Cutting Edge: Humanized Nano-Proresolving Medicines Mimic Inflammation-Resolution and Enhance Wound Healing

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Endogenous microparticles (MPs) were systematically profiled during the time course of self-limited inflammation. Precursors for specialized proresolving lipid mediators were identified in MPs from inflammatory exudates using liquid chromatography tandem mass spectrometry-based metabolomics. Hence, we postulated that formation of anti-inflammatory and proresolving lipid mediators could underlie beneficial effects attributed to MPs and that this process could serve as a basis for biomimicry. Using human neutrophil-derived MPs, we constructed novel nanoparticles (NPs) containing aspirin-triggered resolvin D1 or a lipoxin A4 analog. Enriched NPs dramatically reduced polymorphonuclear cell influx in murine peritonitis, shortened resolution intervals, and exhibited proresolving actions accelerating keratinocyte healing. The enriched NPs protected against inflammation in the temporomandibular joint. These findings indicate that humanized NPs, termed nano-proresolving medicines, are mimetics of endogenous resolving mechanisms, possess potent beneficial bioactions, can reduce nanotoxicity, and offer new therapeutic approaches.

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Uncontrolled inflammation is a fundamental etiology of many pathologies, including cardiovascular diseases, arthritis, and temporomandibular joint (TMJ) disorders (TMDs) (1, 2). Prevalence of TMDs is high, with at least one symptom afflicting a third of United States adults. However, there is an unmet need for effective treatments, as options are limited and often involve behavioral or physical therapies or acute administration of nonsteroidal anti-inflammatory drugs (3). This work was supported by National Institutes of Health Grants DE 019938 (to C.N.S.) and GM38765 (to C.N.S.), Arthritis Research UK Fellowship 18445 (to L.V.N.), and Wellcome Trust Programme Grant 086867/Z/08/Z (to M.P. and R.J.F.).

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Abbreviations used in this article: AT-RvD1, aspirin-triggered resolvin D1; HDHA, hydroxy-docosahexaenoic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LM, lipid mediator; LXA4, lipoxin A4; MP, microparticle; MPO, myeloperoxidase; MRM, multiple reaction monitoring; m/z, mass/charge ratio; NP, nanoparticle; NPRM, nano-proresolving medicine; PMN, polymorphonuclear cell; Rn, resolution interval; TMD, temporomandibular joint disorder; TMJ, temporomandibular joint.
1,3-benzoxadiazol-4-yl (100 µg; Avanti Polar Lipids) and 7S, 8R, 17R-tri-hydroxy-docosa-4Z,9E,11Z,15E,19Z-hexaenoic acid (aspirin-triggered resolvins D1 [AT-RvD1], 1 µg; Cayman Chemical) or e-[9,12]-benzo-oct-epi-lipoxin A₄ (LXA₄) (1 µg) (10) prepared for these studies by custom synthesis. Intercalation of AT-RvD1 or LXA₄ analog and fluorescent phospholipid was performed by aqueous energy dissemination using a sonic dismembrator (output power 15 W, 15 min, 25°C; Fisher Scientific). Humanized NPs were layered on Sephadex G50 columns (Sigma-Aldrich) and fractions collected in 0.2 µm filtered PBS. Incorporation of AT-RvD1 and LXA₄ analog was determined using LC-MS/MS and fluorescent phospholipid content confirmed by flow cytometry (BD FACS Canto II; BD Biosciences). NPs and MPs were sized using calibration beads (Corpuscular) by flow cytometry and conventional electron microscopy following negative staining (Tecnai G² Spirit BioTWIN; Harvard Medical School core facility).

**Mediator lipidomics**

LC-UV/MS/MS-based mediator lipidomics analysis was performed with an HPLC (Shimadzu LC20AD; Shimadzu)-UV (Agilent 1100; Agilent Technologies) coupled to a quadrupole ion-trap mass spectrometer (QTrap3200; Applied Biosystems) equipped with a C18 column (Agilent Eclipse Plus, 4.6 mm × 50 mm × 1.8 µm; Agilent Technologies). Acquisition was conducted in negative ionization mode, and lipid mediators (LMs) were profiled using scheduled multiple reaction monitoring (MRM) and identified using retention time, at least five to six diagnostic ions, and matching criteria (9). Esterified monohydroxy products were assessed in endogenous MPs following methanol/chloroform extraction of phospholipids and overnight saponification with 1 N potassium hydroxide in 90% ethanol. Samples were acidified and extracted with added internal standard d₅-17-hydroxy-docosahexaenoic acid (HDHA; 1 ng) for LC-MS/MS–based lipidomics as above.

For MP phosphatidylcholine analyses, LC-UV/MS/MS–based mediator lipidomic analysis was performed with an HPLC (Shimadzu LC20AD; Shimadzu)-UV (Agilent 1100; Agilent Technologies) coupled to a hybrid quadruple time-of-flight mass spectrometer (QStar XL; Applied Biosystems) equipped with a Phenomenex Luna C18(2) column (2 mm × 150 mm × 3 µm; Phenomenex). Mobile phase consisted of 0.5% ammonium hydroxide in methanol/acetonitrile and 0.1 M ammonium acetate (97:2:1, v/v/v) at 200 µl/min. Operating parameters and collision energies were optimized individually.

**Peritonitis and wound healing**

Human MPs or nano-proresolving medicines (NPRMs) (1 × 10⁸–3 × 10⁹) were given i.v. (or i.p. as indicated) prior to zymosan A (0.1 mg i.p.), and peritoneal leukocytes were assessed at 2, 4, or 12 h. In some experiments, mice were given d₅-DHA (1 µg i.v.) prior to zymosan A (1 mg), and peritoneal macrophages were collected at 4 h. Wound healing was assessed using an Electric Cell-Substrate Impedance Sensing system (Applied Biophysics) (11). Human epidermal keratinocytes (Lonza) were cultured on SW1E electrode arrays and, once confluent (~8000 Ω, using 16 kHz), were wounded (64 kHz, 30 s). Cells were cultured with buffer, AT-RvD1 NPRMs (10 nM), AT-RvD1 (10 nM), or the equivalent NP number.

**TMJ inflammation**

Mice were anesthetized (O₂: 1 L/min, isoflurane: 2.5%), and CFA was administered (10 µg; 20 µl) into the periarticular space of the left TMJ and 0.9% sterile saline (20 µl) into the right TMJ space (as in Ref. 12). Treatments were then delivered i.v. with buffer (100 µl PBS), AT-RvD1 (10 ng) NPRMs, or e-[9,12]-benzo-oct-epi-LXA₄ (10 ng) NPRMs. After 12 h, PMN infiltration was assessed in the TMJ by myeloperoxidase (MPO) activity by ELISA (Hycult Biotechnology).

**Statistics**

Data are mean ± SEM. Multiple group comparisons were made using one-way ANOVA followed by Dunnett’s or Bonferroni post hoc analysis. The p values < 0.05 were considered significant.

**Results and Discussion**

First, we profiled the time course of MP generation in self-limited acute inflammation (i.e., zymosan-induced peritonitis). The endogenous leukocyte-derived MPs were temporally generated in vivo in inflammatory exudates, with maximum MPs identified within the initiation phase, which gradually declined during the resolution phase corresponding with neutrophilic loss (Fig. 1A). Further characterization of these

Annexin A₅⁺ MPs by surface molecule expression demonstrated these were CD11b⁺, CD45⁺, and CD54⁺ (Fig. 1B). To systematically address the LM profiles carried by endogenous MPs, LC-MS/MS–based metabolomics were performed. We discovered that endogenous MPs formed in inflammatory exudates contain esterified biosynthetic precursors of novel specialized proresolving mediators (Fig. 2A). For example, the levels of MP-associated hydroxydocosaheaxenoic acids, namely 14-HDHA and 17-HDHA, were high during the initiation phase of acute inflammatory response, decreased during the peak of inflammation, and accumulated in resolution, the phase in which potent anti-inflammatory and proresolving LMs are biosynthesized (13). Fig. 2B shows a representative mass spectrum of 17-HDHA, with the diagnostic ions used for identification criteria denoted, including mass/charge ratio (m/z) 245 and 273. Notably, endogenous MPs were devoid of unesterified monohydroxy fatty acids, suggesting that once liberated from MP membranes, they are made available to leukocytes within the exudate.

Following i.v. administration of deuterium-labeled d₅-DHA for tracking, circulating d₅-DHA is made rapidly available to exudates (14) and was incorporated into endogenous MPs generated during the onset of acute inflammation. Additionally, these MPs also contained enzymatic metabolites of d₅-DHA, namely d₅-17-HDHA and d₅-14-HDHA, dexteinated biosynthetic pathway biomarkers for δ-series resolvins and maresin biosynthesis, respectively, which reflects activation of these new pathways and their H(p)DHA intermediates (Fig. 2C). These results implicate endogenous MPs as intercellular communicators that can deliver proresolving LM...
precursors to inflammatory loci. Further experiments were performed to assess whether exogenous DHA was stored within MP phospholipids. When human PMNs were incubated with d5-DHA, MPs were generated containing both d5-DHA and d5-HDHA esterified into phosphatidylcholine (Supplemental Fig. 1A, B). Noteworthy, both cytotoxic and secretory phospholipase A2 are induced during the resolution phase of inflammation (15). Secretory phospholipase A2 added to resolving MPs liberated esterified precursors from MPs (Supplemental Table I).

Human PMN-derived MPs display anti-inflammatory properties via ALX/formyl peptide receptor 2, the receptor for LXA4, Annexin-A1, as well as RvD1 (6, 16). Additionally, we demonstrated in this study that MPs enhanced efferocytosis (Supplemental Fig. 2) and contain precursors for proresolving LM, thus likely contributing to the beneficial properties attributed to these MPs. Because many biomaterials used for NP drug delivery can activate the circulatory system and cause nanotoxicity (for example, by uptake and activation of dendritic cells) (8), we sought new means to locally administer and activate proresolving cascades in vivo based on the endogenous MP system. Thus, newly constructed nanomedicines should possess multipronged agonist actions in resolution, activating endogenous ALX, releasing precursors for local proresolving mediators, as well as delivering their cargo of uploaded LM.

Harnessing human neutrophil-derived MPs as scaffolds, we constructed NPs containing either AT-RvD1 (4) or a stable analog of LXA4 (10). Following enrichment procedures and energy-induced conversion of MP to NPs, preparations were layered onto size-exclusion chromatography columns to separate free unincorporated lipids from MP-associated lipids, and eluate fractions were collected and taken for analyses including flow cytometry. Characteristically, enriched NPs (Fig. 3A, blue dot plot) were smaller than their MP counterparts (Fig. 3A, red dot plot) and also contained fluorescent phospholipid (Fig. 3B, blue histogram). NP sizing was accomplished by flow cytometry using nanosphere calibration beads (Fig. 3C, 3D). Sizing was further validated using negative stain electron microscopy of MPs (Fig. 3E) and NPRMs (Fig. 3F). Both methods showed smaller, more uniform sizing of NPs following enrichment procedures. Prior to each experiment, incorporation of AT-RvD1 or the LXA4 analog was determined using LC-MS/MS. Representative tandem mass spectrum of NP-associated AT-RvD1 (m/z 375.2) is shown from scheduled MRM of the m/z 375/215 transition pair at 7:4 min (Fig. 3G).

These humanized NPs were inherently anti-inflammatory, even without enriching with LM, counterregulating PMN infiltration in acute zymosan (0.1 mg) peritonitis by ∼25% following i.v. administration of 1 × 105 NPs (Fig. 4A). A similar reduction in PMN influx was observed with 300 ng stable LXA4 analog consistent with earlier findings (17). Remarkably, when the LXA4 analog was incorporated into human NPs, they drastically limited PMN numbers by ∼60%, showing they possess additional anti-inflammatory properties. Importantly, 1 × 105 NPs equated to an incorporation of 5.0–8.8 ng LX analog, effectively demonstrating that much lower amounts were needed locally to reduce PMN infiltration than analog alone. This proved dose dependent, with maximal NPRMs greatly limiting PMN recruitment (Supplemental Fig. 3A). Noteworthy, we found an inhibitory action on PMN recruitment when NPRMs were administered directly into the peritoneum, suggesting a direct action on resident macrophages, dampening their inflammatory response to zymosan (Supplemental Fig. 3B). We also assessed bioactivity of AT-RvD1–enriched NPs in murine peritonitis. Leukocyte recruitment and differential analyses were assessed at three separate intervals to quantify acute inflammatory response using self-limited inflammation (Fig. 4B). Local microbial administration resulted in a rapid increase in PMN infiltration, which peaked at 4 h postchallenge corresponding to the onset of inflammation. By 12 h, PMNs had declined, and the resolution interval (Ri) was calculated (i.e., time taken for maximal PMN to decrease 50%). NPs carrying AT-RvD1 drastically reduced PMN recruitment in microbial peritonitis, reducing maximal PMN infiltration from 9.4 ± 0.7 × 106 to 4.0 ± 0.8 × 106 and also shortened Ri from 7 h to ∼4.5 h (Fig. 4B).

Proresolving bioactions of these humanized nanomedicines were next evaluated in a keratinocyte wound-healing assay using in vitro epidermal abrasion (11). Following focal clearance of keratinocytes with an elevated electrical field pulse, keratinocyte healing from the perimeter into the clearance zone was assessed in real time by impedance sensing. This highly sensitive method indicated that AT-RvD1-containing
nanomedicines enhanced wound closure rates as compared with either nonenriched NPs or equimolar amounts of AT-RvD1 (10 nM), demonstrating their proresolving properties (Fig. 4C).

TMDs are a significant clinical problem, causing craniofacial pain (2). Effective treatment of pain arising from inflammation of joints and muscles remains an unmet medical need and is implicated not only in TMDs but also in many other human diseases. To this end, we evaluated whether these nanomedicines would confer protection against CFA-induced inflammation of the TMJ. Indeed, 10 ng AT-RvD1 NPRMs or α-[9,12]-benzo-ω6-epi-LXA4 NPRMs drastically reduced the number of infiltrating PMNs into the inflamed TMJ 12 h after CFA-induced inflammation (Fig. 4D). Importantly, MPO levels in the contralateral TMJ were not elevated, and no significant differences were noted between buffer control and NPRM treatment groups (not shown). Of relevance, both RvD1 and RvE1 also attenuate inflammatory pain (18). Together, these results establish that biomimicking endogenous resolution mechanisms centered on anti-inflammatory and proresolving MPs, enhance the bioactions of potent LMs. NP delivery of AT-RvD1 or a stable analog of LXA4 prevented excessive neutrophilic infiltration to inflamed TMJs, thus circumventing ensuing granuloma formation and further joint destruction associated with overzealous leukocyte recruitment in this complex joint disease.
Acknowledgments
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Disclosures
C.N.S. is inventor on composition of matter and patents covering lipoxins, resolvins, and related compounds owned by Brigham and Women’s Hospital-Partners Health Care and licensed for clinical development. C.N.S. retains founder stock in Resolvyx Pharmaceuticals.

References
Supplemental Fig 1: Exogenous $d_5$-DHA is incorporated into MP phospholipids. Human PMN ($25 \times 10^6$) were incubated with $d_5$-DHA (5μg) for 30min, 37°C and MPs were collected to assess whether exogenous DHA could be detected within MP phospholipids. Tandem mass spectra of biogenic (A) hexadecanoyl-docosahexaenoyl phosphatidylcholine and (B) hexadecanoyl-hydroxydocosahexaenoyl phosphatidylcholine. Acyl group positions are arbitrarily assigned. Compounds were identified as acetate adducts in negative ionization mode using Qstar-XL.

Supplemental Fig 2: Microparticles enhance efferocytosis. Human monocyte-derived macrophages (7-day differentiated with 10ng/ml GM-CSF) were seeded at $1 \times 10^5$/well, and vehicle or MPs ($8 \times 10^5$) were added 15min prior to addition of PKH26 (Sigma)-labeled human apoptotic PMN ($3 \times 10^5$). After 1h macrophages were washed and efferocytosis was assessed using a PerkerElmer VICTOR³ plate reader.

Supplemental Fig 3: Nano-pro-resolving medicines reduce peritoneal PMN infiltration. Human NPRMs ($1 \times 10^4$ - $3 \times 10^5$) were given (A) i.v. or (B) i.p. as indicated prior to zymosan A (0.1mg, i.p) and peritoneal PMN infiltration was assessed at 2h.

Supplemental Table I: Microparticle fatty acid liberation with sPLA². Microparticles were collected from zymoan peritonitis (1mg, i.p.) exudates at 48h and incubated with or without human recombinant secretory PLA₂ type V (0.7U, Cayman Chemical) for 30min at 37°C, and liberation of fatty acids was assessed using LC-MS/MS.
Figure S2

% increase in effrocytosis above vehicle control

Vehicle  Microparticles
Figure S3

A

PMN number (x10^6)

Vehicle 10 30 100 300

9,12-benzo-epi-LXA_4 NPRM (x10^3)

B

PMN number (x10^6)

Vehicle i.v. i.p.

9,12-benzo-epi-LXA_4 NPRM (1x10^5)
Supplemental Table I: Microparticle fatty acid liberation with sPLA2.

*Monohydroxy fatty acids were identified by retention time and multiple reaction monitoring. Transition pairs: 17-HDHA 343, 245; 14-HDHA 343, 205; 5-HETE 319, 115; 12-HETE 319,179 and 15-HETE 319, 219.

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