Resolution of Inflammation in Type 2 Diabetes

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Resolution of Inflammation
in Type 2 Diabetes

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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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April 4, 2016

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- GlOSSARY -

AA
Arachidonic acid is an omega-6 essential fatty acid.

BLT1
A member of the rhodopsin-like family of G-protein coupled receptors and is classically known as leukotriene B4 receptor. RVE1 is a partial agonist to this receptor.

CVD
Cardiovascular diseases

DHA
Docosahexaenoic acid is an omega-3 essential fatty acid.

EPA
Eicosapentaenoic acid is an omega-3 essential fatty acid.

ERV-1
Resolvin E1 portein receptor. Initially known as chemokine like receptor (CMKLR1), also known as ChemR23. It is a member of the rhodopsin-like family of G-protein coupled receptors.

GPCR
G-protein coupled receptors.

IL
Interleukin family proteins. Cytokines that mediate cellular communication.

Non-phlogistic
Actions that do not evoke pro-inflammatory stimuli (non-heat causing)

Omega-3 fatty acids
Essential fatty acids require dietary intake since the body cannot synthesize *de novo.* These are characterized by having a double bond located at the 3rd carbon from the omega end and the molecule.

PMN
Polymorphonuclear cell or neutrophil.

PUFA
Poly unsaturated fatty acid.

P. g.
Porphyromonas gingivalis (*P. gingivalis*). Oral bacteria that is involved in the pathogenesis of periodontal disease

Resolvins
Resolution phase interaction products; pro-resolving lipid mediators biosynthesized from omega-3 fatty acids EPA and DHA.

ROS
Reactive oxygen species.

SPM
Specialized pro-resolving lipid mediators; a new genus of pro-resolving mediators including resolvins, protectins and maresins that have dual-inflammatory and pro-resolving actions.

T2D
Type 2 diabetes.

– ABSTRACT –

Unresolved inflammation is a key factor linking metabolic dysregulation and the immune system in type 2 diabetes. Successful regulation of acute inflammation requires biosynthesis of specialized pro-resolving lipid mediators, such as resolvin E1 (RvE1), and activation of cognate g-protein coupled receptors (GPCR). RvE1 binds to BLT-1 on neutrophils and ERV-1/ChemR23 on monocyte/macrophages. The mechanism by which innate immune cells in metabolic diseases fail to respond to endogenous pro-resolution signals is not well understood. The goal of the studies presented was to investigate the expression and function of ERV-1 receptor on neutrophils from type 2 diabetes subjects. Results demonstrate that neutrophils from type 2 diabetic subjects expressed significantly increased ERV-1 receptor on their cell surface that was functional, as determined by phosphorylation of the signalling protein, ribosomal S6 (rS6). Healthy neutrophils express functional BLT-1 and low levels of minimally functional ERV-1. Stimulation with TNFα or LPS increased expression of ERV1 on neutrophils of both healthy and diabetic subjects. Increased expression of ERV1 was reversed by pre- and post- treatment with RvE1. TNFα or LPS stimulation increased phospho-rS6 significantly more in

type 2 diabetic neutrophils than healthy subjects. Receptor antagonism experiments revealed that the phospho-rS6 increase was mediated by BLT-1 in healthy subject neutrophils and ERV1 in diabetic subject neutrophils. Diabetic neutrophil ERV-1 over expression was reversed by RvE1 treatment in a dose response manner (1-10nM). Analysis of the bioactive lipid metabolome revealed higher expression of inflammatory mediators in type 2 diabetes serum than healthy control with slightly reduced levels of RvE1 in type 2 diabetics (by ELISA). Neutrophil phagocytosis of pathogenic bacteria and inert bioparticles was impaired in type 2 diabetes. RvE1, signalling through ERV-1 but not BLT-1, partially rescued deficient phagocytosis by type 2 diabetic neutrophils with a clear shift of the dose response requiring increased RvE1. The dose of RvE1 required to activate resolution signals in type 2 diabetic neutrophils is significantly higher than healthy controls. Overall the findings in this dissertation further our understanding of the importance of expression and function of key resolution receptors in health versus disease and dysregulation of inflammation in type 2 diabetes.

Key words: *Resolvin E1; Type 2 diabetes, ERV-1 receptor, phagocytosis, chronic inflammation, resolution.*

- 1. Introduction -

- INFLAMMATION -

Inflammation is an essential biological response seen across species with particular importance to human health and disease. Historically, descriptions of a localized response to injury and infection were recorded by the ancient Egyptian and Greek cultures. In the first century, Cornelius Celsus documented the first description of sign of inflammation in humans. Four cardinal signs of inflammation were identified: "*rubor et tumor cum calore et dolore*" (redness and swelling with heat and pain). Disease development was defined as an imbalance of the four cardinal signs Majno (2004), (Majno, 1975).

In 1858, Rudolph Virchow's investigations involved understanding the cellular basis of the inflammation as a pathology (Virchow, 1860) ; his observations led to the addition of a new cardinal sign, *fucntio laesa* (loss of function). Subsequent studies included the germ theory of disease, which was introduced in late 19th Century by Robert Koch and Louis Paster in the pursuit to identify microorganisms as major inducers of the acute inflammatory

[1]*A version of this chapter was printed in Periodontology 2000, Volume 63, Issue 1, 149–164.*

response. Élie Metchnikoff noted that when neutrophils were ingested by tissue macrophages acute inflammation resolves (Metchnikoff, 1905), this cellular process is now called "efferocytosis" (Henson et al., 2001). This is particularly important in the context of homeostasis, infection clearance, response to trauma. More than 200 billion neutrophils die everyday, thus efferocytosis is the key cellular action that control health signals. More recently, advanced cellular and molecular mechanisms governing the fate of inflammation have been identified. Acute response initiation is accepted as a physiological response occurring in vascularized tissues as a host defense mechanism to maintain homeostasis.

Inflammation, or "set on fire", response is an active cellular and molecular response that aims to control challenge. Inflammation activation is protective in response to challenges presented to host tissues, such as pathogens, foreign bodies and tissue injury. The initial process is characterized by vascular dilation, enhanced permeability of capillaries, and increased blood flow in addition to leukocyte recruitment. Polymorphonuclear neutrophils (PMN) are among the first leukocyte responders that accumulate in the inflamed niche. These cells are crucial as the first line of defense of the innate

immune system due to their phagocytosis and microbicidal functions. Next, mononuclear cells, monocytes and macrophages, enter the inflammatory site and clear cellular debris and apoptotic PMN by phagocytosis without prolonging inflammation; a non-phlogistic (non-heat or fever producing) process (Savill, 1998).

Although the inflammatory response is protective, chronic inflammation is detrimental to tissue function. Failure to remove noxious materials produced by neutrophils via phagocytosis and/or failure of efferocytosis (phagocytosis of apoptotic inflammatory cells) characterizes the initiation of pathological lesion and disease establishment; see examples of pathways that lead to disease formation or resolution Figure 1. The incomplete elimination of leukocytes from a lesion is observed in susceptible individuals; when acute inflammation fails to resolve, chronic disease and fibrosis develop (Van Dyke, 2007). Accordingly, loss of resolution and failure to return tissue to homeostasis results in neutrophil mediated destruction and chronic inflammation, which is a major cause of human inflammatory pathologies, including arthritis, asthma, cancers, cardiovascular diseases and periodontal diseases.

- Fig 1 -
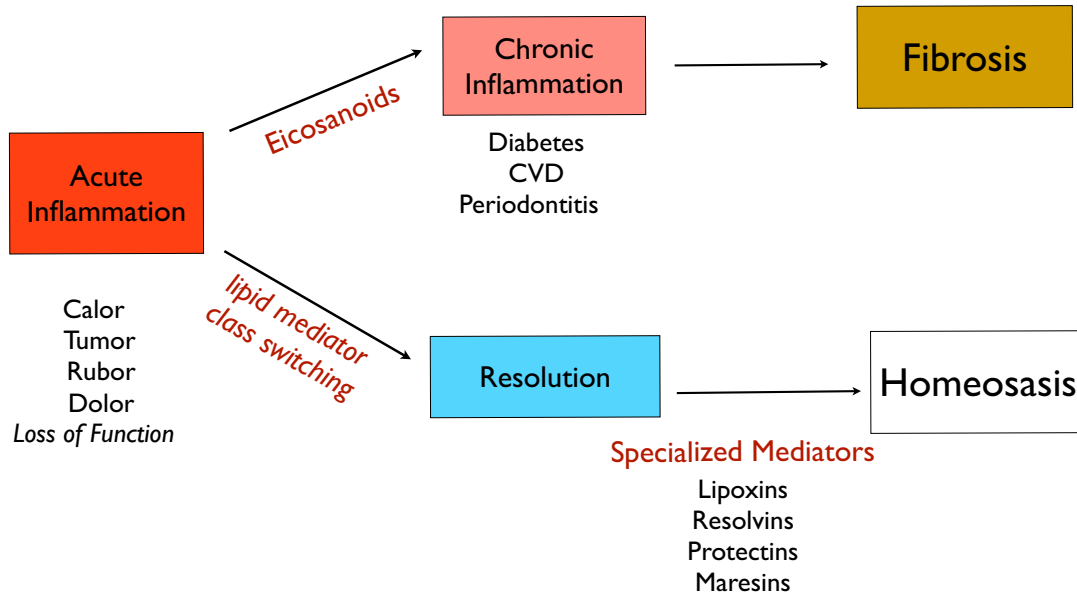
Figure 1. Acute inflammation initiates a cascade of responses. Cardinal signs are manifested when acute inflammation initiates. The outcomes of acute inflammation are influenced by the environmental factors and the type and duration of the challenge. When inflammation initiates the physiological course after is resolution. Inflammation resolution is the reestablishment of normal homeostasis. In resolution, inhibition of leukocyte infiltration, non-phlogistic phagocytosis and vascular and tissue function return to normal. In contrast, when inflammation fails to resolve, chronic signals develop diseases, such as cardiovascular diseases, type 2 diabetes and periodontal diseases.

Adapted from: Freire, 2003 Periodontology 2000.

Innate immunity, characterized by the local inflammatory response, is the early response to identify and eliminate infectious agents or damaged tissues. As an initial and protective response to challenges presented to host tissues, inflammation is characterized by vascular dilation, enhanced permeability of capillaries, and increased blood flow and leukocyte recruitment. This response requires four biological components, including: inflammatory inducers, detecting sensors, downstream mediators and target tissues, see diagrams presented in Figure 2. The type and degree of an inflammatory response is dependent on the nature of the trigger (e.g.: bacterial, viral, parasitic, chemical), and its duration (Medzhitov, 2010). With specific cellular and molecular cues, inducers once detected activate a cascade of events that are tightly regulated.

The major sensors for the inflammatory response are innate immune cells that migrate to the injured site and resident stromal cells. Polymorphonuclear leukocytes (PMN) or neutrophils (named for their staining characteristics with hematoxylin and eosin) are the cellular part of first line of defense of the innate immune system. PMN are professional phagocytes with potent oxidative and

non-oxidative killing mechanisms to combat bacteria. PMN infiltration followed by mononuclear cells, monocytes and macrophages that enter the inflammatory site and clear cellular debris, bacteria and apoptotic PMN by phagocytosis without prolonging inflammation. The degree of inflammation activation is dependent on the nature of the inflammatory trigger, the location and time of the response, (Majno, 2004). Together, innate cells trigger production of mediators that modulate the fate of inflammation. Neutrophils, macrophages, dendritic cells and mast cells produce low molecular weight proteins called cytokines that control initiation of inflammation, maintenance and regulation of its amplitude and duration of the response.

In response to bacteria conserved receptors, expressed in innate immune cells, Toll-like receptors (TLRs), sense molecules expressed on pathogens, such as pathogen-associated molecular patterns (pamps). Binding of TLRs to specific pathogen molecules induce a signal that activate downstream events. In consequence, the production of communicating molecules such as inflammatory cytokines, interleukins (IL), chemokines (CXC), and inflammatory lipid mediators (LM), is key to effectively activate response to the challenge. The inflammatory mediators are the major players in establishing

the "language" responsible to signal clearance of the bacteria. A didactical and hopefully functional classification of mediators classified the inflammatory molecules according to their functions in the context of activation (pro-) or inhibition (anti-) of inflammation. It is important to note that a more dynamic process than described govern the molecules, this is to say one molecule could be pro- and/or anti- inflammation. This dynamic process is controlled by the molecule concentration, the interaction with niche and other molecules, and finally the time of production and action. The binary view of the molecules activity is still the convention and the accepted terminology. Pro-inflammatory mediators, for example, are produced locally on tissues or systemically to the blood stream, e.g.: IL-1β, IL-6, TNF-α and PGE$_2$. In response to the challenge directly or the production of pro-inflammatory molecules, anti-inflammatory cytokines control the response and maintain the response in check, e.g.: IL-10, IL13, TGF-□, IL1RA (Seruga et al., 2008).

- Fig 2 -
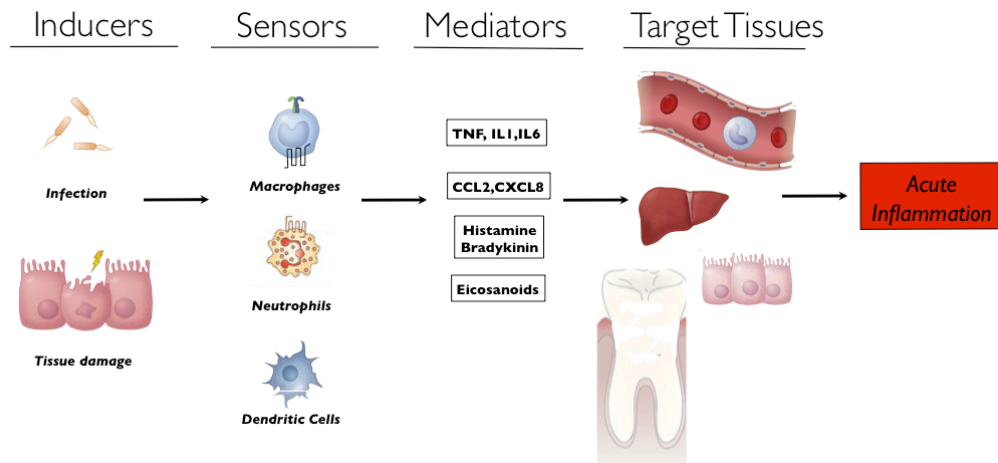
Figure 2. Inflammation pathway components. Inducers trigger the initial cascade that activates inflammation, e.g.: bacteria, viruses, parasites, chemicals, trauma, and particles. Once the triggers invade the tissue barrier, barrier cells and innate immuno cells will detect the challenge through conserved receptors. In response of the chemical and biological detection, mediators are produced to activate or inhibit inflammation. The communicating molecules are able to signal locally and systemically to feed forward the signal received. In consequence, tissues are the target for cells and mediators that receive the message and respond in order to activate acute inflammation.

*Adapted from: Medzhitov, R. (2010). Cell **140**(6): 771-776.*

The regulation leading to the transcription of cytokine genes, their translation and secretion of pro-inflammatory cytokines from a variety of cells is generally dependent on NFκB nuclear protein activation of transcription. Cytokines are low molecular weight proteins that modulate inflammation positively or negatively. Resident cells, such as epithelial cells and fibroblasts, and phagocytes, produce Cytokines in the acute phase and early chronic phase of inflammation, and by immune cells in adaptive immunity.

After microbial recognition, cytokines of the innate response, including TNF-α, IL-1β and IL-6, are the first secreted and are signature innate cytokines. TNF-α is a pleotropic cytokine that has many functions from signaling cell migration to tissue destruction. TNF-α up-regulates the production IL-1β and IL-6. TNF-α is also correlated with extracellular matrix degradation and bone resorption through actions promoting secretion of matrix metalloproteinases (MMPs) and receptor activator or NFκB ligand (RANKL) and coupled bone formation (Freire and Van Dyke, 2013). Chemokines are cytokines with chemoattractant functions that induce cell migration to the site of infection or injury. Once blood leukocytes exit a blood vessel, they are attracted by functional gradients of chemotactic factors. Chemokines are synthesized by a

variety of cells including leukocytes, endothelial, epithelial, and stromal cells. Beyond their chemotactic role, chemokines function as messengers of distinct biological processes, cell proliferation, cell death, angiogenesis and tumor metastasis (Zlotnik and Yoshie, 2000).

Microorganisms that gain access to the blood are usually eliminated by the reticuloendothelial system within minutes (transient bacteremia) with no clinical symptoms. Local bacterial antigens that are systemically dispersed trigger significant systemic inflammation. Leukocytes, endothelial cells and hepatocytes respond to bacteria and their virulence factors with secretion of pro-inflammatory immune mediators called acute phase proteins, like C-reactive protein (CRP). Persistence of infection, with continuous exposure of the host to new antigens, results in the activation of humoral immunity and antibody production. In addition to bacterial challenge, viral infections induce the production of interferon (IFN-α, IFN-β) and cytotoxic activation of lymphocytes, while parasites lead to the production of, IL-4, IL-5, and IL-13 by mast cells and basophiles. Pro-inflammatory cytokines in circulation induce leukocytosis and acute phase proteins produced by the liver. With continued

exposure to the inducers the response become more specific and adapts to the challenge.

Likewise, pro-inflammatory mediators, such as IL-1β, IL-6, TNF-α and PGE_2, produced locally in the inflamed tissues may "spill" into the circulation and have systemic impact, such as induction of endothelial dysfunction (Freire and Van Dyke, 2013, Fredman et al., 2011). If the inflammation response resolves in a temporal manner then tissue homeostasis is reestablished. This is regulated by active molecular pathways.

- NEUTROPHILS IN ACUTE INFLAMMATION -

For many years, our understanding on inflammation was mostly on initiation as an active and resolution as a passive decay of inflammation. We now realize that both the initiation of inflammation and its resolution are active biological processes. Following the classical acute inflammatory response, cellular events are temporally activated. Upon initial challenge, protein exudation increases and polymorphonuclear leukocytes (PMN, neutrophils) accumulate in inflamed tissue. Neutrophil infiltration follows a rapid response from sentinel cells pre-stationed in the tissues at the time of injury, including macrophages and mast cells (Majno, 2004), Figure 3. PMN extravasation into injured or infected tissues requires adhesion to endothelial cells in the capillaries mediated by surface molecules called selectins and integrins on PMN and intracellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) on endothelial cells. Selectins cause rolling of PMN along the capillary wall and integrins mediate firm attachment allowing transcellular diapedesis of neutrophils to the infected tissue (Cotran, 1965b, Cotran, 1965a).

As primary defenders, neutrophils transmigrate into tissues in large numbers to neutralize pathogens and promote clearance of cellular and other debris by phagocytosis. As the lesion matures, neutrophils accumulate in the local tissue and die via apoptosis (programmed cell death). The initial accumulation of neutrophils is followed by a second wave of cellular infiltration; mononuclear phagocytes (monocytes). Differentiation of monocytes into macrophages promotes removal of apoptotic neutrophils and debris by non-phlogistic phagocytosis. Macrophages that have completed the elimination of apoptotic neutrophils are cleared from the inflamed tissue either by egression to the lymphatic system or by efferocytosis.

This temporal regulation of inflammation requires cells to highly clear the original insult (Savill et al., 2002). Failure to resolve the inflammatory response or continuous activation of the responses, become harmful to the tissue and consequently develop into a chronic lesion that we call inflammatory diseases. In addition to pro-inflammatory mediators that turn on inflammation, there is a separate set of lipid mediators that act as endogenous agonists to activate termination of inflammation by stimulating resolution.

A large family of bioactive lipid mediators, the eicosanoids (20-lipid mediators), is involved in pathophysiologic processes, including those associated with host-defense and inflammation. Arachidonic acid (AA) is a common endogenous precursor for the biosynthesis of eicosanoids derived from the sn-2 position of cell membrane phospholipids. The enzyme phospholipase A_2 releases AA from the phospholipid membrane. The release of AA constitutes the rate-determining step in the generation of eicosanoids produced by most phagocytic and immune cells. AA is rapidly converted to various potent lipid mediators in a cell specific manner by cyclooxygenases (COX), lipoxygenases (LO), or epoxygenases to yield prostaglandins, leukotrienes and endoperoxides, respectively.

Locally produced lipid mediators, such as prostaglandins, prostacyclin and thromboxanes, act as autacoids (short lived molecules that signal immediately adjacent or at the site of synthesis). These lipid mediators are synthesized from membrane-released AA when cells are activated by stimuli, such as trauma and cytokines. Arachidonic acids are metabolized by two major enzyme pathways, cyclooxygenases (COX) and lipoxygenases (LO), figure 3.

COX-1 (constitutively expressed COX) is responsible for basal levels of prostaglandin synthesis, whereas COX-2 (inducible COX) catalyze the conversion of AA to lipid mediators during inflammation (Smith, 2000) Prostaglandins have 10 sub-classes, of which D, E, F G, H and I are the most important in inflammation. Specifically PGE_2 is generated via PGE synthase in leukocytes, while PGI_2 is generated by prostacyclin synthase in endothelial cells and thromboxanes (TXA_2) are generated via thromboxane synthase in platelets (Funk and Cyrus, 2001).

Lipoxygenases catalyze the formation of hydroxyeicosatetraenoic acids (HETEs) from AA leading to the formation of leukotrienes (LT) and other biologically active compounds (Weissmann et al., 1980). LTs are predominantly produced by inflammatory cells, including polymorphonuclear leukocytes, macrophages, and mast cells. There are 3 distinct LO that are cell specific; 5-LO in myeloid cells, 12-LO in platelets and 15-LO in epithelial/endothelial cells. Cellular activation by pathogens and immune complexes results in activation of a sequential enzymatic reaction that includes $cPLA_2$ and 5- lipoxygenase (5-LO). 5-LO converts released AA to the epoxide LTA_4 which undergoes transformation by distinct pathways; one to generate LTB_4 which is a potent

regulator of neutrophil chemotaxis and leukocyte adhesion to endothelial cells (Funk, 2001). The end products of 12- and 15-LO are 12- and 15-HETE, which are further metabolized.

Excessive production of pro-inflammatory mediators such as prostaglandins and leukotrienes with an exacerbated sensing response to inflammatory triggers is correlated with progression from acute inflammation to chronic inflammation in many diseases. Favorable inflammatory processes are self-limiting, which implies the existence of endogenous anti-inflammation and pro-resolution pathways (Serhan et al., 2004).
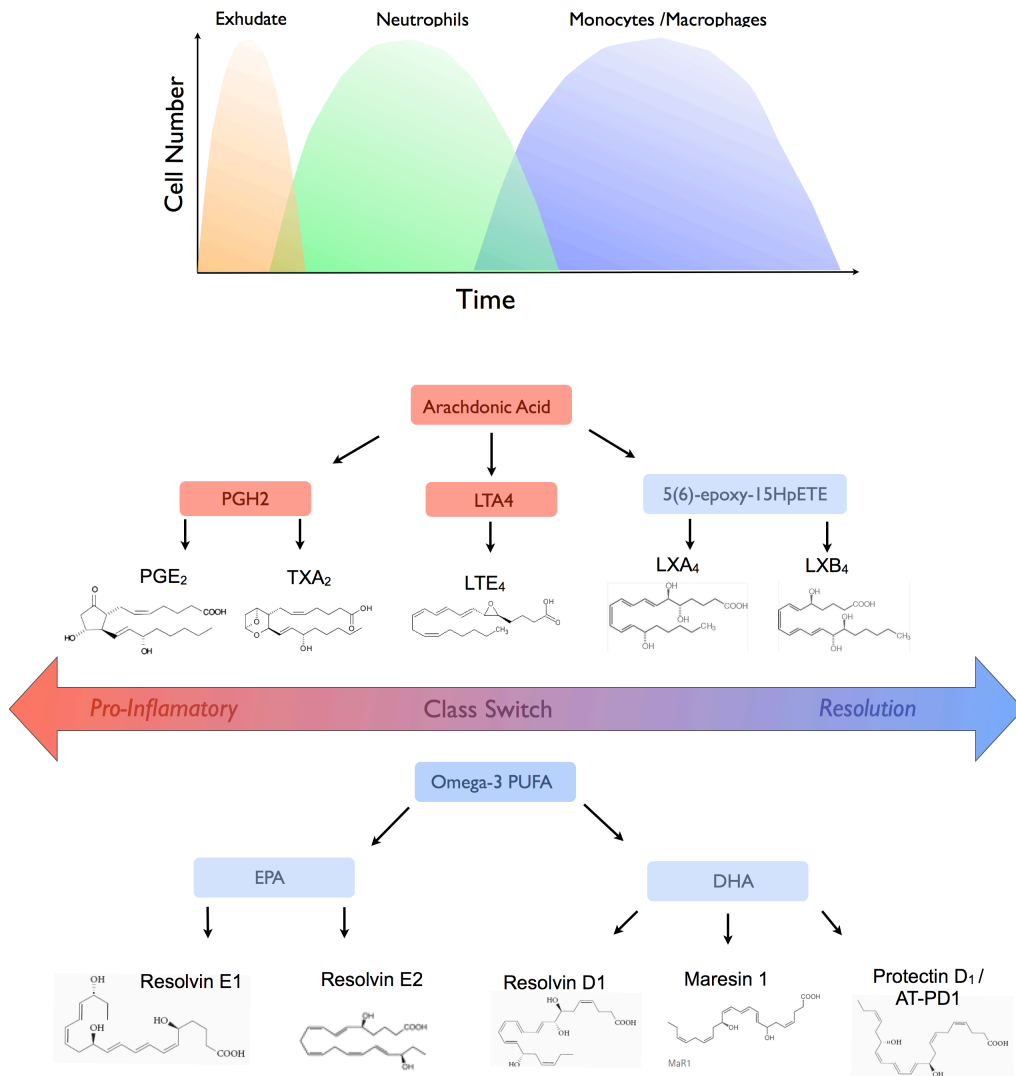
- Fig. 3 -

Figure 3. Cellular and molecular regulation in resolution of inflammation. The local acute inflammatory response is characterized by exudate formation and the initial recruitment of neutrophils followed by the recruitment of monocytes that differentiate into macrophages. Lipid mediators such as PGs and LTs, and cytokines and chemokines coordinatedly regulate the initial events of acute inflammation. Arachidonic acid released from membrane phospholipid upon PLA_2 can be further metabolized by 1) COX1 and 2 to generate prostanoids, including prostaglandins and tromboxanes; 2) lipoxygenase (LO) to generate leukotrienes and 3) LO interaction biosynthetic pathway to generate lipoxins. Lipoxins are generated during transcellular biosynthesis which requires two cell types involving distinct lipoxygenases. Lipid mediator class-switches biosynthesize pro-resolution lipid mediators, such as lipoxin A_4, and the eicosapentaenoic acid (EPA) derived resolvins (i.e. RvE1, RvE2) and docosahexaenoic acid (DHA) derived lipid mediators including, D- series resolvins, protectins and maresins.

Adapted from: Freire, 2003 Periodontology 2000.

- LIPIDS OF INFLAMMATION RESOLUTION -

Inflammation resolution is initiated by an active class switch in the mediators, such as classic prostaglandins and leukotrienes, to the production of immunoresolvents. Endogenous lipid mediators including resolvins, protectins, lipoxins, and maresins, are biosynthesized during the resolution phase of acute inflammation (Bannenberg and Serhan, 2010), showed in Figure 3. The pathways that generate these molecules are complex and will be described in the next section. Lipoxins are derived from endogenous fatty acids (arachidonic acid), while resolvins, protectins and maresins are derived from dietary fatty acids, specifically the ω-3 fatty acids found in fish oil. Functionally, these specialized lipid mediators stimulate and accelerate resolution via mechanisms at the tissue level that are multi-factorial, see Table 1.

- Table 1 -

Resolution Lipid Mediators & Cellular Functions

Lipid Mediator	Cellular Function	References
Lipoxin A$_4$ / ATL	Decreases adherence of leukocytes Reduces vascular leakage Lowers PGE$_2$ levels in exudates Reduces the number of apoptotic neutrophils Inhibits neutrophil recruitment Attenuates NF-κB gene expression Blocks leukocyte adhesion protein-1 and chemotaxis Promotes lymphatic removal of phagocytes Activates FLRP2/ALX receptor internalization and phagocytosis Inhibits T-cell adhesion to vascular and salivary epithelium Enhance microbial phagocytic function	Papayianniet et al. (1995) Takano et al. (1997) Pouliot et al. (2000) Perretti et al. (2002) Serhan et al. (2003) Chiang et al. (2003) Ohira et al. (2004) Bannenberg et al. (2005) Maderna et al. (2010) Chintamani et al. (2012) Tobin et al. (2012)
Resolvin E1	Inhibits neutrophil infiltration Modulate chemokine/cytokine synthesis Promotes inflamed tissues healing and bone regeneration Enhances phagocytosis Activates lymphatic removal of phagocytes Attenuates systemic production of C-reactive protein and IL-1 Reduces eosinophil and lymphocyte recruitment Regulates adipokines Decreases inflammatory actions of COX2 Attenuates NF-κB gene expression Lowers the number of monocytes Increases epithelium cells CD55 and PMN clearance Rescues impaired phagocytosis in LAP patient macrophages Prevents rejection of allograft Activates anti-apoptotic signals	Serhan et al. (2000) Bannenberg et al. (2005) Hasturk et al. (2005) Schwab et al. (2007) Van Dyke et al. (2007) Hasturk et al. (2007) Aoki et al. (2008) Gonzalez-Periz et al. (2009) Li et al. (2010) Ishida et al. (2010) Li et al. (2010) Campbell et al. (2010) Freedman et al. (2011) Levy et al. (2011) El Kebir et al. (2012)
Resolvin D1	Inhibits neutrophil recruitment Anti-hyperalgesic properties Shortens resolution interval Reduces oxidative stress mediated inflammation Attenuates agonist pain molecules Induces macrophages phagocytosis Stimulates M2 macrophage phenotype Temporally regulates miRNAs Reduces cytokines in BALF Ameliorates insuline sensitivity Enhances microbial clearance Reduces levels of prostaglandins and leukotrienes	Serhan et al. (2002) Albert et al. (2002) Hong et al. (2003) Spite et al. (2009) Bang et al. (2010) Krishnamoorthy et al. (201 Titos et al. (2011) Recchiuti et al. (2011) Wang et al. (2011) Hellman et al. (2011) Chiang et al. (2012) Norling et al. (2012)
Protectin D1 / AT-PD1	Decreases inflammatory actions of COX-2 Inhibits neutrophil infiltration Modulate chemokine/cytokine synthesis Regulates T-cell migration Regulates macrophages function Upregulates CCR5 expression on apoptotic leukocytes Prevents hepatocytes steatosis Attenuates NF-κB gene expression Inhibits pain signals Suppresses eosinophils chemotaxis and adhesion	Marcheselli et al. (2003) Bannenberg et al. (2005) Serhan et al. (2006) Ariel et al. (2006) Duffield et al. (2006) Schwab et al. (2007) Gonzalez-Periz et al. (2009) Zhao et al. (2011) Bang et al. (2012) Miyata et al. (2012)
Maresin -1	Reduces neutrophil number in exudate Enhances macrophage phagocytic functions Decreases transendothelial PMN migration	Serhan et al. (2009) Serhan et al. (2012) Shinohara et al. (2012)

Adapted from: Freire, 2003 Periodontology 2000.

- LIPOXINS -

Lipoxins are natural pro-resolving molecules produced from endogenous fatty acids. Derived from arachidonic acid (AA), lipoxins have potent anti-inflammatory and resolution actions. Lipoxins A_4 and B_4 were first isolated and identified as inhibitors of PMN infiltration and stimulators of non-phlogistic recruitment of macrophages. Three main pathways of lipoxin synthesis have been identified. In human mucosal tissues, such as the gastrointestinal tract, airways and the oral cavity, a sequential oxygenation of AA by 15-lipoxygenase and 5-lipoxygenase, followed by enzymatic hydrolysis leads to LXA_4 and B_4 . While in blood vessels, 5-lipoxygenase biosynthesizes LXA_4 and 12-lipoxygenase in platelets produce LXB_4. Lipoxin A_4 regulates cellular functions through specific receptor (ALX/FPR2 and GPR32) activation; these receptors are expressed by neutrophils and monocytes. A third synthetic pathway is triggered by aspirin. Aspirin promotes acetylation of COX-2 leading to a change in COX2 activity and the chirality of the products, which are termed ATL (aspirin triggered lipoxins). Cells that express COX-2 include vascular endothelial cells, epithelial cells, macrophages and neutrophils. In addition to

synthesis of lipoxin, aspirin also blocks prostaglandin synthesis by acetylation of COX2 inhibiting inflammation.

- RESOLVINS -

Resolvins are lipid mediators that are induced endogenously during the resolution phase of inflammation. These lipid mediators are biosynthesized from the precursor essential ω-3 poly-unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from the diet The two major groups of the resolvin family have distinct chemical structures; E-series, derived from EPA, and D-series, derived from DHA. E-series resolvins are produced by vascular endothelium via aspirin modified COX-2 that converts EPA to 18R-hydroperoxyeicoapentaenoic acid (18R-HPEPE) and 18S-hydroperoxyeicoapentaenoic acid (18S-HPEPE). These intermediates are rapidly taken up by human neutrophils and metabolized to RvE1 and RvE2 by 5-lipoxygenase. Resolvin E1 production is increased in plasma of individuals taking aspirin or EPA resulting in amelioration of clinical signs of inflammation Similarly, DHA derived resolvins, D-series, have been shown to reduce inflammation by decreasing platelet-leukocyte adhesion and aspirin triggered

DHA conversion produces molecules with dual anti-inflammatory and pro-resolution function.

Interaction between resolvins and specific receptors modulates the fate of innate immune cells and counter regulates active inflammation. Selective target sites for resolvins are G-protein coupled receptors (GPCR). CMKLR1 (also known as ChemR23) is a GPCR expressed on monocytes and dendritic cells. BLT1, a leukotriene receptor, is the resolvin E1 receptor on neutrophils, figure 5. Upon selective binding to the receptors, RvE1 attenuates NF-κB signaling and production of pro-inflammatory cytokines including TNF-α. D-series resolvins target GPR32 and ALX receptors, and as shown recently CB2 receptor expressed on platelets and PMN. Activation of CB2 receptor leads to inhibition of P-selectin expression decreasing PMN chemotaxis. Resolvins induce hallmark functions of the resolution of inflammation, including preventing neutrophil penetration, phagocytosis of apoptotic neutrophils to clear the lesion, and enhancing clearance of inflammation within the lesion to promote tissue regeneration.

- PROTECTINS -

Protectins are also biosynthesized via a lipoxygenase-mediated pathway. The pathway converts DHA into a 17S-hydroxyperoxide-containing intermediate that is rapidly taken up by leukocytes and converted into 10,17-diHDHA, known as protectin D1 or neuroprotectin. The name accounts for the protective actions observed in neural tissues and within the immune system. Protectin D1 is also produced by human peripheral blood lymphocytes with a Th2 phenotype; reducing TNFα and interferon-□ secretion, blocking T-cell migration and promoting T-Cell apoptosis. Recently, a novel protectin synthesis pathway was found that utilizes aspirin triggered COX-2 to synthesize epimeric 17R-hydroxyperoxide from DHA called AT-PD1 and has shown a positive interaction with CB2 and PPAR family receptors. Both protectins reduce PMN transmigration through endothelial cells, and enhance clearance (efferocytosis) of apoptotic PMN by human macrophages (Shinohara et al., 2012) .

- MARESINS -

Macrophage mediators in resolving inflammation (Maresins, MaR) were recently identified as primordial molecules produced by macrophages with homeostatic functions. Metabolopidomic approaches in peritonitis models led to the identification of a novel pathway of DHA metabolism. Macrophage phagocytosis of apoptotic cells triggers biosynthesis of RvE1, PD1, LXA_4 and MaR1. Conversion of DHA into 14-hydroxy diHA was identified via the 14-lipoxygenase pathway. Freshly prepared 14-H(p)DHA is rapidly converted by macrophages into bioactive products. MaR1 effectively stimulates efferocytosis with human cells and also has regenerative functions.

The local lipid mediators constitute a new genus of anti-inflammatory and pro-resolving endogenous compounds that have proven to be very potent in treating a number of inflammation associated models of human disease. Lipoxin A_4/ATL and resolvin E1 have been shown to inhibit neutrophil recruitment, attenuate pro-inflammatory gene expression and reduce severity of colitis in a murine model. PMN infiltration and lymphatic removal of

phagocytes was observed when resolvin E1, D2, protectin D1, lipoxin and

maresin were used to ameliorate colitis, figure 5.

- Fig. 5 -
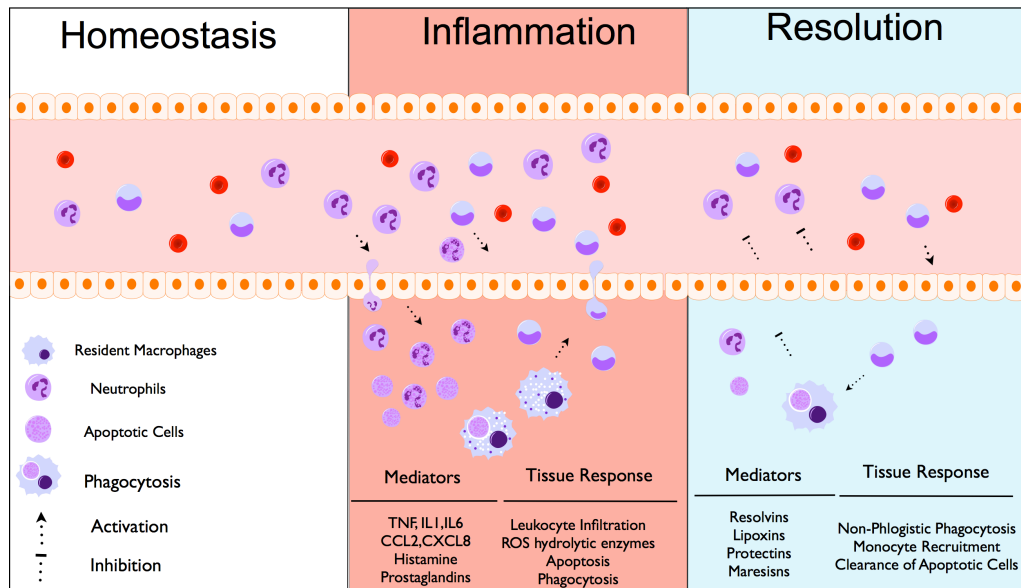
Figure 4. Dynamic transition from inflammation to resolution. Upon challenge, the inflammatory response begins. (B) Cell communication mediators activate chemoattraction, vascular permeability and infiltration of leukocytes to the periphery. Resolution is then activated by increase macrophages and efferocytosis signals. Mediated by immunoresolvents (resolvins, lipoxins, protectins, maresins), resolution is an active biochemical mechanism.

Adapted from: Freire, 2003 Periodontology 2000.

Most recently in similar model, resolvin D1 has been associated with regulation of miRNAs and target genes and reduction of LTB_4, PGD_2, TXA_2, $PGF2_\alpha$, and TXA_2 in peritoneal exudates. Treatment with Maresin 1 has been demonstrated to stimulate tissue regeneration in planaria. Consistently in periodontal disease models, lipoxin A_4/ATL prevented connective tissue and bone loss. Treatment of experimental periodontitis with lipid mediators resulted in complete resolution of inflammation and remarkable regeneration of both soft and bone tissues; a restoration of homeostasis. It has been suggested that defective resolution of inflammation mechanisms underlies the inflammatory phenotype presented in chronic diseases and that lipid mediators can rescue this phenotype. That pro-resolution lipid mediators play a role as natural molecules in maintenance of homeostasis is evident with promising potential as therapeutic agents for human diseases (Table 1).

In summary, the role of acute inflammation is protection of the host. It is initiated by neutrophils in response to challenge. The fate of this process is determined by the balance between presence of mediators and sensors that either amplify the inflammatory process, or that control the return to normal

health. It is now evident that resolution of inflammation is modulated by protective mediators, such as AA-derived lipoxins and ATLs, ω3- EPA-derived resolvins of the E-series, DHA derived resolvins of the D series, protectins and maresins. The selective interaction of the lipid mediators with GPCR receptors of innate immune cells induces cessation of leukocyte infiltration; vascular permeability/edema returns to normal; PMN death (mostly via apoptosis); non-phlogistic infiltration of monocyte/macrophages and macrophage removal of apoptotic PMN, foreign agents (bacteria), and necrotic debris from the site. These cellular events achieve the ideal outcome of inflammation, resolution with return to predisease homeostasis.

- UNRESOLVED CHRONIC INFLAMMATORY DISEASES-

Unresolved inflammation is a hallmark of various human diseases including diabetes, ulcerative colitis, rheumatoid arthritis, cancer, cardiovascular diseases, and periodontitis. The fate of acute inflammation determines return to homeostasis versus disease establishment, figure 1. Diseases associated with uncontrolled acute inflammation are characterized by continuous release of histotoxic substances that results in local tissue damage, prolonged inflammatory response and loss of function. In contrast, in health the fate of inflammation is influenced by endogenous mediators to resolve the acute process and reestablish homeostasis (Bannenberg et al., 2005).

A localized inflammatory response to an injury or infection is a spatially defined and temporally regulated condition that ideally should be self-limited as previously described. If the lesion does not resolve and becomes chronic, the acquired immune system is stimulated including broad activation of lymphocytic pathways, cell-mediated and humoral immunity. The impact of local infection or injury becomes systemic, defined as an extended response

that goes beyond the confined localization of one tissue. The chronicity of the lesion alters molecular, cellular and overall tissue responses in remote regions of the body having a transient or permanent impact on overall health.

In attempt to understand the biological mechanisms behind disease initiation, animal models are established to mimic human diseases, table 2. This notion is certainly key to investigate therapeutic approaches that aim to modulate the biological deficiencies seeing in disease models with the hopes to influence human health. It is hypothesized that diseases associated with uncontrolled acute inflammation are characterized by a combination of excessive, continuous stimuli and insufficient resolution activation. In fact, high levels of toxic substances and pro-inflammatory mediators result in damage to host tissues and prolong the inflammatory response. In experimental animal models presented in table 2 compelling evidence demonstrates the actions of pro-resolution mediators in regulation of both local and systemic inflammatory responses elucidating the role of endogenous mediators in systemic inflammatory processes and disease regulation. Periodontal diseases and type 2 diabetes as two models of chronic unresolved inflammation. Further chapters will characterize the importance of understanding the biological deficiency

seeing in these diseases and their response to exogenous resolution mediators. A clearer picture reveals the central role of Inflammation as the driving mechanism for disease establishment and maintenance. A number of local and systemic chronic diseases share biological gateways in the context of inflammatory response and novel approaches are designed to prevent and treat the biological dysfunctions.

Failure to remove the trigger effectively and inefficient clearance of the innate immune cells (especially dead neutrophils) characterize the initiation of chronic pathological lesions. In susceptible individuals, periodontal inflammation fails to resolve and chronic inflammation becomes periodontal pathology. Accordingly, inadequate resolution and failure to return tissue to homeostasis results initially in neutrophil-mediated destruction and chronic inflammation followed by a complex immune lesion with destruction of extracellular matrix, bone, scarring and loss of periodontal tissue function (Darveau, 2010, Eke et al., 2010).

- Table 2 -

Resolution Lipid Mediators In Chronic Disease Animal Models

Disease Models / Species	Actions	References
Periodontitis / Rabbit	**Lipoxin A$_4$ / ATL** Prevents connective tissue and bone loss Accelerates healing of inflamed tissues Ceases infiltration of neutrophils **Resolvin E1** Reduce bone loss Regenerates lost soft tissue and bone tissue	Serhan *et al.* (2003) Serhan *et al.* (2003) Serhan *et al.* (2003) Hasturk *et al.* (2005) Hasturk *et al.* (2007)
Peritonitis / Mouse	**Lipoxin A$_4$ / ATL** Stops neutrophils recruitment Promotes Lymphatic removal of phagocytes **Resolvin E1** Decrease PMN infiltration Regulates chemokine and cytokine production Promotes lymphatic removal of phagocytes **Resolvin D1** Shortens resolution interval Regulates miRNAs Reduces concentrations of LTB$_4$, PGD$_2$, PGF$_2$ and TXA$_2$ in exudates Lowers antibiotic requirement Increase animal survival Reduces bacterial titers **Protectin D-1** Promotes local clearance of apoptotic cells Regulate lymphatic removal of phagocytes Modulates T-cell migration **Maresin-1** Blocks infiltration of PMN into the peritonium	Bannenberg *et al.* (2005) Schwab *et al.* (2007) Arita *et al.* (2005) Schwab *et al.* (2007) Bannenberg *et al.* (2005) Spite *et al.* (2009) Recchiuti *et al.* (2011) Krishnamoorthy *et al.* (2012) Norling *et al.* (2012) Chiang *et al.* (2012) Chiang *et al.* (2012) Chiang *et al.* (2012) Bannenberg *et al.* (2005) Ariel *et al.* (2005, 2006) Schwab *et al.* (2007) Serhan *et al.* (2009)
Colitis / Mouse	**Lipoxin A$_4$ / ATL** Reduces severe colitis Attenuates pro-inflammatory mediators gene expression Inhibts weight loss Reduces immune dysfunction **Resolvin E1** Improves animal survival rate Reduces weight loss Promotes LPS detoxification Stops neutrophil recruitment **AT-Resolvin D1** Reduces diseases activity index Attenuates pro-inflammatory mediators gene expression Attenuates neutrophil recruitment **Resolvin D-2** Improves diseases activity index Reduces colonic PMN infiltration	Aliberti *et al.* (2002) Gewirtz *et al.* (2002) Gewirtz *et al.* (2002) Wallace *et al.* (2003) Arita *et al.* (2005) Arita *et al.* (2005) Campbell *et al.*(2010) Ishida *et al.* (2010) Bento *et al.* (2011) Bento *et al.* (2011) Bento *et al.* (2011) Bento *et al.* (2011) Bento *et al.* (2011)
Retinopathy / Mouse	**Resolvin E1/ Resolvin D1 / Protectin D1** Protects against neovascularization	Connor *et al.* (2007)
Tissue Regeneration / Mouse	**Resolvin E1** Promotes bone regeneration in calvaria defects	Gao *et al.* (2012)
Tissue Regeneration / Planaria	**Maresin-1** Stimulate tissue regeneration post surgical damage	Serhan *et al.* (2012)

Adapted from: Freire, 2003 Periodontology 2000.

Chronic diseases have reciprocal relationship with associated conditions through inflammation. The impact of a dysregulated local inflammatory response has the potential for harm to the systemic health of individuals, figure 6. Failure in completing self-limited acute inflammation and activate local and systemic signals of inflammation that are detrimental to the host. Loss of resolution and failure to continuous destruction lead to chronic inflammation and disease formation.

-Fig. 6-

Pro-Resolution Signals

TGF-β
IL1-Ra
IL-4
IL-10
GM-CSF
Immunoresolvents:
Resolvins
Lipoxins
Protectins
Maresisns

Local Tissue Inflammation

Pro-Inflammatory Signals

TNF-α
IL-1
IL-6
CCL2
CXCL8
CRP
Histamine
Prostaglandins

Health
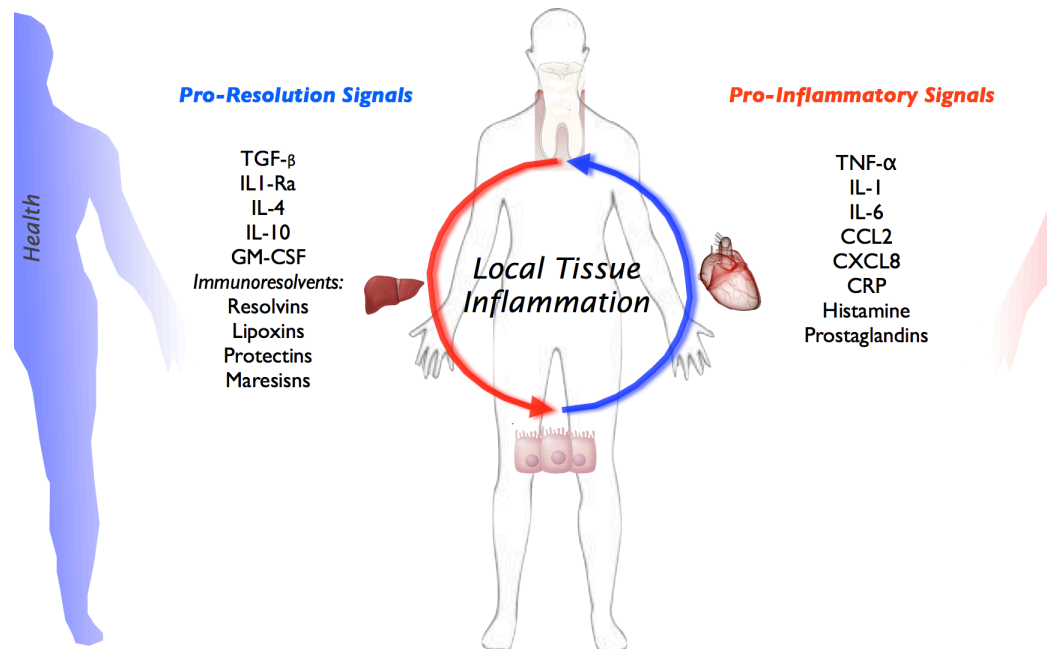
Figure 5. Local and systemic actions of tissue inflammation. Tissue-inflammatory processes link to systemic homeostasis. The nature of challenge and balance between the response from sensors and mediators leads to distinct fates of acute inflammation. Pro-inflammatory mediators such as IL-1, TNF-α, and others listed are important molecules that initiate the tissue response to injury and infection. The termination of acute processes is modulated by immunoresolvents and consequent upregulation of anti-inflammatory cytokines. An unresolved inflammatory process is the primary step leading to development of chronic inflammation and loss of local and systemic function. CCL2, chemokine (C-C motif) ligand 2; CXCL8, interleukin 8; TGF-β, transforming growth factor β; IL1-Ra, interleukin 1 receptor antagonist; GM-CSF, granulocyte-macrophage colony-stimulating factor.

- TYPE 2 DIABETES -

The incidence of type 2 diabetes worldwide has increased drastically during recent decades. According to the International Diabetes Federation about 642 million adults have diabetes and unfortunately the current prevalence is estimated to increase by year 2040 (Maertens, 2016; Guariguata, 2011). The problem is global and impacts quality of life, lifespan and overall healthcare costs. The long term consequences of uncontrolled metabolic disorders such as type-2 diabetes include a cluster of pathologies associated with chronic inflammation, such as obesity, cardiovascular diseases, blindness, chronic kidney diseases and periodontal diseases (Hotamisligil, 2006, Li and Hotamisligil, 2010).

Inflammation is a key component in protecting the host against injury and infections; protective acute inflammation is self-limited, but can lead to pathology when prolonged or excessive (Serhan, Vhiang and Van Dyke, 2008). The resolution phase of inflammation is activated temporall after an acute

challenge and involves eicosanoid class switching from pro-inflammatory to pro-resolution lipid mediators (LM).   The failure of resolution of inflammation leads to chronic oxidative stress, tissue damage, scar formation and fibrosis (Samuelsson et al., 1987, Serhan and Drazen, 1997, Fierro and Serhan, 2001, Serhan and Chiang, 2002, Serhan et al., 2002).   Continuous unresolved inflammation establishes a pathological response leading to chronic diseases (Van Dyke and Kornman, 2008).

A relationship between inflammatory pathways and metabolic diseases, such as type 2 diabetes or insulin resistance, is one line of active investigation (Chapple, 2013). Attempts to understand the molecular pathways that regulate diabetes and insulin resistance require the study of adipose tissue. For example, adipose tissue is now recognized as a biologically active endocrine organ and not simply a site of inert lipid storage. Adipocytes, like any stromal cell, secrete cytokines, that are now collectively known as adipokines, into the circulation, including adiponectin, TNF-α, plasminogen activator inhibitor type 1 and resistin (Bharti, 2013). The realization that adipocytes have a molecular influence on the entire system helps explain why obesity is frequently associated with inflammatory disease, type 2 diabetes and atherosclerosis.

Evidence suggests that inflammatory cells may be found within adipose tissue; perhaps the fat itself is actively involved in inflammatory processes, contributing through the release of proinflammatory mediators.

Adiponectin has many actions in the muscle, liver, and blood vessel walls and is believed to play an important role in modulating glucose and lipid metabolism. Although adipose tissue may release a host of proinflammatory cells, adiponectin is of interest because it seems to be anti-inflammatory and, therefore, protective. For example, lower levels of plasma adiponectin are found in patients with diabetes and CHD compared to patients with diabetes without CHD, suggesting that adiponectin may be anti-atherogenic. Humans in insulin-resistant states also have lower levels of adiponectin; this was reversed by the administration of thiazolidinedione, an insulin-sensitizing compound. There also seems to be a negative relationship between adiponectin and CRP, again suggesting that adiponectin has an anti-inflammatory role.

Patients with diabetes are at two to three times greater risk for developing chronic periodontitis and those with elevated HbA1c have a significantly higher prevalence of periodontitis and more tooth loss than those

with better metabolic control. Acute and chronic infections may adversely influence glycemic control. Furthermore, it has been established that HbA1c is adversely affected by systemic inflammation. In this context, a biologically plausible link between metabolic control and periodontitis has been established. If effective treatment of periodontitis can modify glycemic control, as suggested in some studies, then periodontal therapy may be an important contribution to a patient management program that incorporates life style changes and medications.

As more studies are published, the question of the impact of periodontal therapy on diabetic control becomes clearer. Recent meta-analyses included studies with at least 3 months of follow-up after periodontal therapy measuring type 2 diabetes outcomes. Equally noteworthy is that the effect size of −0.36% HbA1c (95% CI −0.66, −0.19) observed across all nine studies is comparable to the two most recent and extensive systematic reviews [−0.40% HbA1c, CI −0.77, −0.04 and −0.40% HbA1c, CI −0.78, −0.01], and remains statistically significant. Hence, conclusions made previously that periodontal therapy may improve metabolic parameters, as measured by HbA1c, as a result of periodontal therapy are consistent with the findings of the most recent meta-

analysis. Of note, a review and meta-analysis reached a similar (albeit non-significant) effect level (−0.38% HbA1c, CI −1.5, 0.7); none of the studies in that review were part of the present analysis due to inclusion restraints. A major limitation, as before, is that no single randomized clinical trial reported here would be defined as a large randomized controlled trial (RCT, pivotal study) and hence, validation of these findings in a large clinical trial is still needed.

Also of importance is that while periodontal therapy across studies included in the most recent analysis were fairly uniform, the issue of treatment to a clinical end-point has not yet been addressed. In two of these nine studies, periodontal conditions did not change significantly, whereas in seven of these nine studies statistically significant periodontal treatment effects were observed. To what extent these treatment effects can be considered clinically relevant for diabetes outcomes cannot be determined, because the authors reported mean values for clinical attachment loss and probing depths and outcomes are open to interpretation from a clinical perspective.

Reductions of HbA1c have been the standard treatment outcome for diabetes and the development of new treatment modalities, because reducing

HbA1c has been shown to prolong the onset of diabetic complications. However, there is currently no evidence of a "threshold" for the benefit of reducing HbA1c (Atouf, 2007; Bharti, 2013; Borgnakke, 2013). Hence, any reduction in HbA1c might be expected to decrease the risk of diabetic complications regardless of the baseline HbA1c. Regarding the clinical relevance of periodontal treatment on metabolic control, it must be understood that dental treatment is an add-on therapy to pharmacotherapy, and lifestyle changes (Atouf, 2007). Metformin is often used as the drug of first choice in the treatment of type 2 diabetic patients. Around 50% of all type 2 diabetes patients are treated with one or more additional drugs to achieve metabolic control. Thus, to weigh the clinical relevance of any "dental" HbA1c reduction, we have to compare its impact with a second drug in addition to metformin. As add-on treatments to metformin, several agents have been used. The additional reduction of HbA1c over metformin alone was 0.85%, 0.61%, and 0.42% for sulphonylureas, α-glucosidase inhibitors and thiazolidinediones, respectively. Thus, if periodontal therapy can improve HbA1c levels by 0.4 to 0.5%, then its effect may be comparable to additional pharmacotherapy and therefore may find a place in the treatment of diabetic patients.

In type 2 diabetes, dysregulation of resolution is a possible link to inflammatory pathology (Spranger et al., 2003). The exact deficiencies are not known. In a monogenic murine model of obesity and type-2 diabetes, *db/db* transgenic mice were shown to exhibit decreased neutrophil chemotaxis, delayed wound healing, deficient phagocytosis, delayed neutrophil apoptosis and deficient clearance of inflammatory lesions (Herrera et al., 2015).

Here, the expression and function of a key receptor in resolution, ERV-1 was investigated in human neutrophils from subjects with type 2 diabetes. We discovered that ERV1 was upregulated and functional on neutrophils in T2D and BLT-1 signaling and function was lost. The goal of the studies described was to determine the basis for inflammatory dysregulation in type 2 diabetes.

- 2. Hypothesis & Specific Aims -

Loss of regulation of inflammation is a biological event common to many human diseases including Type 2 *diabetes*. Acute inflammation is a well-coordinated process that is self-limited; the termination sequence is mediated by endogenous lipids that orchestrate a return to homeostasis (resolution of inflammation) through cellular stimulus. For example, a well-characterized pathway of activation of resolution of inflammation is mediated by interaction between resolvin E1 (RvE1) and GPCR receptor ERV-1 (CMKLR1 or ChemR23). The receptor ERV-1 binds the agonist ligand RvE1 leading to attenuation of NF-KB mediated pro-inflammatory cytokines. The expression and function of ERV-1on various cell types in patients with chronic inflammatory diseases is partially known; however, how ERV-1 activation dictates the path of innate cells to resolution, rather than development of a chronic lesion, remains unknown.

A central characteristic of effective acute inflammation is the rapid return to homeostasis. Protective endogenous inflammation is tightly regulated by a genus of specialized pro-resolving lipid mediators (SPM). Much attention has been given to these lipid ligands and their downstream functions, but how the receptors of resolution behave in human chronic diseases has not been explored.

The goal of the experiments outlined in this proposal will test the central hypothesis that ERV-1 expression and function is altered in patients with chronic inflammatory diseases, including type 2 diabetes. *The* long term goal *of this proposal is to understand the molecular mechanisms that control expression and function that activate resolution of inflammation receptor, ERV-1.* The *rationale* that underlies the proposed research is that a better understanding of the biology of activation of resolution of inflammation will lead to improved clinical management and prevention in the future. We plan to objectively test our central hypothesis and thereby attain the objective of this application by pursuing the following *specific aims*:

Specific Aim 1: is to characterize the expression of ERV-1 receptors on neutrophils of subjects with type 2 diabetes.

- Do patients with uncontrolled chronic inflammatory diseases present differential expression of ERV-1 receptor?

- Is the expression of ERV-1 on neutrophils and monocytes influenced by pro-inflammatory or pro-resolution receptor agonists?

Specific Aim 2: is to characterize the molecular pathways that are regulated by ERV-1 in disease.

- Are the specific intracellular signaling pathways activated through ERV-1 abnormal in diabetes or periodontitis?

- Does ERV-1 activation by resolvins influence cellular fate?

- 3. Materials and Methods -

SUBJECT SAMPLES

The patients were recruited to the Center for Clinical and Translational Research at the Forsyth Institute and samples were obtained under consent approved by the Forsyth Institute Review Board (FIRB, protocol #11-03). Peripheral venous blood (~60 ml) of individual patients diagnosed with type 2 diabetes (T2D) or not was collected (n=83,T2D; n=83,healthy). The diagnosis followed American Association of Diabetes guidelines (American Diabetes, 2016) and HbA1c levels were used to confirm the glycemic levels. All blood donors were otherwise healthy, denied taking any non-steroidal anti-inflammatory drugs (NSAIDs) or antimicrobials for at least two weeks prior to the experiment.

ISOLATION & CULTURE OF HUMAN NEUTROPHILS

Human neutrophils were isolated from human whole blood by Ficoll-Histopaque density-gradient centrifugation (Histopaque 107 1077& 1119, Sigma-Aldrich). Neutrophils were isolated after isotonic lysis of red blood cells followed by two washes in phosphate buffered saline (Sigma-Aldrich). In

culture, experiments, cells were obtained and incubated with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (v/v) (Life Technologies) at 37°C.

After cell isolation as described above, neutrophils and monocytes from subjects with diabetes and normal controls were be incubated with stimulants including, LPS (10ng/ml), RvE1 (0.1nM-100nM), TNF-□ (10ng/ml). Various combinations of the agonists were explored. When two compounds were used the first was incubated for 15min at 37° C, 5% CO2 and the subsequent treatment for 15 in similar conditions.

In order to understand whether ERV-1 receptor is functional in type 2 diabetes, peripheral blood neutrophils were isolated, and treated with RvE1 (10nM), Blt-1 receptor antagonist, U-compund (1ng/ml) and RvE-1 receptor antagonist,ERV1/Ab(1ng/ml). Various combinations of the RvE1 and antagonists were explored. When two compounds were used the first was incubated for 15min at 37° C, 5% CO2 and the subsequent treatment for 15 in similar conditions. The total stimulation time was 30 mins and Rs6 signaling was evaluated by FACS analysis

CELL LINES

Recombinant Human ERV-1 was inserted to Chinese hamster ovarian cells positive for ERV-1 receptor (CHO $^{ERV1\ +}$, Genscript) and negative for ERV-1 receptor CHO $^{ERV1\ -}$ cells, Sigma). Cells were cultured in Ham's 12 medium supplemented with 10% fetal bovine serum (GIBCO), 100U/ml of Zeocin and Hydromycin antimicrobiasl and maintained 37°C/5% CO2. Cells were incubated with resolvin E1 for one hour before total RNA was extracted.

RESOLVIN

RvE1 was prepared by total organic synthesis, as in Arita et al. The structural integrity of RvE1 was monitored using UV tandem LC-MS/MS. Immediately before use, RvE1 was diluted in phosphate-buffered saline to final ethanol concentration of <1%.

SURFACE MARKER EXPRESSION

 Peripheral blood PMN were isolated from control and type 2 diabetes adult individuals. Isolated cells were stained with anti-human ERV-1 ab or anti-IgG

alexafluor 488 (isotype control, R&D Systems).   Expression of ERV-1 of neutrophils were evaluated by immunofluorescence and quantified by flow cytometry. Anti-human Cd11 and Cd18 antibodies (BD) revealed similar expression patterns when comparing both groups, data represented as overlapping histograms. Expression levels of the proteins were monitored by flow cytometry (FACSCanto II, BD Biosciences) and analyzed with FlowJo (Tree Star).

CYTOSPIN & GIEMSA STAINNING

Isolated human cells were centrifuged at 2000 rpm for 5 min, cell pellets were suspended in PBS (200 µL), counted and 50 µL of each cell suspension was mixed with 150 µL of 30% BSA and centrifuged onto microscope slides at 500 rpm for 5 min using a cytospin centrifuge, air-dried, and stained with Wright-Giemsa to identify individual cell type.

PCR

Total RNA was isolated from human neutrophils through a TRIzol (Life Technologies, Carlsbad, CA, USA) extraction method (M. Portillo, 2006) and then the purity was tested using a NanoDrop ND-1000 spectrophotometer

(Thermo Scientific, Waltham, MA, USA). After the purity and concentration was tested, the RNA was then stored for later use at -80 C cDNA Synthesis. The RNA extracted using the TRIzol RNA extraction method (M. Portillo, 2006) was then reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) (S.C. Lee, 2005). The quantitative real-time PCR used to analyze gene expression was the Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The detection assay used was the SYBR Select Master Mix for CFX (Applied Biosystems, Foster City, CA, USA).

LC-MS/MS–BASED LIPID MEDIATOR (LM) METABOLOLIPIDOMICS

Blood samples were collected, centrifuged at 2,300 rpm, and frozen at −80 °C until analysis. Methanol (4 volumes, 4 °C, 30 min) containing 500 pg of deuterated internal standards d4-LTB4, d8-5S-HETE, d5-LXA4, and d4-PGE2 to facilitate quantification of sample recovery were added to serum. LMs were extracted using C18-silica reverse-phase cartridges as in Dalli and Serhan (2012). Samples were eluted with 6 mL methylformate and dried using

Speedvac and suspended in methanol/water for LC-MS-MS. The liquid chromatography-UV coupled with tandem mass spectrometry system includes QTrap 5500 equipped with a Shimadzu SIL-20AC auto-injector and LC-20AD binary pump. An Agilent Eclipse Plus C18 column (100 mm × 4.6 mm × 1.8 μm) was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (v/v/v) to 100:0:0.01 at a 0.5-mL/min flow rate. To monitor and quantify the levels of the various LMs, we developed a multiple reaction–monitoring (MRM) method with signature ion fragments for each molecule. Identification was conducted using published criteria (Dalli and Serhan 2012). Calibration curves were obtained using synthetic and authentic LM mixtures, including d8–5S-HETE, d4-LTB4, d4-PGE2, LXA4, LXB4, LTB4, PGE2, PGD2, PGF2α, TxB2, RvE1, RvE2, RvD1, RvD2, RvD3, RvD5, PD1, and MaR1 at 12.5, 25, 50, and 100 pg. Linear calibration curves for each were obtained with $r2$ values in the range of 0.98 to 0.99. Quantification was based on peak area of the MRM transition and the linear calibration curve for each compound. Reverse phase chiral LC-MS-MS was conducted as described (Dalli and Serhan 2012).

PHAGOCYTOSIS ASSAY

Human neutrophils were cultured into RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS, 5.5 mM glucose, 1% DMSO (Sigma) and incubated at 37°C to differentiate cells. Porphyromonas gingivalis (W38, 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 LaboratoryServices), 5 µg/ml of hemin, and 0.5 µg/ml vitamin K (Sigma-Aldrich). 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) and grown for 4 days. Bacterial titers were determined at 600 nm using a spectrometer (SmartSpec 3000, Bio-Rad) and adjusted to OD=1.0 (approximately130 CFU/ ml) prior to experiments. Human neutrophils were then counted and aliquot in 1 x 10^6 increments. Bacteria was then cultured (as estimated by an OD_{600} of 1 = 10^9 cells) and labeled with BacLight Green (Molecular Probes) for 20 m at RT with gentle agitation and washed twice with PBS. Labeled bacteria were opsonized in heat inactivated normal serum (Sigma) for 30 m at RT and incubated with human neutrophils (1:20 ratio) for 1 h in

serum and antibiotic-free medium at 37°C. Cells were gently washed, extracellular fluorescence was quenched by Trypan blue and phagocytosis was determined by flow cytometry analysis (BD FACS Aria). Similar protocol was followed for labeled zymosan fluorescent bioparticles (Life sciences). Positive labeled cells with internalized particles or bacteria were evaluated by FlowJo and data expressed as phagocytic index: Phagocytic index = % phagocytic neutrophils × mean fluorescence intensity.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using Prism 6 (GraphPad). Wilcoxon Test and Student unpaired *t* test were used to compare measurements. Values of $P \leq 0.05$ were considered statically significant.

- 4. Results -

CLINICAL CHARACTERISTICS OF RESEARCH SUBJECTS

To investigate the role of the RvE1 - ERV-1, ligand – receptor axis in type 2 diabetes, peripheral whole blood was collected from subjects enrolled in the study (healthy subjects: n = 83; T2D subjects: n=83) after obtaining informed consent. The experimental protocol was approved by the Institutional Review Board of the Forsyth Institute (IRB#13-07). Serum glucose was evident in patients with type 2 diabetes when compared to controls ($p<0.0001$, Figure 6A), and HbA1c levels confirmed serum measurements (Supplementary Figure 1). Serum cholesterol and BMI were elevated in subjects with type 2 diabetes ($p<0.05$, Figure 6 B and $p<0.0001$, Figure 6C). Total neutrophil counts were increased in subjects with type 2 diabetes ($p<0.05$, Figure 6 D); no difference was found in total monocyte count ($p= 0.176$, Figure 6E). Healthy age, gender and race matched volunteers served as controls.
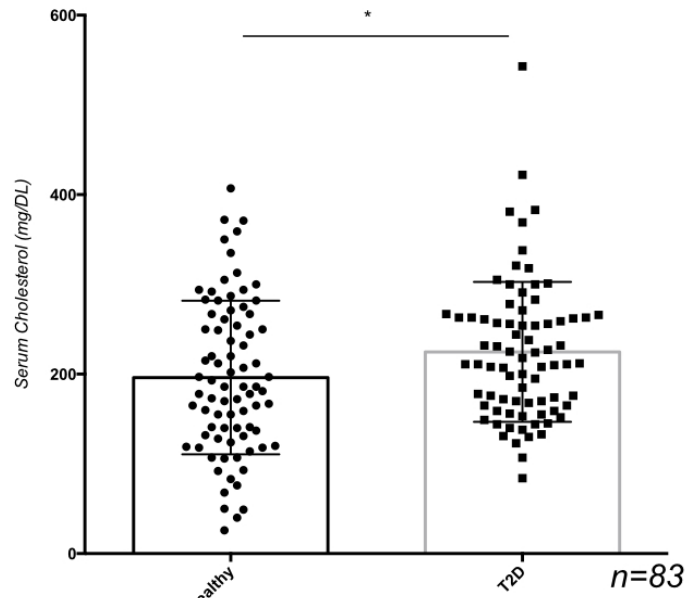
- Fig. 6 -

A
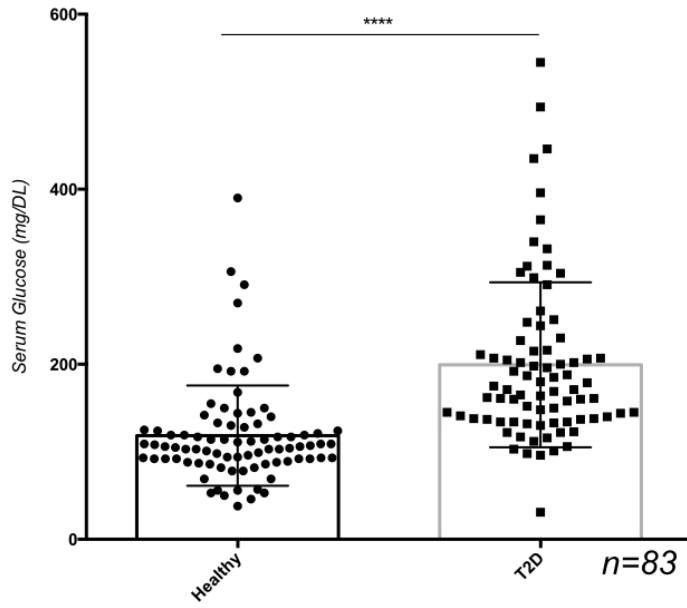
B
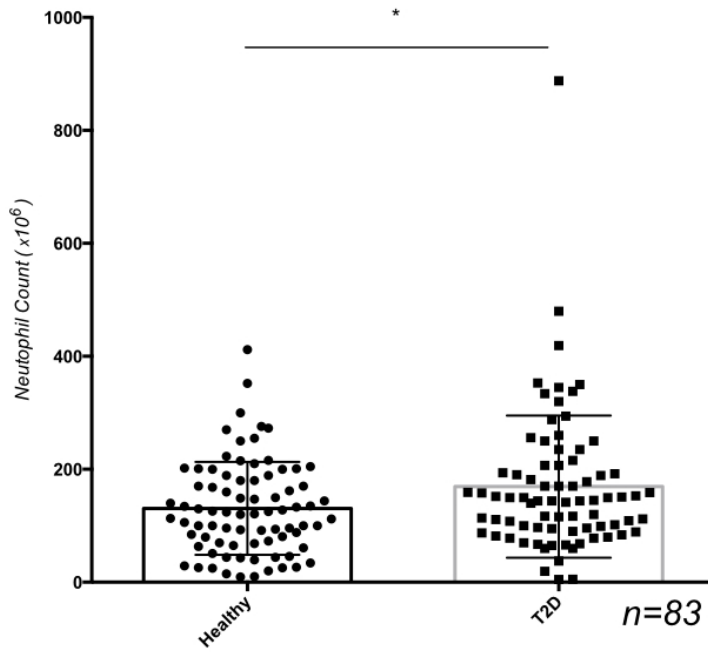
C

Figure 6: Clinical characteristics of study subjects. (A) Serum glucose, (B) total neutrophil count, (C) Serum cholesterol. Statistical significance was evaluated by Wilcoxon test (total number of individuals n=166, healthy, n=83, T2D, n=83; * p<0.05, ns = non-significant).
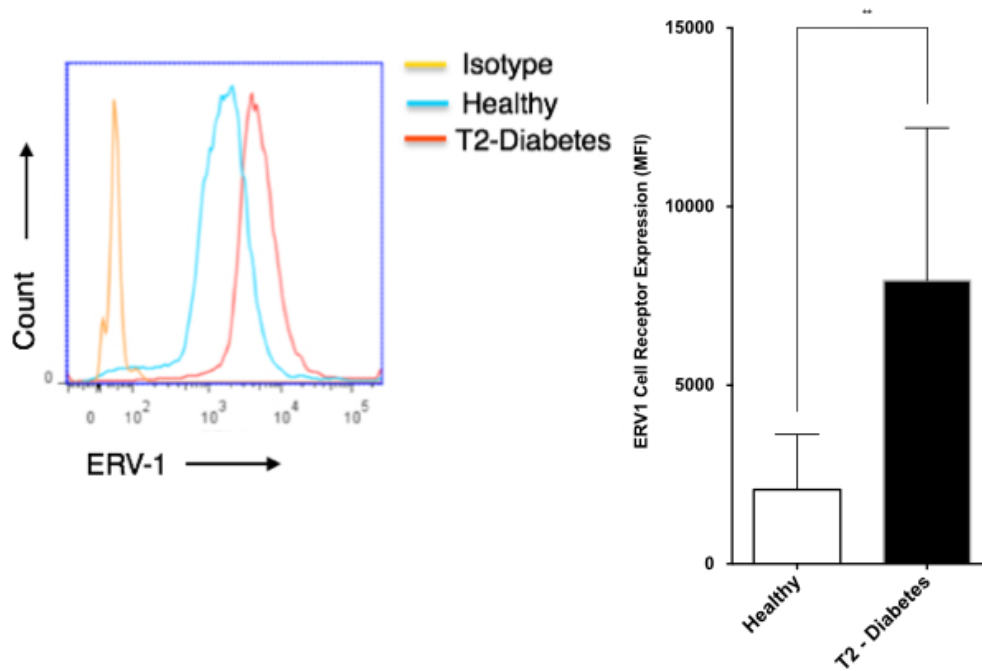
ERV1 IS UPREGULATED ON HUMAN NEUTROPHILS IN TYPE 2 DIABETES

Figure 7A illustrates known receptors active on monocytes and neutrophils in health (Arita et al., 2007). To determine cell surface ERV1 receptor expression pattern of human neutrophils in type 2 diabetes, isolated cells were labeled with anti-ERV-1 antibody for quantification by flow cytometry and immunofluorescence (Figure 7 and 7C). ERV-1 expression was significantly increased on neutrophils isolated from type 2 diabetics (Figure 7B and C, n = 23 p<0.001). The distinct expression pattern of neutrophil ERV-1 and BLT-1 receptors demonstrates that in type 2 diabetes there is high expression of ERV-1 and low expression of BLT-1, while in healthy individuals there is lower expression of ERV-1 and higher expression of BLT-1. CD11b and CD18 expression by neutrophils from Type 2 diabetics and healthy controls is not different. These results indicate a distinct profile of in resolution receptor expression on neutrophils in type 2 diabetes.

Resolution of Inflammation - RvE1/Receptor Activation
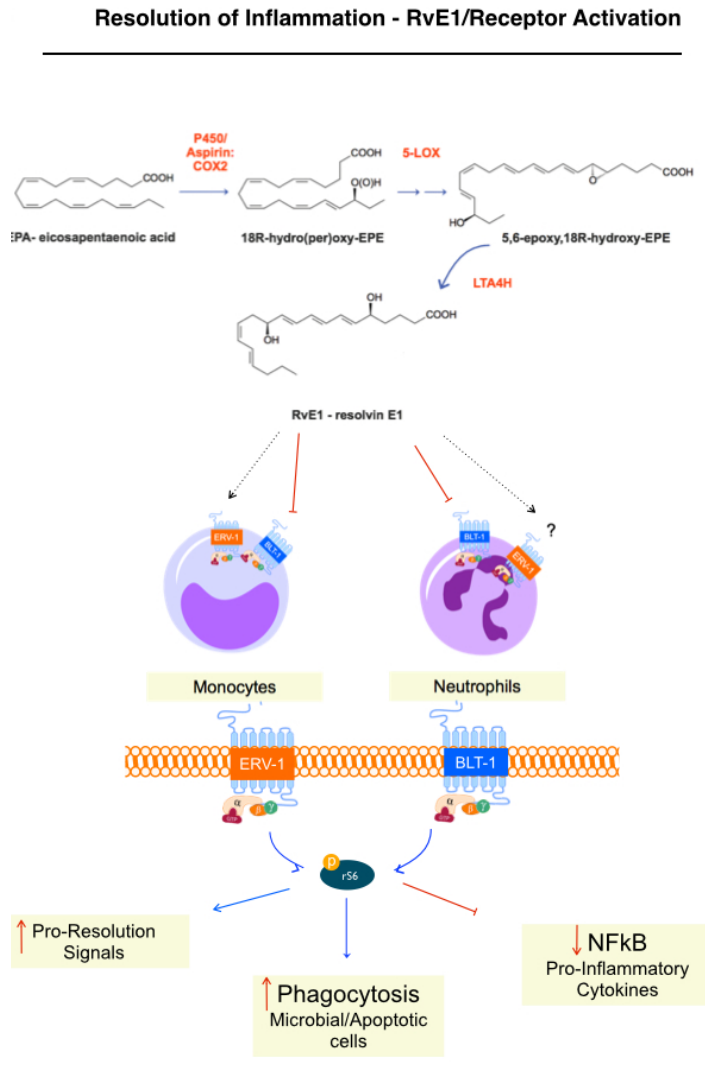
Figure 7A. Schematic representation of resolution of inflammation activation by G-protein receptors ERV-1 and BLT1 interaction with RvE1.

Figure 7. Human ERV-1 receptor is upregulated on type 2 diabetic neutrophils.

(B) Peripheral blood neutrophils were isolated from healthy and type 2 diabetic

adult individuals. Isolated cells were stained with anti-human ERV-1 ab or anti-

IgG alexafluor 488 (isotype control). Expression of ERV-1 by neutrophils was

evaluated by immunofluorescence and quantified by flow cytometry. Data is

expressed as mean fluorescence intensity (MFI). Wilcoxon test was used

evaluate statistical significance (n=23; * p<0.05, ns = non-significant). (n=23;

***p<0.001, **p<0.01, * p<0.05, ns, non-significant).
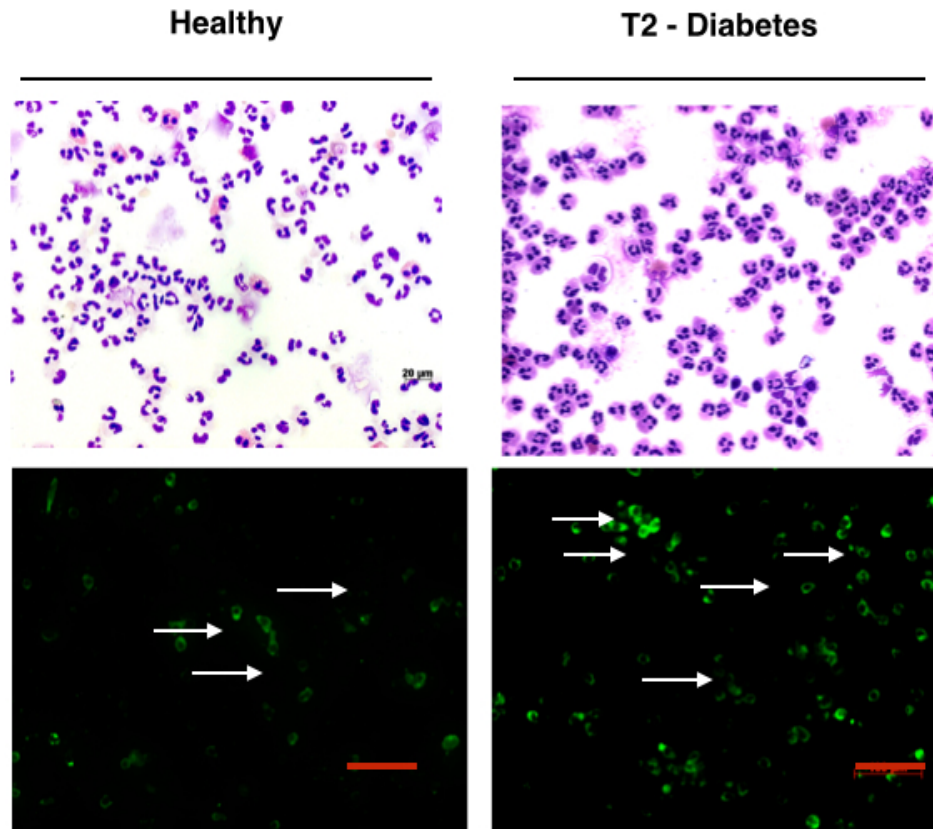
Figure 7C: Cellular staining of isolated neutrophils showed similar morphology among the groups (upper panels) and representative immunofluoresnce staining with anti-human ERV-1 ab (green) showed increased staining of ERV-1 receptors per cell (white arrows).

RvE1 stimulated healthy neutrophils have been shown to signal through BLT-1 and also inhibit LTB_4 function through competitive inhibition (Arita et al., 2007). Since surface BLT-1 is downregulated in T2D and ERV1 is upregulated, we performed experiments to determine how ERV1 expression is regulated. We first stimulated T2D and control neutrophils with pro-inflammatory cytokine TNF-α or bacterial LPS and measured surface expression of ERV1 with FACS. When neutrophils are treated with TNF-□ (10ng/mL) for 1 hour, both healthy and T2D diabetics increased ERV-1 protein expression; T2D was significantly greater than control (n=10, p<0.01, Figure 8A). Interestingly, LPS stimulation did not upregulate ERV1 on healthy neutrophils (slight reduction), but significantly upregulated ERV1 on T2D neutrophils (n=6, p<0.01, Figure 8B). Neutrophils were also exposed to RvE1 15 minutes before or 15 minutes after TNFα or LPS. RvE1 treatment of neutrophils under all conditions returned ERV1 surface expression to levels of unstimulated healthy neutrophils (Figure 8A, 8B). Since the kinetics of ERV1 clearance and re-expression are known (Arita et al., 2005, Arita et al., 2007, Ishida et al., 2010, Ishizuka et al., 2008, Ohira et al., 2010) and not likely to account for downregulation of the receptor, we investigated transcriptional regulation to determine whether expression was

controlled at the RNA level. To answer this question, we transfected Chinese

Hamster Ovarian cells (CHO) with the human ERV-1 receptor (designated CHO

$^{ERV1\,+}$). Expression of ERV-1 protein was confirmed compared to CHO^{ERV1-} by

immunofluorescence (Figure 8C) and by qPCR (Figure 8D). We further

confirmed that regulation was directly in response to RvE1 in a dose response

experiment (1-100nM RvE1). Lower concentrations of RvE1 (1nM) trended to

reduce the overexpression of ERV-1 on CHO $^{ERV1\,+}$ cells, (n=6, ns, Figure 8E). 10

nM RvE1 significantly reduced the receptor expression (n=6, $p<0.01$, Figure 8E)

as did 100nM RvE1 (n=6, $p<0.01$, Figure 9E).

- Fig. 8 A,B -

A
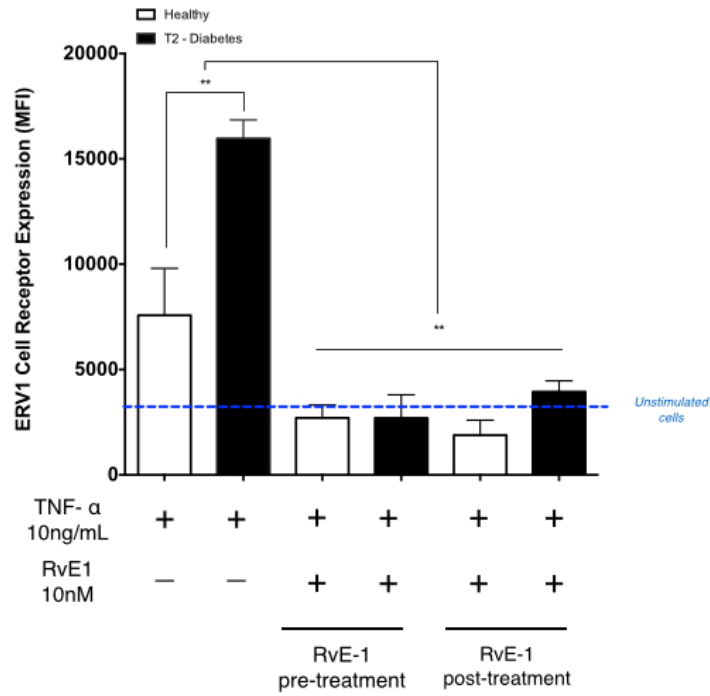
B
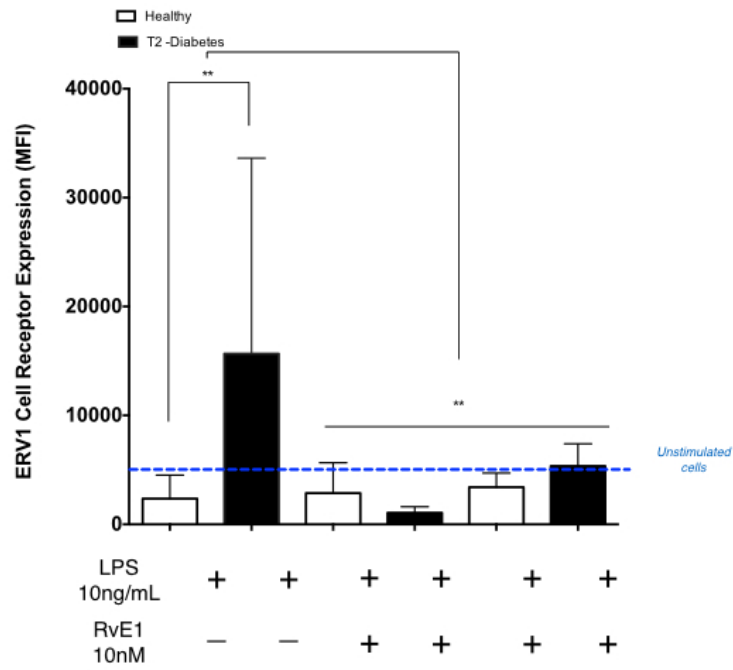
Figure 8. ERV1 receptor upregulation on neutrophils is stimulated by TNF-▢ and LPS, and reversed by RvE1. Neutrophils were stimulated with **(A)** TNF-α (10ng/ml) or **(B)** LPS (10ng/ml) and the impact of 15 minute pretreatment or 15 minute post-treatment with RvE1 (10nM) on ERV-1 expression after 60 minutes was assessed. Neutrophils were stained with alexafluor 488 labeled anti-human ERV-1 or anti-IgG alexafluor 488 (isotype control). Expression of ERV-1 was quantified by immunofluorescence and flow cytometry. Results are the mean ± SEM of mean fluorescence intensity (MFI, Wilcoxon test, n=8, **p<0.01, ns = non-significant).

- Fig. 8 C -

C
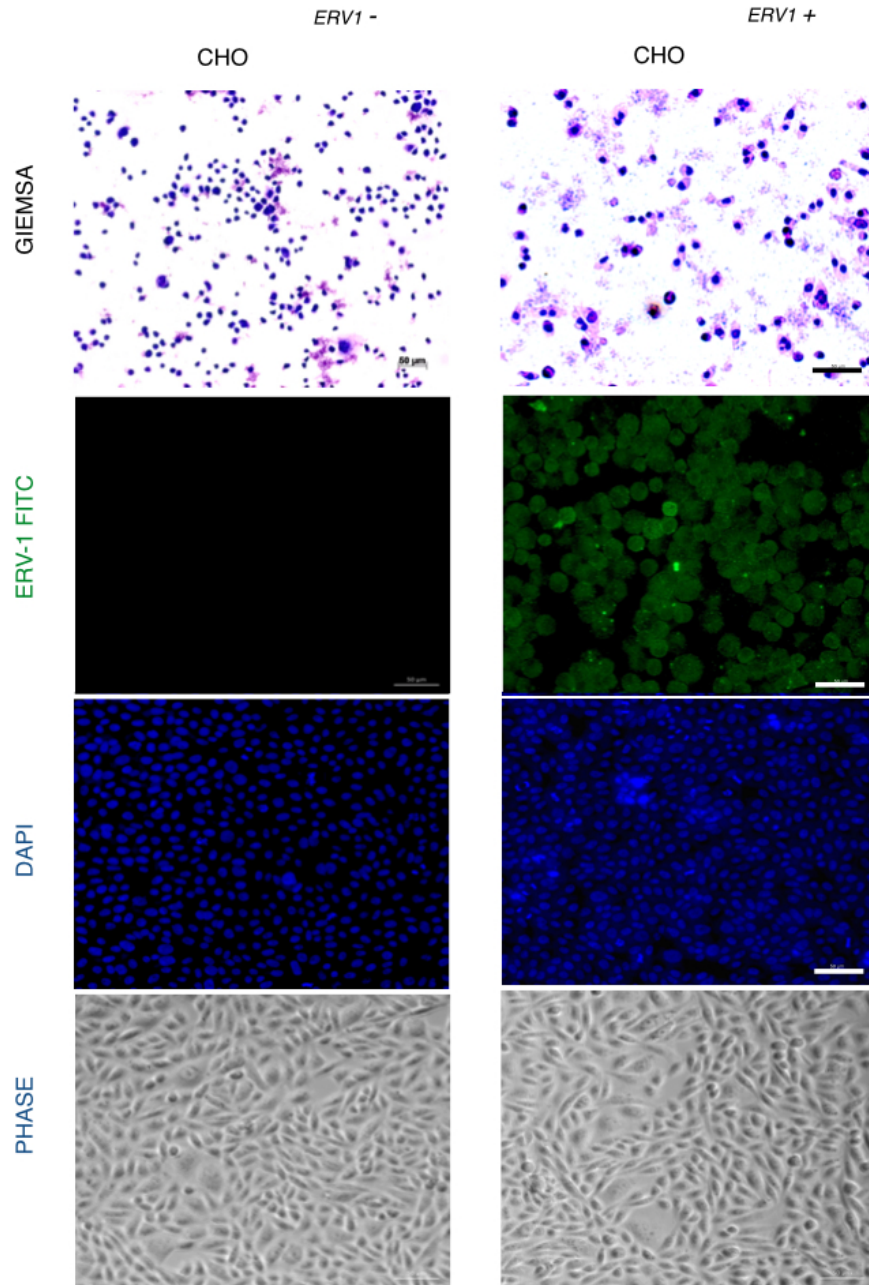
Figure 8. Overexpression of ERV-1 in CHO cell line. **(C)** Chinese hamster ovarian cells were transfected with human ERV1 (CHO $^{ERV1+}$) and were stained with Giemsa, FITC labeled anti-ERV1 and DAPI to demonstrate successful transfection compared to un-transfected (CHO $^{ERV-}$).

- Fig. 8 D, E -

D
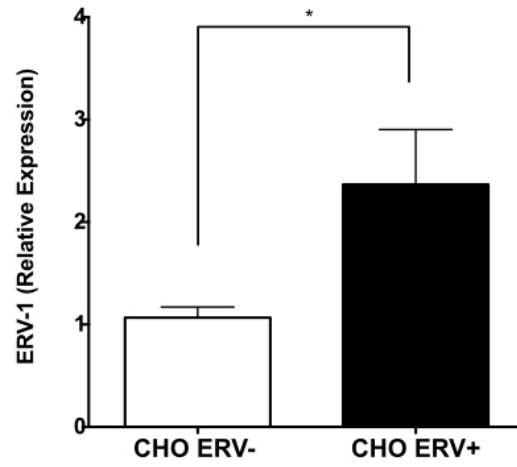
E
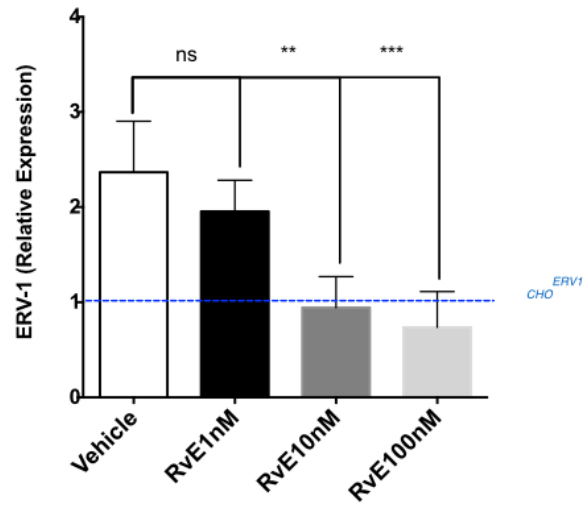
Figure 8. PCR analysis of ERV-1 receptor expression. **(D)** Quantification of relative expression of ERV-1 by CHO $^{EVRV1+}$ cells by real time PCR. **(E)** CHO $^{EVRV1+}$ cells were treated RvE1 (1-100nM) for 1 hour and ERV1 gene expression assessed by qPCR. Cells treated with RvE1 (10nM) or RvE1 (100nM) exhibited down regulation of ERV1 (Wilcoxon test, n=6, **p<0.01, ns = non-significant).

Stimulation of neutrophils with RvE1 activates BLT-1 signal transduction through the Akt/ ribosomal protein S6 (rS6)/ mTOR pathway (Ohira, 2007); human macrophages transduce ERV1 signals through the identical pathway (Ohira, 2007). To determine which receptor(s) is active in T2D neutrophils, we monitored phosphorylation of rS6 protein after alternatively blocking each receptor. Figure 9A demonstrates that when ERV1^{+} neutrophils are treated with resolvin E1 (10nM) intracellular rS6 signaling increases (right panel) when compared to baseline controls (left panels). Because ERV-1 receptor is overexpressed in type 2 diabetes, we investigated the baseline rS6 phosphorylation levels of unstimulated cells. We observed increased levels on neutrophils from T2D when compared to healthy controls (n=8, $p<0.05$, Figure 9 B). Rv E1 treatment significantly increased rS6 phosphorylation in both T2D and healthy cells (n=6, $p<0.001$, Figure 9 B) with T2D expression being significantly greater (n=6, $p<0.001$, Figure 9 B). To assess functional transduction of signals, neutrophils were stimulated with TNF-α or LPS and blocking agents (ERV-1 blocking antibody and the BLT-1 antagonist U230495, Figure 9 C). The results indicate that RvE1 activates rS6 phosphorylation

through BLT-1 in neutrophils from healthy donors and ERV1 in neutrophils from T2D subjects.

To determine which receptor is a transducing signal in health and T2D neutrophils, we used specific blockers of the receptor (Figure 9 D). A mono-specific ERV-1 blocking antibody was used to block ERV1 signaling and the antagonist U230495 was used to block BLT-1. In healthy neutrophils, blocking ERV1 receptor partially blocked Rs6 phosphorylation and completely inhibited T2D neutrophils signaling suggesting that in healthy neutrophils, another functional receptor plays a role in activation signal trasnduction while in T2D neutrophils, ERV1 is the functional receptor (n=8, p<0.001, Figure 9 D). To understand the role of BLT-1, antagonist U230495 treatment depleted signaling while in T2D neutrophils it did not change phosphorylation expression from baseline (n=4, p<0.001, Figure 9 D). Treating neutrophils with both blockers simultaneously completely obliterated rS6 phosphorylation in both T2D and health suggesting that BLT-1 and ERV1 are the key receptors transducing RvE1 signals through this pathway.

- Fig. 9 A, B -

A

Cells   Vehicle   RvE1-10nM

prS6

ERV1

B

rS6 Phosphorylation (MFI)

Healthy   T2- Diabetes   Healthy   T2- Diabetes

Unstimulated Cells   RvE1 10nM
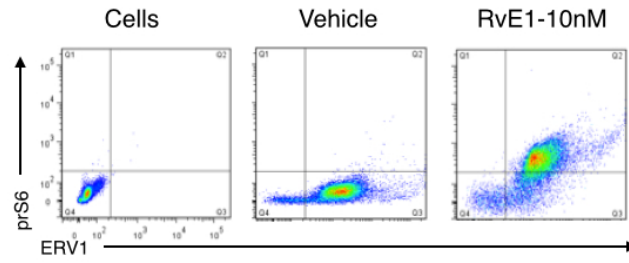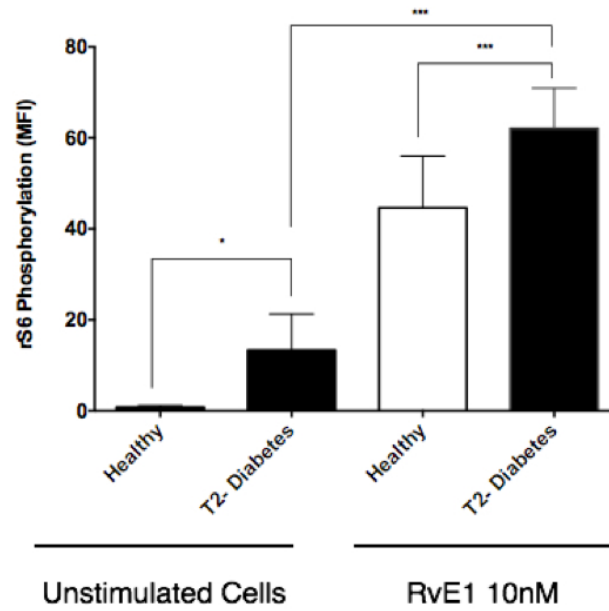
Figure 9. ERV1 signals through rS6 phosphorylation in neutrophils from healthy and type 2 diabetics. **(A)** Representative plots demonstrate rS6 phosphorylation of neutrophils positive for ERV-1 receptor when treated with RvE1 (10nM) for 30m min. After RvE1 treatment, cells were permeabilized, fixed and anti-phospho-rS6-APC antibodies stained cytosolic phosphorylation detected by flow-cytometry. **(B)** Quantification of flow cytometry analyses demonstrates increased phospho-rS6 in type 2 diabetes when compared to healthy controls. Wilcoxon test (n=16, * p<0.05).

- Fig. 9 C, D -

C

RvE1

D

rS6 Phosphorylation (MFI)

Healthy
T2 - Diabetes

ERV1-Ab	+	+	−	−	+	+
U230495	−	−	+	+	+	+
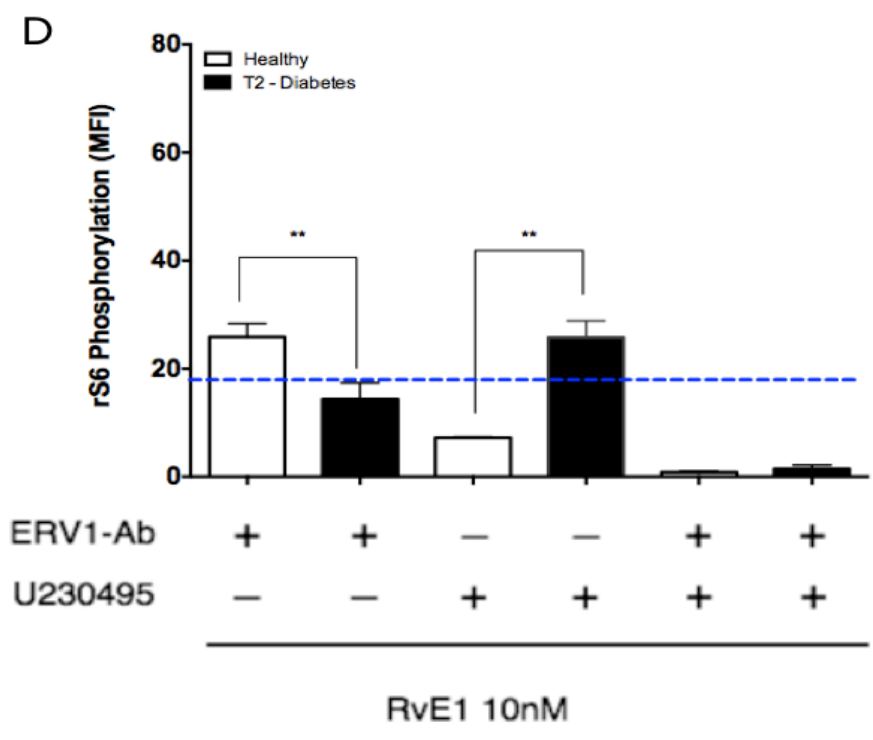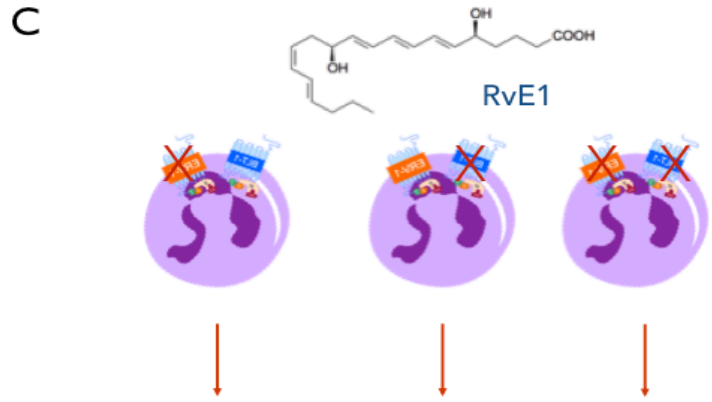
RvE1 10nM

Figure 9. Blocking neutrophils ERV-1 and BLT-1 receptors interrupt RvE1 signal. **(C)** Schematic diagram demonstrates blocking of each receptor individually. Neutrophils were treated with RvE1 (10nM), 15 minutes prior to or after stimulation with TNF-α (10ng/ml) or LPS (10ng/ml). **(D)** Neutrophils were treated with RvE1 (10nM) alone, or in combination with BLT-1 receptor antagonist (U-230495) or ERV1 receptor antagonist (ERV1/Ab) or both. After 30 minutes treatment, FACS quantification of phospho-rS6 signaling was evaluated. Blocking antibody to ERV1 ablates the response suggesting that the T2D neutrophils are responding to stimulation rather than having been pre-activated. Results are expressed as mean fluorescence intensity (MFI, mean ± SEM, Wilcoxon test, n=10, **p<0.01).

TYPE 2 DIABETES EPA BIOACTIVE AND AA BIOACTIVE METABOLIPIDOMICS

To understand the profile of LM-SPM in human peripheral blood, human sera from healthy controls and type 2 diabetic individuals were analyzed by LC-MS-MS . We indentified LM from selected DHA, EPA and AA bioactive metabolomes. Representative MRM chromatograms for each of the identified LM in serum from diabetic patients and healthy volunteers (Figure 11A, left panel). MS-MS fragmentation spectra employed for identification of RvD5, RvD1 and RvE1 (Figure 10A top panel). Lipid mediators from DHA bioactive metabolome (RvD1-6, apirin triggered,AT-, RvD1, protectin D1 and maresin D1, MAR1) identified in diabetic patient samples and control. For the EPA bioactive metabolome in healthy, lipid mediators resolving were quantified (RvE1-3). In addition the arachdonic acid, AA, metabolome was investigated including 5S,15S-diHETE, leukotrine B4 (LTB4), 20-OH-LTB4,20-COOH-LTB4, prostaglandins (PG) D2, E2 and F2a, lipoxin (LX) A4, B4 and tromboxane (TxB)2. Quantification of mediators from the EPA metabolome demonstrated in healthy sera, RvE1 (0.6± 0 pg/ml), and non detectable for RvE2 RvE3 lipid mediators. While in type 2 diabetic sera a slight decrease of the RvE1 (0.4± 0.1 pg/ml), and non detectable for RvE2, RvE3 lipid mediators. Since endogenous resolvin E1 is a key target to our study, we further investigated it's concentration by ELISA.

Results demonstrated a decrease in type 2 diabetes compared to healthy controls (n=10, p<0.001).

The AA bioactive metabolome from healthy serum indentified a remarkable increase of LTB_4 (16.8 ± 4.0 pg/ml) in serum from type 2 diabetes when compared to healthy controls LTB_4 (5.8 ± 2.1 pg/ml) (n=5, p<0.05). While TxB_2 was higher in healthy serum (148.5 ± 63.9 pg/ml when compared to type 2 diabetes (63.9± 37.7 pg/ml) (n= 4, p<0.001). For health sera LXA_4 (0.2± 0.1pg/ml), and LXB_4 (2.6 ± 1.2 pg/ml), $5S,15S$-diHETE (7.6 ± 3.2 pg/ml), 20-OH-LTB4 (5.8 ± 2.4 pg/ml), 20-COOH-LTB4 (1.7 ± 0.6 pg/ml), PGD_2 (1.2± 0.5pg/ml), PGE_2 (2.7± 1.0pg/ml), PGF_{2a} (2.7± 1.0pg/ml), TxB_2 (148.6± 43.0pg/ml). For type 2 diabetic serum, increase of inflammatory mediators and slight decrease in the resolution mediators was found. LXA_4 (0.2± 0pg/ml), and LXB_4 (1.4 ± 0.3 pg/ml), $5S,15S$-diHETE (4.3± 1.4 pg/ml), 20-OH-LTB4 (6.6 ± 1.8 pg/ml), 20-COOH-LTB4 (1.6 ± 0.3 pg/ml), PGD_2 (1.2± 0.7pg/ml), PGE_2 (1.4± 0.5pg/ml), PGF_{2a} (2.1± 1.1pg/ml).

- Fig.10 A -
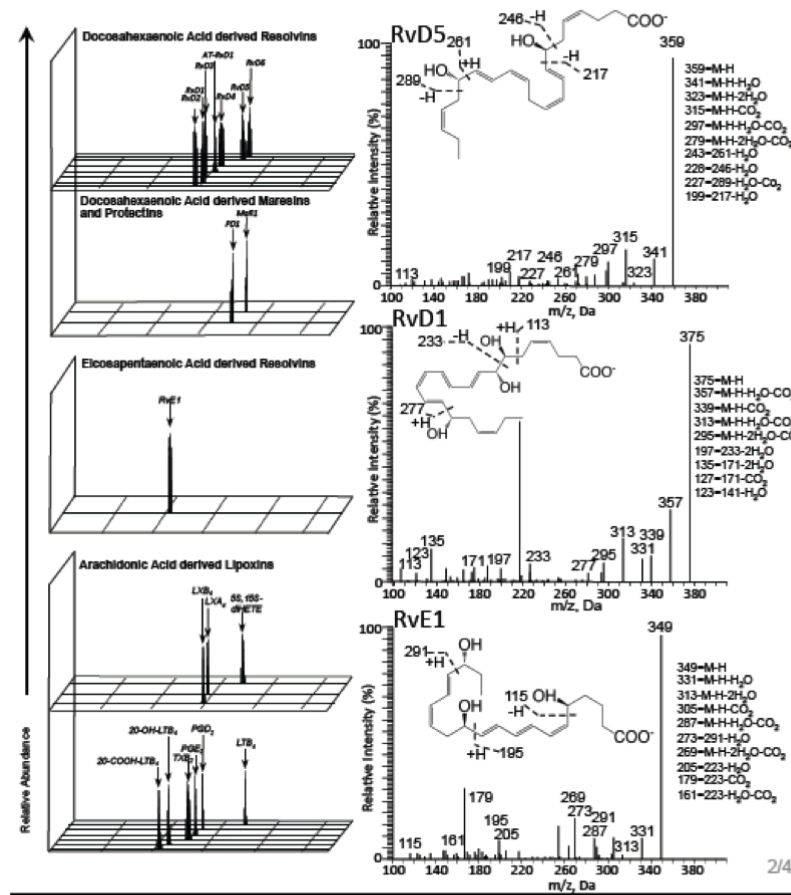
Figure 10. (A) Representative MRM chromatograms for each of the identified LM in serum from Type 2 diabetes patients and healthy volunteers, left panel MS-MS fragmentation spectra employed for identification of RvD5, RvD1 and RvE1, right panel.

- Fig.10 B -

B

Healthy

T2 Diabetes

DHA - Metabolome

- RvD1
- AT-RvD1
- RvD2
- RvD3
- RvD4
- RvD5
- RvD6
- PD1
- MAR1

EPA - Metabolome

- RvE1
- RvE2
- RvE3

AA - Metabolome

- 5s,15S-diHETE
- LTB4
- 20-OH-LTB4
- 20-COOH-LTB4
- PGD2
- PGE2
- PGF2a
- LXA4
- LXB4
- TxB2
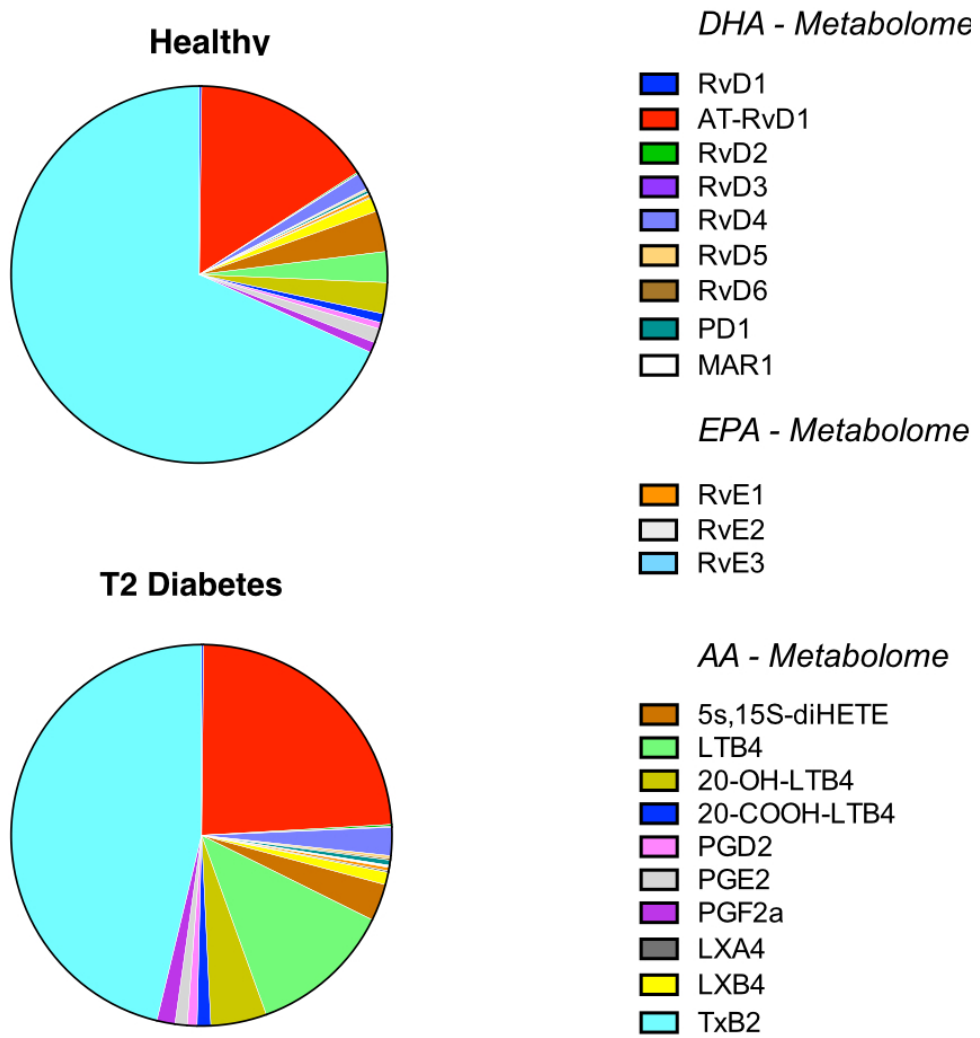
Figure 10. EPA bioactive and AA bioactive metabolomics from serum of healthy and type 2 diabetes. **(B)** Proportions of lipid mediators from AA bioactive metabolome identified in Type 2 diabetes patient samples and healthy volunteers. Results are expressed in parts of whole pie chart, (n=5; *p<0.05; **p<0.01). Quantification of lipid mediators are expressed in mean ± SD pg/mL of serum.

RESOLVIN E1 RESCUES PHAGOCYTOSIS IN A DOSE DEPENDENT MANNER

SPMs like resolvin E1 have the ability to influence cell behavior and act as agonist for functional in response to infection and inflammation (Cotran, 1965b, Ramon et al., 2014, Schwab et al., 2007, Spite et al., 2009, Widmann et al., 1972). Phagocytosis is a key biological process for neutrophils and macrophages to promote clearance and return to homeostasis. To understand functional actions of neutrophils in type 2 diabetes, we have treated cells with resolvin E1 and assayed phagocytosis *in vitro* upon bacterial challenge and zymosan bioparticles. In a time point assay, neutrophils from T2D had decreased phagocytosis after 1-4 hours when compared to controls (n=4, Figure 11A). The deficient phagocytosis seen in T2D is clear hallmark in diabetes and formation of ulcers and periodontal abscesses are major consequence of this deficiency. Cells in T2D presented increased phagocytosis when resolvin E1 treatment was done prior to the bacterial infection. We treated resting primary human neutrophils with 1, 10, and 100nM RvE1 and investigated their action on activation of the ERV-1 receptor and phagocytosis (n=12, Figure 11B).

The results were consistent for bacteria and zymozan bioparticles (n=8, Figure 11C and 11D). For neutrophils from healthy subjects low dosages of treatment (1, 10 nM RvE1) induced phagocytosis in a dose response manner, while for diabetics subjects higher therapeutic dose showed the highest induction (100 nM RvE1). In healthy neutrophils, blocking ERV1 blocked resolving E1 induced phagocytosis and completely inhibited T2D neutrophils cell function (n=4, p<0.01, Figure 11E). By blocking BLT-1, with antagonist U230495, healthy neutrophils were had highly induced phagocytosis while in T2D neutrophils the ceullular function was depleted (n=4, p<0.001, Figure 11E). Treating neutrophils with both blockers simultaneously obliterated resolvin E1 agonist function in phagocytosis for T2D diabetes, demonstrating that the receptor ERV1 is a key regulator for agonist functions activation by resolving E1.

- Fig.11 A,B -

A

B
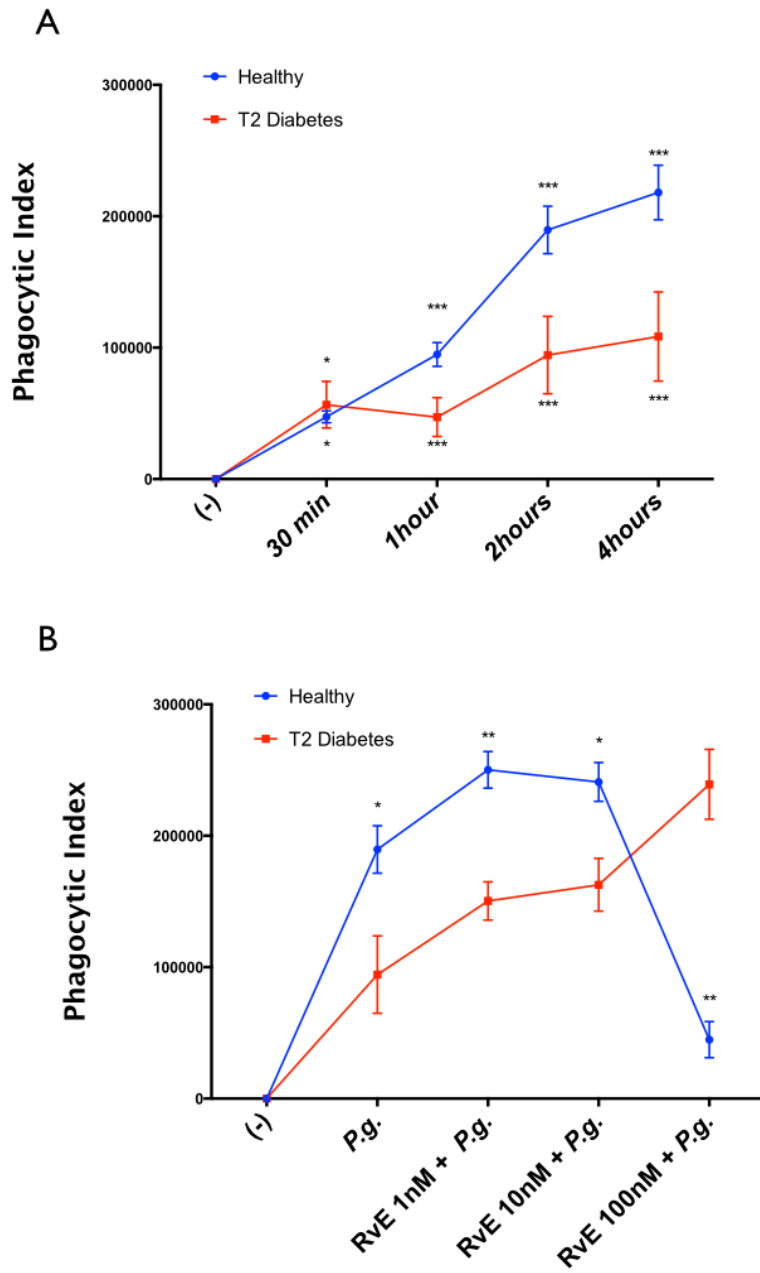
Figure 11. Human ERV-1 mediated phagocytosis. Peripheral blood PMN from the subjects were incubated with labeled *Porphyromonas gingivalis* (*P.g with BacLight Green*). **(A)** Positive labeled neutrophils were evaluated in a time point (30mins-4hours) and data expressed as phagocytic index: Phagocytic index = % phagocytic neutrophils × MFI. Results are expressed as mean ± SD. **(B)** Cells were stimulated with *P.g.* only or pretreated with RvE1 (1-10onM) for 2 hours. Cells were gently washed, extracellular fluorescence was quenched by Trypan blue, and phagocytosis was determined by flow cytometry analysis (FACS Canto II). Wilcoxon test was used evaluate statistical significance (n=11; *p<0.05; **p<0.01, ***p<0.001).

- Fig.11 C,D,E -

C

Control	Healthy	T2 Diabetes

Bioparticles

D

E

ERV1-Ab	+	+	−	−	+	+
U230495	−	−	+	+	+	+

RvE1 10nM
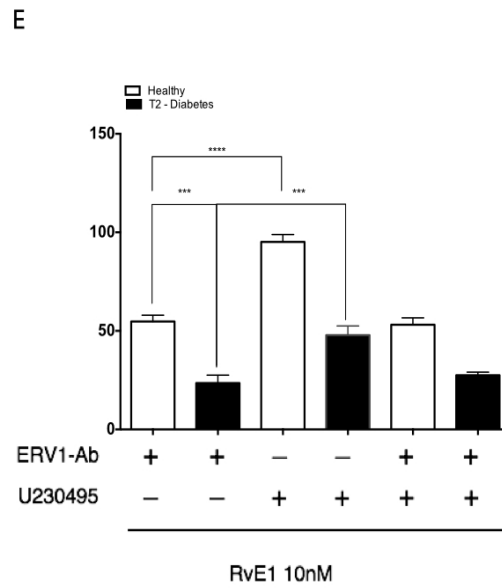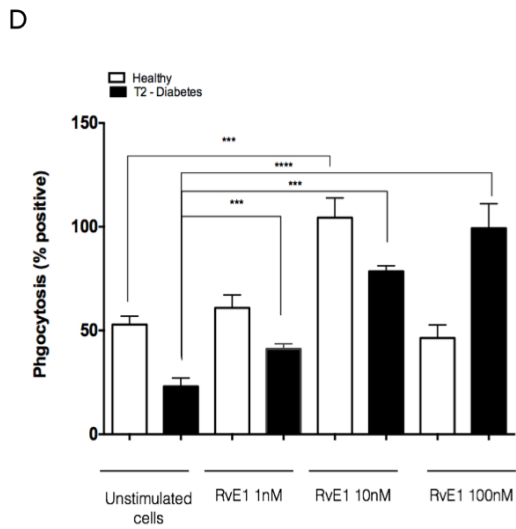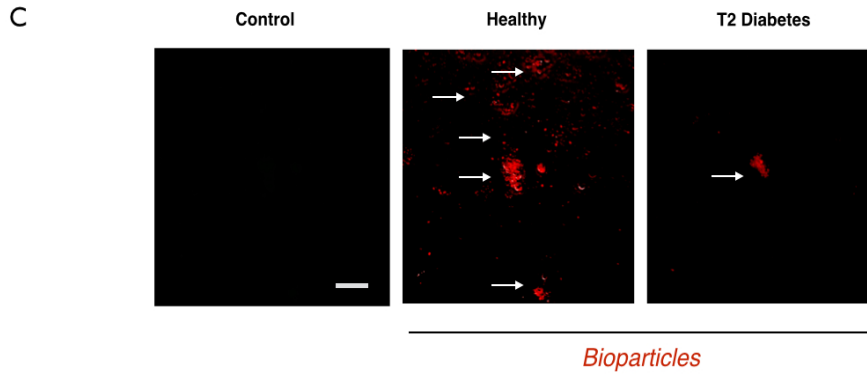
Figure 11. PMN from study subjects were incubated with labeled zymosan bioparticles (deep red). **(C)** Upon zymosan challenge, cells from both health and diabetic were evaluated by immunofluerescent microscopy. **(D)** Quantification of flow cytometry phagocytosis index was evaluated among individual groups upon resolvin E1 dose response (1-100nM). **(E)** Cells were treated with RvE1 (10nM), alone, or with BLT-1 receptor blocking agent (U-230495) and ERV1 receptor blocking agent (ERV1/Ab) and combinations. After 15 min pre-treatment with blocking agents biopartciles were plated to the cells. Quantification was done by flow cytometry analysis. Results are the mean ± SD and are expressed as mean fluorescence intensity (MFI). Wilcoxon test was used evaluate statistical significance (n=18; ***p<0.01; ****p<0.001).

- 5. Discussion -

Here, we demonstrated that uncontrolled type 2 diabetes impact the phenotypic expression of the RvE1 receptor, ERV1, on neutrophils. ERV-1 expression was originally reported as an active, signaling receptor on macrophages and dendritic cells (Samson et al., 1998). We report here that ERV-1 expression is low on healthy human neutrophils and BLT-1 mediated signaling dominates. The expression and function of ERV-1 in type 2 diabetes has not been previously investigated. However, Cash et al. reported in 2013 that another ERV1 ligand, chemerin 15, actively signals in chronic coronary lesions.

Development of metabolic syndrome prior to the onset of type 2 diabetes is characterized by changes in the metabolism of glucose and fatty acids that activate innate immune responses that give rise to systemic insulin resistance, which in turn establishes a state of chronic systemic inflammation (Guariguata et al., 2011, Li and Hotamisligil, 2010, Spranger et al., 2003). As a result, type 2 diabetes is associated with dysbiosis of healing cascades, including deficiencies in clearing microbial infections, impaired phagocytosis and chronic inflammation. Individuals with type 2 diabetes in this study,

presented increase neutrophil total count, high cholesterol and glucose levels (Figure 7). Due to increased inflammation in type 2 diabetes, prolonged activation of PMN with ineffective clearance of bacteria consequently increasing susceptibility to infection, such as cardiovascular, retino infections and high incidence of oral manifestation like periodontal diseases (Herrera et al., 2015).

The exact mechanism by which neutrophils fail to activate resolution cascade in type 2 diabetes are not known. Neutrophils activities during inflammation and resolution phases are tighlty regulated by pro-inflammatory mediators and pro-resolution mediators. Here, we demonstrated that resolution G-protein coupled receptor ERV-1 is upregulated (Figure 7B) in type 2 diabetes neutrophils. This was specific to neutrophils as monocytes did not show similar increase. The unbalance seen in type 2 diabetes has been mostly justified by chronic inflammation, however deficient resolution programees have not been yet identified. Inflammatory stimulants, LPS and TNF-a have shown to modulate the patterns of ERV-1 expression (Figure 8 A and B). The fact that both healthy and diabetic netrophils express ERV-1, it demonstrates that RvE1 is a possible therapeutic agent to these individuals when endogenous signals fail and chronic inflammation persists. Upon RvE1 pre-treatment was able to rescue

ERV-1 expression. When neutrophils were treated by TNF-a, Fmlp, IL-8 a rapid

upregulation of the cell receptor occurred (Cash et al., 2013). Interestingly, only

neutrophil ERV-1 was stimulated by TNF- a, but not ERV-1 from monocytes and

lymphocytes (Cash et al., 2013). Consistently, our results demonstrated that

ERV-1 from neutrophils responded to the stimulation in both groups and LPS

only influenced T2D diabetic neutrophils. While previous results, demonstrated

that anti-inflammatory ligands such chemrin, annexin A1, α – melanocyte

stimulating hormone did not influence ERV-1 phenotype, RvE-1 was able to

influence the receptor expression in a dose response manner (Figure 8). CHO

$^{ERV1\ +}$ expression decreased when cells were treated with 10-100nM resolvin E1

(Figure 9E). The results indicate the importance of RvE-1 lipid ligand ERV-1

receptor axis in inflammation resolution.

Endogenous activation of resolution of inflammation is mediated by

interaction between resolvin E1 (RvE1) and its receptor ERV1. The activation of

ERV-1 signaling is partially known, upon ligand interaction and ribosomal s6

phosphrylation signals cellular biological functions. Here, both type 2 diabetic

and healthy patients demonstrated response to receptor activation (Figure 9),

but type 2 diabetic samples significantly showed higher expression in the

baseline (Figure 9A), following receptor over expression patterns. Upon exogenous RvE1 activation, cells were responsive by signal transduction by rS6 phosphorylation (Figure 9B). LPS and TNF-a treatment were able to influence ERV-1 expression phenotype, but not rS6 signaling (Figure 9C). Upon exogenous RvE1 activation, part of the abnormal phenotype has been rescued; both overexpression of the receptor and increased phosphorylation was decreased and increased cytokines activations has been dismissed. In addition, To ERV-1 overexpression type 2 diabetes cells have shown to have functional but downexpress BLT-1 receptor. The understanding of the molecular mechanisms which regulate expression and function of resolution receptor, ERV1 and BLT-1, leads to molecular regulation of RvE1 exogenous biological functions. The signaling in healthy human neutrophils were through ERv-1 mostly and partially through BLT-1 (Figure 10E). Protective acute inflammation phenotype clearly requires lipid mediators to activate the resolution phase. In the presence of lipid mediators such as resolvin E1, functional resolution signals are activated.

Unresolved chronic inflammation, leads to continuous response, scar formation, fibrosis and disease initiation, innate immune cells such as

neutrophils sense the environment to modulate the subsequent cascade of biological events that guide healing. Activation of ERV-1 receptor on neutrophils are known to attenuate NF-κB activation and production of IL-1 and TNF-α. Activation of, ERV1, by the agonist ligand RvE1 is known to lead to attenuation of NF-KB mediated proinflammatory cytokines, increasing phagocytosis of pathogens, non-phlogistic efferocytosis and clearance, consequently resolution of inflammation. In this study, ERV-1 activation led to change of cell phenotype and activated phagocytes in a dose-response manner. In healthy individuals it was evident that a stimulatory concentration (1-10nM) is able to increase phagocytosis (Figure 11A, 11B). However, a higher dose was necessary to activate the cells. In addition, RvE1 is known to interact with the leukotriene B_4 receptor (BLT1), and not ERV-1, which is known to be expressed in human neutrophils ameliorating leukotriene-B_4 dependent pro-inflammatory signals (Figure 11C). However, the reason endogenous ERV-1 present in innate cells fails to activate resolution in chronic inflammatory diseases remains unclear. It is possible that resolution receptors in chronic inflammatory diseases are oversaturated. This was confirmed by PCR analysis of CHO ERV1+ cells dose dependent application of resolvin E1 (Figure 9D). The cellular function is also dependent on specific dose, for example 100nM

application of RvE1 was the most potent in rescuing phagocytosis, while in healthy patients 1nM achieved similar results (Figure 9D). Among the individuals diagnosed with diabetes, one-third present with severe periodontal disease and adult patients with severe periodontitis had increased risk of poor glycemic control, increasing the risk of oral and systemic complications Not limited to this disease we have also found similar patterns using a "generic" molecule to activate the cells such zymosan. RvE-1 rescue the deficient phagocytosis seen when cells were challenged with both the pathogenic bacteria and zymosan.

Taken all together, loss of regulation of inflammation is a biological event common to human chronic diseases including type 2 diabetes. Understanding deficient programes in resolution of inflammation is key to influence treatment for individuals with type 2 diabetes. The traditional focus in managing type 2 diabetes has been the control of hyperglycemia and insulin; not resolution of inflammation. The results here demonstrate clearly that the lipid ligand resolvin E1 improve the deficient phenotype seen in uncontrolled diabetes. The discovery of distinct cell population requires RvE1 to control receptor expression and activate regular functions is key to improve type 2

diabetetic cells to respond to inflammation and infection. Perhaps chronic disease treatment required specific dose of RvE1 to efficiently activate resolution signals. Further studies are necessary to characterize the dysfunction seeing here in ERV-1 receptor in other disease models. These are important concepts for personalized medicine approaches against inflammatory diseases. These findings further our understanding of the importance of expression and function of key resolution receptors in health vs. disease.

- 6. Conclusions -

-

- CONCLUSIONS -

The overall goal of these studies was the investigation of ERV-1 receptor actions on human neutrophils to identify a potentially new component in the resolution of acute inflammation. The hypothesis tested was that ERV-1 from neutrophils play a role in resolution and that resolvins act directly on the receptor. To achieve this goal, three specific aims were proposed, 1) Characterize the actions of ERV-1 expression on human neutrophils 2) Elucidate functional mechanisms and components underlying ERV-1 in human type 2 diabetes on neutrophils.

The actions of RvE1 were thought to be through BLT-1 receptor expressed on neutrophils, this notion was challenged in Chapter 3. The first observation was that in type 2 diabetic cells, ERV-1 was overexpressed. The regulation of the receptor was mostly controlled by inflammatory marker TNF- α and slightly by LPS. This fast regulation induced by inflammation demonstrates that the resolution receptor expression is plastic and responsive to environmental changes. In fact, this was evident when the ligand RvE-1 was used together or isolated from the inflammatory stimulant. The notion that ERV-

1 is the key regulator of neutrophils is novel. Also, it has a role in chronic diseases such as type 2 diabetes as demonstrated by functional assays seen in human and murine studies, in chapter 2 and 3 respectively. Phagocytosis is a key cell function to promote clearance of microbes and other pathogens. Once clearance signals initiates activation of resolution pathways begin. In contrast, type 2 diabetes phagocytosis is deficient. Exogenous RvE-1 was able to influence the neutrophils and activate phagocytosis.

There is solid evidence in the literature that exogenous use of specialized lipid mediators such as RvE1 activates resolution signals. Animal models and studies with humans demonstrated rescue of deficient phenotype with remarkable cellular and molecular activation. This dissertation presents the importance of biological activation of resolution receptor ERV-1. This concept may apply not just to one resolution receptor but to a family of receptors responsible for resolution of inflammaiton. While lipid ligands are necessary, the environment of chronic disease patients changes the conventional expression of the receptors. This was clearly seeing in human cells. Importantly, understanding the receptor leads to better use of the ligand. In fact, high concentrations (100nM) of RvE1 were necessary to fully activate the downstream signals, rS6 phosphorylation in humans and AKT, MAK in mice.

While in healthy conditions, this was different and even with 1nM the cells were responsive. It is possible that the ligand was first regulating the amount of receptor on the surface of the diabetic cells. This was seen when CHO cells that were genetically engineered to overexpress the receptors, showed downregulation after RvE-1 applications. Upon controlling the receptor expression, resolvin was able to signal in both health and diabetes and stimulate resolution signals. Weather these findings extend to other disease models will be of interest in further studies.

The new concepts presented in this dissertation have applications beyond RvE1-ERV-1 axis. A family of receptors involved in resolution need to be investigated in the context of each disease. Pro-resolving lipid mediators are produced endogenously, and the fact that in chronic disease the endogenous molecules are not able to resolve inflammation is still a question to pursue. Certainly, the continuous stimulation by the trigger and exacerbated inflammatory response over time maintains the system saturated in which the feedback mechanisms are no able to maintain the balance. In response to the toxic environment, resolution receptors may require increased amounts of ligands or even continuous exposure, which the endogenous machinery may not be able to produce. Consistent with this notion, exogenous application of

lipid ligands to an acute or saturated chronic biological system is able feed forward resolution signal and return to homeostasis.

In future clinical trials, exogenous application of resolution mediators may be used for prevention of diseases. Also, different concentrations may be necessary to be effective for human chronic diseases. In control of inflammation, pro-resolution mediators will have therapeutic actions, locally and systemically. Altogether, these findings may lead to the understanding of how to control inflammatory processes, especially those that lead to chronic diseases.

- LIST OF ABBREVIATED JOURNAL TITLES -

Am. J. Cardiol.	American journal of Cardiology
Am. J. Cardiol.	American Journal of Clinical Pathology
Chem Biol	Chemical Biology
J. Clin. Invest.	Journal of Clinical Investigation
J. Exp. Med.	Journal Experimental Medicine
J. Immunol.	Journal of Immunology
J. Perio.	Journal of Periodontology
Nat Immunol.	Nature Immunology
Nat. Med.	Nature Medicine
PLoS ONE	Public Library of Science ONE
Perioddontol 2000	Periodontology 2000

AMERICAN DIABETES, A. 2016. 2. Classification and Diagnosis of Diabetes. *Diabetes Care,* 39 Suppl 1**,** S13-22.

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