Aging Delays Resolution of Acute Inflammation in Mice: Reprogramming the Host Response with Novel Nano-Proresolving Medicines

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Aging is associated with an overt inflammatory phenotype and physiological decline. Specialized proresolving lipid mediators (SPMs) are endogenous autacoids that actively promote resolution of inflammation. In this study, we investigated resolution of acute inflammation in aging and the roles of SPMs. Using a self-resolving peritonitis and resolution indices coupled with lipid mediator metabololipidomics, we found that aged mice had both delayed resolution and reduced SPMs. The SPM precursor docosahexaenoic acid accelerated resolution via increased SPMs and promoted human monocyte reprogramming. In aged mice, novel nano-proresolving medicines carrying aspirin-triggered resolins D1 and D3 reduced inflammation by promoting efferocytosis. These findings provide evidence for age-dependent resolution pathways in acute inflammation and novel means to activate resolution.

Materials and Methods

Materials

Materials included the following: zymosan A (from Saccharomyces cerevisiae), BSA, RPMI 1640, PBS (with and without calcium and magnesium), Ficoll-Histopaque 1077-1, and FITC goat anti-mouse IgG (Sigma-Aldrich); FBS (Life Technologies); rat anti-mouse Lyg6-Pe and FITC, CD16/CD32 purified (mouse Fc block), and mouse anti-human CD14, CD68, CD74 (BD Biosciences); F4/80-Pe and -PerCP-Cy5.5 CD11b (eBioscience); mouse recombinant GM-CSF, mouse anti-human CD206, CD3, CD19-PE (BioLegend); 15-lipoxygenase (LOX)-1 (OriGene; mouse anti-human ChemR23, mouse recombinant human GM-CSF, and recombinant human TNF-α (R&D Systems); human monocyte isolation kits (StemCell Technologies); liquid chromatography–grade solvents (Fisher Scientific, Wald-Than, MA); Eclipse Plus C18 (Agilent Technologies); C18 solid phase extraction (SPE) columns (Waters); EPA (Sigma-Aldrich); DHA (Nu-Check Prep); and AT-RvD1 and synthetic and deuterium-labeled LM standards (Cambay Chemical).

Animals

Male BALB/c mice (2 mo old and 20 mo old) were obtained from the National Institute on Aging, and male FVB mice (6–8 wk old) were obtained from Charles River Laboratories. All mice were maintained in a temperature- and light-controlled environment and were fed standard diet (Picolab rodent diet 20 5053; LabDiet) and water ad libitum. Mice were allowed to acclimate for at least 1 wk prior to experimentation. All experiments were approved by the Committee on the Care and Use of Laboratory Animals at Harvard Medical School (protocol 02570) and performed in accordance with institutional guidelines.

Self-resolving peritonitis

Resolution of acute inflammation in aged mice. Young and aged mice were injected with zymosan (0.1 mg/mouse, i.p.) (9, 10) and peritoneal exudates were collected at indicated time intervals (0–24 h). In some experiments aged mice received saline or Resolvin-NPRM (see details in “Construction of NPRMs” below) either i.p. or p.o. (9–10) prior to zymosan administration, and peritoneal exudates were collected at 4 h. Exudate aliquots were placed in two volumes of cold MeOH containing deuterium-labeled internal standards and taken to liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based LM metabololipidomics following SPE (as outlined in “LC-MS/MS–based LM metabololipidomics” below).

Impact of DHA and SPMs on resolution time interval. Mice received either 1) DHA (1, 10, or 100 μg, i.v.) or 2) RvD3 (50 ng, i.p.) prior to zymosan injection (1 mg/mouse), and peritoneal exudates were collected at indicated time intervals (0–48 h). Leukocyte numbers were determined using Turk’s solution, and differential counts were assessed with flow cytometry (FACS Canto II) as outlined in “Flow cytometry assessment of exudate cells” below. Cytokine and chemokine levels were assessed in cell-free supernatants by multiplex ELISA.

Principal component analysis

Principal component analysis (PCA) was performed using SIMCA 13.0.3 software (Umetsee) 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 LMs/SPMs contribute to the cluster separation in the score plot (11).

Macrophage effecrocystosis in vitro

To obtain apoptotic PMNs, human PMNs were isolated by density-gradient Ficoll-Histopaque from human peripheral blood. Blood was obtained from healthy white volunteers, according to Protocols Committee Protocol no. 1999-P-00297. PMNs were labeled with bisbenzimide H 33342 (Sigma-Aldrich), a fluorescent nuclear dye (10 μg/ml, 30 min, 37˚C) and cultured overnight (5 × 10⁶ cells/ml in PBS¹⁷). Mouse bone marrows were obtained by flushing mouse femurs and tibiae with RPMI 1640; cells were then centrifuged (300 × g, 10 min, 4˚C), suspended in PBS¹⁷, and seeded in a 10-ml dish at 15 × 10⁶ cells/dish (60 min, 37˚C, pH 7.4). Adherent cells were cultured in RPMI 1640 (10% FBS), penicillin and streptomycin, and mouse rGM-CSF (10 ng/ml) for 6 d. Mouse bone marrow-derived macrophages (BMDMs) were plated onto 96-well plates (Costar) at 5 × 10⁶ cells/well followed by phagocytosis of fluorocein-labeled apoptotic PMNs (3:1 ratio) as described in Dalli and Serhan (12). Phagocytosis was assessed using a SpectraMax M3 plate reader.

Human monocyte isolation and incubations

Human PBMCs were obtained by density-gradient Ficoll-Histopaque from deidentified healthy human volunteers from the Children’s Hospital Boston blood bank. Monocytes were purified using negative magnetic separation specific for CD14⁺CD16⁻ monocytes (StemCell Technologies), and purity was assessed by flow cytometry after cell staining with fluorescently conjugated Abs to CD3-FITC, CD19-PE, and CD14⁻ with >95% of the cells positive for CD14. This suggests little to no lymphocyte contamination. The monocytes were incubated (1 × 10⁶ cells in 2 ml, 24 h, 37˚C) in RPMI 1640 (1% human serum) with vehicle or EPA mixture (10 μM) containing different ratios of DHA and EPA. The ratios were designated as follows: R0 was vehicle alone, R1 contained a 10:90 ratio, R2 contained a 50:50 ratio, and R3 contained a 90:10 ratio of DHA to EPA. Monocytes and supernatants were collected and administered to mice (see “Peritonitis with reprogrammed monocytes” below) and aliquots were suspended in 2 volumes cold MeOH containing deuterium-labeled internal standards and subjected to LC-MS/MS–based LM metabololipidomics following SPE.

15-LOX-1 enzyme expression. Monocytes were collected following 24 h incubation with vehicle or mixture of DHA and EPA and fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences). Intracellular labeling was carried out using unlabeled mouse anti-human 15-LOX-1 (30 min, 4˚C), followed by goat anti-mouse IgG-FITC (30 min, 4˚C). Cells were washed and resuspended in staining buffer (PBS containing 3% BSA), and staining was assessed using a FACS Canto II (BD Biosciences) and FlowJo (Tree Star).

Macrophage differentiation. In designated experiments, monocyte supernatants were discarded and replaced with RPMI 1640 medium supplemented with 10% FCS (v/v) and 20 ng/ml GM-CSF. These cells were then cultured for 6 d and then stimulated for 24 h with 20 ng/ml IFN-γ and 1 ng/ml LPS for M1 polarization. Expression of CD206, CD163, HLA-DR, and CD3 was assessed using a FACSCanto II (BD Biosciences) and FlowJo (Tree Star).

Peritonitis with reprogrammed monocytes

Human monocytes and monocyte supernatants containing their LM products were collected following 24 h incubation with vehicle or indicated ratios of DHA and EPA (as detailed in “Human monocyte isolation and incubations” above). These were then transferred to mice (~1.5 × 10⁶ monocytes in 400 μl, i.p.) that had been injected with zymosan (1 mg/mouse) 12 h prior and exudates were collected at 24 and 48 h. Peritonitis plus vehicle mice received PBS without monocytes. In some experiments, mice received monocyte incubations prior to initiation of peritonitis, and exudates were collected at 4 h. Neutrophil numbers were determined using the solution and flow cytometry as detailed in “Flow cytometry assessment of exudate cells” below.

Construction of NPRMs

R3 monocytes (see “Human monocyte isolation and incubations” above) were stimulated with TNF-α (10 ng/ml, 20 min, 37˚C) and cells were removed by centrifugation (3000 × g; 10 min, 4˚C) before pelleting microparticles by ultracentrifugation (100,000 × g; 1 h, 4˚C). NPRMs were prepared as in Norling et al. (10). Briefly, microparticles were added to thin lipid films in glass flasks (after organic solvent removal by rotary evaporation; 10 min, 25˚C) containing fluorescent 1,2-dioleoyl-sn-glycero-3-phospho-l-serine-N-7-nitro-2-1,3-benzoxadiazol-4-yl (100 μg; Avanti Polar Lipids, Alabaster, AL), AT-RvD1 (1 μg), and AT-RvD3 (1 μg; prepared by total organic synthesis by Dr. Nicolas Petasis) (2). Irradiation of synthetic AT-RvD1, AT-RvD3, and fluorescent phospholipids was carried out using aqueous energy dissemination via a sonin deissipator (output power 15 W, 15 min, 25˚C; Fisher Scientific). The preparations were layered on Sephadex G50 columns (Sigma-Aldrich) and fractions were collected in 0.2-mm-filtered PBS. The concentrations of AT-RvD1 and AT-RvD3 associated with Resolvin-NPRM were determined using LC-MS/MS.

Flow cytometry assessment of exudate cells

Murine exudate cells (0.2 × 10⁶) harvested at indicated time intervals were washed in staining buffer (PBS containing 3% FBS, incubated with Fc block for 30 min at 4˚C, and then labeled with mAbs against CD11b, Lyg6-Pe and F4/80-Pe or appropriate isotype controls for 30 min at 4˚C. Cells were washed and stained was assessed using a FACS Canto II (BD Biosciences) and FlowJo (Tree Star, Ashland, OR).
For assessment of macrophage efferocytosis of apoptotic PMNs in vivo, inflammatory exudate cells were labeled for extracellular F4/80-FITC, fixed and permeabilized (Cytofix/Cytoperm; BD Bioscience), and then labeled for intracellular Ly6G-PE (13). Cells were washed and staining was assessed using a FACS Canto II (BD Biosciences) and FlowJo (Tree Star).

**LC-MS/MS–based LM metabololipidomics**

Deuterated internal standards d₅-5S-HETE, d₅-leukotriene (LT)B₄, d₅-LXA₄, d₅-PGE₂, and d₅-RvD2 representing each chromatographic region of identified LMs (500 pg each) were added to samples to facilitate quantification. Samples were extracted by SPE on C18 columns as in Dalli and Serhan (12) and subjected to LC-MS/MS. The system consisted of a QTrap 5500 (AB Sciex) equipped with Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). An Agilent Technologies 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 (v/v/v) to 100:0:0.01 at 0.4 ml/min flow rate. To monitor and quantify levels of targeted LMs, a multiple reaction monitoring (MRM) method was developed with signature ion pairs Q1 (parent ion)/Q3 (characteristic daughter ion) for each molecule (see Fig. 2, Supplemental Tables I–III). Identification was conducted using published criteria (12) where a minimum of six diagnostic ions were employed. When the synthetic or biogenic standard for a given LM was not available, the calibration curve of the LM with most similar physical properties to the analyte was used. A linear calibration curve for each compound was obtained with R² values ranging from 0.98 to 0.99, and the detection limit was <0.1 pg in this system.

**Statistics**

Data are presented as means ± SEM. The criterion for statistical significance was p < 0.05 using a Student t test (two groups), one-way ANOVA (multiple groups), or two-way ANOVA (two groups over time) followed by a Bonferroni post hoc test using GraphPad Prism 6.

**Results**

**Resolution of inflammation is delayed in aged mice**

To investigate resolution programs in aged (20 mo old) mice we compared their responses to young (2 mo old) mice using a systems approach combined with LM metabololipidomics. First, self-limiting acute inflammation was initiated by i.p. injection of yeast cell wall particles (zymosan, 0.1 mg/mouse) that initiate acute inflammation, including leukocyte infiltration (9, 10, 14). To quantitate resolution, we used defined resolution parameters of acute inflammation (13).

In young mice, acute challenge initiated a rapid influx of PMNs that peaked (Cmax = 5.2 ± 0.9 × 10⁶ cells/murine exudate) at 4 h (Tmax), which was followed by steady decline, reaching 50% of maximum (C50) at 14 h (Fig. 1A, 1B). This gave a resolution interval (Ri; the difference between Tmax and T50) of 10 h in young mice (Fig. 1A, 1B). In comparison, aged mice displayed exacerbated PMN recruitment (Cmax = 12.3 ± 2.1 × 10⁶ cells/murine exudate) at 4 h (Tmax), which was followed by steady decline, reaching 50% of maximum (C50) at 14 h (Fig. 1A, 1B). This gave a resolution interval (Ri; the difference between Tmax and T50) of 10 h in young mice (Fig. 1A, 1B). In comparison, aged mice displayed exacerbated PMN recruitment (Cmax = 12.3 ± 2.1 × 10⁶ cells/murine exudate).

![FIGURE 1. Resolution of inflammation is delayed in aged mice.](http://www.jimmunol.org/)

**TABLE 1.**

<table>
<thead>
<tr>
<th></th>
<th>Young mice</th>
<th>Aged mice</th>
<th>% increase in aged mice</th>
</tr>
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<tbody>
<tr>
<td>ψmax (×10⁶)</td>
<td>5.2±0.9</td>
<td>12.3±2.1</td>
<td>136%</td>
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<tr>
<td>Tmax (h)</td>
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<td>4.0</td>
<td></td>
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<tr>
<td>T50 (h)</td>
<td>~14.0</td>
<td>~22.5</td>
<td>61%</td>
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<tr>
<td>Ri (h)</td>
<td>~10.0</td>
<td>~18.5</td>
<td>85%</td>
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* p < 0.05 versus young mice at indicated time interval; ** p < 0.01; ***p < 0.001 versus young mice at indicated time interval; # p < 0.05, ##p < 0.01, ####p < 0.0001 versus 0 h within age group.
exudate) following acute challenge, with subsequent delay in resolution. This was evidenced by elevated PMN levels at 24 h in aged mice and 61% increase in the time to reach $\Psi_{50}$, giving an 85% increase in the $R_i$ (Fig. 1A, 1B). Also, the proinflammatory cytokine IL-6 and the subunit IL-12p40 (associated with IL-12 and IL-23) were higher in the exudates from aged mice com-

**FIGURE 2.** Aged mice have reduced SPMs and increased proinflammatory LM levels. (A) Representative MRM chromatograms of DHA and AA bioactive metabolomes and EPA-derived pathway markers in aged mouse peritoneal lavage before challenge (0 h). (B) Accompanying MS/MS spectra employed for identification of, for example, RvD5, MaR1, and LXB4 (inset, diagnostic ions; M, molecular mass). Cumulative levels of (C) $\delta$-series resolvins, protectins, and maresins (left), AA-derived lipoxins (middle), and AA-derived PGs and thromboxane (right) in peritoneal lavages (pg/ml) from young (gray bar) and aged (black bar) mice before challenge. *$p < 0.05$ versus young mice. Results are (A and B) representative of six mice or (C) mean ± SEM from three mice per age group combined from three separate experiments.
pared with young mice following initiation of peritonitis (Fig. 1C). These results demonstrate that aged mice have increased acute PMN influx and delayed resolution of acute inflammation.

Because macrophage clearance of apoptotic PMNs and cell debris is a critical process in promoting resolution of inflammation (2), we next examined monocyte/macrophage numbers (cells expressing CD11b and F4/80) in these exudates. Aged mice had higher total number of monocytes/macrophages both prior to challenge (0 h) and during the resolution phase (12 and 24 h) compared with young mice (Fig. 1D). We next investigated whether the ability of macrophages from aged mice to clear apoptotic PMNs was altered. Indeed, BMDMs from aged mice displayed a significantly lower ability to uptake apoptotic PMNs compared with BMDMs from young mice (Fig. 1E). Taken together, these results indicate that aged mice exhibit heightened and prolonged inflammatory response, increased macrophage recruitment, elevated proinflammatory cytokines, and impaired uptake of apoptotic PMNs. These findings suggested that resolution programs might be altered in aged mice.

**SPMs are reduced and proinflammatory LM levels increased in aged mice**

Next, we assessed the LM profiles in young and aged mice peritoneal lavages (Fig. 2, Supplemental Table I). In this study, we identified 22 proresolving and proinflammatory LMs as well as biosynthetic pathway markers from LOX and cyclooxygenase (COX) pathways that included RvD1, PD1, and MaR1, as well as PGs and LTB4 (Fig. 2A, Supplemental Table I). All LMs were identified in accordance with published criteria (12), as illustrated for RvD5, MaR1, and LXB4 (Fig. 2B; see Materials and Methods for details). LM quantification, using MRM, demonstrated that even before inflammatory challenge aged mice gave higher levels of LTB4 and PGs, including PGF2α, but lower levels of SPMs, such as RvD1, MaR1, and LXB4, in their peritoneal lavages compared with young mice (Supplemental Table I).

Having found that young and aged mice displayed distinct LM/SPM profiles, we next compared the cumulative amounts of different LM families identified in peritoneum prior to zymosan challenge. This approach permitted us to assess potential effector functions that these mediators may endow on the resident leukocytes. In aged mice, levels of SPMs from both DHA and AA metabolomes were lower compared with young mice, whereas PGs and thromboxane were elevated in the aged mice.

**FIGURE 3.** Temporal LM levels are dysregulated in aged mice: PCA. LMs obtained from (A) young and (B) aged mouse peritoneal exudates at indicated time intervals following zymosan administration (0.1 mg/mouse) were identified by LM metabololipidomics (see Materials and Methods for details). (A and B) Score plots (left) and loading plots (right) for young and aged mice with 0 h (baseline) in green, 4 h (peak of inflammation) in blue, 12 h (resolution phase) in red, and 24 h (resolution phase) in aqua. Results are from three mice per age group at given time interval.

**FIGURE 4.** SPM precursor elevates local proresolving LM levels and shortens resolution. (A) Exudate PMN numbers and (B) resolution indices from mice administered DHA (10 μg, i.v., gray open circles) or vehicle (black squares) 1 min prior to (T0) zymosan challenge (1 mg, i.p.). Inset shows representative flow cytometry zebra plot; PMNs are identified as CD11b+Ly6G+ events (n = 3). Resolution indices were calculated as in Fig. 1. *p < 0.05 versus zymosan plus vehicle for indicated time interval. (C) Shortened resolution expressed as percentage change in R from peritonitis plus vehicle mice. (D) Increase in RvD1 and RvD3 in 12 h exudates following administration of DHA (1, 10, or 100 μg, i.v., T0), expressed as percentage increase from peritonitis plus vehicle mice. *p < 0.05 versus peritonitis plus vehicle mice. Results are (A, B, and D) means ± SEM or (C) mean from three mice in each group at each time interval.
(Fig. 2C). These findings indicate that, even prior to challenge, the aged mice possess proinflammatory LM profiles.

**Temporal LM levels are dysregulated in aged mice**

Temporal SPM biosynthesis contributes to regulate the onset of resolution programs (2, 13). Therefore, we next used PCA to investigate the temporal LM regulation in inflammatory exudates of aged mice compared with young mice following self-limited challenge. In young mice, PCA score plots gave a distinct cluster for each time interval after inflammatory challenge (Fig. 3A, left). The 0 h (baseline) in young mice was characterized by high levels of RvD1, PD1, and MaR1 as demonstrated in the loading plot (Fig. 3A, right). Upon zymosan challenge (4 h; peak of inflammation) there was an increase in both proinflammatory and proresolving LMs, including RvD5, LXB4, LT B4, and PGE2, indicated by a right and downward shift in the 4 h cluster in the score plot (Fig. 3A). This was followed by a clockwise shift in the 12 h cluster (resolution phase) toward the 0 h (Fig. 3A, left). In comparison, in aged mice PCA analysis demonstrated that thromboxane B2 was associated with the 0 h cluster. Whereas in young mice we found a clockwise shift for the 12 h cluster, in aged mice this gave an anti-clockwise shift relative to the 4 h cluster with high proinflammatory mediator levels, including PGF2α, being associated with this cluster (Fig. 3B). Additionally, proresolving mediators, including RvD1, were found to be associated with later time intervals in aged mice (Fig. 3B). These findings suggest that heightened inflammation and delayed resolution in aged mice are associated with dysregulated LM production contributing to a proinflammatory phenotype.

**SPM precursor enhances SPMs and shortens resolution in vivo**

We next examined whether increased availability of the biosynthetic precursor to d-series resolvins, protectins, and maresins (2) accelerates resolution of acute inflammation. Initiation of acute inflammation (zymosan; 1 mg/mouse; i.p.) led to a rapid PMN infiltration that reached a maximum (Tmax) at 12 h and a time-dependent reduction in PMN numbers within the subsequent 32 h giving an Rτ of ~20 h (Fig. 4A, 4B). Intravenous administration of DHA (10 μg) prior to challenge reduced PMN numbers by 32% at 12 h and shortened Rτ by 50% (Fig. 4A, 4B). We next assessed the impact of increasing doses of DHA on the Rτ. In this study, we found that whereas the lowest dose tested (1 μg) apparently did not reduce the Rτ, both 10 and 100 μg DHA each shortened the Rτ ~50 and 60%, respectively (Fig. 4C). The 100 μg dose also accelerated Tmax from 12 to 4 h (n = 3 per treatment at each time interval). These results demonstrate that increased availability of the SPM precursor DHA shortens resolution of acute inflammation.

Because edema supplies the local inflammatory environment with SPM precursors (2), we next questioned whether the in-

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**FIGURE 5.** Reprogrammed monocytes activate resolution. Monocytes (1 × 10⁶ cells) were incubated with vehicle (R0) or different ratios of DHA and EPA (R1, 10:90; R2, 50:50; R3, 90:10; 10 μM, 24 h, 37˚C, pH 7.45). (A) R3-incubated monocytes and supernatants were collected and injected to mice (~1.5 × 10⁶ cells in 400 μL, i.p.) 12 h after initiation of zymosan peritonitis. Peritonitis mice were injected with PBS alone. Exudate PMNs were enumerated at indicated time intervals as well as the resolution interval (Rτ) calculated as in Fig. 1. (B) Shortening of Rτ for each time interval after inflammatory challenge (Fig. 3A, right). Upon zymosan challenge (4 h; peak of inflammation) there was an increase in both proinflammatory and proresolving LMs, including RvD5, LXB4, LT B4, and PGE2, indicated by a right and downward shift in the 4 h cluster in the score plot (Fig. 3A). This was followed by a clockwise shift in the 12 h cluster (resolution phase) toward the 0 h (Fig. 3A, left). In comparison, in aged mice PCA analysis demonstrated that thromboxane B2 was associated with the 0 h cluster. Whereas in young mice we found a clockwise shift for the 12 h cluster, in aged mice this gave an anti-clockwise shift relative to the 4 h cluster with high proinflammatory mediator levels, including PGF2α, being associated with this cluster (Fig. 3B). Additionally, proresolving mediators, including RvD1, were found to be associated with later time intervals in aged mice (Fig. 3B). These findings suggest that heightened inflammation and delayed resolution in aged mice are associated with dysregulated LM production contributing to a proinflammatory phenotype.

**FIGURE 6.** RvD3 shortens resolution of acute inflammation. (A) Exudate PMN numbers in mice administered vehicle or RvD3 (50 ng/mouse, i.p.) prior to (T0) zymosan challenge (1 mg/mouse). Inset shows representative flow cytometry zebra plot (n = 4); PMNs are identified as CD11b⁺ Ly6G⁺ events. (B) Resolution indices determined as in Fig. 1. ***p < 0.01 versus vehicle-treated mice. Results are means ± SEM from four mice per treatment at each time interval. (C) Enhanced uptake of fluorescently labeled apoptotic PMNs (1.5 × 10⁶ cells) by RvD3-treated (1 nM, 15 min, 37˚C) BMDMs (5 × 10⁶ cells, 60 min, 37˚C) from aged mice. **p < 0.01 versus vehicle-treated BMDMs. Results are means ± SEM from four mice per treatment.
creased substrate availability also elevated local SPMs in self-resolving inflammation. Employing LM metabololipidomics we identified 26 LMs from both LOX and COX pathways, including RvD1 and PGE2 in inflammatory exudates collected 12 h after challenge (Supplemental Table II). We compared endogenous levels of LMs in 12 h exudates between mice administered vehicle or DHA (1, 10, or 100 μg, i.v.) prior to zymosan challenge (Supplemental Table II). Systemic administration of the lowest dose tested (1 μg/mouse) did not increase the local levels of DHA-derived SPMs. Exudate levels of RvD1 were significantly increased following i.v. DHA at both 10 and 100 μg per mouse whereas RvD3 levels were significantly increased only at the highest dose tested (Fig. 4D). Similarly, RvD2, RvD5, MaR1, and PD1 showed a trend toward increased levels following i.v. DHA at either 10 or 100 μg (Supplemental Table II). Taken together, these results demonstrate that increased substrate availability selectively enhanced specific local DHA-derived SPM levels (e.g., RvD1 and RvD3) and shortened Ri.

Reprogrammed monocytes activate resolution

Because cells of the monocyte/macrophage lineage regulate key processes in the resolution of inflammation (2, 3), we next tested whether incubation of human monocytes with SPM biosynthetic substrates could enhance their ability to promote resolution of inflammation. Human CD14+CD162 monocytes were incubated with vehicle (R0) or a mixture containing different ratios of DHA and EPA to assess the optimal substrate ratio using 10:90 (R1), 50:50 (R2), or 90:10 (R3) DHA plus EPA (10 μM, 37˚C, 24 h; Fig. 5). Cells and their supernatants were then collected and administered (~1.5 × 10^5 cells in 400 μl, i.p.) to mice 12 h after zymosan challenge (i.e., at peak of inflammation: 12 h). R3-incubated monocytes reduced the number of PMNs recovered from peritoneum at 24 h (Fig. 5A, Supplemental Fig. 1A) and resulted in the greatest reduction in Ri of 70% (Fig. 5A, 5B). A lesser reduction of ~55% was observed with R2 monocytes, and administration of R1 monocytes led to an ~10% reduction in Ri. Of interest, vehicle-treated (R0) monocytes did not reduce Ri (Fig. 5B). The reduction in Ri correlated with DHA availability in the monocyte incubations (R^2 = 0.94; Fig. 5B, inset). R3 incubations were also most efficacious in limiting PMN infiltration when administered prior to the onset of peritonitis (Supplemental Fig. 1B). Taken together, these results indicate that R3, containing a 90:10 ratio of DHA to EPA, is the optimal ratio limiting PMN accumulation and shortening Ri.

We next assessed whether these reprogrammed monocytes displayed an altered phenotype. Monocytes incubated with DHA and EPA for 24 h displayed increased protein expression of 15-LOX-1, a fundamental enzyme for SPM biosynthesis (2), with highest expression in the R2 and R3 monocytes (Supplemental Fig. 1C). Because SPMs, including RvD1, skew macrophages toward an M2-like phenotype (15), we next investigated whether these reprogrammed human monocytes would lead to altered macrophage profiles upon differentiation. All reprogramming ratios (R1, R2, and R3) led to elevated levels of HLA-DR, a marker associated with an rM phenotype (16), and the E-series resolvin 1 receptor ChemR23 (2), compared with human macrophages differentiated from R0 (vehicle)-incubated human monocytes (Supplemental Fig. 1D). Also, when we looked at the expres-

FIGURE 7. Construction of novel Resolvin-NPRMs. Human monocyte-derived NPRMs were obtained by energy-induced conversion of microparticles from reprogrammed monocytes that were enriched with AT-RvD1 and AT-RvD3. (A) Flow cytometry dot blot showing human monocyte-derived nanoparticles. Incorporation of AT-RvD1 and AT-RvD3 into nanoparticles was determined using LC-MS/MS–based LM metabololipidomics. (B) MRM chromatograms of selected ion pairs for AT-RvD1 and AT-RvD3 and (C) representative MS/MS spectra employed in the identification of AT-RvD1 and AT-RvD3 in NPRMs.
sion of the M2 markers CD206 and CD163 we found that the R2 and R3 incubated human monocytes gave rise to macrophages with higher levels of CD206, whereas only the R3 ratio led to elevation in the M2 marker CD163 (Supplemental Fig. 1D). These findings suggest that DHA, and to a lesser extent EPA, plays a role in human monocyte reprogramming to a pro-resolving phenotype.

Using LM metabololipidomics we identified LMs from both the LOX and COX bioactive metabolomes in the monocyte incubations (Supplemental Table III). We found that R3 incubations had the highest levels of cumulative SPM and pathway markers from the DHA metabolome in the incubations (Fig. 5C), which were positively correlated with substrate availability ($R^2 = 0.96$; Fig. 5C, inset) as well as shorter $R_i$ in vivo ($R^2 = 0.98$; Supplemental Fig. 1E). Of these SPMs, RvD1 and RvD3 correlated with both substrate availability ($R^2 = 0.71$ and 0.88, respectively; Fig. 5D) and reduction in $R_i$ ($R^2 = 0.88$ and 0.97, respectively; Supplemental Fig. 1F). Taken together, these results suggest that reprogramming of human monocytes toward a proresolving phenotype also involves upregulation of SPMs, including RvD1 and RvD3.

**RvD3 enhances resolution of acute inflammation**

Because RvD3 was identified in supernatants from reprogrammed monocytes (Fig. 5D, Supplemental Table III), we next determined its impact on $R_i$ in peritonitis. Local administration of RvD3 prior to initiation of acute peritonitis (50 ng/mouse; i.p.) reduced PMN numbers at 12 h by 47% (Fig. 6A, 6B) and shortened $R_i$ by 91% (23 versus 2 h) (Fig. 6A, 6B). We next tested whether RvD3 retained its proresolving actions with aged macrophages. Incubation of BMDMs (5 x 10^4 cells) from aged mice with RvD3 (1 nM, 37°C, 15 min) prior to addition of fluorescently labeled apoptotic PMNs (1.5 x 10^5 cells, 37°C, 60 min) led to enhanced efferocytosis (Fig. 6C). These results demonstrate the potent pro-resolving actions of RvD3 with cells from young and aged mice.

In aged mice, Resolvin-NPRMs reduce inflammation and stimulate resolution

Biomimicking endogenous resolving pathways using NPRMs constructed from neutrophil microparticles enhances the bioactions of SPMs (17). Therefore, we next questioned whether NPRMs could rectify the overt inflammatory response in aged mice, restoring homeostasis. We constructed novel humanized NPRMs from reprogrammed human monocytes (see Materials and Methods) for local delivery of SPMs (Fig. 7). Because both RvD1 and RvD3 were enhanced in reprogrammed monocytes (Fig. 5D), we constructed the Resolvin-NPRMs using their AT 17R epimer form that displays enhanced resistance to further enzymatic conversion (2). The amounts of AT-RvD1 and AT-RvD3 in the Resolvin-NPRMs were ascertained using LC-MS/MS (Fig. 7C).

We next assessed the potential actions of these new Resolvin-NPRMs in age-associated inflammation when administered i.p. either prior to (T0) or 2 h after (T2) zymosan challenge (Fig. 8A). Resolvin-NPRMs (2 x 10^5 NPRMs containing ~20 ng each of AT-RvD1 and AT-RvD3; i.p.) significantly increased macrophage efferocytosis of apoptotic PMNs monitored by Ly6G^+Ly6C^-events in peritoneal exudates (Fig. 8B). Also, PMN numbers were reduced in these exudates (Fig. 8C). This was observed both when Resolvin-NPRMs were administered before zymosan challenge or 2 h after. Using LM metabololipidomics we also found that administration of Resolvin-NPRMs reduced proinflammatory LTB_4 levels when administered 2 h after but not prior to zymosan challenge (Fig. 8D). Taken together, these results demonstrate that Resolvin-NPRMs possess potent anti-inflammatory and proresolving actions in aged mice, reducing exacerbated inflammatory response and stimulating resolution.

**Discussion**

In the present study, we report age-associated alterations in inflammation and resolution programs that were counterregulated by Resolvin-NPRM. Using LM metabololipidomics we established basal and temporal LM profiles in aged mice that had lower local levels of SPMs and increased PMN numbers in exudates. Increased substrate availability elevated SPM levels in inflammatory exudates in mice as well as in human monocyte supernatants, accelerated resolution in vivo, and promoted reprogramming of monocytes. These findings suggest an age-related change in resolution pathways in acute inflammation and potential new means to activate resolution in an aged innate immune system.

Aging is associated with heightened basal inflammatory status that is driven by hypothalamic NF-kB activation, a prototypical proinflammatory signaling pathway (8, 18). This underlying proinflammatory phenotype influences disease pathogenesis and prevalence of infectious and chronic inflammatory diseases seen in aged individuals (8). Of note, SPMs, such as resolvins, protectins, and lipoxins, are endogenous LMs that actively counterregulate...
proinflammatory signals, including NF-kB signaling (19), cytokines, and leukotrienes (2). Of interest, aggregated NETs promote resolution of neutrophilic inflammation via degrading chemokines and cytokines (20). Also, apoptotic leukocytes bind chemokines and cytokines that are cleared by macrophages, a process enhanced by SPMs (21). In the present studies, both RvD2 and RvD3 at nanomolar concentrations inhibited zymosan-stimulated human PMN release of NETs (data not shown), suggesting that SPMs can regulate several steps in the resolution program. Additionally, SPMs also augment host-directed defenses, including microbial containment (22, 23), actions that are impaired in aged individuals (8). Along these lines, lower urinary levels of LXA₄ and reduced ratio of lipoxins to leukotrienes were found in centenarians (24). Also, aged horses have reduced expression of the LXA₄ receptor, ALX/FPR2 (25). Hence, taken together with our present findings that resolution programs are diminished in aged mice, SPMs and their pathways may have implications in modulating age-related inflammation.

During resolution of acute inflammation there is a temporal shift in exudate leukocyte composition with accumulation of nonphlogistic monocytes and macrophages in the resolving exudate (2, 16, 26). However, higher prevalence of M1 macrophages is associated with a proinflammatory milieu (15). LM signature profiles have shown that M1 macrophages produce higher amount of proinflammatory LMs, such as, LTβ4 and COX-mediated PGs, but lower levels of SPMs, including RvD5, PD1, and MaR1, compared with M2 macrophages (12). Hence our findings that aged mice have higher exudate proinflammatory LM levels and lower SPM levels suggest that aged mice may have disrupted M1 to an M2/rM class-switch that in turn may delay exudate dissolution.

SPMs are now appreciated as regulators, as well as markers, of M1 and M2 macrophage phenotypes (2), where they promote class-switch toward an anti-inflammatory and proresolving M2-like phenotype (12, 15, 27, 28). Macrophage switch to an M2 phenotype involves upregulation of the enzyme 15-LOX-1 (29), a pivotal enzyme for conversion of EFAs to SPMs (2). In the present study, human monocytes incubated with DHA and EPA upregulated the expression of 15-LOX-1 (Supplemental Fig. 1C) as well as SPM production, including RvD1 and RvD3 (Fig. 5C, 5D, Supplemental Table III). These displayed elevated proresolving actions in vivo (Fig. 5A, 5B) and skewed toward an mM2-like macrophage phenotype when differentiated in M1 skewing conditions (12) (Supplemental Fig. 1D), functions that correlated with DHA availability and levels of RvD1 and RvD3 in the supernatants (Fig. 5B, Supplemental Fig. 1E, 1F). Taken together, these findings suggest that increased substrate availability enhances endogenous SPM levels and promotes reprogramming of human monocytes to a homeostatic phenotype that may have implications in regulation of acute inflammation and resolution.

There is increasing evidence that the ω-3 fatty acids DHA and EPA exert beneficial actions in several human diseases with underlying inflammatory pathology, including Alzheimer’s disease and rheumatoid arthritis (30–32). In murine arthritis, AT-RvD1, as well as one of its precursors 17R-hydroxy-DHA, alleviate joint stiffness, inflammation, and inflammatory pain (33). SPMs (RvD5, MaR1, and LXA₄) are found at bioactive concentrations in synovial fluid from patients with arthritis (34). Along these lines, RvD1, RvD2, and AT-RvD1 were found within their bioactive range in human plasma from healthy volunteers taking ω-3 supplements (35). The present results suggest that increasing substrate availability enhances resolving exudate biosynthesis of potent SPMs in vivo in mice, which play key roles in orchestrating resolution (2). These findings may be relevant to humans, which remain to be established (30–32).

Neutrophil-derived NPRM carrying SPMs, that is, LXA₄ or RvD1, exert anti-inflammatory and proresolving actions contributing to resolution (10, 17). Because reprogrammed monocytes displayed potent proresolving actions (Fig. 5), their microparticles were used as scaffolds for constructing new biomimetics for SPM delivery in aged mice. Monocyte-derived Resolvin-NPRMs enriched with AT-RvD1 and AT-RvD3 were used (Fig. 7), because their 17R-epimers were both upregulated in reprogrammed monocyte incubations (Fig. 5D). These new Resolvin-NPRMs ameliorated the excessive inflammatory response observed in aged mice, demonstrated by enhanced effectorcytosis (uptake of apoptotic PMNs by macrophages) in vivo as well as reduced PMN numbers and LTB₄ levels in the peritoneum (Fig. 8). Thus, these new Resolvin-NPRMs display potent actions in inflammation and stimulated resolution.

In summary, in aged mice, LM metabololipidomic profiling of exudates uncovered an unappreciated aberrant endogenous resolution program in acute inflammation that was associated with deregulation of local SPM levels. These included RvD1, PD1, and MaR1. Reprogrammed monocytes accelerated resolution in vivo that was associated with elevated levels of RvD1 and RvD3. Resolvin-NPRMs constructed from reprogrammed monocytes reduced the exacerbated inflammatory response in aged mice. Hence, the present results implicate a role for SPMs in modulating aged-associated inflammation and rescuing failed resolution in aged mice. Taken together, they provide a new paradigm for improving age-related diseases characterized by excessive innate inflammatory responses.

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Disclosures
C.N.S is an inventor on patents (resolvins) assigned to Brigham and Women’s Hospital and licensed to Resolvyx Pharmaceuticals and owns equity in the company. The interests of C.N.S. were reviewed and are managed by the Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict-of-interest policies. The other authors have no financial conflicts of interest.

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