Human milk proresolving mediators stimulate resolution of acute inflammation

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Human milk contains nutrients and bioactive products relevant to infant development and immunological protection. Here, we investigated the proresolving properties of milk using human milk lipid mediator isolates (HLMIs) and determined their impact on resolution programs *in vivo* and with human macrophages. HLMIs reduced the maximum neutrophil numbers ($14.6 \pm 1.2 \times 10^6$–$11.0 \pm 1.0 \times 10^6$ cells per exudate) and shortened the resolution interval (R_i; 50% neutrophil reduction) by 54% compared with 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-specialized proresolving mediator (LM-SPM) signature profile, containing SPMs (e.g. resolvins (Rv), protectins (PDs), maresins (MaRs), and lipoxins (LXs)) at bioactive levels (pico-nanomolar concentrations) that enhanced human macrophage efferocytosis and bacterial containment. SPMs identified in human milk included D-series Rvs (e.g., RvD1, RvD2, RvD3, AT-RvD3, and RvD4), PD1, MaR1, E-series Rvs (e.g. RvE1, RvE2, and RvE3), and LXs (LXA_4 and LXB_4). Of the SPMs identified in human milk, RvD2 and MaR1 (50 ng per mouse) individually shortened R_i by ~75%. Milk from mastitis gave higher leukotriene B_4 and prostanoids and lower SPM levels. Taken together, these findings provide evidence that human milk has proresolving actions via comprehensive LM-SPM profiling, describing a potentially novel mechanism in maternal–infant biochemical imprinting.

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 (LMs) that drive the influx and function of immune cells, and eventually cell efflux and tissue repair.[1,2] Newly identified families of bioactive LMs, biosynthesized from essential fatty acids (EFAs), that actively stimulate resolution of inflammation were uncovered in self-resolving exudates and their structures elucidated.[1] Collectively, they are coined specialized proresolving mediators (SPMs).[1] SPMs comprise several families that include arachidonic acid (AA)-derived lipoxins (LXs), eicosapentaenoic acid (EPA)-derived resolvins (RvEs), and docosahexaenoic acid (DHA)-derived resolvins (RvD), protectins (PDs), and maresins (MaRs); 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 Serhan[1]). SPMs are evolutionarily conserved biochemical signals, as they are present in trout, salmon, and planaria (reviewed in Serhan[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

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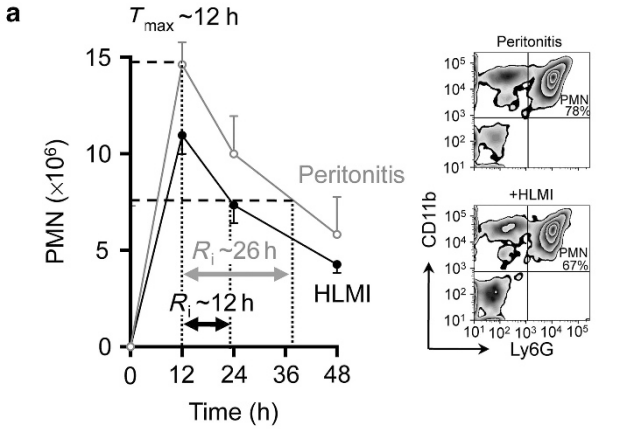
can be detrimental.[12] Hence, protective mechanisms for resolving infection and inflammation in a timely manner and educating the innate immune system in early life are critical and of general interest. In this report, we present evidence for new immunoresolving properties of human milk. Using self-limited 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 polymorphonuclear neutrophil (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), PDs (PD1 and AT-PD1), MaR1, E-series Rvs (RvE2 and RvE3), and LXs (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 resolution interval (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

HLMIs stimulate resolution of inflammation

To investigate whether human milk exerts proresolving actions, we used human milk chromatographic isolates with self-limited acute inflammation *in vivo* and mapped leukocyte trafficking. Because SPMs, including Rvs, PDs, and MaRs, 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 (HLMIs)) and assessed their ability to accelerate resolution of acute inflammation *in vivo*. First, self-limiting acute inflammation was initiated by intraperitoneal injection of yeast cell wall particles (zymosan, 1 mg per mouse), and to quantitate resolution we used defined resolution parameters of acute inflammation.[13,14] The self-limited response reached maximal neutrophil numbers ($\Psi_{max} = 14.6 \pm 1.2 \times 10^6$ cells per murine exudate) at 12 h (T_{max}), which was followed by subsequent decline (**Figure 1a**). Administration of HLMIs immediately before inflammatory challenge gave an $\sim 23.1 \pm 8.9\%$ reduction in Ψ_{max} ($11.0 \pm 1.0 \times 10^6$ cells per exudate; **Figure 1a, b**). Reduction in neutrophil levels was observed throughout the course of inflammation resolution in mice administered HLMIs, with $31.3 \pm 4.4\%$ and $24.5 \pm 10.9\%$ fewer neutrophils at 24 and 48 h, respectively, compared with peritonitis plus vehicle (**Figure 1c**).

To quantify the regulation of leukocyte trafficking at the site of inflammation, we investigated the 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 to 12 h (**Figure 1a, b**). These results



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Resolution Indices	Peritonitis + vehicle	Peritonitis + HLMI	% reduction
Ψ_{max}; (×10^6)	14.6 ± 1.2	11.0 ± 1.0	**23.1 ± 8.9 %***
T_{max}; (h)	12.0	12.0	–
T_{50}; (h)	~ 38.0	~ 24.0	**~ 37%**
R_i; (h)	~ 26.0	~ 12.0	**~ 54%**

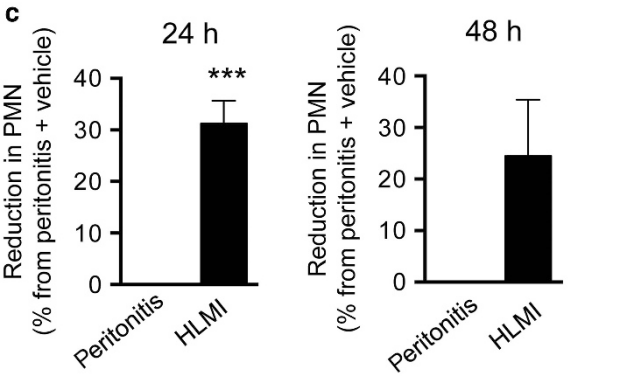


Figure 1 Human milk lipid mediator isolates (HLMIs) shorten resolution of acute inflammation. (**a**) Exudate polymorphonuclear cell (PMN) numbers in mice administered vehicle or HLMIs (in 200 µl saline, intraperitoneally) immediately before self-limited inflammatory challenge (zymosan; 1 mg, intraperitoneally). 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 Methods for details). (**c**) Reduction in PMN numbers at 24 and 48 h from peritonitis plus vehicle mice. Results are mean ± s.e.m.; ***$P < 0.001$ vs. vehicle; $n = 4$ mice per treatment at each time interval.

demonstrate that human milk possesses proresolving properties contained in the HLMIs.

Human milk LM-SPM signature profile: LM metabololipidomics

Because isolates from human milk accelerate resolution (**Figure 1**), we next sought to investigate the LM profile of human milk. Using liquid-chromatography tandem-mass spectrometry (LC-MS-MS)-based LM metabololipidomics (see Methods for details), we identified a profile signature

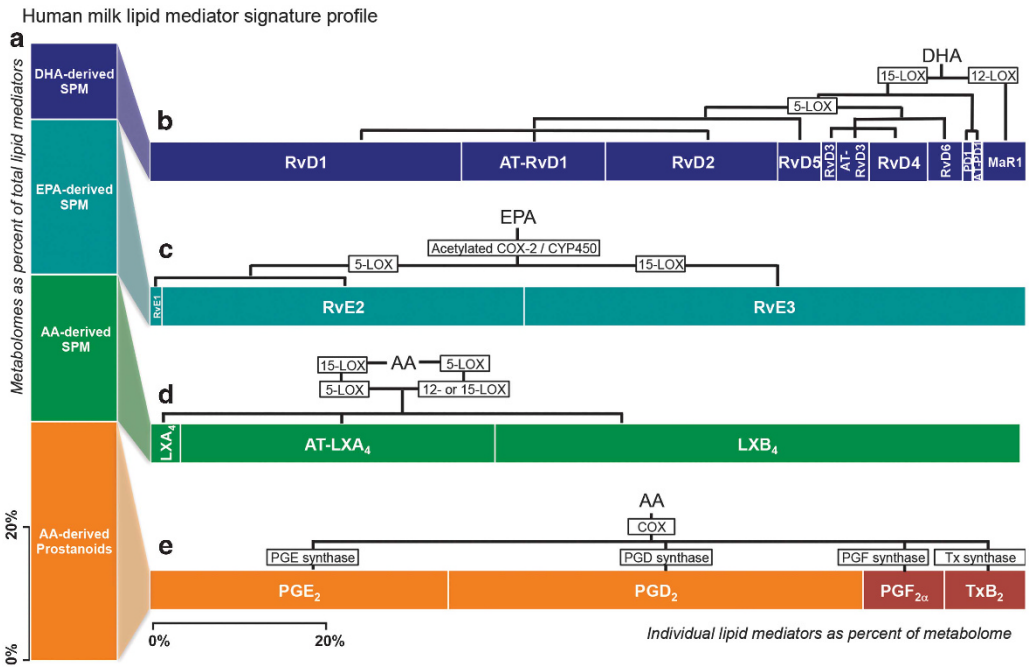


Figure 2 Signature lipid mediator-specialized proresolving mediator (LM-SPM) profile of human milk. LMs obtained from human milk (4–8 weeks postpartum) were identified by liquid-chromatography tandem-mass spectrometry (LC-MS-MS)-based LM metabololipidomics (see Methods for details). (**a**) Percentage of docosahexaenoic acid (DHA)-derived SPM, eicosapentaenoic acid (EPA)-derived SPM, arachidonic acid (AA)-derived SPM, and AA-derived prostanoids in human milk from healthy volunteers. (**b**–**e**) Contribution of individual LMs and SPMs within each metabolome. Biosynthetic pathways are indicated above each major essential fatty acid (EFA) metabolome (DHA, EPA, and AA). Vertical bars represent % of total LMs (ng) from $n = 4$ healthy human milk donors. COX-2, cyclooxygenase-2; LOX, lipoxygenase; LX, lipoxin; MaR, maresin; PD, protectin; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGD_2, prostaglandin D_2; RvD, D-series Rv; RvE, E-series resolvin; TxB_2, thromboxane B_2.

of LMs consisting of 20 bioactive LMs (**Figure 2**, **Table 1**, and **Supplementary Figure 1** online) from both lipoxygenase and cyclooxygenase pathways, including Rvs, PDs, MaRs, LXs, and prostanoids (**Figure 2**, **Table 1**, **Supplementary Figure 1**, and **Supplementary Table 1**). Each LM was identified by matching LC retention time and at least six diagnostic ions, and quantification was achieved using multiple reaction monitoring in accordance with published criteria,[3] and as illustrated with representative results obtained for all identified LMs (**Supplementary Figure 1b**).

LM quantification, using multiple reaction monitoring, demonstrated that SPMs in healthy mature human milk (4–8 weeks postpartum) include AT-RvD1 ($67.4 \pm 11.7\,pg\,ml^{-1}$), RvD2 ($82.4 \pm 28.0\,pg\,ml^{-1}$), RvD3 ($7.2 \pm 2.7\,pg\,ml^{-1}$), AT-RvD3 ($15.0 \pm 2.9\,pg\,ml^{-1}$), RvD4 ($27.4 \pm 7.5\,pg\,ml^{-1}$), RvD5 ($19.9 \pm 8.9\,pg\,ml^{-1}$), RvD6 ($6.7 \pm 2.4\,pg\,ml^{-1}$), PD1 ($4.3 \pm 2.3\,pg\,ml^{-1}$), AT-PD1 ($3.8 \pm 0.9\,pg\,ml^{-1}$), and MaR1 ($20.8 \pm 6.3\,pg\,ml^{-1}$) from the DHA metabolome, RvE2 ($321.2 \pm 129.2\,pg\,ml^{-1}$) and RvE3 ($444.9 \pm 179.8\,pg\,ml^{-1}$) from the EPA metabolome, and AT-LXA_4 ($370.0 \pm 176.6\,pg\,ml^{-1}$) and LXB_4 ($267.1 \pm 93.9\,pg\,ml^{-1}$) from the AA metabolome (**Table 1**). These are in addition to RvD1 ($147.0 \pm 47.2\,pg\,ml^{-1}$), RvE1 ($8.8 \pm 3.6\,pg\,ml^{-1}$), and LXA_4 ($25.7 \pm 8.6\,pg\,ml^{-1}$). These confirm the identification of RvD1, RvE1, and LXA_4 in human milk, at values consistent with those recently reported.[6] From the cyclooxygenase pathways, we also identified prostaglandin E_2 (PGE_2) ($409.7 \pm 146.6\,pg\,ml^{-1}$), prostaglandin D_2 (PGD_2) ($568.3 \pm 188.9\,pg\,ml^{-1}$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ($111.1 \pm 36.2\,pg\,ml^{-1}$), and thromboxane B_2 (TxB_2) ($111.8 \pm 44.4\,pg\,ml^{-1}$) in these samples in accordance with published findings.[15] These results demonstrate that human milk contains SPMs 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 (**Figure 2**). LM metabololipidomics of human milk AA, EPA, and DHA identified bioactive metabolome demonstrated that SPMs represented ~61.6% of the human milk LM profile (**Figure 2**), consisting of DHA-derived Rvs, PDs, and MaRs (13.1%), AA-derived LXs (23.5%), and EPA-derived Rvs (24.9%; **Figure 2**). AA-derived prostanoids amounted to ~38.4% of the LMs identified (**Figure 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 LMs (**Figure 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

Table 1 Bioactive LM profile of human milk (4–8 weeks postpartum)

			Healthy human milk, 4–8 weeks postpartum	
	Q1	Q3	Lipid mediator levels (pg ml^{-1})	pM
DHA bioactive SPM metabolome				
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	1,260
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	1,160
PGD_2	351	189	568.3 ± 188.9	1,610
$PGF_{2\alpha}$	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	1,330

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-MS-MS, liquid-chromatography tandem-mass spectrometry; LM, lipid mediator; LTB_4, leukotriene B_4; LXB_4, lipoxin B_4; MaR, maresin; PD, protectin; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RvD, D-series resolvin; RvE, E-series resolvin; SPM, specialized proresolving mediator; TxB_2, thromboxane B_2.
Quantification of bioactive LMs in human milk (4–8 weeks postpartum), as assessed by LC-MS-MS based LM metabololipidomics. Results are expressed as pg ml^{-1} 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 four donors. Complete LC-MS-MS, retention times, and MS-MS spectra for each eicosanoid and SPMs listed here are presented in **Supplementary Figure 1.**

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 LMs and SPMs with proresolving properties at concentrations commensurate with their known bioactions.[1,17,18]

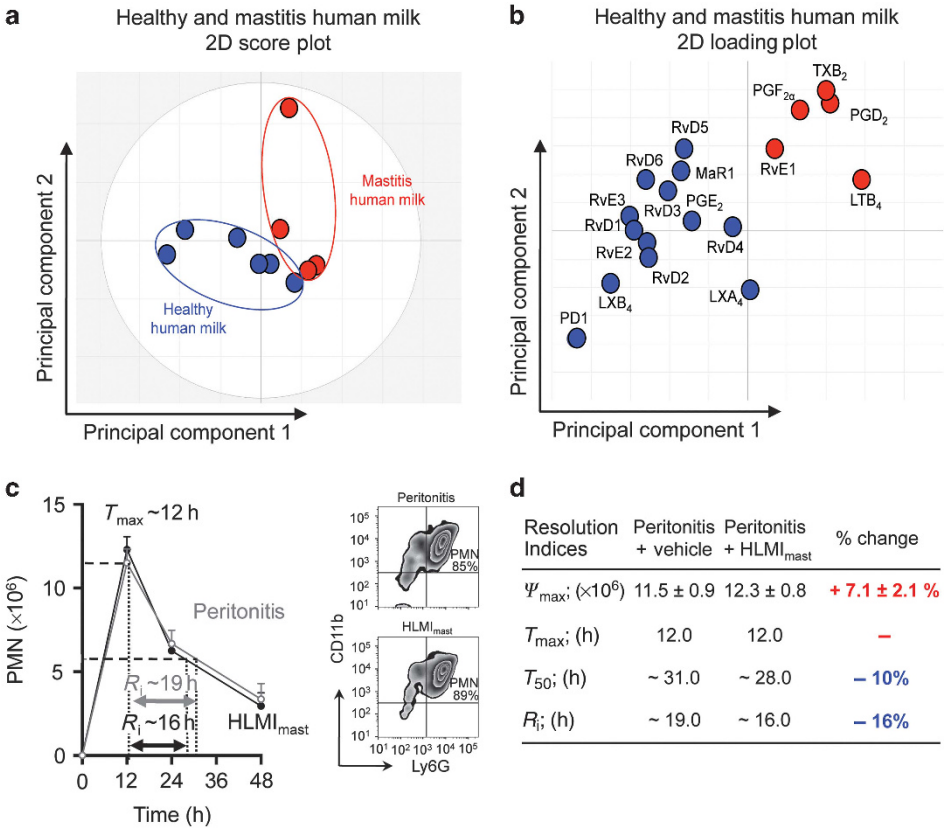
Human milk LM-SPM profile is altered in mastitis

SPMs 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 with milk from healthy subjects (**Figure 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. 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 (**Figure 3a**). The healthy milk cluster was characterized by higher levels of SPMs, including RvD1, RvD2, RvD3, MaR1, PD1, RvE2, and LXA_4 and LXB_4 as demonstrated in the loading plot (**Figure 3b**). Conversely, principal component 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 LMs and reduced SPMs.

As mastitis milk had an altered LM-SPM profile, we next investigated the ability of HLMIs from mastitis milk (referred to as $HLMI_{mast}$) to accelerate resolution of acute inflammation. HLMI from mastitis milk was obtained as described above for HLMIs from healthy milk (see Methods for details). Administration of $HLMI_{mast}$ immediately before challenge (1 mg zymosan per mouse) did not limit neutrophil numbers at T_{max} (12.3 ± 0.8 vs. 11.5 ± 0.9 cells per exudate compared with peritonitis plus vehicle), and only slightly shortened the R_i by 16%, or from 19 h observed in peritonitis plus vehicle to 16 h (**Figure 3c and d**). Taken 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 SPMs identified in human milk (**Figure 2** and **Table 1**), we sought to assess its potential contribution to the regulation of leukocyte trafficking and the R_i. Mice were administered RvD2 (50 ng per mouse, i.e., 2 μg kg^{-1}; intraperitoneally) before initiation of a self-limited inflammatory challenge and resolution parameters quantified (**Figure 4**). RvD2 gave ~40% reduction in Ψ_{max} ($10.0 \pm 0.8 \times 10^6$ vs. $17.0 \pm 2.4 \times 10^6$ cells per exudate) compared with peritonitis plus vehicle mice and shortened the R_i by 74%, or from 25 to 6.5 h (**Figure 4**). DHA also serves as a substrate for MaRs,[1] and as MaR1 was identified in human milk at bioactive concentrations (**Figure 2** and **Table 1**) we compared its actions



Resolution Indices	Peritonitis + vehicle	Peritonitis + $HLMI_{mast}$	% change
Ψ_{max}; (×10^6)	11.5 ± 0.9	12.3 ± 0.8	+ 7.1 ± 2.1 %
T_{max}; (h)	12.0	12.0	–
T_{50}; (h)	~ 31.0	~ 28.0	– 10%
R_i; (h)	~ 19.0	~ 16.0	– 16%

Figure 3 Mastitis human milk has altered lipid mediator-specialized proresolving mediator (LM-SPM) profiles and reduced ability to accelerate resolution. LMs obtained from healthy and mastitis-affected human milk (1–6 months postpartum) were identified by liquid-chromatography tandem-mass spectrometry (LC-MS-MS) metabololipidomics (see Methods for details). (**a**) Two-dimensional (2D) score plot of human milk from healthy donors ($n = 7$) compared with mastitis donors ($n = 4$). (**b**) 2D loading plot. (**c**) Exudate polymorphonuclear cell (PMN) numbers in mice administered vehicle or HLMI from mastitis milk ($HLMI_{mast}$; in 200 µl saline, intraperitoneally) immediately before self-limited inflammatory challenge (zymosan; 1 mg, intraperitoneally). Inset, representative flow cytometry zebra plot; PMN identified as $CD11b^+Ly6G^+$ events. (**d**) Resolution indices were calculated as in **Figure 1** (see Methods for details). Results are mean ± s.e.m.; $n = 3$ mice per treatment at each time interval. LTB_4, leukotriene B_4; LX, lipoxin; MaR1, maresin 1; PD1, protectin D1; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGD_2, prostaglandin D_2; RvD, D-series Rv; TxB_2, thromboxane B_2.

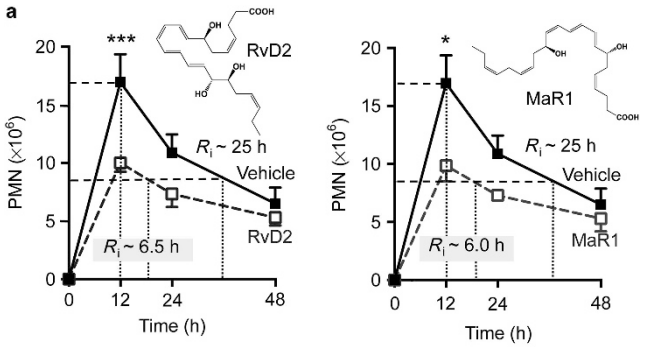
on regulating leukocyte trafficking to RvD2. MaR1 (50 ng per mouse, intraperitoneally) gave a maximal PMN number of $9.9 \pm 1.3 \times 10^6$ cells per exudate and shortened the R_i to 6 h, or by 76% (**Figure 4**). We also assessed the ability of RvD2 and MaR1 to accelerate resolution of established peritonitis (**Supplementary Figure 2a, b**). RvD2 and MaR1 (50 ng per mouse) administered 12 h after zymosan challenge (1 mg per mouse) each accelerated resolution, reducing neutrophil numbers and shortening the R_i by 33% and 40%, respectively (**Supplementary Figure 2a, b**). Thus, both RvD2 and MaR1, at physiologic range, that is, nanograms per mouse, regulate neutrophil trafficking and shorten the R_i.

HLMIs and MaR1 stimulate resolution of infectious peritonitis

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

HLMIs enhance human macrophage phagocytosis

Given the key actions of SPMs in resolution are enhancing macrophage clearance of apoptotic cells and debris,[1] we next questioned whether HLMIs have direct impact on phagocytosis with isolated human cells. Incubation of human macrophages



b

Resolution Indices	Peritonitis + vehicle	Peritonitis + RvD2	Peritonitis + MaR1
Ψ_{max}; (×10^6)	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 Resolvin D2 (RvD2) and maresin 1 (MaR1) accelerate resolution of inflammation. (**a**) Exudate polymorphonuclear cell (PMN) numbers in mice administered vehicle, RvD2 (left) or MaR1 (right; 50 ng each per mouse, intraperitoneally) before injection of zymosan (1 mg per mouse, intraperitoneal). Inset: molecular structure for RvD2 (left) and MaR1 (right). (**b**) Resolution indices were calculated as in **Figure 1** (see Methods for details). Results are mean ± s.e.m.; $^{*}P<0.05$ and $^{***}P<0.001$ vs. vehicle; $n=6$ mice (vehicle and RvD2) or $n=3$ mice (MaR1) at each time interval.

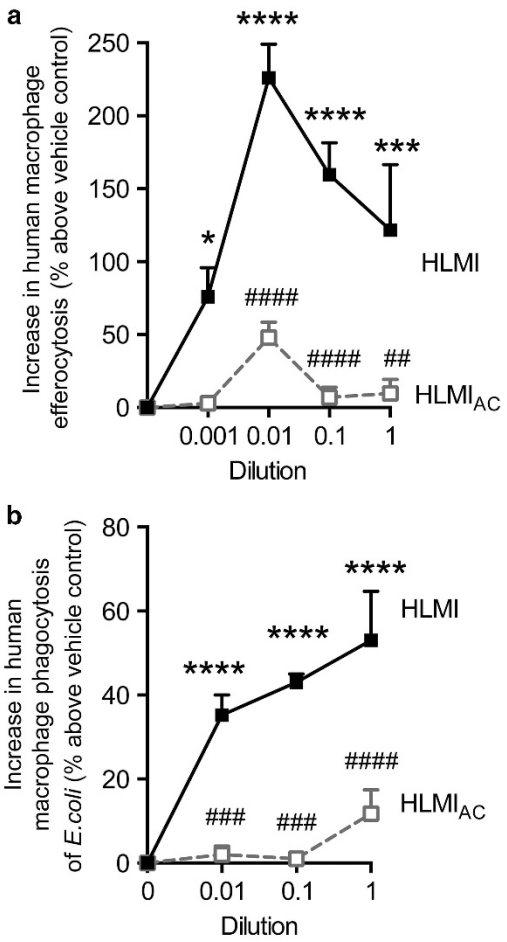


Figure 5 Human milk lipid mediator isolates (HLMIs) enhance human macrophage phagocytosis. Enhanced phagocytosis of (**a**) apoptotic polymorphonuclear cell (PMN) and (**b**) *Escherichia coli* (*E. coli*) with human macrophages expressed as increase in phagocytosis above vehicle. Macrophages (5×10^4 cells per well) were incubated with indicated concentration of HLMIs or HLMI depleted of LMs 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 at 37 °C for 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 at 37 °C). Nonphagocytosed apoptotic PMN or *E. coli* were washed off, extracellular fluorescence quenched, and phagocytosis determined using a fluorescence plate reader. Results are mean ± s.e.m.; $^{*}P<0.05$, $^{***}P<0.001$, $^{****}P<0.0001$ vs. vehicle; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ vs. HLMI. (**a**) $n=3$ and (**b**) $n=4$ macrophage donors.

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

Based on these and the *in vivo* findings, and as SPM, including RvD1, RvD2, and RvD5, directly enhance human phagocyte containment of *E. coli*,[19,20] we next questioned whether HLMIs have direct impact on bacterial containment with isolated human cells. HLMIs increased human macrophage phagocytosis of fluorescent *E. coli* by ~35–55% compared with vehicle-treated macrophages (**Figure 5b**). The ability of HLMIs to enhance macrophage containment of *E. coli* was significantly reduced after LM depletion with activated charcoal (**Figure 5b**). Taken together, these results demonstrate that HLMI possesses bioactive LMs/SPMs 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 Rvs, PDs, MaRs, and LXs at bioactive concentrations in healthy human milk. For comparison, in mastitis, milk LM-SPM levels were altered showing elevated proinflammatory LMs and lower levels of SPMs. 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, HLMIs 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] Owing 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 Rvs, PDs, MaRs, and LXs, are endogenous LMs found in many tissues that actively counterregulate proinflammatory signals, including nuclear factor-κB,[9] cytokines, and leukotrienes.[1] They exert their potent actions via activating specific G-protein-coupled receptors in cell-specific and tissue-dependent manner. Several SPM receptors are identified, for example, RvE1 specifically binds both ChemR23 and BLT1, and LXA_4 and RvD1 bind and activate the LX A_4 receptor ALX and human GPR32, which also binds RvD3 and RvD5 (reviewed in Serhan[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 interleukin-8 secretion by intestinal epithelial cells.[24] Enterocytes also express ChemR23, where RvE1 induces intestinal alkaline phosphatase expression and enzyme activity that attenuates lipopolysaccharide-induced nuclear factor-κB signaling.[25] Hence, together with our present results SPMs 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 G-protein-coupled receptors, they may activate specific and potentially additive responses in the newborn gut mucosa; such actions remain of interest.

Proresolution 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 SPMs 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 *P*450 in the mastitis microenvironment, for example, cytochrome *P*450 can produce the RvE1 precursor 18-HEPE (hydroxyicosapentaenoic acid) from EPA, which in turn is converted to RvE1 by human PMN (reviewed in Serhan[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 SPMs.

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 **Supplementary Table 2**). For example, RvD1 and RvD3 (50 ng per mouse, i.e., 2 μg kg^{-1}, each) shorten R_i in murine peritonitis (**Table 2**). Also, RvD1, PD1, and AT-LXA_4 at 300 ng per mouse (i.e., 12 μg kg^{-1}) each reduce the R_i, whereas RvE1 accelerates the onset (T_{max}) of resolution (**Supplementary 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 SPMs, including RvD2 and MaR1, are present in human milk at biologically relevant levels, SPMs and their pathways may have implications in the regulation of acute inflammation and resolution in maternal–infant transferred protection.

Table 2 Comparison of SPM impact on the R_i in mouse peritonitis[a]

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

Abbreviations: LM, lipid mediator; MaR, maresin; R_i, resolution interval; RvD, D-series resolvin; SPM, specialized proresolving mediator.
[a]The impact of SPMs administered intraperitoneally (at initiation of inflammation) on resolution interval in murine self-limited peritonitis initiated by intraperitoneal injection of 1 mg zymosan or *E. coli* (10^5 colony-forming units, c.f.u). For direct comparison, see Chiang *et al.*[19] and Arnardottir *et al.*[41]

Emerging evidence indicates that breastfeeding is correlated with lower prevalence of inflammatory conditions in early life (e.g., necrotizing enterocolitis) 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 have 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 SPMs 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 β-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 SPMs may be relevant in infant neurological development.

In summation, human milk LM metabololipidomic profiling uncovered specific LM signature with physiologically relevant levels of endogenous SPMs 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 SPMs herein, RvD2 and MaR1 each accelerated resolution of acute inflammation and infection (**Figure 4**, **Table 2**, and **Supplementary Figure 2**). With human macrophages, HLMIs stimulate 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 SPMs in modulating inflammation, infection, and stimulating resolution during early immune development, as SPMs display potent actions in the innate immune system.

METHODS

Extraction of HLMIs for murine peritonitis. Deidentified 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 r.p.m. at 4 °C for 10 min). Supernatants were extracted using two volumes of diethyl ether, and LMs were further isolated using solid-phase extraction as in Colas *et al.*[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 weeks; Charles River Laboratories, Newton, MA) by intraperitoneal injection of 1 mg zymosan A (Z4250; Sigma-Aldrich, St. Louis, MO).[38] For infectious peritonitis, mice were injected with self-limited inoculum of *E. coli* (10^5 colony-forming unit). Immediately before zymosan injection, mice were administered (intraperitoneally) HLMIs (levels representative of ~1 ml human milk), RvD2 (50 ng per mouse), MaR1 (50 ng per mouse), or vehicle (saline containing 0.2% ethanol). In some experiments, mice were administered treatments at T_{max} (12 h). Isolates pooled from three 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 before each experiment according to published criteria.[20] Peritoneal exudates were collected at indicated time intervals by lavaging with 5 ml PBS (phosphate-buffered saline). Exudate PMN numbers were assessed using Turk's solution, light microscopy, and flow cytometry (FACSCanto II; BD Bioscience, San Jose, CA). PMNs were determined as Ly6G- (clone 1A8; BD Bioscience) and CD11b- (clone M1/70; eBioscience, San Diego, CA) positive events and F4/80- (clone BM8; eBioscience) negative events from events as assessed by forward scatter and side scatter. Resolution indices were calculated as in Schwab *et al.*[13] and Bannenberg *et al.*,[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 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 no. 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 postpartum; Lee Biosolutions, Maryland Heights, MO) or matched mastitis and healthy donors (1–6 months postpartum; Creative Bioarray, Shirley, NY) was extracted using solid-phase extraction with C18 columns (Waters, Milford, MA), following the addition of three volumes of cold methanol containing deuterated internal standards (1 ng d4-PGE_2, d4-LTB4, and d8-5S-HETE, as well as d5-RvD2) and protein precipitation. LM levels were assessed by a LC-MS-MS system, QTrap 5500 and QTrap 6500 (ABSciex, Concord, Ontario, Canada) equipped with Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). 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^{-1} vol^{-1}) to 100:0:0.01 at 0.4 ml min^{-1} flow rate. To monitor and identify various LM, a multiple reaction monitoring 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 six diagnostic ions were used in each MS-MS. The complete stereochemistry of resolvin D4 was recently determined,[39] 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 to 0.99. Detection limits were ~0.1 pg.

Principal component analysis. Principal component analysis was performed using SIMCA 13.0.3 software (Umetrics, San Jose, CA) following mean centering and unit variance scaling of LM amounts. Principal component analysis 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 LMs/SPMs contribute to the cluster separation in the score plot.[40]

Depletion of milk LMs 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 room temperature. Activated charcoal was washed out, three volumes methanol were added to the milk, and proteins precipitated at -20 °C. Precipitate was pelleted by centrifugation (3,000 r.p.m. at 4 °C for 10 min), and LMs isolated using C18 columns and solid-phase extraction.[3] Products were eluted in methyl formate, solvent was evaporated under N_2, and suspended in 500 μl ethanol. For human macrophage phagocytosis, 20 μl HLMIs were dried down and resuspended in 1 ml $PBS^{+/+}$ (highest dilution = 1) followed by indicated dilutions (10–1,000-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 PMNs 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. PMNs were labeled with Bisbenzimide H 33342 (Sigma-Aldrich), a fluorescent nuclear dye (10 μg ml^{-1} for 30 min at 37 °C) and cultured overnight (5×10^6 cells per ml in $PBS^{+/+}$). Human primary macrophages were differentiated from peripheral blood monocytes[19] and plated onto 96-well plates (5×10^4 cells per well). Macrophages were

incubated with either HLMIs or $HLMI_{AC}$ at indicated dilutions (1–1,000 fold dilutions, pH 7.45, at 37 °C for 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, Sunnyvale, CA).

Statistics. Data are presented as individual values or mean ± s.e.m. The criterion for statistical significance was $P<0.05$ using nonparametric Mann–Whitney test or two-way analysis of variance, followed by a *post hoc* Bonferroni test using GraphPad Prism 6 (La Jolla, CA).

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

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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. The remaining authors declare no conflicts of interest.

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