1 hour. Whole blood was diluted with PBS and plated on agar for 7 days. Colony forming units (CFU) were counted using ImageJ. Total WBC counts were obtained from CBC reports and results are expressed as the correlation between bacterial titers and WBC. *Insets* show the coefficient of determination (R^2).

MaR1 enhanced killing of *P. gingivalis* and *A. actinomycetemcomitans* in LAP and healthy human whole blood

Given that MaR1 rescued impaired phagocytosis and killing with isolated LAP phagocytes, we next investigated if MaR1 could enhance bacterial killing with human whole blood *ex vivo*. Incubation of HC and LAP whole blood with the bacteria for 60 min gave similar remaining bacteria titers (Fig. 7 insets) MaR1 dose-dependently (0.1nM-100nM) enhanced bacterial killing (4-32% reduction in bacterial titers) in the whole blood from LAP patients. In comparison, MaR1 at 10nM also reduced bacterial titers with whole blood from HC (37% reduction in *P. gingivalis* titers and 22% for *A. actinomycetemcomitans* at 10nM) (Fig. 7). These results demonstrated that MaR1 retained its biological actions in human whole blood and enhanced bacterial killing within nanomolar concentrations.

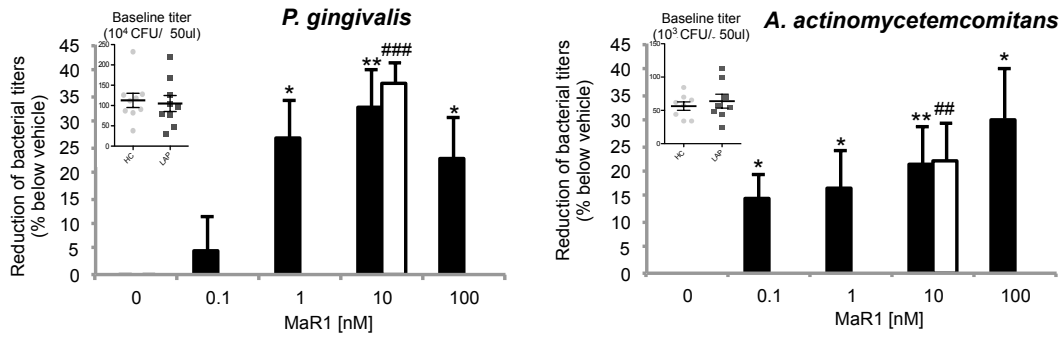


FIGURE 7. MaR1 enhanced killing of *P. gingivalis* and *A. actinomycetemcomitans* in whole blood from LAP patients and healthy volunteers. Whole blood from HC and LAP patient was incubated with vehicle (PBS) or MaR1 (0.1-100nM) for 15 min followed by bacteria (10^7 CFU/ml) for 1 hour. Whole blood was diluted with PBS and plated on agar for 7 days culture. Colony forming units (CFU) were counted using ImageJ. Results are expressed as percent reduction of bacteria titers compared to vehicle; mean \pm SEM, n=4-9 (n=9 for 10nM; n=4-6 for other concentrations), *p<0.05, **p<0.01 vs. LAP vehicle; ###p<0.01, ####p<0.001 vs. HC vehicle. *Insets* demonstrate bacterial titers at baseline with vehicle alone.

Discussion

In the present chapter, we investigated the biosynthesis of MaR1 in LAP patients with LM metabololipidomics and its role in regulating phagocyte functions in killing periodontal pathogens. Results from these experiments demonstrate that LAP patients have dysregulated endogenous MaR1 biosynthesis and that their phagocytes (neutrophils and macrophages) display an impaired ability to phagocytize and kill periodontal pathogens (*P. gingivalis* and *A. actinomycetemcomitans*). Exogenous MaR1 rectified this impairment in LAP patients increasing phagocyte ingestion and killing of the periodontal pathogens. MaR1 also enhanced these functions of phagocytes from healthy controls.

Maresin 1 biosynthesis in human was recently investigated by our group using recombinant human macrophage 12-LOX enzyme and synthetic intermediates (Dalli et al., 2013, Deng, Wang et al., 2014). It was confirmed that 12-LOX as the key initiating enzyme converting docosahexaneic acid (DHA) to 14*S*-hydroperoxy-docosahexaneic acid (14*S*-HpDHA) intermediate (Deng et al., 2014). 14*S*-HpDHA then goes through 12-LOX-mediated epoxidation to 13*S*, 14*S*-epoxy- docosahexaneic acid (13, 14-epoxy-maresin; 13,14-eMaR) and further converted to MaR1 by human macrophage (Dalli et al., 2013) (Fig. 1A). 14-HDHA is a marker for the maresin pathway reduced from 14-HpDHA. In this study we found that macrophages from LAP patients express lower levels of 12-LOX (70%) and a reduced production of 14-HDHA (69%) and MaR1 (37%) levels compared to healthy control donors (Fig. 1B, 1E). The lower levels of 14-HDHA and MaR1 in macrophages from LAP patients corresponded with lower levels of 12-LOX enzyme expression. These results suggested that patients with localized aggressive

periodontitis have dysregulated MaR1 biosynthesis.

MaR1 enhances macrophage phagocytosis of zymosan and apoptotic PMN (Serhan et al., 2009; Serhan et al., 2012), in the present study, we assessed if MaR1 could enhance phagocytosis of periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*. We found MaR1 enhances phagocytosis of both bacteria and this leads to the next question if LAP macrophages with lower levels of MaR1 give impaired phagocytosis of these pathogens. Earlier it was discovered that LAP neutrophils display defective functions in phagocytosis of *Staphylococcus aureus* (Cainciola et al., 1977, Van Dyke et al., 1986). Recently, macrophages from LAP patients were also found to display impaired phagocytosis of zymosan (Fredman et al., 2011). It is unknown whether these phagocytes also give reduced phagocytosis of periodontal pathogens. In this study, we found an impaired phagocytosis of the periodontal pathogens (*P. gingivalis* and *A. actinomycetemcomitans*) by phagocytes (neutrophils and macrophages) from LAP patients (Fig. 2B, 4). In addition, exogenous MaR1 rescued the impaired phagocytosis of both periodontal pathogens with LAP macrophages and neutrophils (Fig. 2C, 5A). However, it is now appreciated that periodontal disease is a polymicrobial infection modulated by host response (Darveau, 2010). Besides *P. gingivalis* and *A. actinomycetemcomitans*, there is growing knowledge about the potential role of other commensal oral bacteria in the biofilm. *S. intermedius* is a gram positive, facultative anaerobic commensal oral bacteria that have antagonistic relationship to periodontal pathogens like *P. gingivalis* and *A. actinomycetemcomitans* (previously known as *Bacteroides gingivalis* and *Actinobacillus actinomycetemcomitans* in the reference) (Hillman et al., 1985). Additionally, previously neglected *F. nucleatum* have been

recently uncovered as a potential target pathogen (Han, 2015). *F. nucleatum* is also prevalent in the deep periodontal pockets of LAP patients (Oettinger-Barak et al., 2014) and its number is associated with the severity of periodontal inflammation in adolescents (Yang et al., 2014). In addition, *F. nucleatum* is essential for the growth of the periodontal pathogens (Bradshaw et al., 1998) and can synergistically give stronger inflammatory response and tissue destruction when co-infection with *P. gingivalis* in a mouse periodontitis model (Polak et al., 2009). In current study, we investigated LAP neutrophil phagocytosis of four oral bacteria and compared with the levels obtained from HC. We demonstrated that the LAP neutrophils give different levels of impairment for each bacteria and the rank order for the extent of phagocytosis at 60 min resemble the rate of kinetics in phagocytosis. The most prominent defect is in the extent of phagocytosis of *F. nucleatum* (38% reduction), followed by *P. gingivalis* (31% reduction), *A. actinomycetemcomitans* (26% reduction), and giving the least for *S. intermedius* (21% reduction) compared to HC after one hour incubation (Fig. 4B). It was reported that LAP neutrophils give 10-20% reduced phagocytosis of *Staphylococcus aureus* within one hour (Van Dyke et al., 1986). The reduction in maximal phagocytosis with LAP neutrophils is also about 20% except *F. nucleatum* is 46%. The results from the differential impairment in phagocytosis with neutrophils from LAP patients suggest that *F. nucleatum* may play an important role and this imbalanced kinetics in patients with localized aggressive periodontitis may postulate a potential mechanism for host-mediated disruption of the biofilm homeostasis and dysbiosis (Hajishengallis, 2014). Of note, MaR1 also enhanced macrophage phagocytosis of *F. nucleatum* (~30%, 1nM) (Fig. S6).

Following phagocytosis, intracellular ROS generation is critical for oxidative bacterial

killing within the phagocytes (Majno and Joris 2004). In the present study, we reported that MaR1 enhanced phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* and also increased intracellular ROS generation with phagocytes from HC and LAP reaching maximal effect at 1nM consistently for both functions. In addition, LAP neutrophils and macrophages produced similar levels of intracellular ROS compared to HC when incubating with both bacteria. However, in the same condition, fewer bacteria were ingested into the LAP phagocytes indicating LAP phagocytes may give a hyper-responsive intracellular ROS generation for bacterial killing. After incubating with MaR1, HC neutrophils enhanced similar levels of intracellular ROS for both bacteria yet LAP neutrophils enhanced lower levels of intracellular ROS with *A. actinomycetemcomitans* and higher levels for *P. gingivalis*. Earlier it was observed that LAP neutrophils have an impaired intracellular killing of *A. actinomycetemcomitans* (Kalmar et al., 1987). In this study, LAP neutrophils give similar intracellular ROS production for *A. actinomycetemcomitans* compared to HC but was enhanced by MaR1 to a lower extent. In addition, uncontrolled intracellular ROS production may result in leakage to extracellular ROS causing collateral tissue damage and indeed LAP neutrophils release higher levels of extracellular ROS after activation (Gronert et al., 2004), which is counter-regulated and reduced by Resolvin E1 (RvE1), another family of pro-resolving lipid mediators from eicosapentaenoic acid (EPA) (Hasturk et al., 2006). In addition, topical RvE1 resolves inflammation and restores tissue homeostasis in a rabbit *P. gingivalis*-infected periodontitis model (Hasturk et al., 2007).

Another finding in the present investigation is that neutrophil and monocyte counts are elevated in LAP patients' whole blood compared to HC (Fig. 6A). Given that all the LAP

patients recruited are African descendants, the white blood cell counts for ethnicity-matched healthy controls are within the reported range, which is lower than seen in other population (Lim et al., 2010; Beutler et al., 2005). This suggested that localized oral inflammation might have a systemic impact on elevating peripheral leukocyte numbers, which observation is also evident in patients with generalized aggressive periodontitis (Gaddale et al., 2014). In addition, LAP patients recruited in this study had a trend to have lower red blood cell (RBC) counts and larger mean corpuscular volume (MCV) resembling an anemia condition (Supplemental Table 1). However, it was reported that African Americans normally have higher values for MCV (Beutler et al., 2005). Given that there were more neutrophils and monocytes in the whole blood from LAP patients (Fig. 6A) and the same volume of whole blood gave similar bacterial killing capacity as seen in HC (Fig 7 insets); it may suggest a lower ability on a per leukocyte basis to clear bacteria in LAP whole blood. Of note, we found that MaR1 retained its biological actions and enhanced bacterial killing when added to whole blood from HC and LAP patients (Fig. 7). Since viable *A. actinomycetemcomitans* and *P. gingivalis* were identified in human atherosclerotic plaque (Kozarov et al., 2005), these periodontal pathogens have been implied to involve in the development of systemic inflammatory diseases like cardiovascular disease and arthritis through the circulation (Hajishengallis, 2014, Kozarov et al., 2005; Genco and Van Dyke, 2010) and thus the implication from these results may be extended for further investigation in intercepting the impact of oral infection to systemic diseases (Genco and Van Dyke, 2010).

Conclusion

Taken together, we identified functional defects in LAP phagocytes with impaired bacterial containment and killing along with reduced MaR1 levels in the macrophages. Administration of MaR1 corrected this defect, enhancing phagocytosis and killing of periodontal pathogens as well as stimulating intracellular ROS generation for bacterial killing. In addition, these results suggest that MaR1 biosynthetic pathway may underlie some of the pathogenesis in LAP and that potentially therapeutics targeting the maresin pathway may have clinical application in treating localized aggressive periodontitis and other oral diseases associated with bacterial infection and uncontrolled inflammation.

Chapter 3

The regulation of leukocyte-platelet aggregation in human whole blood by MaR1

Introduction

Platelet-leukocyte aggregates form when platelets are activated and it is a sign of systemic vascular inflammation (Michelson et al., 1996). Increased circulating monocyte-platelet and neutrophil-platelet aggregates have also been reported in numerous other diseases, including diabetes mellitus, cystic fibrosis, asthma, pre-eclampsia, migraine, systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel disease (Michelson et al., 2007). These chronic excessive aggregates may be regarded as a crucial pathophysiological mechanism linking inflammation and thrombosis (Michelson et al., 1996). Furthermore, monocyte-platelet aggregates have been suggested as early markers of acute myocardial infarction (Furman et al., 2001) and type 2 diabetes mellitus (Patko et al., 2012).

The formation of leukocyte-platelet aggregates is mediated through several adhesion molecules. The initial adhesion of platelets to neutrophils and monocytes occurs via platelet surface P-selectin binding to its constitutively expressed leukocyte counter-receptor, P-selectin glycoprotein ligand 1 (PSGL-1). Stabilization of leukocyte-platelet aggregates occurs via the binding of leukocyte surface Mac-1 (also known as CD11b/CD18) to platelet surface glycoprotein Ib (GPIb) (de Gaetano et al., 1999).

Excessive platelet-leukocyte aggregation was also reported in patients with LAP (Fredman et al., 2011) and may be a potential link between oral infection and systemic inflammation. Although several SPMs have been shown to reduce adhesion molecules (Fiore et al., 1995; Filep et al., 1999; Dona et al., 2008; Oh et al., 2012) and PMN aggregation (Fiore et al., 1995), little is known if SPMs or MaR1 regulate leukocyte-platelet aggregation. The aim of this study is to investigate the role of MaR1 in regulating leukocyte-platelet aggregations and if MaR1 could reduce these aggregates in LAP whole blood.

Materials and methods

Human whole blood

Fresh peripheral venous blood (10 U/mL sodium heparin) was collected from healthy nonsmoking volunteers for Platelet-activating factor (PAF) induced leukocyte-platelet aggregation. Informed consent was obtained from each volunteer in accordance with the Declaration of Helsinki. The protocol was approved by the Partners Human Research Committee Protocol (1999P001297). Additional whole blood were collected from patients with a diagnosis of localized aggressive periodontitis (LAP) and age/ gender - matched healthy volunteers (HC) with no signs of periodontal disease following informed consent under a Forsyth Institute Review Board (FIRB, no. 11-05). All the participants denied taking any drugs for at least 2weeks before the experiments.

Oral bacteria preparation

Porphyromonas gingivalis (A7436, *P. gingivalis*; *P.g*) was cultured on 2% trypticase soy agar (TSA) supplemented with 2.6% Brain Heart Infusion Agar (BD BBL), 1% (w/v) yeast extract (BD BACTO), 5% defibrinated sheep RBC (Northeast Laboratory Services), 5ug/ml of hemin, and 0.5µg/ml vitamin K (Sigma-Aldrich). Cultures were taken placed in an anaerobic chamber (85% N₂, 10% CO₂, 5% H₂ at 37°C). Colonies were transferred from the plate to Wilkin's broth (OXOID) to grow for 4 days. Bacterial titers were determined at 600nm using a spectrometer (SmartSpec 3000, Bio-Rad) and adjusted to OD=1.0 (approximates 10⁹ CFU/ ml) prior to the experiments.

Incubation of MaR1 with healthy and LAP whole blood and stimulus for aggregation

Heparinized human whole blood was incubated *ex vivo* (30 min, 37°C) with vehicle alone (saline, Teknova) or increasing concentrations of MaR1 (1-100nM) then activated with PAF (100nM) or *P. gingivalis* (10^7 CFU/ ml) (30 min, 37°C) before determining the levels of the leukocyte-platelet aggregation (See below).

Determination the levels of leukocyte-platelet aggregates and adhesion molecules in human whole blood

Flow cytometry analysis was performed after cell staining with select antibodies and RBC lysed (RBC Lysis Buffer, Biolegend). Anti-human CD14 (APC/Cy7, Biolegend) was used to identify monocyte populations and CD16 (PE) or CD18 (PE, Biolegend) for PMNs together with the cell morphology (forward and side scatter). Aggregation levels were determined by the mean of MFI for CD41 (FITC, Biolegend) within double positive cells, PMN ($CD16^+ CD41^+$) and monocyte ($CD14^+ CD41^+$) aggregates. Adhesion molecule expression levels are determined with the MFI of CD18 and CD62P (P-selectin) (APC, Biolegend) on the aggregates. CD11b (FITC), L-selectin (APC, Biolegend) were also examined.

Statistics

All the results were expressed as mean \pm SEM. One- way ANOVA and student t-test were carried out to determine the difference between groups. $p < 0.05$ was considered significant.

Results

MaR1 counter-regulated PAF-induced leukocyte-platelet aggregation and adhesion molecules in human whole blood

MaR1 dose-dependently (1-100nM) attenuated PAF-activated neutrophil-platelet and monocyte-platelet aggregation in human whole blood reaching the maximal effect at 100nM (Fig. 1A). PAF at 100nM induced higher levels of monocyte-platelet aggregates than neutrophils (>60% vs. 40%). MaR1 had higher potency in counter-regulating monocyte-platelet aggregation and completely abolished the stimulation from PAF at 100nM (Fig. 1A). Within the aggregates, P-selectins were highly up-regulated by 100nM PAF stimulation (4 times higher above the vehicle), whereas CD18 was differentially regulated in different cell types (75% increase on neutrophils, 18% on monocytes above vehicle). MaR1 at 1nM marginally induced higher expression of the adhesion molecules in neutrophil-platelet aggregates yet the levels of aggregation remained stable. MaR1 (>10nM) reduced the levels of both CD18 and P-selectin within the aggregates.

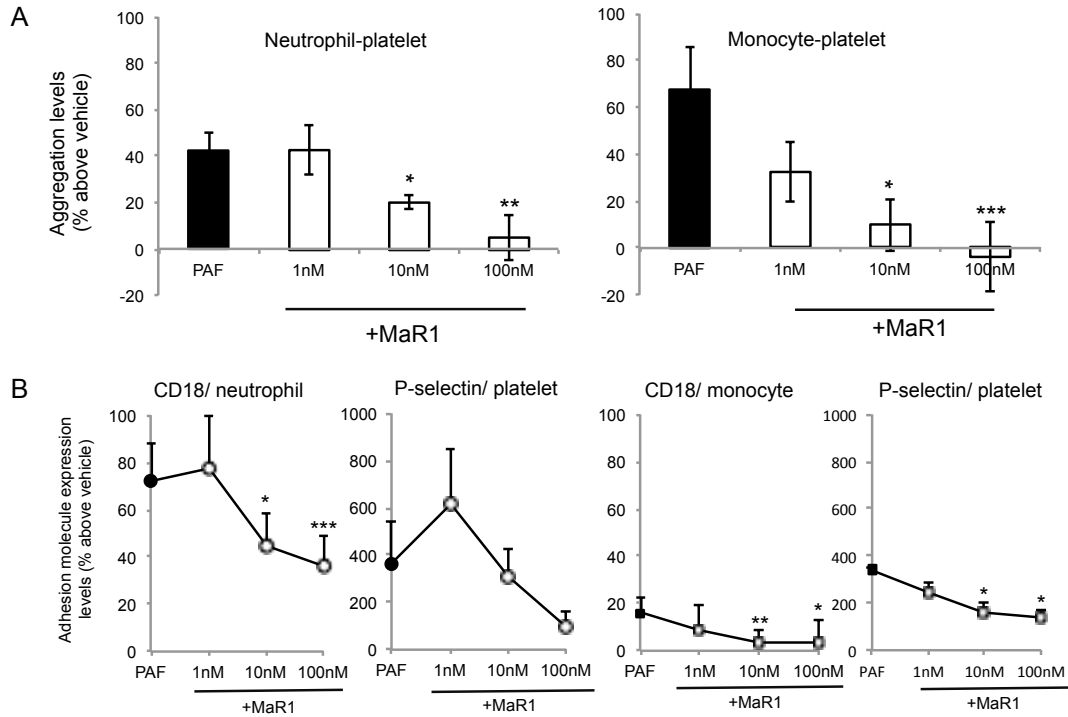


Figure 1. MaR1 counter-regulated PAF-induced neutrophil/monocyte-platelet aggregates and adhesion molecules in human whole blood. Heparinized human whole blood was incubated *ex vivo* (30 min, 37°C) with vehicle alone (saline) or increasing concentrations of MaR1 then activated with PAF (100nM, 30 min, 37°C). Flow cytometry analysis was performed after staining with select antibodies and RBC lysed. CD14 was used to identify monocyte populations and CD18 for PMNs. **(A)** Aggregation levels were determined by MFI of CD41 on double positive cells, PMN (CD16⁺ CD41⁺) and monocyte (CD14⁺CD41⁺)-aggregates. **(B)** Adhesion molecule expression levels were determined with the MFI of CD18 and CD62P (P-selectin) within the aggregates. Results are expressed as mean± SEM, percent above vehicle, n=3-4 healthy separate donors. *p<0.05, **p<0.01, ***p<0.001, PAF plus MaR1 vs. PAF alone.

MaR1 counter-regulated P. gingivalis -induced monocyte-platelet aggregation in LAP and healthy human whole blood

After incubating of *P. gingivalis* with the whole blood at 10^7 CFU/ ml for one hour, it induced about 30% increase in monocyte-platelet aggregation in HC whole blood and 21% in LAP whole blood. Incubation of MaR1 (1-100nM) with the whole blood for 30 min attenuated the aggravation from *P. gingivalis* with a different dose response in LAP and HC whole blood. In HC whole blood, MaR1 had no effect at 1nM but almost abolished the induction from *P. gingivalis* at 10nM and reduced about 23% of the excess aggregates at 100nM. In LAP whole blood, starting as low as 1nM, MaR1 exert high potency in reducing the aggregates and the effect maintained up to 100nM (Fig. 2).

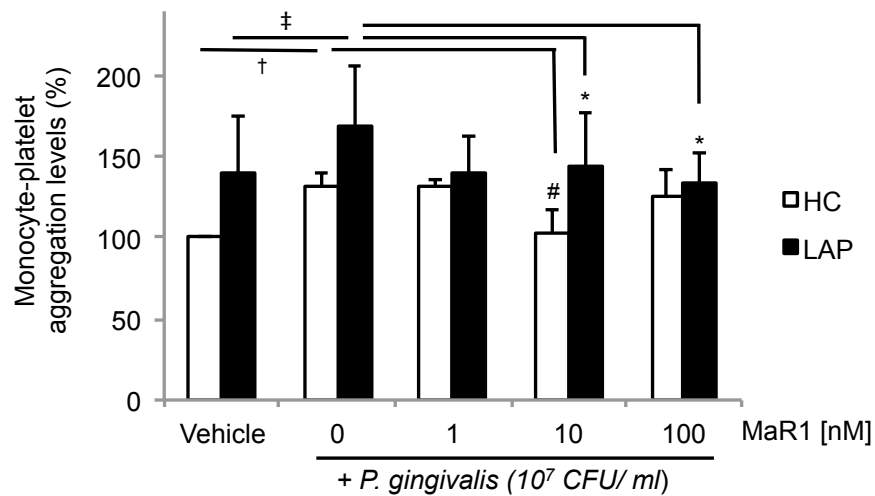


Figure 2. MaR1 attenuated *P. gingivalis* – stimulated monocyte-platelet aggregation in healthy and LAP whole blood. Heparinized LAP and HC whole blood was incubated *ex vivo* (30 min, 37°C) with vehicle alone (saline) or increasing concentrations of MaR1 then activated with *P.gingivalis* (10⁷ CFU/ml, 30 min, 37°C). Flow cytometry analysis was performed after staining with select antibodies and RBC lysed. CD14 and cell morphology was used to identify monocyte populations. Aggregation levels were determined by MFI for CD41 within double positive (CD14⁺CD41⁺) aggregates. Results are expressed as mean± SEM, percent normalized to HC with vehicle alone, n=3-4 paired donors. HC †p<0.05, LAP ‡p<0.05, *P. gingivalis* vs. vehicle alone; HC #p<0.05, LAP *p<0.05, *P. gingivalis* plus MaR1 vs. *P. gingivalis* alone.

MaR1 reduced excessive leukocyte-platelet aggregates in LAP whole blood

Peripheral blood collected from patients with LAP had elevated leukocyte-platelet aggregation compared to HC. Both neutrophil-platelet and monocyte-platelet aggregates were about 40% higher in LAP whole blood compared to HC (Fig. 3 *Insets*). Adding exogenous MaR1 (1-100nM) with LAP whole blood dose-dependently reduced excessive leukocyte-platelet aggregates (40-60%) giving the maximal effect at 10nM (Fig. 3).

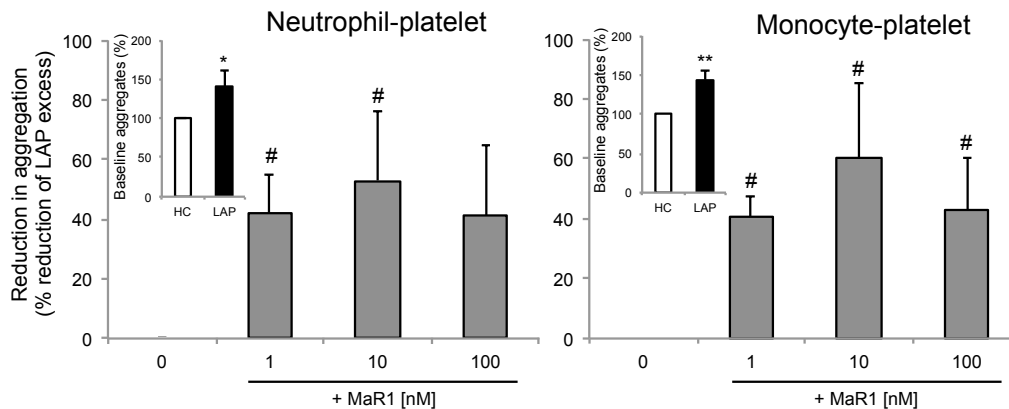


Figure 3. MaR1 reduced excessive leukocyte-platelet aggregates in LAP whole blood.

Heparinized LAP whole blood was incubated *ex vivo* (60 min, 37°C) with vehicle alone (saline) or increasing concentrations of MaR1. Flow cytometry analysis was performed after staining with select antibodies and RBC lysed. CD14 was used to identify monocyte populations and CD16 for PMNs. Aggregation levels were determined by MFI of CD41 within double positive cells, PMN (CD16⁺ CD41⁺) and monocyte (CD14⁺CD41⁺) aggregates. *Insets* show aggregation levels with vehicle alone. Results are expressed as mean± SEM, percent above vehicle, n=4 paired donors.

*p<0.05, HC vs. LAP; #p< 0.05 vs. vehicle alone.

Discussion

In the current study, we demonstrated the role of MaR1 in regulating leukocyte-platelet aggregation in human whole blood at nano-molar (nM) concentrations. Incubation of MaR1 with the whole blood attenuated the effect of PAF and *P.gingivalis*-induced leukocyte-platelet aggregations. In addition, MaR1 reduced the higher levels of aggregates in LAP whole blood. These results demonstrated the potent function of MaR1 in regulating leukocyte-platelet aggregation in human whole blood and its potential therapeutic use in reducing aggregates in LAP patients or other systemic diseases associated with elevated levels.

Earlier it was discovered that LXA4, the first family in SPMs could inhibit the levels of CD11b and homotypic PMN aggregation after FMLP-stimulation (Fiore et al., 1995). Later with LXA4 analog, it counter-regulated the increase in CD18 expression after PAF induction (1 μ M, 30min) (Filep et al., 1999). It was also demonstrated that RvE1 has the highest potency at 30nM in regulating the shredding of L-selectin (CD62L) and reducing the expression of CD18 from 7- 30 minutes with increasing effects on both neutrophil and monocyte-platelet aggregates (Dona et al., 2008). Later, too, RvE2 dose-dependently attenuated the up-regulation of CD18 after PAF stimulation (50nM, 15min) (Oh et al., 2012). The results from current study demonstrated that MaR1 counter-regulated the adhesion molecules after PAF (100nM, 30min) stimulation (Fig. 1). Given the different concentrations and activation time from PAF, the potency between these SPMs in regulating adhesion molecules cannot be compared.

In terms of the effect of SPMs in regulating leukocyte-platelet aggregation, the aspirin-

triggered lipoxin A4 analog, decreases both neutrophil-platelet and neutrophil-neutrophil adhesions in human whole blood (Filep et al., 2005). Recently, it was also shown that this stable analog could also regulate neutrophil-platelet aggregation and attenuates acute lung injury in mice (Ortiz-Munoz et al., 2014). LXA4 was also shown to inhibit *P. gingivalis*-induced aggregation in a mice model (Borgeson et al., 2011). Results from this study uncovered a new action of MaR1 in regulating both neutrophil-platelet and monocyte-platelet aggregation and attenuated *P. gingivalis*- aggravated aggregation. In addition, LAP patients have elevated levels of aggregates yet the cause is unknown and may require further investigation. Of note, MaR1 also reduced the aggregates in LAP whole blood in a dose-dependent manner (1-100nM).

Conclusion

Results from this study demonstrated that MaR1 counter-regulated leukocyte-platelet aggregation with two different stimuli (PAF and *P. gingivalis*). In addition, MaR1 regulated the expression levels of surface adhesion molecules (CD18 and CD62P) that are essential for aggregation. Patients with localized aggressive periodontitis patients had elevated leukocyte-platelet aggregation that could be reduced by MaR1. The present study provided additional evidence for the potential therapeutic use of MaR1 in human whole blood in regulating leukocyte-platelet aggregates that may be important in intercepting oral infection spread and treating cardiovascular inflammation.

CONCLUSIONS AND FUTURE DIRECTIONS

The overarching goal of these studies was to investigate the biosynthetic pathway of MaR1 in human macrophage and its novel bioactions with leukocytes and oral pathogens using localized aggressive periodontitis (LAP) as a model disease. The central hypothesis of the thesis tested was that ***“There is a defect in the leukocyte maresin biosynthetic pathway in localized aggressive periodontitis (LAP) patients and reduced maresin production contributes to the abnormal leukocyte functions and excessive platelet leukocyte aggregation in this disease.”*** Three specific aims and different approaches were conducted to address this hypothesis. In chapter one, we studied the biosynthesis of MaR1 in human macrophage and established that 12-LOX is the key-initiating enzyme converting DHA into 14S-HpDHA. Soluble epoxide hydrolase (sEH) was tested for the enzymatic hydrolysis of eMaR producing a novel anti-inflammatory and pro-resolving MaR2 which could also be identified endogenously in macrophage mediators metabolome. To this end, a future search for the hydrolase enzyme responsible for the formation of MaR1 is still of interest and synthetic MaR2 will be needed to confirm its chirality and explore its specialized role and actions in maresins metabolome.

In chapter two, we investigated this maresin pathway in LAP patients and uncovered that LAP macrophage expressed lower 12-LOX levels and produced significantly lower levels of MaR1. Both LAP neutrophils and macrophages display impaired phagocyte functions to periodontal pathogens (*P. gingivalis* and *A. actinomycetemcomitans*). Exogenous MaR1 rectified this defect by enhancing phagocytosis, intracellular ROS production, and killing of these pathogens. It also further enhanced these functions in

phagocytes from healthy donors. In addition, MaR1 retained its biological actions and enhanced bacterial killing in human whole blood which may have extended clinical implications involving therapeutics for bacteremia and sepsis which could be further explored. Future directions may also involve the efferocytosis equation in resolution, examining the apoptotic LAP neutrophils and investigate the potential link between the defective DAG kinase lipid signaling (Tyagi, Van Dyke et al., 1992; Gronert et al., 2004; Batista et al., 2014) with these impaired phagocyte functions. The imbalanced kinetics in LAP neutrophils phagocytosis of the oral bacteria postulate a potential mechanism for host-mediated disruption of biofilm homeostasis would require further investigation in a poly-microbial infection model.

In chapter three, we demonstrated that MaR1 counter-regulated PAF and *P. gingivalis* induced leukocyte-platelet aggregation in the whole blood along with regulating the adhesion molecules on the aggregates. LAP patients had elevated leukocyte-platelet aggregation levels and could be reduced by MaR1 within nanomolar concentrations, which is similar to the dose-response in enhancing bacterial killing in human whole blood. These are new actions for MaR1 in human whole blood that are related to bacteremia and cardiovascular inflammation. Future directions may include studies on the mechanistic relationship between the formation of the aggregates and *P. gingivalis* as well as the clearance of leukocyte-platelet aggregates *in vivo*.

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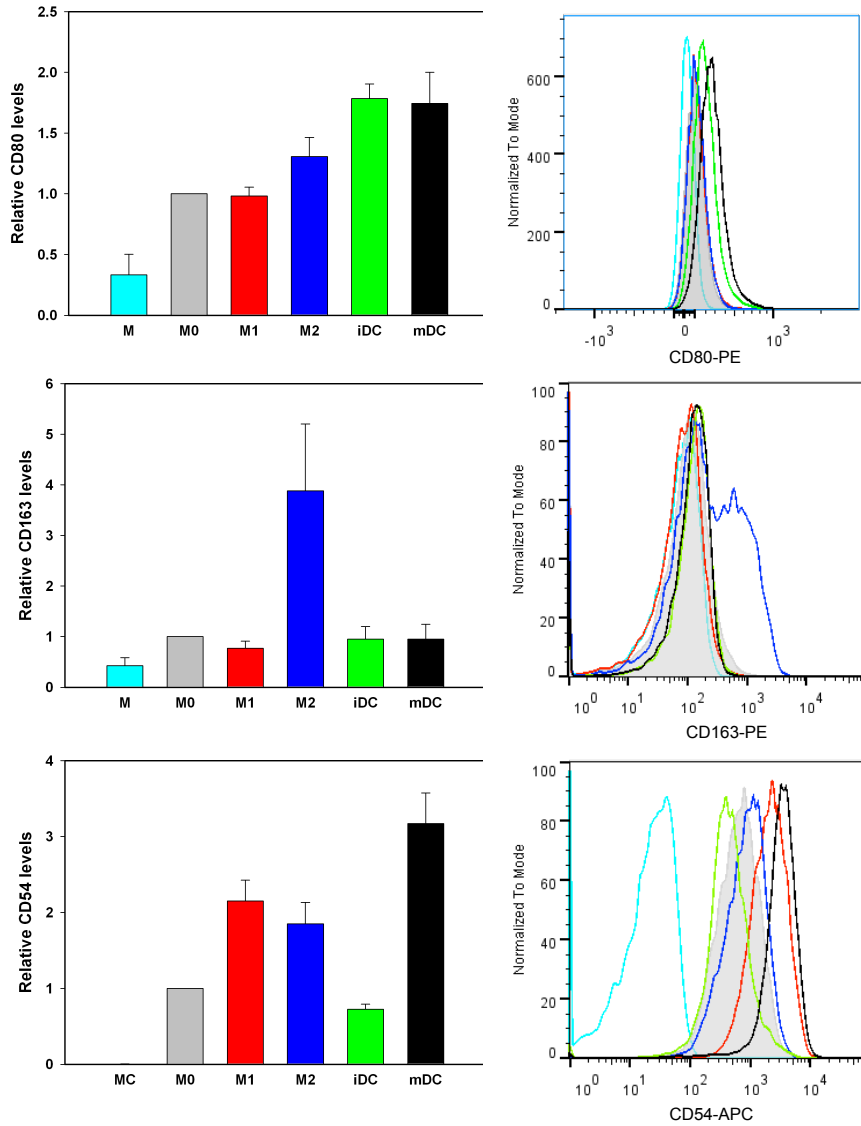
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APPENDIX SUPPLEMENTAL DATA

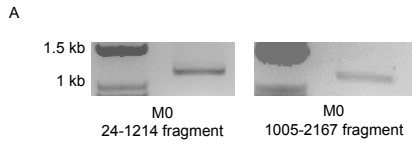
Supplemental Table 1. Complete blood count report for LAP patients and ethnicity/age/gender-matched HC

	HC		LAP		Unit	P-value
	Mean	±SE	Mean	±SE		
WBC	4.7	±1.2	7.3	±0.6	10 ³ / ul	0.089
RBC	5.2	±0.1	4.7	±0.3	10 ³ / ul	0.074
Hemoglobin	14.1	±0.3	13.6	±1.1	g/ dL	0.306
Hematocrit	42.9	±0.8	42.0	±2.7	%	0.348
MCV	82.8	±1.8	90.1	±1.0	fl	*0.026
MCH	27.2	±0.8	29.2	±0.7	pg	0.090
MCHC	32.8	±0.3	32.5	±0.4	g/ dL	0.276
RDW	14.5	±0.3	13.6	±1.2	%	0.247
Platelets	220.3	±29.1	289.5	±27.3	10 ³ / ul	0.141
MPV	9.4	±0.8	9.3	±0.5	fl	0.458
Neutrophils	47.7	±4.8	58.1	±4.2	%	*0.031
	2231.3	±807.6	4288.3	±296.6	cells/ ul	*0.026
Lymphocytes	44.1	±5.1	34.1	±0.9	%	0.087
	2117.5	±607.8	2506	±424.1	cells/ ul	0.347
Monocyte	4.8	±0.7	5.5	±0.2	%	0.248
	215.3	±48.6	386.8	±29.9	cells/ ul	*0.049
Eosinophils	2.8	±0.7	1.8	±0.4	%	0.192
	131.5	±35.8	117	±29.9	cells/ ul	0.378
Basophils	0.7	±0.2	0.5	±0.2	%	0.320
	29.0	±10.9	27.5	±2.0	cells/ ul	0.457

*Footnote: Complete blood count reports from age/ gender/ ethnicity-matched HC and LAP donors. Results are expressed as mean ± SEM, n=4 *p<0.05, HC vs. LAP. MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration; RDW= red cell distribution width; MPV= mean platelet volume.



Supplemental Figure 1. Peripheral blood mononuclear cells (PBMC) were isolated from human whole blood by density gradient centrifugation using Histopaque-1077 and Phenotypic differentiations were obtained by culture as described in methods. Each lineage was confirmed by flow- cytometry using the surface markers. Results are representative of n=4. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

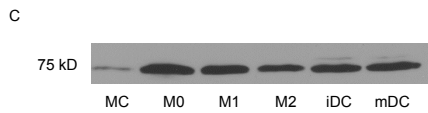


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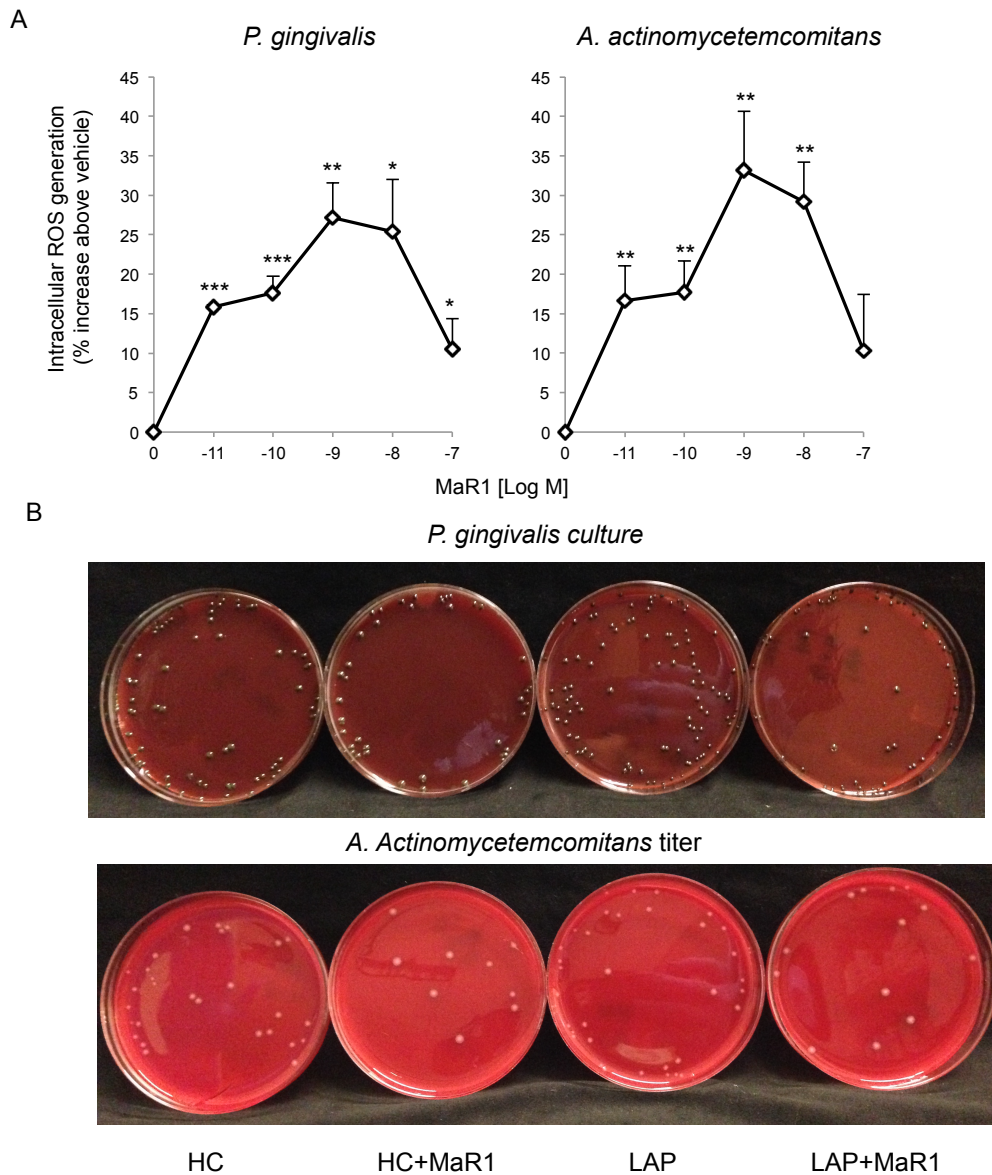
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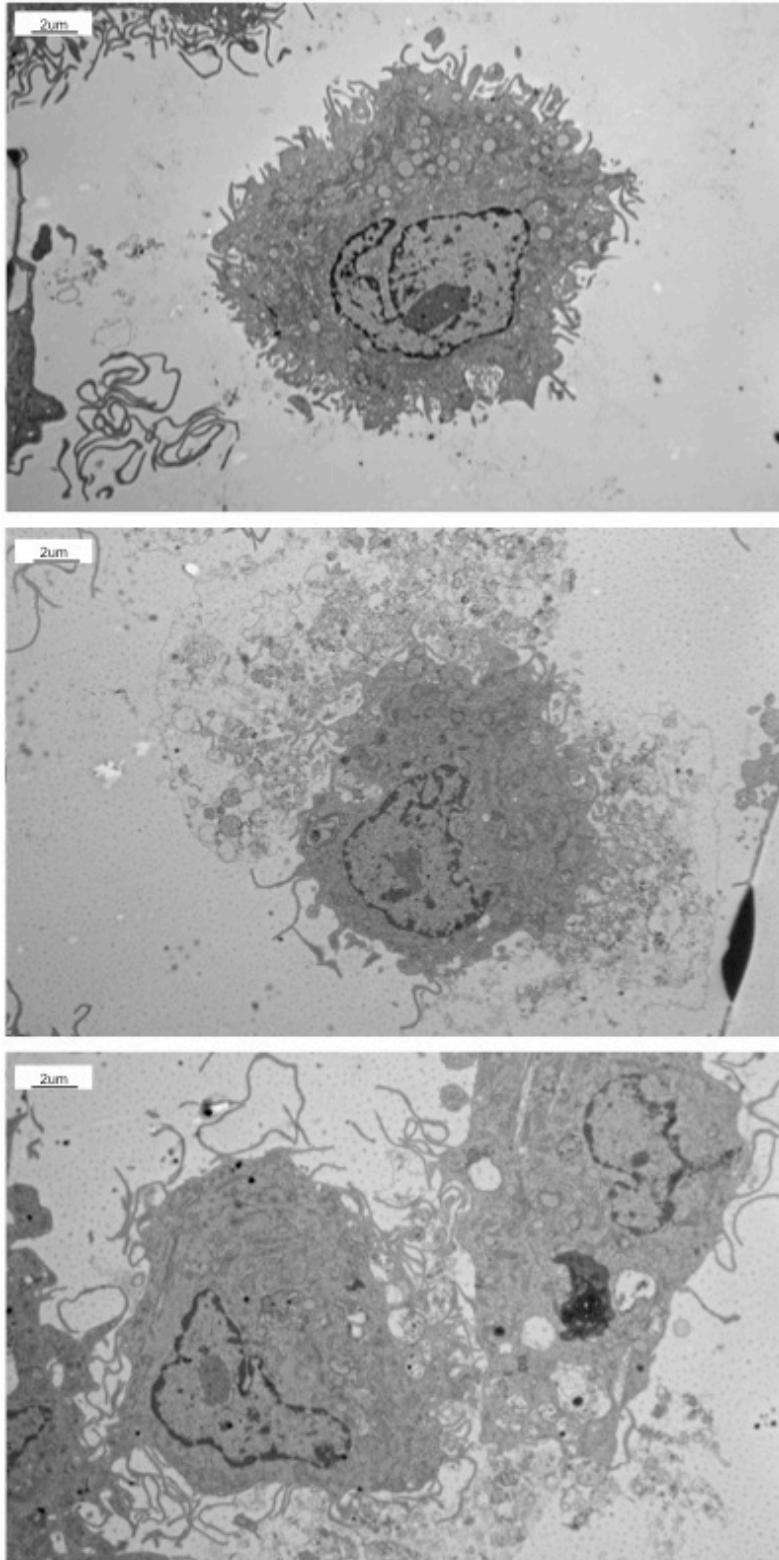
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Supplemental Figure 2. (A) Two fragments of h12LOX were obtained by using cDNA from M0 as template. Two fragments together cover the entire coding area in h12LOX cDNA with ~200 bp overlap. **(B)** Human macrophage 12-LOX cDNA was cloned to pET20b vector and sequenced. The result matches the platelet type of 12-LOX sequence. **(C)** Expression of 12-LOX protein in macrophages and DCs was detected by western blotting with anti-human platelet 12-LOX antibody. 75 kD bands were present in each cell lineage, and the size is consistent with platelet type. (Figure adapted from Deng, Wang et al., 2014 PLoS ONE)

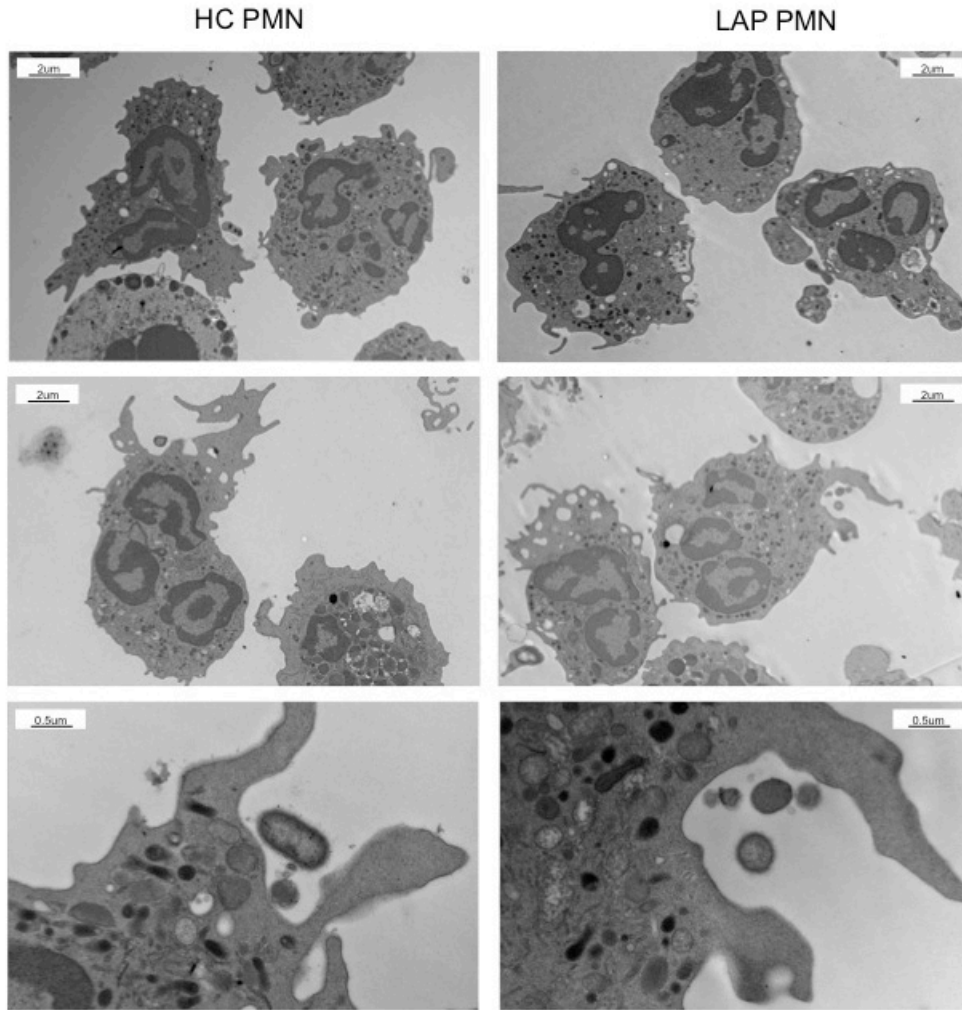


Supplemental Figure 3. MaR1 enhanced macrophage intracellular ROS generation and rescued impaired killing of *P. gingivalis* and *A. actinomycetemcomitans* by LAP macrophages. **(A)** Macrophages from healthy donors (5×10^4 cells/ well) were incubated with fluorescent carboxy- H_2DCF -DA for 30 min before incubation with vehicle (PBS) or MaR1 (10pM-100nM) for 15 min followed by addition of bacteria (1:50) for 60mins. Intracellular ROS generation was assessed using a fluorescence plate reader. Results are expressed as percent increase above vehicle; mean \pm SEM, n=5, *p<0.05, **p<0.01, ***p<0.001 vs. vehicle. **(B)** Representative images of the bacterial cultures following incubation with macrophages.



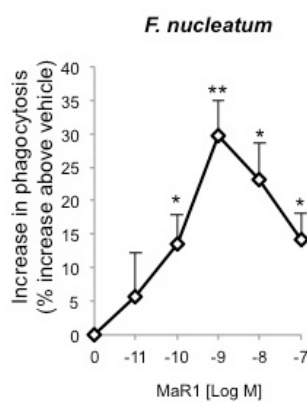
Supplemental Figure 4.

Representative TEM images of macrophages from LAP patients with *P. gingivalis*.



Supplemental Figure 5.

Representative images of neutrophils from HC and LAP patients with *P. gingivalis* showing no significant structural difference under TEM.



Supplemental Figure 6. MaR1 enhanced macrophage

phagocytosis of *F. nucleatum*. Incubation of vehicle (PBS) or MaR1 (10p-100nM) with macrophages from healthy donors (5×10^4 cells/well) for 15 min prior to addition of fluorescence-labeled bacteria (1:50). Phagocytosis was assessed by a fluorescence plate reader. Results are expressed as percent increase above vehicle; mean \pm SEM, n=4 healthy donors, *p<0.05, **p<0.01 vs. vehicle.

APPENDIX

ABBREVIATION GLOSSARY

AA= Arachidonic acid

DHA= Docosahexaenoic acid

EPA= Eicosapentaenoic acid

HC= healthy controls

HpDHA= Hydroperoxy- docosahexaenoic acid

HDHA= Hydroxy-docosahexaenoic acid (eg. 14-HDHA)

LAP= localized aggressive periodontitis

LT= Leukotriene (eg. LTB₄)

LM= lipid mediator LOX= Lipoxygenase (eg. 12-LOX)

LX= Lipoxin (eg. LXA₄)

MaR= Maresin (eg. MaR1, MaR2)

PG= Prostaglandin (eg. PGE₂)

ROS= reactive oxygen species

SPM= Specialized pro-resolving lipid mediators

Oral bacteria

P. g = *Porphyromonas gingivalis* (*P. gingivalis*)

A. a = *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*)

F. n = *Fusobacterium nucleatum* (*F. nucleatum*)

S. i = *Streptococcus intermedius* (*S. intermedius*)

DEDICATION

This dissertation is dedicated to my family, Shing-Yaw Wang, Chung-Hey Chen, and my sister Tina Wang, for their love and unconditional support; as well as my colleagues and best friends Tae Kwon and Mindy Gil for their accompany during this journey.

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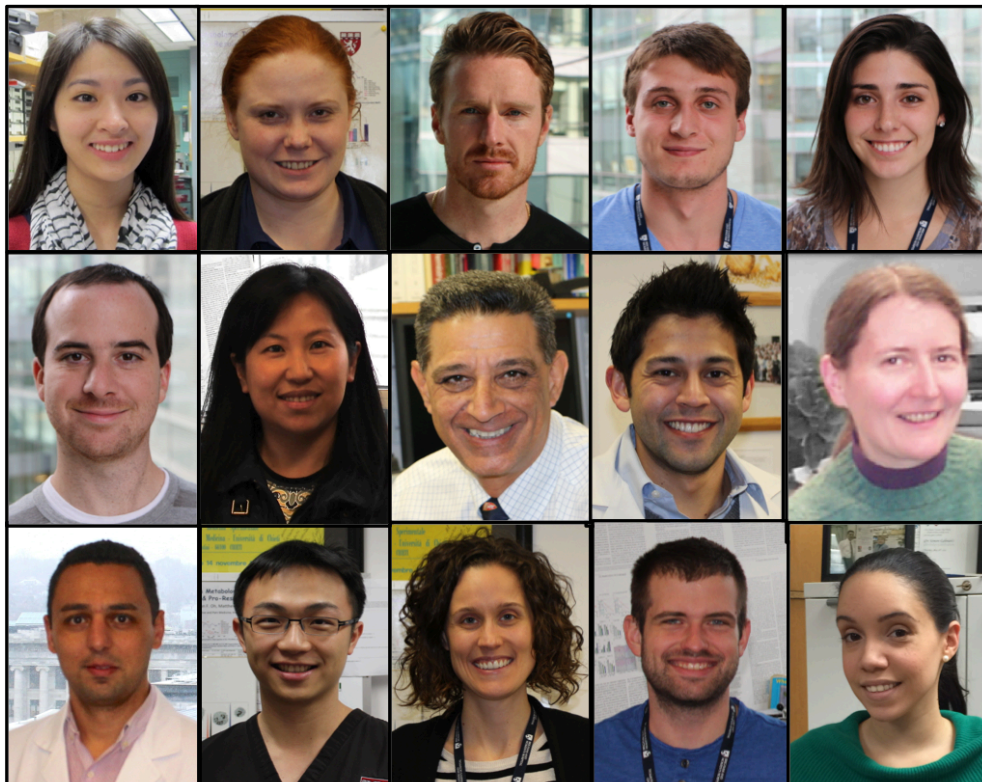
In addition, this thesis would be incomplete without acknowledging Nan Chiang as another valuable mentor from drafting proposals to guiding me throughout the years. Especially when I changed to work on a new project and when times got tough, I greatly appreciated her patient guidance, compassion and support. And I am really thankful to Jesmond Dalli as well for him training me in rigorous self-directed scientific scrutiny and self-criticism. Together, the mentorship I got in Serhan's lab was exceptional and the high standards pushed me beyond my expectation to a certain level.

For chapter one, I am appreciative of all the co-authors: Bin, Hildur, Yongsheng, Cindy, Jesmond, and especially my mentor Dr. Serhan for their guidance in working towards publication (Deng, Wang et al., 2014). Special gratitude I would express for Bin, Yongsheng, and Hildur for their patience and teaching during my early stage of learning the laboratory techniques. In addition, the work from chapter two was selected as a finalist for Hatton Award (AADR 2015), which I would also sincerely pay my gratitude

for all the contributors: Romain, Jesmond, Hildur, Daniel, Hatice, Nan, Dr. Van Dyke, and Dr. Serhan for all their help and input. I would extend my great appreciation to Dr. Van Dyke and Dr. Hasturk for their fully support and guidance at Forsyth giving me the privilege to study a legendary cohort of patients with LAP. I have to also thank Daniel for his guidance from the very beginning and Yannis for his assistance. It was a wonderful collaboration between the institutes and I am glad I was able to contribute to the mission.

Furthermore, I would also like to acknowledge all other Serhan Lab members: Cindy, Masa, Megumi, Julia, Illiam, Mary, Norma, Sesquile, Paul, Jeremy, and Sarah for their guidance, support, and friendship. It has been an amazing experience to be trained in such a rigorous yet supportive professional lab. I am very fortunate and honored to be part of the team and witness unprecedented passion, productivity, discoveries and contributions to the world of specialized pro-resolving lipid mediators.

Serhan Lab members



I am also grateful for my program director, Dr. David Kim, and all mentors in HSDM: Dr. Giuseppe Intini, Dr. Roland Baron, and Dr. Howard Howell and previous director Dr. Nadeem Karimbux for their support and encouraging remarks.

In addition, the journey would not be complete without acknowledging all the committee members for their time, effort and advice on my pathway towards DMSc.

NIH Proposal Defense Committee

April 6th, 2012

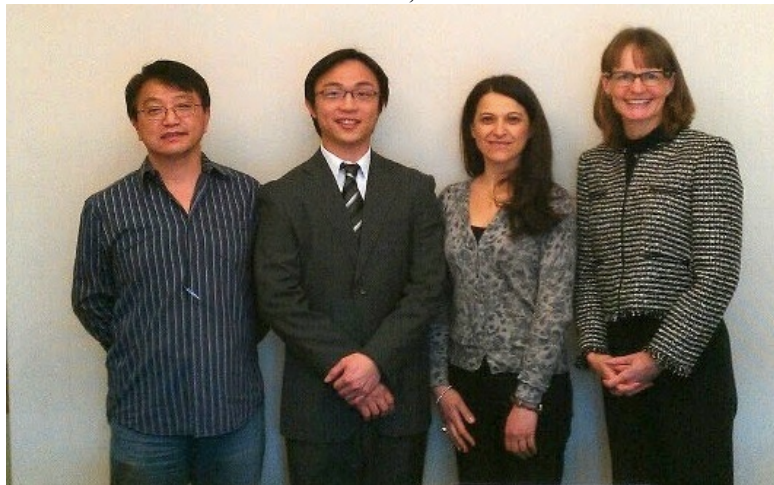


Dr. Bruce Paster (Left) and Dr. Robert White (Right)

For the first project “Inflammatory to Pro-Resolving Mediator Class Switching via 5-Lipoxygenase in Localized Aggressive Periodontitis Patients”

Oral Qualifying Examination Committee

Feb 20th, 2013



(Left to Right) Dr. Toshi Kawaii, Dr. Hatice Hasturk, and Dr. Laurie McCauley

Thesis Advisory Committee

Dec 11th, 2013

May 14th, 2014

Dec 9th, 2014



(Left to Right) Dr. Bjorn Olsen, Dr. Nan Chiang, and Dr. Hatice Hasturk

Thesis Defense committee

March 27th, 2015



(Left to Right) Dr. Roland Baron, and Dr. Thomas Van Dyke, and Dr. Bruce Donoff