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Human Milk Proresolving Mediators Stimulate Resolution of Acute Inflammation

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Abstract

Human milk contains nutrients and bioactive products relevant to infant development and immunological protection. Here, we investigated the pro-resolving properties of milk using human milk lipid mediator isolates (HLMI) and determined their impact on resolution programs *in vivo* and with human macrophages. HLMI reduced maximum neutrophil numbers ($14.6\pm1.2\times10^6$ to $11.0\pm1.0\times10^6$ cells/exudate) and shortened the resolution interval (R_i; 50% neutrophil reduction) 54% compared to peritonitis. Using rigorous liquid-chromatography tandem-mass spectrometry (LC-MS-MS)-based lipid mediator (LM) metabololipidomics, we demonstrated that human milk possesses a proresolving LM-SPM signature profile, containing specialized proresolving mediators (SPM; e.g. resolvins, protectins, maresins and lipoxins) at bioactive levels (pico-nanomolar concentrations) that enhanced human macrophage efferocytosis and bacterial containment. SPM identified in human milk included D-series resolvins, (e.g. Resolvin (Rv) D1, RvD2, RvD3, AT-RvD3 and RvD4), Protectin (PD)1, Maresin (MaR)1, E-series resolvins (e.g. RvE1, RvE2 and RvE3) and lipoxins (LXA_4 and LXB_4). Of the SPM identified in human milk, RvD2 and MaR1 (50 ng/mouse) individually shortened R_i~75%. Milk from mastitis gave higher LTB_4 and prostanoids and lower SPM levels. Taken together, these findings provide evidence that human milk has pro-resolving actions via comprehensive LM-SPM profiling, describing a potentially novel mechanism in maternal-infant biochemical imprinting.

Keywords

resolvins; protectins; maresins; eicosanoids; human tissue; leukocytes

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DISCLOSURE

CNS is an inventor on patents [resolvins] assigned to BWH and licensed to Resolvyx Pharmaceuticals. CNS is a scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. CNS' interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

INTRODUCTION

The acute inflammatory response is critical in infection and injury. The initiation and resolution of inflammation are important in host defense; each governed by bioactive lipid mediators (LM) that drive the influx and function of immune cells, and eventual cell efflux and tissue repair [1, 2]. Newly identified families of bioactive LM, biosynthesized from essential fatty acids (EFA), that actively stimulate resolution of inflammation were uncovered in self-resolving exudates and these structures elucidated [1]. Collectively they are coined specialized pro-resolving mediators (SPM)[1]. SPM comprise several families that include arachidonic acid (AA) derived lipoxins (LX), eicosapentaenoic acid (EPA) derived resolvins (RvE), and docosahexaenoic acid (DHA) derived resolvins (RvD), protectins (PD), and maresins (MaR); these structurally distinct families are each host protective with defining actions in anti-inflammation (e.g. limit further neutrophil inflammation), proresolution (e.g. enhancing macrophage clearance of apoptotic cells, debris and bacteria), pain reduction and wound healing (Reviewed in [1]). SPM are evolutionary conserved biochemical signals, as they are present in trout, salmon and planaria (Reviewed in [1]), and have already been identified in human organ systems, including plasma (RvD1, RvD5, RvD6 and RvE2) [3], adipose tissue (RvD1, RvD2, PD1, RvE1 and LXA_4) [4], placenta (RvD1, AT-RvD1, RvD2 and PD1) [5] and recently human milk (RvD1, RvE1 and LXA_4) [6]. LXA_4, RvE1, RvD1 and RvD2 each reduce mucosal inflammation, stimulate the innate immune response and activate resolution of periodontal disease, colitis and dermal inflammation [7-9].

Human milk is recognized as being important for infant development, providing essential nutrients and bioactive products relevant for maternal-mucosal immune defense and immune system maturation [10]. The n-3 EFA including EPA and DHA are enriched in human milk [11]. For infants, and particularly premature infants, injurious and infectious insult can be detrimental [12]. Hence protective mechanisms for resolving infection and inflammation in a timely manner and educating the innate immune system in early life are critical and of general interest. In this report, we present evidence for new immunoresolving properties of human milk. Using self-limited acute inflammation and LM metabololipidomics we found that isolates from human milk contain chemical signals with proresolving actions, namely limiting neutrophil trafficking *in vivo,* enhancing human macrophage phagocytosis of apoptotic PMN (efferocytosis) and bacterial containment. These actions were attributed to the proresolving LM-SPM signature profile of identified bioactive mediators that included D-series resolvins (AT-RvD1, RvD2, RvD3, AT-RvD3, RvD4, RvD5 and RvD6), protectins (PD1 and AT-PD1), maresins (MaR1), E-series resolvins (RvE2 and RvE3) and lipoxins (AT-LXA_4 and LXB_4). The LM-SPM profile was altered in human milk from inflamed mammary glands (mastitis) with higher prostanoids and leukotriene B_4 (LTB_4) and lower SPM levels, and had reduced ability to accelerate R_i. Hence the present results provide evidence for bioactive resolution signals in human milk that are linked to homeostasis, resolution of inflammation and innate host responses.

RESULTS

Human milk lipid mediator isolates (HLMI) stimulate resolution of inflammation

To investigate whether human milk exerts pro-resolving actions, we used human milk chromatographic isolates with self-limited acute inflammation *in vivo* and mapped leukocyte trafficking. Because SPM, including resolvins, protectins and maresins, stimulate resolution [1] and elute within the methyl formate chromatographic fractions from C18 solid-phase extraction [3], we obtained human milk isolates from these fractions (referred to as human milk lipid mediator isolates (HLMI)) and assessed their ability to accelerate resolution of acute inflammation *in vivo*. First, self-limiting acute inflammation was initiated by *i.p.* injection of yeast cell wall particles (zymosan, 1 mg/mouse), and to quantitate resolution we used defined resolution parameters of acute inflammation [13, 14]. The self-limited response reached maximal neutrophil numbers (Ψ_{max}= 14.6 ± 1.2 ×10^6 cells/murine exudate) at 12 h (T_{max}) that was followed by subsequent decline (Fig. 1a). Administration of HLMI immediately prior to inflammatory challenge gave a ~23.1 ± 8.9% reduction in Ψ_{max} (11.0 ± 1.0 ×10^6 cells/exudate; Fig. 1a,b). Reduction in neutrophil levels was observed throughout the course of inflammation-resolution in mice administered HLMI, with 31.3 ± 4.4% and 24.5 ± 10.9% fewer neutrophils at 24 and 48h, respectively, compared to peritonitis plus vehicle (Fig. 1c).

To quantify the regulation of leukocyte trafficking at the site of inflammation we investigated the resolution interval (R_i) that quantitates the local kinetics of leukocyte infiltration, with the R_i being defined as the time interval between T_{max} and T_{50} (the time interval when the number of infiltrated PMN drops to half of the peak number)[13, 14]. We found that HLMI administration gave 54% reduction in R_i, from 26 h to 12 h (Fig. 1a,b). These results demonstrate that human milk possesses proresolving properties contained in the HLMI.

Human milk LM-SPM signature profile: LM metabololipidomics

Because isolates from human milk accelerate resolution (Fig. 1), we next sought to investigate the lipid mediator profile of human milk. Using LC-MS-MS-based LM metabololipidomics (see Methods for details) we identified a profile signature of LM consisting of 20 bioactive LM (Fig. 2; Table 1; Supplemental Fig. 1) from both lipoxygenase (LOX) and cyclooxygenase (COX) pathways, including resolvins, protectins, maresins, lipoxins and prostanoids (Fig. 2; Table 1; Supplemental Fig. 1 and Supplemental Table 1). Each LM was identified by matching LC retention time and at least six diagnostic ions, and quantification achieved using multiple reaction monitoring (MRM) in accordance with published criteria [3], and as illustrated with representative results obtained for all identified LM (Supplemental Fig. 1b).

LM quantification, using MRM, demonstrated that SPM in healthy mature human milk (4-8 weeks postpartum) include AT-RvD1 (67.4 ± 11.7 pg/mL), RvD2 (82.4 ± 28.0 pg/mL), RvD3 (7.2 ± 2.7 pg/mL), AT-RvD3 (15.0 ± 2.9 pg/mL), RvD4 (27.4 ± 7.5 pg/mL), RvD5 (19.9 ± 8.9 pg/mL), RvD6 (6.7 ± 2.4 pg/mL), PD1 (4.3 ± 2.3 pg/mL), AT-PD1 (3.8 ± 0.9 pg/mL), and MaR1 (20.8 ± 6.3 pg/mL) from the DHA metabolome, RvE2 (321.2 ± 129.2

pg/mL) and RvE3 (444.9 ± 179.8 pg/mL) from the EPA metabolome and AT-LXA_4 (370.0 ± 176.6 pg/mL) and LXB_4 (267.1 ± 93.9 pg/mL) from the AA metabolome (Table 1). These are in addition to RvD1 (147.0 ± 47.2 pg/mL), RvE1 (8.8 ± 3.6 pg/mL) and LXA_4 (25.7 ± 8.6 pg/mL). These confirm the identification of RvD1, RvE1 and LXA_4 in human milk, at values consistent with those recently reported [6]. From the COX-pathways we also identified PGE_2 (409.7 ± 146.6 pg/mL), PGD_2 (568.3 ± 188.9 pg/mL), $PGF_{2\alpha}$ (111.1 ± 36.2 pg/mL) and TxB_2 (111.8 ± 44.4 pg/mL) in these samples in accordance with published findings [15]. These results demonstrate that human milk contains SPM at biologically relevant concentrations.

Next we determined the contribution of each of the major bioactive metabolomes (DHA, EPA and AA) as well as individual mediators within each metabolome to the human milk LM signature profile (Fig. 2). LM metabololipidomics of human milk AA, EPA and DHA identified bioactive metabolome demonstrated that SPM represented ~61.6% of the human milk LM profile (Fig. 2), consisting of DHA-derived resolvins, protectins and maresins (13.1%), AA-derived lipoxins (23.5%) and EPA-derived resolvins (24.9%; Fig. 2). AA-derived prostanoids amounted to ~38.4% of the LM identified (Fig. 2), consisting primarily of PGE_2 and PGD_2 (~81.5% of total prostanoids) that are key in LM mediator class switching and initiation of resolution [16]. Of primary proinflammatory LM, $PGF_{2\alpha}$ and TxB_2, an inactive further metabolite of TxA_2 [17], combined amounted to <10% of total milk LM (Fig. 2). LTB_4 is a potent proinflammatory neutrophil chemoattractant [18] and was not identified in appreciable amounts in these milk samples (Table 1). This approach permitted us to assess the potential effector functions that human milk LM-SPM may endow locally within the mammary gland or on the infant during maternal-infant transfer. Taken together, these results demonstrate that human milk contains a proresolving LM-SPM signature profile, comprised predominantly of LM and SPM with pro-resolving properties at concentrations commensurate with their known bioactions [1, 17, 18].

Human milk LM-SPM profile is altered in mastitis

SPM are endogenous chemical signals that actively stimulate resolution of inflammation [1]; therefore we next sought to investigate the LM profiles of human milk from inflamed mammary glands (mastitis) and compare it to milk from healthy subjects (Fig. 3). Differences in LM-SPM profiles obtained with human milk from healthy individual donors (1-6 months postpartum) and donors with mastitis (1-4 months postpartum) were assessed using principal component analysis (PCA). The two principal components, calculated using the data matrix, showed clear separation between the healthy milk cluster and mastitis milk cluster on the score plot (Fig. 3a). The healthy milk cluster was characterized by higher levels of SPM, including RvD1, RvD2, RvD3, MaR1, PD1, RvE2 and LXA_4 and LXB_4 as demonstrated in the loading plot (Fig. 3b). Conversely, PCA analysis of the LC-MS-MS results demonstrated that the mastitis milk cluster was associated with higher levels of RvE1, LTB_4, PGD_2, $PGF_{2\alpha}$ and TxB_2. These findings indicate that the human milk LM profile is altered in mastitis, with elevated proinflammatory LM and reduced SPM.

Since mastitis milk had an alerted LM-SPM profile, we next investigated the ability of HLMI from mastitis milk (referred to as $HLMI_{mast}$) to accelerate resolution of acute

inflammation. HLMI from mastitis milk was obtained as described above for HLMI from healthy milk (see Material and Methods for details). Administration of $HLMI_{mast}$ immediately prior to challenge (1 mg zymosan/mouse) did not limit neutrophil numbers at T_{max} (12.3 ± 0.8 cells/exudate vs. 11.5 ± 0.9 cells/exudate compared to peritonitis plus vehicle), and only slightly shortened the R_i by 16%, or from 19 h observed in peritonitis plus vehicle to 16 h (Fig. 3c, d). Together these findings indicate that mastitis milk has altered LM-SPM signature profile and reduced ability to accelerate resolution *in vivo.*

RvD2 and MaR1 potently accelerate resolution of acute inflammation

Because DHA is recognized to be critical for neonatal development [10] and RvD2 was one of the more abundant DHA-derived SPM identified in human milk (Fig. 2, Table 1), we sought to assess its potential contribution to regulation of leukocyte trafficking and the R_i. Mice were administered RvD2 (50 ng/mouse, i.e. 2 μg/kg; *i.p.*) prior to initiation of a self-limited inflammatory challenge and resolution parameters quantified (Fig. 4). RvD2 gave ~40% reduction in Ψ_{max} (10.0 ± 0.8 ×10^6 cells/exudate vs. 17.0 ± 2.4 ×10^6 cells/exudate) compared to peritonitis plus vehicle mice and shortened the R_i by 74%, or from 25 h to 6.5 h (Fig. 4). DHA also serves as a substrate for Maresins [1], and since MaR1 was identified in human milk at bioactive concentrations (Fig. 2, Table 1) we compared its actions on regulating leukocyte trafficking to RvD2. MaR1 (50 ng/mouse, *i.p*) gave a maximal PMN number of 9.9 ± 1.3 ×10^6 cells/exudate and shortened the R_i to 6 h, or by 76% (Fig. 4). We also assessed the ability of RvD2 and MaR1 to accelerate resolution of established peritonitis (Supplemental Fig. 2a, b). RvD2 and MaR1 (50 ng/mouse) administered 12 h after zymosan challenge (1 mg/mouse) each accelerated resolution, reducing neutrophil numbers and shortening the R_i by 33 and 40%, respectively (Supplemental Fig. 2a, b). Thus both RvD2 and MaR1, at physiologic range, i.e. nanograms per mouse, regulate neutrophil trafficking and shorten the R_i.

HLMI and MaR1 stimulate resolution of infectious peritonitis

Given these *in vivo* findings and since HLMI contain SPM that enhance host-directed responses to infection, such as RvD1, RvD5 and RvD2 [19, 20], we next investigated whether HLMI enhanced resolution of infectious peritonitis (Supplemental Fig. 3 a, b). Mice were inoculated with a resolving dose of *E. coli* (10^5 c.f.u.) and administered vehicle or HLMI (*i.p.*) 12 h later. HLMI gave reduced PMN numbers at 24 h by 33% (9.8 ± 1.1 cells/exudate vs. 14.6 ± 1.8 cells/exudate compared to peritonitis plus vehicle; Supplemental Fig. 3a) and enhanced leukocyte uptake of *E. coli* (Supplemental Fig. 3b). Since MaR1 potently accelerated resolution of sterile inflammation and is present in human milk, we assessed its ability to enhance resolution of infection (Supplemental Fig. 3c, d). We found that MaR1 (50 ng/mouse) reduced PMN numbers at 24 h by 40% (Supplemental Fig. 3c) and enhanced leukocyte uptake of *E. coli* (Supplemental Fig. 3d). Similar results were obtained with RvD2 (n=2, data not shown) used for direct comparison [20]. Together these results demonstrate that HLMI and MaR1 accelerate resolution of infection, limiting neutrophil numbers and enhancing *in vivo* bacterial clearance.

HLMI enhance human macrophage phagocytosis

Given the key actions of SPM in resolution are enhancing macrophage clearance of apoptotic cells and debris [1], we next questioned whether HLMI have direct impact on phagocytosis with isolated human cells. Incubation of human macrophages with HLMI gave an enhanced efferocytosis (i.e. phagocytosis of fluorescently-labeled apoptotic neutrophils) compared to vehicle treated macrophages (Fig. 5a). To provide evidence whether the LM found in HLMI are responsible for the potent bioactions, we depleted LM from human milk using activated charcoal [21] (referred to here as $HLMI_{AC-}$) and compared its actions to that of HLMI. Charcoal treatment depleted more than ~97% of the bioactive LM content of human milk (DHA derived SPM: 23.3 vs. 0.1 pg/20μl isolate, AA-derived SPM: 35.2 vs. 1.3 pg/20μl isolate; EPA-derived SPM: 78.5 vs. 3.3 pg/20 μl isolate; AA-derived prostanoids: 155.8 vs. 10.6 pg/20μl isolate) and significantly reduced the ability of the HLMI to stimulate macrophage efferocytosis by ~80-95% (Fig. 5a). Thus, HLMI possess bioactive SPM that stimulate key resolution programs in human macrophages, namely efferocytosis.

Based on these and the *in vivo* findings, and since SPM, including RvD1, RvD2 and RvD5, directly enhance human phagocyte containment of *Escherichia coli* (*E. coli*) [19, 20], we next questioned whether HLMI have direct impact on bacterial containment with isolated human cells. HLMI increased human macrophage phagocytosis of fluorescent *E. coli* by approximately 35-55% compared to vehicle treated macrophages (Fig. 5b). The ability of HLMI to enhance macrophage containment of *E. coli* was significantly reduced after LM depletion with activated charcoal (Fig. 5b). Together, these results demonstrate that HLMI possesses bioactive LM/SPM that enhance bacterial containment with isolated human macrophages.

DISCUSSION

In the present study, we report the human milk LM-SPM signature profile that signals resolution of inflammation and bacterial clearance. Using LC-MS-MS-based LM metabololipidomics we identified resolvins, protectins, maresins and lipoxins at bioactive concentrations in healthy human milk. For comparison, in mastitis, milk LM-SPM levels were altered showing elevated proinflammatory LM and lower levels of SPM. RvD2 and MaR1 were identified in human milk, and each individually accelerated resolution of inflammation, shortening the R_i from 26 to 12 h. Also, HLMI had infection-resolving actions *in vivo,* enhanced efferocytosis, and phagocytosis of *E. coli* with isolated human macrophages.

Human milk is a dynamic biologically active fluid that in addition to delivering essential nutrients provides passive protection for the immature mucosal immune system [10]. Due to the immaturity of the intestinal immune system in newborns, they have enhanced susceptibility to excessive inflammation and infection [12]. Recently, chemical signals that actively stimulate resolution of inflammation and infection [1] were identified in human milk [6]. Of note, SPMs, such as resolvins, protectins, maresins and lipoxins, are endogenous LM found in many tissues that actively counterregulate proinflammatory signals, including NF-κB [9], cytokines and leukotrienes [1]. They exert their potent actions via activating specific G-protein coupled receptors (GPCR) in cell-specific and tissue-dependent manner. Several

SPM receptors are identified; e.g. RvE1 specifically binds both ChemR23 and BLT1, LXA_4 and RvD1 bind and activate the lipoxin A_4 receptor ALX and human GPR32, which also binds RvD3 and RvD5 (reviewed in [1]). RvD2 was recently found to exert its tissue-protective actions via GPR18 [22]. Along these lines, enterocytes express ALX [23] and LXA_4 stable analogs inhibit bacterial-induced IL-8 secretion by intestinal epithelial cells[24]. Enterocytes also express ChemR23, where RvE1 induces intestinal alkaline phosphatase expression and enzyme activity that attenuates LPS induced NF-κB signaling[25]. Hence, together with our present results SPM in human milk may be relevant for infant mucosal responses. Given their presence at bioactive levels in human milk (pM to nM) and their ability to engage GPCRs, they may activate specific and potentially additive responses in the newborn gut mucosa; such actions remain of interest.

Pro-resolution is a distinct process from anti-inflammation, where agonists of resolution, such as SPM, augment non-phlogistic clearance from sites of inflammation and infection, augmenting host–directed defenses including microbial containment [19, 26]. In the present report, we found that human milk isolates containing SPM accelerate resolution of acute inflammation and infection *in vivo* and with isolated human leukocytes. Mastitis milk gave altered SPM levels and reduced ability to accelerate resolution of acute inflammation. The higher RvE1 levels in mastitis milk may reflect an increased cytochrome P450 in the mastitis microenvironment, e.g. cytochrome P450 can produce the RvE1 precursor 18-HEPE from EPA, which in turn is converted to RvE1 by human PMN (reviewed in [1]), which are known to be abundant in mastitis-affected milk [27]. In addition to the known beneficial properties of human milk, our current results extend its protective roles to now include proresolving properties, namely accelerating resolution of acute inflammation and infection as well as stimulating macrophage phagocytic functions with the LC-MS-MS-based identification of human milk SPM.

Resolution of acute inflammation can be quantitated using defined resolution indices introduced by this laboratory [13, 14]. These permit direct assessment of proresolving properties of endogenous mediators (Table 2 and Supplemental Table 2). For example, RvD1 and RvD3 (50 ng/mouse, i.e. 2 μg/kg, each), shorten R_i in murine peritonitis (Table 2). Also, RvD1, PD1 and AT-LXA_4 at 300 ng/mouse (i.e. 12 μg/kg) each reduce the R_i, while RvE1 accelerates the onset (T_{max}) of resolution (Supplemental Table 2). In these experiments, RvD2 and MaR1 accelerate resolution of acute inflammation, reducing the magnitude of PMN infiltration (Ψ_{max}) and shortening R_i. Of note, RvD2 and MaR1 each limit intestinal inflammation and tissue damage in experimental colitis [9, 28]. Of interest, oral administration of RvD1 shortens the R_i [29]. Hence taken together with our finding that SPM, including RvD2 and MaR1, are present in human milk at biologically relevant levels, SPM and their pathways my have implications in regulation of acute inflammation and resolution in maternal-infant transferred protection.

Emerging evidence indicates that breastfeeding is correlated with lower prevalence of inflammatory conditions in early life (e.g. NEC) and later life (e.g. obesity, diabetes and cardiovascular disease) [30]. Human milk contains high levels of EFA, such as DHA, which are derived from maternal dietary and endogenous pools (e.g. adipose tissue) [31]. Increased maternal intake of n-3 EFA during gestation and lactations has been associated with

beneficial outcome for infants [10]. Also, DHA in breast milk is thought to play a role in early neural development, [10] and some studies have found that DHA may be associated with better cognitive outcome and higher IQ; however, further investigation is needed [32]. Of note, evidence in humans indicated that n-3 EFA intake can elevate RvD1, RvD2, PD1 and 17-HDHA levels in healthy individuals [33]. Increases in specific SPM after n-3 EFA intake followed by aspirin are associated with enhanced functional outcome in whole blood (i.e., increased phagocytosis) demonstrating functional metabolomic profiling [3]. Omega-3 intake elevated RvD1 levels in diabetic mice [34] and in patients with minor cognitive impairment and was associated with enhanced uptake of beta-amyloid [35]. AT-RvD1 improves postoperative cognitive decline in mice, [36] and RvE1 and AT-RvD1 differentially improve functional outcome following diffuse traumatic brain injury [37]. Hence, taken together with present findings that human milk contains a proresolving LM-SPM signature profile, human milk SPM may be relevant in infant neurological development.

In summation, human milk LM metabololipidomics profiling uncovered specific LM signature with physiologically relevant levels of endogenous SPM associated with accelerated resolution of acute inflammation *in vivo*. By profiling LM-SPM in human milk, we identified several potent bioactive proresolving mediators including AT-RvD1, RvD2, RvD3, AT-RvD3, RvD4, RvD5, RvD6, MaR1, PD1, AT-PD1, RvE2, RvE3, AT-LXA_4 and LXB_4 in human milk, as well as confirmed the earlier identification of RvD1, RvE1 and LXA_4 [6]. Mastitis milk had higher prostanoids, lower SPM and reduced ability to accelerate resolution. Of these newly identified SPM herein, RvD2 and MaR1 each accelerated resolution of acute inflammation and infection (Fig. 4, Table 2, Supplemental Fig. 2). With human macrophages, HLMI stimulated efferocytosis and containment of *E. coli*, key actions in resolution of inflammation and infection, and accelerate resolution of infection in vivo. Hence, the present results implicate a role for SPM in modulating inflammation, infection and stimulating resolution during early immune development, since SPM display potent actions in the innate immune system.

MATERIALS AND METHODS

Extraction of human milk lipid mediator isolates (HLMI) for murine peritonitis

Human milk from healthy donors was purchased from Biological Specialty Corporation (Colmar, PA) or from healthy and matched mastitis donors from Creative Bioarray (Shirley, NY). Two volumes of methanol were added to milk, and proteins were precipitated for 30 min on ice. Precipitate was pelleted by centrifugation (10,000 rpm, 4°C, 10 min). Supernatants were extracted using two volumes diethyl ether, and LM were further isolated using solid phase extraction as in [3]. Products were eluted in methyl formate; solvent was evaporated under N_2, and resuspended in ethanol. Aliquots of the ethanol fractions were taken to LC-MS-MS-based metabololipidomics for LM profiling.

Peritonitis and resolution indices

Sterile self-limited peritonitis was initiated in male FVB mice (6-8 wks; Charles River Laboratories) by *i.p.* injection of 1 mg zymosan A (Z4250; Sigma) [38]. For infectious peritonitis, mice were injected with self-limited inoculum of *E. coli* (10^5 c.f.u.). Immediately

prior to zymosan injection, mice were administered (*i.p.*) HLMI (levels representative of ~ 1mL human milk), RvD2 (50ng/mouse), MaR1 (50ng/mouse), or vehicle (saline containing 0.2% ethanol). In some experiments, mice were administered treatments at T_{max} (12 h). Isolates pooled from 3 human milk donors were used in determining the impact on the R_i of acute peritonitis. RvD2 and MaR1 for each experiment were prepared by total organic synthesis, and matched to authentic RvD2 and MaR1[20]. Physical properties of RvD2 and MaR1 were validated prior to each experiment according to published criteria [20]. Peritoneal exudates were collected at indicated time intervals by lavaging with 5mL PBS. Exudate PMN numbers were assessed using Turk's solution, light microscopy, and flow cytometry (FACSCanto II; BD Bioscience). PMN were determined as Ly6G (clone 1A8; BD Bioscience) and CD11b (clone M1/70; eBioscience) positive events and F4/80 (clone BM8; eBioscience) negative events from events as assessed by FSC and SSC. Resolution indices were calculated as in [13, 14], where Ψ_{max} is the maximal PMN count, T_{max} the time interval when PMN reaches maximum, T_{50} the time interval corresponding to 50% PMN reduction (or Ψ_{50}) and the resolution interval (R_i) is the interval between T_{max} and T_{50}. All animal experiments were approved by the Standing Committee on Animals of Harvard Medical School (protocol 02570) and performed in accordance with institutional guidelines.

LC-MS-MS based LM metabololipidomics of human milk

For quantification of LM, human milk from four healthy donors (1-2 months post-partum; Lee Biosolutions) or matched mastitis and healthy donors (1-6 months post-partum; Creative Bioarray) was extracted using solid-phase extraction with C18 columns (Waters), following addition of 3 volumes of cold methanol containing deuterated internal standards (1 ng d4-PGE2, d4-LTB4, and d8-5S-HETE, and d5-RvD2) and protein precipitation. Lipid mediator levels were assessed by a LC-MS-MS system, QTrap 5500 and QTrap 6500 (ABSciex) equipped with Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu Corp.). 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 55:45:0.01 (vol/vol/vol) to 100:0:0.01 at 0.4 ml/min flow rate. To monitor and identify various LM, a multiple reaction monitoring (MRM) method was developed with signature ion pairs, Q1 (parent ion) – Q3 (characteristic daughter ion) optimized for each molecule. Identification was conducted using published criteria [3] where a minimum of 6 diagnostic ions were employed in each MS-MS. The complete stereochemistry of resolvin D4 was recently determined (manuscript in preparation), and the synthetic standard was used here for identification and quantitation from human milk. Linear calibration curves for each compound were obtained with r^2 values ranging from 0.98–0.99. Detection limits were ~0.1 pg.

Principal component analysis

Principal component analysis (PCA) was performed using SIMCA 13.0.3 software (Umetrics) following mean centering and unit variance scaling of LM amounts. PCA is an unbiased, multivariate projection designed to identify the systematic variation in a data matrix (the overall bioactive LM profile of each sample) with lower dimensional plane using score plots and loading plots. The score plot shows the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plots

describe the magnitude and the manner (positive or negative correlation) in which the measured LM/SPM contribute to the cluster separation in the score plot [39].

Depletion of milk LM using activated charcoal adsorption

Human milk from three healthy donors (10 mL from each donor) was combined and incubated with or without 4% activated charcoal (Sigma) for 1 h at RT. Activated charcoal was washed out, 3 volumes methanol were added to the milk and proteins precipitated at −20°C. Precipitate was pelleted by centrifugation (3,000 rpm, 4°C, 10 min), and LM isolated using C18 columns and solid phase extraction [3]. Products were eluted in methyl formate, solvent was evaporated under N_2, and suspended in 500μl ethanol. For human macrophage phagocytosis, 20 μl HLMI were dried down and resuspended in 1 mL $PBS^{+/+}$ (highest dilution=1) followed by indicated dilutions (10-1000 fold). Aliquots of the ethanol fractions were taken to LC-MS-MS-based metabololipidomics for quantification of LM profiling.

Human macrophage phagocytosis and efferocytosis

To obtain apoptotic PMN, human PMN were isolated by density-gradient Ficoll-Histopaque from human peripheral blood. Blood was obtained from healthy volunteers giving informed consent according to Partners Human Research Committee Protocol no. 1999-P-001297. PMN were labeled with Bisbenzimide H 33342 (Sigma-Aldrich), a fluorescent nuclear dye (10μg/mL, 30 min, 37°C) and cultured overnight (5×10^6 cells/mL in $PBS^{+/+}$). Human primary macrophages were differentiated from peripheral blood monocytes [19] and plated onto 96-well plates (5×10^4 cells/well). Macrophages were incubated with either HLMI or $HLMI_{AC}$ at indicated dilutions (1-1000 fold dilutions, pH 7.45, 37 °C, 15 min) followed by a phagocytic challenge with either fluorescently labeled apoptotic PMN (3:1 PMN:macrophage) or *E. coli* (50:1 *E. coli*:macrophage). Incubations were continued for 45 min at 37 °C [19], macrophages washed and remaining extracellular fluorescence quenched using Trypan Blue (1:15 Trypan blue:$PBS^{+/+}$). Phagocytosis was assessed using a SpectraMax M3 plate reader (Molecular Devices).

Statistics

Data are presented as individual values or mean ± SEM. The criterion for statistical significance was $p<0.05$ using nonparametric Mann Whitney test or two-way ANOVA followed by a post hoc Bonferroni test using GraphPad Prism 6.

Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

AA	arachidonic acid
ALX	lipoxin A_4 receptor
COX	cyclooxygenase
d	deuterated
DHA	docosahexaenoic acid
E. coli	Escherichia coli
EFA	essential fatty acid
EPA	eicosapentaenoic acid
GPCR	G-coupled receptor
LC-MS-MS	liquid chromatography tandem mass spectrometry
LM	lipid mediators
LOX	lipoxygenase
LT	leukotriene
LTB_4	leukotriene B_4, (5*S*, 12*R*-dihydroxy-eicosa-6*Z*, 8*E*, 10*E*, 14*Z*-tetraenoic acid)
LX	lipoxin
LXA_4	lipoxin A_4 (5*S,* 6*R,* 15*S*-trihydroxy-eicosa-7*E*, 9*E*, 11*Z*, 13*E*-tetraenoic acid)
AT-LXA_4	lipoxin A_4 (5*S,* 6*R,* 15*R*-trihydroxy-eicosa-7*E*, 9*E*, 11*Z*, 13*E*-tetraenoic acid)
LXB_4	lipoxin B_4: (5*S,* 14*R,* 15*S*-trihydroxy-eicosa-6*E*, 8*Z*, 10*E*, 12*E*-tetraenoic acid)
MaR1	maresin 1 (7*R*, 14*S*-dihydroxy-docosa-4*Z*, 8*E*, 10*E*, 12*Z*, 16*Z*, 19*Z*-hexaenoic acid)
MRM	multiple reaction monitoring
PCA	principal component analysis
PD	protectin
PD1	protectin D1 (10*R*, 17*S*-dihydroxy-docosa-4*Z*, 7*Z*, 11*E*, 13*E*, 15*Z*, 19*Z*-hexaenoic acid), also known as neuroprotectin D1 (NPD1)

AT-PD1	protectin D1 (10*R*, 17*R*-dihydroxy-docosa-4*Z*, 7*Z*, 11*E*, 13*E*, 15*Z*, 19*Z*-hexaenoic acid), also known as neuroprotectin D1 (NPD1)
PGD_2	11-oxo-9α, 15*S*-dihydroxy-prosta-5*Z*, 13*E*-dien-1-oic acid
PGE_2	9-oxo-11α, 15*S*-dihydroxy-prosta-5*Z*, 13*E*-dien-1-oic acid
$PGF_{2\alpha}$	9α, 11α, 15*S*-trihydroxy-prosta-5*Z*, 13*E*-dienoic acid
PMN	polymorphonuclear leukocyte
R_i	resolution interval
Rv	resolvin
RvD1	Resolvin D1 (7*S*, 8*R*, 17*S*-trihydroxy-docosa-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
AT-RvD1	Resolvin D1 (7*S*, 8*R*, 17*R*-trihydroxy-docosa-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvD2	Resolvin D2 (7*S*, 16*R*, 17*S*-trihydroxy-docosa-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*, 19*Z*-hexaenoic acid)
RvD3	Resolvin D3 (4*S*, 11*R*, 17*S*-trihydroxy-docosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
AT-RvD3	Resolvin D3 (4*S*, 11*R*, 17*R*- trihydroxy-docosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvD4	Resolvin D4 (4*S*, 5R, 17*S*- trihydroxy-docosa-*6E*, 8*E*, *10Z*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvD5	Resolvin D5 (7*S*, 17*S*-dihydroxy-docosa-4*Z*, 8*E*, 10*Z*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvD6	Resolvin D6 (4*S*, 17*S*-dihydroxy-docosa-5*E*, 7Z, 10*Z*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid)
RvE1	Resolvin E1 (5*S*, 12*R*, 18*R*-trihydroxy-eicosa-6*Z*, 8*E*, 10*E*, 14*Z*, 16*E*-pentaenoic acid)
RvE2	Resolvin E2 (5*S*, 18*R*-dihydroxy-eicosa-6*E*, 8*Z*, 11*Z*, 14*Z*, 16*E*-pentaenoic acid)
RvE3	Resolvin E3 (17*R*,18*R*-dihydroxy-eicosa-5*Z*, 8*Z*, 11*Z*, 13*E*, 15*E*-pentaenoic acid)
SPM	specialized pro-resolving mediator

Tx	thromboxane
TxB_2	9α, 11, 15*S*-trihydroxy-thromba-5*Z*, 13*E*-dien-1-oic acid

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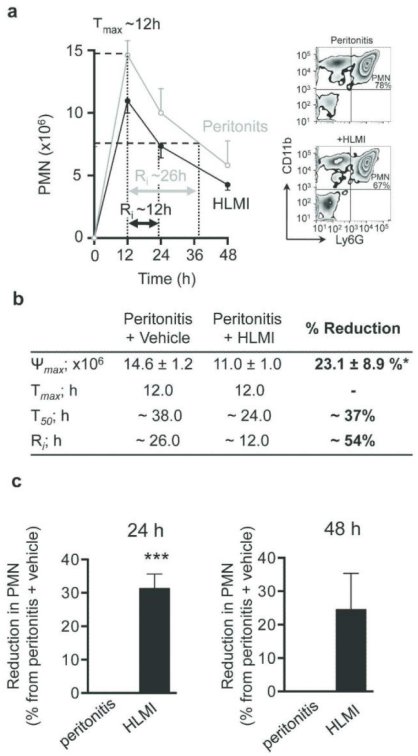


FIGURE 1. Human milk lipid mediator isolates (HLMI) shorten resolution of acute inflammation

(a) Exudate polymorphonuclear cell (PMN) numbers in mice administered vehicle or HLMI (in 200 μl saline, *i.p.*) immediately prior to self-limited inflammatory challenge (zymosan; 1 mg, *i.p.*). Inset, representative flow cytometry zebra plot; PMN identified as $CD11b^{+}Ly6G^{+}$ events. (b) Resolution indices were determined: Ψ_{max} (maximal PMN counts), T_{max} (the time interval when PMN reach maximum), T_{50} (the time interval corresponding to 50% PMN reduction, or Ψ_{50}) and R_i (resolution interval, the interval between T_{max} and T_{50}; see Materials and Methods for details). (c) Reduction in PMN numbers at 24 and 48 h from peritonitis plus vehicle mice. Results are mean ± SEM; *p<0.05 and ***p<0.001 vs. vehicle, n=4 mice per treatment at each time interval.

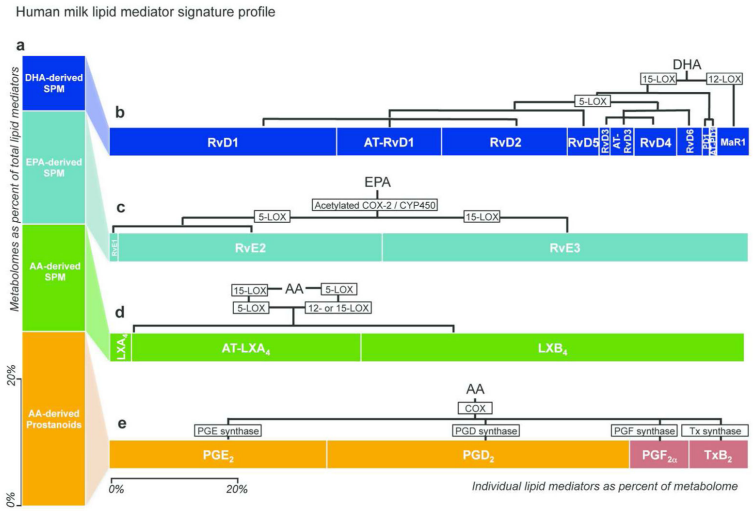


FIGURE 2. Signature LM-SPM profile of human milk

LM obtained from human milk (4-8 weeks post-partum) were identified by LC-MS-MS-based LM metabololipidomics (see Materials and Methods for details). (a) Percentage of DHA-derived SPM, EPA-derived SPM, AA-derived SPM, and AA-derived prostanoids in human milk from healthy volunteers. (b-e) Contribution of individual LM and SPM within each metabolome. Biosynthetic pathways are indicated above each major EFA metabolome (DHA, EPA and AA). LOX=lipoxygenase; COX-2=cyclooxygenase-2. Bars represent % of total LM (ng) from n=4 healthy human milk donors.

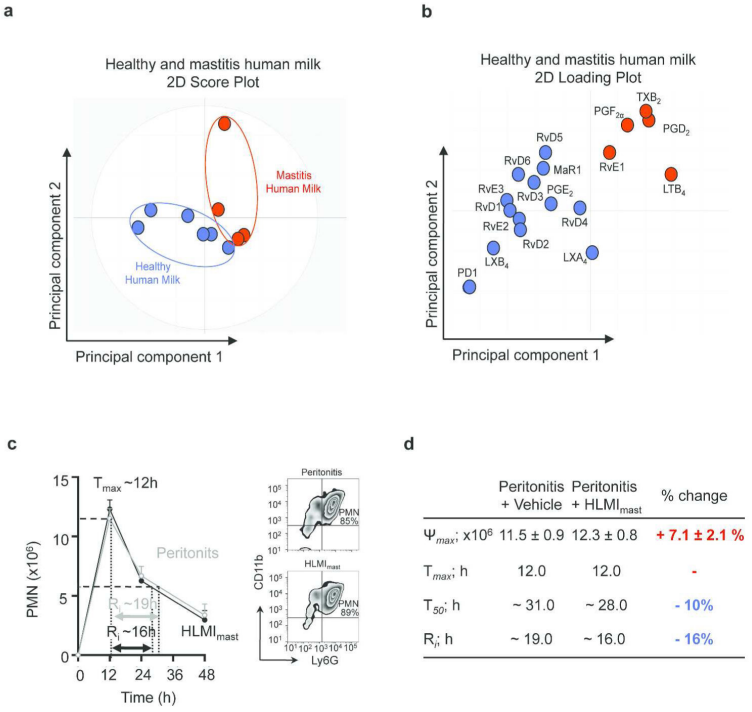
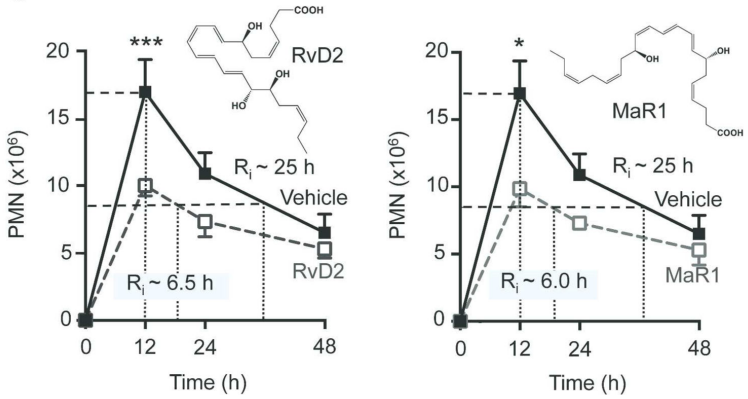


FIGURE 3. Mastitis human milk has altered LM-SPM profiles and reduced ability to accelerate resolution

LM obtained from healthy and mastitis-affected human milk (1-6 months postpartum) were identified by LC-MS-MS metabololipidomics (see Materials and Methods for details). (a) 2-dimentional score plot of human milk from healthy donors (n = 7) compared to mastitis donors (n=4). (b) 2-dimentional loading plot. (c) Exudate PMN numbers in mice administered vehicle or HLMI from mastitis milk ($HLMI_{mast}$; in 200 μl saline, i.p.) immediately prior to self-limited inflammatory challenge (zymosan; 1 mg, i.p.). Inset, representative flow cytometry zebra plot; PMN identified as $CD11b^{+}Ly6G^{+}$ events. (d) Resolution indices were calculated as in Figure 1 (see Materials and Methods for details). Results are mean ± SEM, n = 3 mice per treatment at each time interval.

a



b

	Peritonitis + Vehicle	Peritonitis + RvD2	Peritonitis + MaR1
Ψ_{max}; x10^6	17.0 ± 2.4	10.0 ± 0.8	9.9 ± 1.3
T_{max}; h	12.0	12.0	12.0
T_{50}; h	~ 37.0	~ 18.5	~ 18.0
R_i; h	~ 25.0	~ 6.5	~ 6.0
Shortening of R_i	**-**	**74%**	**76%**

FIGURE 4. RvD2 and MaR1 accelerate resolution of inflammation

(a) Exudate PMN numbers in mice administered vehicle, RvD2 (left) or MaR1 (right; 50 ng each/mouse, *i.p.*) before injection of zymosan (1 mg/mouse, *i.p.*). Inset: molecular structure for RvD2 (left) and MaR1 (right). (b) Resolution indices were calculated as in Figure 1 (see Materials and Methods for details). Results are mean ± SEM; *p<0.05 and ***p<0.001 vs. vehicle, n=6 mice (vehicle and RvD2) or n=3 mice (MaR1) at each time interval.

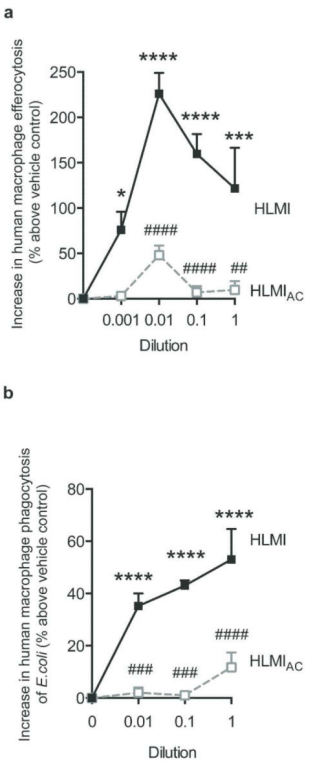


FIGURE 5. HLMI enhance human macrophage phagocytosis

Enhanced phagocytosis of a) apoptotic PMN and b) *E. coli* with human macrophages expressed as increase in phagocytosis above vehicle. Macrophages (5×10^4 cells/well) were incubated with indicated concentration of HLMI or HLMI depleted of LM by activated charcoal ($HLMI_{AC}$) (1=highest concentration, 0.1=1:10 dilution, 0.01=1:100 dilution, 0.001=1:1000 dilution; pH 7.45, 37 °C, 15 min). Subsequently, fluorescently labeled a) apoptotic PMN (3:1 PMN:macrophage) or b) *E. coli* (50:1 *E. coli*:macrophages) were added (45 min, 37°C). Non-phagocytosed apoptotic PMN or *E. coli* were washed off, extracellular fluorescence quenched, and phagocytosis determined using a fluorescence plate reader. Results are mean ± SEM; $^{**}p<0.01$, $^{****}p<0.0001$ vs. vehicle; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#}p<0.0001$ vs. HLMI. (a) n=3 and (b) n=4 macrophage donors.

Table 1

Bioactive LM profile of human milk (4-8 weeks post-partum)*

			Healthy human milk, 4-8 wks post-partum	
DHA Bioactive			Lipid mediator levels	
SPM Metabolome	*Q1*	*Q3*	(pg/mL)	pM
RvD1	*375*	*215*	147 ± 47.2	391
AT-RvD1	*375*	*215*	67.4 ± 11.7	180
RvD2	*375*	*215*	82.4 ± 28.0	219
RvD3	*375*	*147*	7.2 ± 2.7	19.1
AT-RvD3	*375*	*147*	15.0 ± 2.9	39.9
RvD4	*375*	*101*	27.4 ± 7.5	72.9
RvD5	*359*	*199*	19.9 ± 8.9	52.9
RvD6	*359*	*101*	6.7 ± 2.4	17.8
PD1	*359*	*153*	4.3 ± 2.3	11.9
AT-PD1	*359*	*153*	3.8 ± 0.9	10.6
MaR1	*359*	*221*	20.8 ± 6.3	57.8
AA Bioactive LM				
Metabolome				
LXA_4	*351*	*115*	25.7 ± 8.6	72.9
AT-LXA_4	*351*	*115*	370.0 ± 176.6	1260
LXB_4	*351*	*115*	267.1 ± 93.9	759
AT-LXB_4	*351*	*115*	-	-
LTB_4	*335*	*195*	-	-
PGE_2	*351*	*189*	409.7 ± 146.6	1160
PGD_2	*351*	*189*	568.3 ± 188.9	1610
$PGF_{2\alpha}$	*353*	*193*	111.1 ± 36.2	314
TxB_2	*369*	*169*	111.8 ± 44.4	302
EPA Bioactive				
SPM Metabolome				
RvE1	*349*	*195*	8.8 ± 3.6	25.1
RvE2	*333*	*253*	321.2 ± 129.2	962
RvE3	*333*	*201*	444.9 ± 179.8	1330

*Quantification of bioactive lipid mediators (LM) in human milk (4-8 weeks post-partum) assessed by LC-MS-MS based LM metabololipidomics. Results are expressed as pg/mL human milk. Q1: M-H (parent ion) and Q3 (daughter ion): diagnostic ion in the MS-MS. Detection limit was ~ 0.1 pg; - denotes below limits. Results are mean ± s.e.m. from 4 donors. Complete LC-MS-MS, retention times and MS-MS spectra for each eicosanoid and SPM listed here are presented in Supplemental Figure 1.

Table 2

Comparison of SPM impact on the resolution interval (R_i) in mouse peritonitis *

Agonist	Dose	Shortening of R_i (%)	Reduction of ψ_{max} (%)
RvD1	50 ng/mouse[19]	40	40
RvD2	50 ng/mouse	74	41
RvD3	50 ng/mouse[40]	92	47
MaR1	50 ng/mouse	76	58

* The impact of SPM administered *i.p.* at initiation of inflammation) on resolution interval in murine self-limited peritonitis initiated by i.p. injection of 1mg zymosan *i.p. or E. coli*, (10^5 colony forming units, c.f.u). For direct comparison see [19, 40].