Omega-3 polyunsaturated fatty acids promote inflammation resolution and affect natural killer cells

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Ómega-3 fjölómetteðar fitusýrur auka hjöðnun bólgu og hafa áhrif á náttúrulega drápsfrumur

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Ágrip

Bráð bólga getur þróast yfir í langvinna bólgu ef bólguhjöðnun er ábótavant. Langvarandi bólga er talin eiga þátt í allt að helmingi dauðsfalla vegna sjúkdóma í heiminum. Bólguhjöðnun er ferli sem er vel stjórnað og einkennist af tíu aðgreindum kennimerkjum sem taka til bæði frumna og sameinda. Lípíðafleidd bólguhjöðnunarboðefni er hópur sameinda sem er upprunnin frá ómega-3 eða ómega-6 fjölómettuðum fitusýrum (FÓFS) og hvetja bólguhjöðnun. Því er talið að ójafnvægi í neyslu á ómega-6 og ómega-3 FÓFS geti verið orsakaþáttur í þróun langvarandi bólgu. Niðurstöður rannsókna sýna að ómega-3 FÓFS í fæði geta ýtt undir bólguhjöðnun í tilraunadýrum. Auk þess hefur verið sýnt fram á að náttúrulegar drápsfrumur (NK frumur) taka virkan þátt í bólguhjöðnun. Þó er lítið vitað um áhrif ómega-3 FÓFS á íferð NK frumna á bólgustað og hvernig þær geta miðlað bólguhjöðnun.

Markmið verkefnisins var að kanna áhrif ómega-3 FÓFS á bólguhjöðnun, íferð NK frumna á bólgustað, virkni þeirra og samspil við daufkyrninga. Til að rannsaka þetta var framkölluð vakamiðluð kviðarholsbólga í músum og bólguhjöðnun efld með því að gefa músunum fóður með ómega-3 FÓFS. Fjöldi og svipgerð NK frumna í kviðarholi var metin. Jafnframt voru áhrif ómega-3 FÓFS á NK frumur og samspil þeirra við daufkyrninga rannsökuð, sem og myndun þeirra á lípíðafleiddum boðefnum.

Kvenkyns C57Bl/6 mýs fengu viðmiðunarfæði með íbættu 2,8% af meinhaddsfiskolíu (fiskolíufæði) í samtals 5 vikur. Þær voru bólusettar tvisvar með metýleruðu nautgripaalbúmíni með tveggja vikna millibili og sama sameind síðan notuð til að mynda kviðarholsbólgu. Músum var lógað fyrir og 1,5, 3, 6 og 12 klst eftir bólgumyndun og kviðarholsvöka og -frumum safnað ásamt garnahengiseitlum. NK frumur úr mönnum voru forræktaðar með FÓFS og síðan ræktaðar áfram einar og sér eða með nýeingruðum daufkyrningum. Fjöldi daufkyrninga og NK frumna og tjáning þeirra á yfirborðssameindum var mæld með frumuflæðisjá; styrkur bólgumyndandi og bólguhjöðnunar sameinda með ELISA, Luminex og LC-MS/MS aðferðum; tjáning á lípoxýgenösum með SimpleWestern aðferð; og fjöldi frumna í stýrðum frumudauða í eitilvef með TUNEL litun.

Fiskolía í fæði fækkaði daufkyrningum í kviðarholi og minnkaði tjáningu þeirra á „ekki-éta-mig“ sameindinni CD47 á sama tíma og hún fjölgaði daufkyrningum sem voru í stýrðum frumudauða miðað við það sem var í músum sem fengu viðmiðunarfæði. Fiskolíufæðið minnkaði einnig styrk bólguhvetjandi boðefna og flakkboða, s.s. CXCL1, -2, CCL20, TNF-α, IL-6, og IL-6Rα, og eikósanóíða upprunnum úr ómega-6 FÓFS, þ.e. prostaglandína og þromboxans B_2. Samtímis jókst styrkur IGF-1, TGFβ1, og TNFRII í kviðarholsvökva músa sem fengu fiskolíu í fæði miðað við það sem var í músum sem fengu viðmiðunarfæði. Mýs sem fengu fiskolíufæði höfðu einnig hærra hlutfall af ómega-3 FÓFS á móti ómega-6 FÓFS í kviðarholsvökva og -frumum á öllum tímapunktum í bólgunni.

Fiskolíufæði jók fjölda $CD11b^{+}CD27^{-}$ NK frumna í kviðarholi músa á þeim tíma sem bólgan var í hámarki og sömuleiðis styrk CCL5, CXCL10, og CXCL12 í kviðarholsvökva. NK frumur úr músum sem fengu fiskolíufæði tjáðu meira af CCR5 en NK frumur úr músum sem fengu viðmunarfæði. Auk þess var hærra hlutfall, heildarfjöldi og tjáning á CD107a á NK frumum úr músum sem fengu fiskolíufæði en hlutfall, heildarfjöldi og tjáning á CD62L á NK frumum lægra en það sem var í NK frumum úr músum sem fengu viðmiðunarfæði.

Ómega-3 fjölómettaða fitusýran dókósahexaen sýra (DHA) dró úr áhrifum NK frumna til að auka tjáningu daufkyrninga á CD11b og CD47. Þá leiddu DHA meðhöndlaðar NK frumur ekki til auknins fjölda $CD11b^{+}CD47^{+++}$ daufkyrninga eins og NK frumur án DHA meðhöndlunar gerðu. DHA meðhöndlun NK frumna dró úr getu þeirra að auka stýrðan frumudauða daufkyrninga eftir 18 klst í rækt. DHA meðhöndlun á NK frumum leiddi einnig til minni tjáningar á NKp46 í kjölfar samræktar með daufkyrningum. Að lokum leiddi DHA meðhöndlun NK frumna til minni seytunar þeirra á bólguboðefnum í kjölfar samræktar með daufkyningum.

Óræstar NK frumur tjáðu 5-, 12-, and 15-lípoxýgenasa sem eru ensím sem þarf til að mynda lípíðafleidd bólguhjöðnunarboðefni. Myndun lípíðafleiddra boðefna var mismunandi eftir því hvort NK frumurnar voru meðhöndlaðar með arakídon sýru (AA), eikósapentaen sýru eða DHA. Í ljós kom að NK frumur sem voru meðhöndlaðar með ómega-3 FÓFS gátu myndað lípíðafleidd bólguhjöðnunarboðefni og forvera þeirra án hjálpar frá daufkyrningum. Að lokum kom í ljós að NK frumur sem voru meðhöndlaðar með AA gátu bara myndað prostaglandín og þromboxan ef daufkyrningar voru með í ræktinni.

Niðurstöðurnar sýna að fiskolía í fæði eykur mörg af kennimerkjum bólguhjöðnunar, m.a. stýrðs frumudauða daufkyrninga, át þeirra og færslu í eitla. Þessum áhrifum getur verið miðlað af aukinni íferð frumudrápsvaldandi $CD11b^{+}CD27^{-}$ NK frumna. Auk þess sýna niðurstöðurnar að DHA hefur áhrif á hvernig NK frumur eiga samskipti við daufkyrninga. Að lokum sýna þær að NK frumur tjá lípoxýgenasa og geta myndað lípíðafleidd bólguhjöðnunarboðefni sem hvetja til bólguhjöðnunar.

Lykilorð:

Bólguhjöðnun, bólguhjöðnunarboðefni, náttúrulegar drápsfrumur, daufkyrningar.

Abstract

Acute inflammation can progress into chronic inflammation when its resolution is impaired. Chronic inflammation is estimated to contribute to up to half of all disease-associated deaths worldwide. Resolution of inflammation is a tightly regulated process characterized by ten distinct cellular and molecular hallmarks. Lipid-derived specialized pro-resolving mediators (SPMs) are a group compounds derived from omega-3 or omega-6 polyunsaturated fatty acids (PUFAs) that effectively promote inflammation resolution. Thus, it is speculated that an imbalanced consumption of omega-6 and omega-3 PUFAs can contribute to the development of chronic inflammation. Research has shown that dietary omega-3 PUFAs can promote resolution of inflammation in murine inflammatory models. Additionally, natural killer (NK) cells have been described as potential effector cells in resolving inflammation. However, the effects of omega-3 PUFAs on NK cell recruitment and on NK cell resolution effector functions is still being investigated.

We aimed to determine the effects of omega-3 PUFAs on inflammation resolution and NK cell recruitment, function, and interactions with neutrophils. To investigate this, we used an antigen-induced peritonitis model and enhanced its resolution using dietary fish oil rich in omega-3 PUFAs. We also evaluated the numbers and phenotype of the NK cells accumulated in the inflamed peritoneum. We then assessed the effects of omega-3 PUFAs on human NK cell crosstalk with neutrophils and their production of oxygenized lipids.

Female C57Bl/6 mice were fed a Westernized diet (control, C) enriched with 2.8% menhaden fish oil (Fo) for a total of 5 weeks. They were immunized twice with methylated bovine serum albumin (mBSA) with a two-week interval and subsequently injected intraperitoneally with mBSA to induce inflammation. Mice were sacrificed prior to and 1.5, 3, 6, and 12 h after inflammation induction and peritoneal exudate and lymph nodes collected. Human NK cells were pre-incubated with PUFAs and cultured alone or with freshly isolated neutrophils. Neutrophil and NK cell numbers and their expression of surface molecules were analyzed by flow cytometry; pro-inflammatory and pro-resolving mediator concentration by ELISA, Luminex, and LC-MS/MS; lipoxygenase expression by SimpleWestern; and apoptotic cell numbers in lymph nodes by TUNEL staining.

We found that dietary fish oil can decrease peritoneal neutrophil numbers and their expression of the 'eat-me-not' molecule CD47, while increasing their apoptosis levels. We also detected dampened concentrations of the pro-inflammatory chemokines and cytokines; CXCL1, -2, CCL20, TNF-α, IL-6, and IL-6Rα, and omega-6 PUFA-derived eicosanoids; prostaglandins (PGs) and thromboxane (Tx)B_2 in inflamed mice fed the Fo

diet compared to that in mice fed the C diet. Concurrently, dietary fish oil enhanced peritoneal concentrations of insulin-like growth factor-1, transforming growth factor-β1, and soluble TNF receptor II during inflammation. Inflamed mice fed the Fo diet had higher peritoneal omega-3 to omega-6 ratio in peritoneal exudate throughout acute inflammation compared to that in mice fed the C diet.

Dietary Fo promoted accumulation of $CD11b^{+}CD27^{-}$ NK cells in the peritoneum at the peak of inflammation and increased peritoneal concentrations of CCL5, CXCL10, and CXCL12. The accumulated peritoneal NK cells were also found to express higher levels of CCR5 in inflamed mice fed the Fo diet compared to those fed the C diet. Interestingly, dietary fish oil increased percentages, numbers, and expression levels of peritoneal $CD107a^{+}$ NK cells in inflamed mice, while the peritoneal percentages, numbers, and expression levels of $CD62L^{+}$ NK cells were lowered.

We found that the omega-3 PUFA, docosahexaenoic acid (DHA), attenuated human NK cell induction of CD11b and CD47 expression on neutrophils. Specifically, NK cells pre-incubated with DHA did not induce the $CD11b^{+}CD47^{high}$ neutrophil subset as seen when untreated NK cells and neutrophils were cultured together. DHA also dampened NK cell-induced neutrophil apoptosis after extended co-culture of the two cell types. Interestingly, NK cells pre-incubated with DHA expressed lower levels of NKp46 than untreated NK cells when cultured with neutrophils. Finally, DHA tempered neutrophil-induced production of pro-inflammatory cytokines by NK cells.

Interestingly, we found that unstimulated NK cells expressed 5-, 12-, and 15-lipoxygenases required for the synthesis of lipid-derived SPMs. Further, distinct oxylipidomic profiles were observed in NK cells cultured with arachidonic acid (AA), eicosapentaenoic acid, or DHA. In fact, NK cells cultured with omega-3 PUFAs produced both fully formed SPMs and their intermediates regardless of the presence of neutrophils. Finally, we found that NK cell cultured with AA only contained PGs or TXs if neutrophils were present.

In this thesis we show that dietary fish oil enhances several hallmarks of inflammation resolution, including neutrophil apoptosis and efferocytosis. This effect may be mediated through enhanced peritoneal accumulation of degranulating $CD11b^{+}CD27^{-}$ NK cells. In addition, the data illustrate that DHA modifies NK cell effects on and crosstalk with neutrophils. Finally, we show that NK cells express lipoxygenases and synthesize lipid-derived SPMs that promote resolution of inflammation.

Keywords:

Resolution of inflammation, specialized pro-resolving mediators, natural killer cells, neutrophils.

Acknowledgements

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I am deeply grateful to Professor Freysdottir and Professor Hardardottir for their invaluable supervision, guidance, and collaboration throughout this project, which has been instrumental in my success. Their critical questions, constructive discussions, and unwavering support have helped shape me as a person and a scientist, and for that, I will always be grateful.

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Contents

List of Abbreviations

2B4	Signaling lymphocyte activation molecule family receptor
AA	Arachidonic acid
AA-NK	Arachidonic acid-treated NK
ACK	Ammonium-chloride-potassium
AD	Alzheimer's disease
ALA	α-Linolenic acid
Arg-1	Arginase-1
ATP	Adenosine 5'phosphate
BAFF	B cell-activating factor
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CFA	Complete Freund's adjuvant
ChemR23	Chemerin receptor 23
CHI3-L1	Chitinase-3-like protein 1
CLP	Common lymphoid progenitor
C-NK	Control NK
COX	Cyclooxygenase
CR	Complement receptor
CRP	C-reactive protein
CX_3CL1	Fractalkine, C-X_3-C motif chemokine ligand
CXCL	C-X-C motif chemokine ligand
CYP450	P450 cytochrome epoxygenase
DAMP	Damage associated molecular pattern
DHA	Docosahexaenoic acid
DHA-NK	Docosahexaenoic acid-treated NK
DKK-1	Dickkopf-1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAM	DNAX accessory molecule
DPA_{n-3}	Omega-3 docosapentaenoic acid
DPP	Dipeptidyl peptidase
DSS	Dextran sulfide sodium
EDTA	Ethylenediaminetetraacetic acid

EET	Epoxyeicosatetraenoic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Prostaglandin E receptor
EPA	Eicosapentaenoic acid
EPA-NK	Eicosapentaenoic acid-treated NK
FACS	Fluorescence-associated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
G-CSF	Granulocyte colony stimulating factor
GDF	Growth/differentiation factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HDHA	Hydroxydocosahexaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HRP	Horse-radish peroxidase
HSC	Hematopoietic stem cell
i.p.	Intraperitoneal
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IL-1R	Interleukin 1 receptor
ILC	Innate lymphoid cell
JAK	Janus kinase
kDa	Kilodalton
KETE	Ketoeicosatetraenoic acid
KIR	Killer-cell immunoglobulin-like receptor
LA	Linoleic acid
LAMP	Lysosomal-associated membrane protein
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LIX	Lipopolysaccharide-induced C-X-C chemokine
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
LT	Leukotriene
LX	Lipoxin
MaR	Maresin
mBSA	Methylated bovine serum albumin
M-CSF	Macrophage colony stimulating factor

MHC	Major histocompatibility complex
MMP	Metallomatrix protease
MS	Multiple sclerosis
MSR	Macrophage scavenging receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD	Nucleotide oligomerization domain
NRF	Nuclear factor erythroid 2-regulating factor
NSAIDs	Non-steroid anti-inflammatory drugs
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD	Protectin
PG	Prostaglandin
PKA	Proteinase kinase A
PL	Phospholipase
PPAR	Peroxisome proliferator-activation receptor
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycosylation endproducts
RANKL	Receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells
R_i	Resolution interval
RIPA	Radio-immunoprecipitation
ROS	Reactive oxygen species
RPMI	Rosswell Park Memorial Institute
RSV	Respiratory syncytial virus
s.c.	Subcutanous
S1P	Sphingosine 1-phosphate
SEM	Standard error of the mean
SimpleWes assay	Simple Western Immunoassay
SLAM	Signaling lymphocyte activation molecule
SLE	Systemic lupus erythematosus
SPM	Specialized pro-resolving mediator
STAT	Signal transducer and activator of transcription
sTNFR	Soluble tumor necrosis factor receptor
T_{50}	Time at which the number of neutrophils has halfed
TGF	Transforming growth factor
T_H	T helper
TIM	T-cell immunoglobulin and mucin domain
TIMP	Tissue inhibitor of metalloproteinase

TLR	Toll-like receptor
T_{max}	Time at which the number of neutrophils peak
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
T_{reg}	T regulatory
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
Tx	Thromboxane
VEGF	Vascular endothelial growth factor

List of Figures

List of Tables

List of Original Papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I, II, III):

I. Kirstine Nolling Jensen, Marieke Heijink, Martin Giera, Jona Freysdottir, and Ingibjorg Hardardottir. "Dietary Fish Oil Increases the Number of CD11b+CD27— NK Cells at the Inflammatory Site and Enhances Key Hallmarks of Resolution of Murine Antigen-Induced Peritonitis". Journal of Inflammation Research, 2022.

II. Kirstine Nolling Jensen, Sunnefa Yeatman Omarsdottir, Margret Sol Reinhardsdottir, Ingibjorg Hardardottir, and Jona Freysdottir. "Docosahexaenoic Acid Modulates NK cell Effects on Neutrophils and Their Crosstalk". Frontiers in Immunology, 2020.

III. Kirstine Nolling Jensen, Marieke Heijink, Martin Giera, Jona Freysdottir, and Ingibjorg Hardardottir. "Natural Killer Cells are a Novel Cellular Source of Specialized Pro-Resolving Mediators". Ready for submission.

In addition, some unpublished data may be presented.

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Declaration of Contribution

Paper I: In this paper, we described the effects of dietary omega-3 polyunsaturated fatty acids on key hallmarks of resolution of inflammation as well as natural killer cell recruitment, phenotype, and function. I designed the hypothesis, experiments, and flow cytometry panels with my mentors Dr. Freysdottir and Dr. Hardardottir. I monitored the mice and exchanged their diets, immunized, euthanized, and dissected the mice with help from Sunnefa Yeatman Omarsdottir, Sigridur Eyglo Unnarsdottir, and Hronn Gudmundsdottir. The immunofluorescence stainings of mesenteric lymph nodes was conducted by M.Sc. students Sigridur Eyglo Unnarsdottir and Sara Rut Bjorgvinsdottir. Flow cytometric analysis of neutrophil apoptosis and expression of CD47 was conducted by previous Ph.D. student Hronn Gudmundsdottir. Lipid mediator measurements on peritoneal exudate was performed by Marieke Heijink in the research group of Dr. Martin Giera. I performed all cell-related and protein assays (flow cytometry, Luminex, and ELISA), analyzed the data, and designed the figures included in the paper. Finally, I wrote the manuscript in collaboration with Dr. Freysdottir, Dr. Hardardottir, and Dr. Giera.

Paper II: In this paper, we investigated the effect of docosahexaenoic acid on human natural killer cell effects on and crosstalk with neutrophils. I designed the hypothesis and flow cytometry panels with Dr. Freysdottir and Dr. Hardardottir. I optimized the cell isolation, cell culture model, and flow cytometry assay with M.Sc. students Sunnefa Yeatman Omarsdottir, Irena Bjork Asgeirsdottir, Sandra Dogg Gudnadottir, and Margret Sol Reinhardsdottir. We all contributed to the collection of data that went into this manuscript. I analyzed the data and drafted the manuscript in collarboration with my supervisors Dr. Freysdottir and Dr. Hardardottir.

Paper III: In this paper, we evaluated the potential of human natural killer cells to contribute to produce lipid mediators from polyunsaturated fatty acids to promote inflammation resolution. I designed the hypothesis and experimental setup in collaboration with Dr. Freysdottir, Dr. Hardardottir, and Dr. Giera. NK cell cultures with or without neutrophils that were enriched with polyunsaturated fatty acids for lipid measurements were performed by M.Sc. student Sunnefa Yeatman Omarsdottir. The lipid concentrations in cell cultures were measured by Evelyne Steenvorden, Marieke Heijink, and Kevin A. J. Brewster in the research group of Dr. Giera. Protein isolation from NK cells and SimpleWestern ImmunoAssays were performed by me. I conducted the data analysis and wrote the paper in collaboration with Dr. Freysdottir, Dr. Hardardottir, and Dr. Giera.

1 Introduction

Inflammation is a protective response initiated in response to insults such as pathogens and tissue damage. The inflammatory response is mediated by four central components: inflammatory inducers, sensors, mediators, and target tissues (1). Inflammatory inducers are comprised of infectious agents, sterile tissue injury, hypoxia, and metabolic stressors. Tissue-resident antigen-presenting cells, including macrophages, dendritic cells, and endothelial cells, act as inflammatory sensors by ligation of pathogen and damage associated molecular patterns (PAMPs and DAMPs, respectively) to pattern recognition receptors (PRRs). Inflammatory mediators, such as eicosanoids, cytokines, chemokines, bioactive amines, and proteolytically generated molecules, are subsequently released (1). The cardinal signs of acute inflammation can be observed in the affected target tissue, which is determined by the specific combination of inducer, sensor, mediator, and target tissue mounting the response (1). Thus, acute inflammation comprises a major part of the innate arm of the immune system and provides the organism with a broad protection against inflammatory insults. It is later resolved by a tightly regulated sequence of cellular and molecular events that ensure the target tissue's return to homeostasis (2).

Failure to resolve acute inflammation can result in its progression to chronic inflammation (3). It is characterized by either continuous low-grade inflammation or occasional acute inflammatory flare-ups and has been implicated as an underlying pathology in many diseases, including cancer, rheumatoid arthritis, asthma, obesity, psoriasis, and autoimmunity (4). Chronic inflammation is estimated to contribute to as many as 50% of all disease-associated deaths worldwide and poses a major burden on societies across the globe (5).

1.1 Inflammation and its resolution

1.1.1 Acute inflammation

The acute inflammatory response is triggered by the recognition of PAMPs or DAMPs by PRRs, which are comprised of a diverse family of receptors, including toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene-I-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (6). Recently, deoxyribonucleic acid (DNA) sensors and inflammasomes have also been identified as PRRs (7). The engagement of PRRs triggers the release of cytokines, chemokines, and acute-phase proteins, leading to redness, swelling, heat, pain, and loss of function (8). These are commonly known as the five cardinal signs of acute inflammation. This, in turn, promotes the migration of immune cells, including neutrophils, lymphocytes, monocytes, across

the endothelial barrier (8). As neutrophils are abundant in circulation and rapidly migrate in response to these mediators, their numbers are often used to assess the degree of ongoing inflammation (3).

Redness is the first cardinal sign of inflammation, and it results from hyperemia, an excess of blood in the vessels at the inflamed site (9). Hyperemia is caused by inflammation-induced angiogenesis, which is the formation new micro blood vessels after the ligation of PRR (9). This process is further promoted through the production of growth factors and related molecules, such as vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α (7). The enhanced angiogenesis and blood flow ultimately facilitate the recruitment of immune cells by enabling increased rolling.

Swelling, also known as edema, is a common occurrence in response to inflammation-induced endothelial barrier dysfunction (7). This dysfunction can be triggered by the engagement of PRRs, which activates the inflammasome, leading to the secretion of interleukin (IL)-1β and IL-18. These cytokines, in turn, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which promotes the production of additional pro-inflammatory mediators (7). The resulting acute phase cytokines and proteins then promote endothelial barrier dysfunction, characterized by the loss of tight junctions between neighbouring endothelial cells. This allows for the extravasation of plasma proteins, cells, and soluble mediators (7). Additionally, endothelial cells will upregulate their expression of cell adhesion molecules (CAMs) and metallomatrix proteases, which facilitate the rolling of immune cells and subsequent excavasion to the inflamed site (10). This process is critical for the rapid influx of neutrophils, natural killer (NK) cells, and other immune cells to the site of inflammation (7).

Heat or fever constitutes another cardinal sign of inflammation (11). The most well-established fever inducing signaling axis for inducing fever is the IL-6-prostaglandin (PG)E_2 axis (11, 12). Tissue-resident macrophages and dendritic cells produce pyrogenic cytokines, i.e., IL-1, IL-6, and TNF-α, which bind to their respective receptors on brain endothelial cells, inducing the expression of cyclooxygenase (COX)-2 for PGE_2 production (12). Similarly, tissue-resident macrophages will produce PGE_2, which is released into circulation (13). Regardless of its cellular source, PGE_2 crosses the blood-brain barrier and ligate to PGE receptor (EP)3 on neuronal cells in the hypothalamus, thereby initiating a fever response (11, 12).

Inflammation-associated pain is mediated by nociceptors, specialized sensory neurons that respond to signals releaed by damaged and inflamed tissues including cytokines, growth factors, or eicosanoids (14, 15). These signals are promoted by macrophages or microglia by their production of IL-1, IL-6, TNF-α, C-X-C chemokine motif ligand (CXCL)1, and CXCL12 (14). Eicosanoids, such as PGE_2, PGI_2, and leukotriene (LT)B_4 further promotes nociception (15). To target these signals non-steroid anti-inflammatory drugs (NSAIDs) have been used for many years to reduce pain and inflammation (15).

Due to the toxic nature of inflammation, cells undergo apoptosis or necrosis in response to various inflammatory stimuli (16, 17). Apoptosis can be induced by two distinct mechanisms; the intrinsic and the extrinsic pathways (18). External stimuli, such as reactive oxygen species (ROS), nitric oxide, or heat, triggers caspase cleavage and controlled cell death (18). Release of cytolytic proteins or ligation of death-inducing receptors induce apoptosis following the recognition of abberant cells by cytotoxic lymphocytes (18). Apoptosis is regarded as a controlled, non-inflammatory mean of cell death, and the removal of apoptotic bodies is critical in limiting the gradual transition from acute to chronic inflammation (16, 18). In contrast, necrosis results from the loss of mitochondrial barrier integrity, which leads to cell swelling and failure to induce apoptosis (17, 18). During necrosis, mitochondrial DNA and intracellular proteins spill into the surrounding interstitial space due to cell rupture (18). Necrosis results in a temporary loss-of-function at the inflamed or injured site, further amplifying the release of inflammatory mediators and creating a positive inflammatory feed-forward loop (17).

1.1.2 Resolution of inflammation

Resolution of inflammation is a highly orchestrated sequence of cellular and molecular events to ultimately return the inflamed tissues to homeostasis (2). This process is marked by ten interrelated hallmarks, with six occurring early on and four appearing later in the resolution phase (19). These processes counteract the five cardinal signs of inflammation following the elimination of an inciting agent. Figure 1 provides an overview of the ten hallmarks of inflammation resolution.

The first step in resolution of inflammation is the restriction of granulocyte recruitment (20). This is achieved by eliminating inflammatory factors that promote neutrophil recruitment from the inflamed site (20). Decoy receptors, such as IL-1 receptor (IL-1R)2, IL-1R8, and atypical chemokine receptors play an integral role in this process by contributing to the scavenging and removal of these inflammatory factors (21). Additionally, most soluble forms of the receptors for TNF (sTNFR), with the exception of sTNFRI, are signaling incompetent, acting as *de facto* decoy receptors (22). By binging to and neutralizing pro-inflammatory cytokines, these soluble decoy receptors effectively limit neutrophil migration and facilitate inflammation resolution. In fact, synthetic versions of sTNFRII are routinely used to treat a variety of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis (23).

In the initiation of resolution of inflammation, an early switch in lipid mediator synthesis can be detected (2, 3, 20). This switch involved the preferential synthesis of specialized pro-resolving mediators (SPMs) derived from polyunsaturated fatty acid (PUFA), as opposed to eicosanoids (24). Simultaneously, the production of growth factors, such as insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β, increases to counteract pro-inflammatory signaling (25). Studies have shown that IGF-1 attenuates the pro-inflammatory profile of neutrophils in myocardial infarctions in mice (26), as well as

mitigates hyperoxia-induced lung injury (27), lipopolysaccharide (LPS)-induced inflammation (28), and murine colitis (29). TGF-β, on the other hand, has been shown to suppress pro-inflammatory cytokine production (30, 31) and stimulate resolution of several diseases, including influenza A-induced (32) and monohydrate crystal-induced inflammation (33). Another important pro-resolving mediator is IL-10, which enhances resolution of inflammation in conjuntion with IL-4 and TGF-β in a variety of murine inflammatory models and human diseases (19).

The induction of neutrophil apoptosis, as opposed to pyroptosis and necroptosis, is considered a critical step towards resolution of inflammation (20, 25, 34). Neutrophils phagocytose infectious agents and cell debris at the site of inflammation, and subsequently undergo apoptosis spontaneously (34). In addition, cytotoxic NK cells contribute to the resolution of inflammation by inducing neutrophil apoptosis through the ligation of cytotoxic receptors and the release of cytolytic proteins (35). For example, to become more susceptible to NK cell-induced apoptosis, neutrophils downregulate their expression of human leukocyte antigen (HLA) molecules (36). This induction may be mediated through an NKp46-Fas ligand (FasL) dependent signal (37). Moreover, apoptotic neutrophils release 'find-me' signals, such as sphingosine 1-phosphate (S1P), fractalkine (C-X_3-C chemokine motif ligand, CX_3CL1), and adenosine 5'triphosphate (ATP) (38). These signals assist macrophages in localizing and clearing apoptotic neutrophils, further promoting inflammation resolution (38).

Apoptotic bodies must be removed from inflamed sites to avoid the induction of an inflammatory positive feed-forward loop (34). Macrophages migrate in response to the 'find-me' signals released from apoptotic cells as described above (38). Once they localize the apoptotic cells, they bind phosphatidylserine (PS) with CD91, CD300f, brain angiogenesis inhibitor 1, T-cell immunoglobulin and mucin domain (TIM)-1, TIM-4, and Mer tyrosine kinase (38). This binding trigger actin formation and subsequently the engulfment of apoptotic bodies (38). As macrophages engulf these apoptotic cells, they upregulate their expression of arginase-1 (Arg-1) to metabolize arginine found in apoptotic bodies (39). This metabolism subsequently polarize macrophages towards an M2 phenotype, thereby further strengthening the inflammatory resolution response (39). Additionally, T regulatory (T_{reg}) cells promote M2 polarization of macrophages through the signal tranducer and activator of transcription (STAT)6/Arg-1 signaling pathway (40, 41). Apoptotic neutrophils also their removal from the inflamed site, and promotes resolution of inflammation, by downregulating 'eat-me-not' molecules, i.e., CD47, CD31, CD24, and major histocompatibility complex (MHC)-I (38).

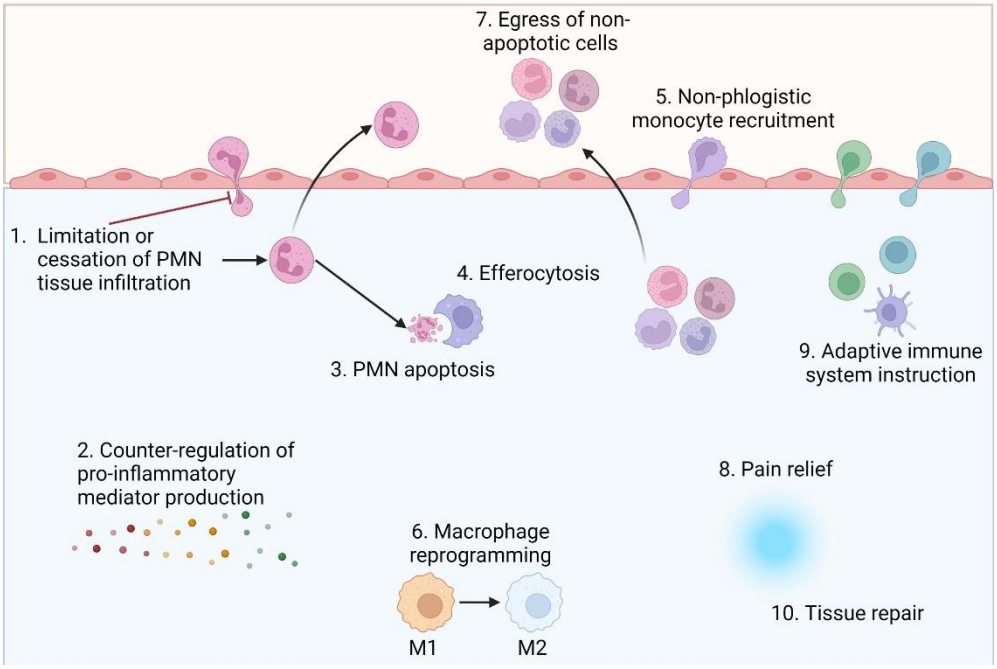

Figure 1. Molecular and cellular events in resolution of inflammation.

Resolution of inflammation is a cascade of cellular and molecular events involved in appropriate return of inflamed tissues to homeostasis. Firstly, neutrophil (PMN) influx is limited or cessated (**1**) along with a counter-regulation of pro-inflammatory mediator production (**2**). Subsequently, inflammatory neutrophils undergo apoptosis (**3**) and are removed from the inflammatory site through efferocytosis (**4**). Non-phlogistic monocyte recruitment is initiated (**5**) leading to reprogramming and polarization of macrophages/monocytes towards an M2-like phenotype that promotes tissue repair (**6**). In addition, pro-inflammatory cells that do not undergo apoptosis leave the inflamed site through reverse migratory patterns (**7**). The resolution process also includes the dampening and relief of inflammation-induced pain (**8**), instruction of the adaptive immune system (**9**), and promotion of tissue regeneration and repair (**10**). The ten hallmarks of inflammation resolution is adapted from the literature (2, 3, 19, 20) and the figure was created using **BioRender.com**.

Research has shown that nonphlogistic, or non-inflammatory, monocyte recruitment promotes resolution of inflammation responses (19). However, the exact mechanisms that drive this recruitment are not yet fully understood. One potential mechanism is provided by free cyclic adenosine monophosphate (cAMP) that signals through protein kinase A (PKA) to enhance nonphlogistic monocyte recruitment by the C-C motif chemokine receptor (CCR)2 and C-C motif chemokine ligand (CCL)2 signaling axis (42). In addition to this pathway, it has recently been suggested that nonphlogistic monocyte recruitment may be mediated by angiotensin-1-7 binding of the Mas receptor (43).

Macrophages can polarize to a number of different activation states depending on the inflammatory microenvironment (44). A well-established polarization state, that promote

resolution of inflammation, is the M2 phenotype (2). They engulf apoptotic cells and bodies to promotes the resolution of inflammation and M2 polarization as described above (44). In both neuroinflammation (45) and atherosclerotic lesions (46), macrophages can polarize to an M2-like phenotype through IL-4 signaling by the Janus kinase (JAK)/STAT6 pathway to ameliorate clinical symptoms.

In late stages of inflammation resolution, non-apoptotic immune cells are drained from the inflamed site via the lymphatic system or reverse migration to the blood stream (19). Then, inflammatory pain is ameliorated through decreased production and increased scavenging of pro-inflammatory cytokines and lipid mediators in the inflammatory microenvironment around nociceptors (47). Once the inciting agent is removed from the inflammatory site, the adaptive immune system is instructed to avoid reinfections. However, in cases where the inflammation resolution is impaired, T lymphocytes may be less responsive, potentially perpetuating the gradual progression to chronic inflammation (48). Ultimately, regeneration and return to homeostasis of inflamed tissues is required as the final step in the inflammation resolution cascade (19). This process is facilitated by growth factors, such as TGF-β and IGF-1, which stimulate the expansion of local stem cells to repopulate the inflamed site and heal damaged tissue (49-51).

1.2 Long-chain polyunsaturated fatty acids

Long-chain PUFAs are fatty acids that contain 18 or more carbons and are categorized into three main groups, namely omega-3, -6, and -9, based on the location of a double bond from the methyl end of the carbon-chain (52). While higher animals do not possess the enzymes required for *de novo* synthesis of omega-3 or -6 PUFAs, these fatty acids are essential for maintaining homeostasis (53). Ingestion of the essential omega-6 PUFAs such as linoleic acid (LA) and arachidonic acid (AA), as well as the omega-3 PUFAs including α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), is crucial to ensure the health of the organism (54). Once incorporated into cellular membranes, PUFAs can modulate membrane phospholipid composition, membrane order, and lipid raft assembly (55). Further, PUFAs serve as energy sources, modify protein structure, regulate gene expression and signaling, and serve as precursors for bioactive eicosanoids and SPMs (56).

Historically, prior to the industrialization of societies, the ratio of consumed omega-6 to omega-3 PUFAs ranged between 1:1 – 2:1 (57). However, estimates indicate that in industrialized countries, the current omega-6:omega-3 ratio ranges from ~8:1 – 30:1 (57-62). This imbalance in the consumption of PUFAs may directly modulate inflammatory responses and its resolution, as AA can give rise to eicosanoids that can contribute to inflammatory processes, whereas EPA and DHA mainly give rise to SPMs (63). Additionally, omega-3 and omega-6 PUFAs act as competitive antagonists for each other in the enzymatic conversions by lipoxygenases (LOXs) and COXs (64). Due to this, an imbalanced omega-6:omega-3 ratio may be detrimental to human health (54).

Dietary supplementation of omega-3 PUFAs in humans is being investigated as a preventative measure and complementary treatment against chronic degenerative diseases. In healthy individuals, supplementation of omega-3 PUFAs subtle to no detectable effects (65, 66). However, in chronically inflamed subjects, dietary interventions or supplementation of omega-3 PUFAs have been shown to lower the levels of inflammatory serum proteins, decrease disease scores, and protect against disease progression in multiple sclerosis (MS) (67, 68), Alzheimer's disease (AD) (69), rheumatoid arthritis (RA) (70, 71), obesity (72), chronic kidney disease (73), and systemic lupus erythematosus (SLE) (73). The reduction in pro-inflammatory serum proteins is attributed to dampened pro-inflammatory NF-κB, TLR, TGF-β, and peroxisome proliferator-activation receptor (PPAR) signaling (74).

Studies have shown that dietary fish oil ameliorates sepsis-induced liver injury (75) and diabetic renal inflammation (76) in rodent models. Previous studies conducted by our research group demonstrated that dietary fish oil modulates neutrophil and monocyte phenotypes prior to and after the induction of endotoxin-induced inflammation (77, 78). Furthermore, our group demonstrated that dietary fish oil enhances inflammation resolution and B1 immune responses in antigen-induced peritonitis (79, 80). These findings suggest that dietary omega-3 PUFA can modulate the composition of immune cells at steady states and attenuate responses to various inflammatory stimuli in mice and humans.

1.2.1 Eicosanoids

Long-chain PUFAs are converted to eicosanoids through enzymatic oxygenation pathways involving LOXs, COXs, and P450 cytochrome epoxygenases (CYP450) (56, 81, 82). The oxygenation of 20-carbon PUFAs generates a variety of eicosanoids, including hydroxy-, hydroperoxy-, epoxy-, and oxo-eicosanoids, PGs, LTs, and thromboxanes (Txs) (82). Upon acute inflammatory signaling, the expression of phospholipase (PL)A_2, COX-2, and 5-lipoxygenase (LOX) is rapidly induced (56, 82-84). This induction promotes the synthesis of eicosanoids, which exert diverse effects on tissue homeostasis, pain, host defence mechanisms, and inflammation depending on their concentrations and target cells that aid in the inflammatory signaling (56).

The enzymatic oxidation of AA by COX and LOX is summarized in Figure 2. Prostanoids includes both PGs and Txs and constitute a major group of eicosanoids (63). They are synthetized from AA by the enzymatic actions of COX-1 and -2, prostaglandin synthase, and thromboxane synthase (82). TxB_2 is the stable but biologically inactive product of TxA_2 (85). As TxA_2 has a half-life of only six to eight seconds *in vivo,* TxB_2 is measured as an indirect evaluation of its presence (85). TxA_2 is a potent vasoconstrictor that also stimulates the aggregation of activated platelets during tissue injury by signaling through the TxA receptor (86). Thus, TxA_2 directly enhances the acute inflammatory response to inflammatory insults and is integral in the initiation but also the limitation of inflammation-

associated tissue damage.

PGs exert their effects by binding specific G-protein coupled receptors (GPRs), affecting a wide range of both immune and non-immune cells (63). They modulate the function and crosstalk between dendritic cells, T cells, and NK cells (56). For instance, PGE_2 promotes immunoglobulin class-switching of B cells, suppresses T helper (T_H)1 effector functions, and enhances pro-inflammatory functions of T_H17 cells (56). Moreover, PGE_2 induces the production of IL-1, IL-6, and murine CXCL1 (87), possibly through inflammasome activation (88, 89). PGE_2 signaling in NK cells and cytotoxic T cells inhibits their cytotoxic functions in the decidua, uterine lining (90), and several tumors (91, 92). Consequently, PGE_2 feeds into the positive feed-forward inflammatory loop, propagating neutrophil-driven inflammation while inhibiting induction of neutrophil apoptosis through inhibition of cytotoxic effector cells.

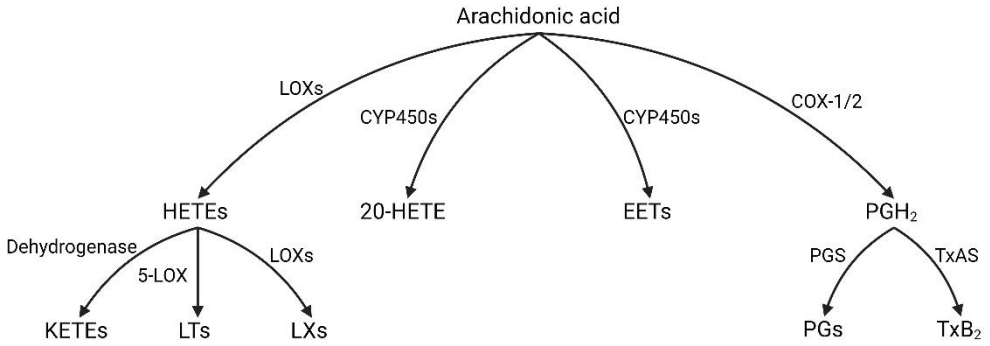

Figure 2. Arachidonic acid metabolism.
Arachidonic acid is metabolized by the enzymatic actions of lipoxygenases (LOXs), P450 cytochrome epxygenases (CYP450s), cyclooxygenase (COX)-1/2, dehydrogenases, prostaglandin synthase (PGS), and thromboxane A_2 synthase (TxAS). These enzymatic reactions lead to the production of bioactive metabolites, such as hydroeicosatetraenoic acids (HETEs), oxoeicosatetraenoic acids (KETEs), leukotrienes (LTs), lipoxins (LXs), epoxyeicosatetraenoic acids (EETs), prostaglandins (PGs), and thromboxane (Tx)B_2. This figure was created using **Biorender.com**.

Another major subclass of eicosanoids is composed of LTs, which are synthetized by 5-LOX-dependent enzymatic actions and primarily produced by neutrophils and macrophages (55). Among them, LTB_4 contributes to the cardinal signs of inflammation by enhancing vascular permeability, pain reception, and production of ROS and pro-inflammatory cytokines (55). Furthermore, LTB_4 amplifies the positive inflammatory feed-forward loop by promoting the infiltration of neutrophils, monocytes, and lymphocytes to the inflamed site (93). LTs have been strongly associated with the pathology of several chronic inflammatory degenerative diseases, including atherosclerosis, myocardial infarctions, and asthma (94, 95).

In addition to the traditional eicosanoids, non-classical hydroxyeicosatetraenoic acids (HETEs) have recently garnered attention for their role in chronic inflammatory diseases. Inflammation-associated obesity, determined by body mass index, is positively correlated with increased serum concentrations of 5- and 11-HETE (96). High concentrations of 12-HETE have been identified in hypertension (97), type 1 diabetes (98), hepatic ischemia-reperfusion injury (99), and cancer (100). Additionally, 15-HETE promotes adipose tissue angiogenesis via induction of VEGF production (101). Thus, non-classical eicosanoids may enhance inflammation independent of PG and LT production and effects.

1.2.2 Specialized pro-resolving mediators

Early in the inflammatory response, a switch in the lipid mediator synthesis from PG, LT, and Tx to SPM can be observed (20). Lipid-derived SPMs are derived from AA, EPA, DHA, and as has been recently recognized, omega-3 docosapentaenoic acid (DPA_{n-3}) (102). In addition to lipid-derived SPMs, other molecules, including proteins (annexin A1 and growth factors), gaseous mediators, nucleotides, and neuromodulators, can also act as pro-resolving mediators (19). Lipid-derived SPMs can be divided into four major subgroups; lipoxins (LXs), E- and D-series resolvins (Rvs), protectins (PDs), and maresins (MaRs) (2). These mediators promote inflammation resolution by directly promoting several of its hallmarks.

LXs are derived from AA and produced by the enzymatic actions of 5- and 15-LOX (103). They are mainly produced by activated platelets (104) or by platelets and neutrophils in unison (105). LXs downregulate inflammasome activation to promote resolution of inflammation (106). Additionally, LXs inhibit tumor progression by inducing monocyte phenotypic changes (107). Notably, LXA_4 promotes M2-polarization by n-formyl peptide 2 receptor and interferon regulatory factor signaling (108). Furthermore, LXA_4 has been shown to reduce pathogenic T_H1 and T_H17 effector functions in patients with relapsing remitting MS (109).

Rvs are divided into two subgroups based on their precursor PUFA as shown in Figure 3: E- and D-series derived from EPA or DHA, respectively (2). E-series Rvs are produced through a series of enzymatic steps, beginning with the oxygenation of EPA by COX-1/2 to give rise to 18-hydroxyeicosapentaenoic acid (HEPE), which is subsequently oxygenated by 5- and 15-LOX to yield the final product (110). This synthesis primarily occurs in human leukocytes (111), particularly in macrophages following the engulfment of apoptotic bodies (112) and apoptotic neutrophils (113). Rvs can alleviate inflammatory pain by competitively binding of the LTB_4 receptor 1 (114) and ameliorates LPS-induced depression in mice via chemerin receptor (ChemR)23 (115). RvE_1, in particular, mitigates atherosclerotic disease progression by inhibiting osteoclastogenesis through IL-17 and receptor activator of NF-κB ligand (RANKL) signaling (116). Additionally, RvE_1 regulates T_H17 functions and T cell activation, thereby modulating pathogenic autoimmune signaling (117). Finally, RvE_1 stimulates NK cell migration by binding its receptor

ChemR23, which may act as resolution effector cells in lung inflammation (118).

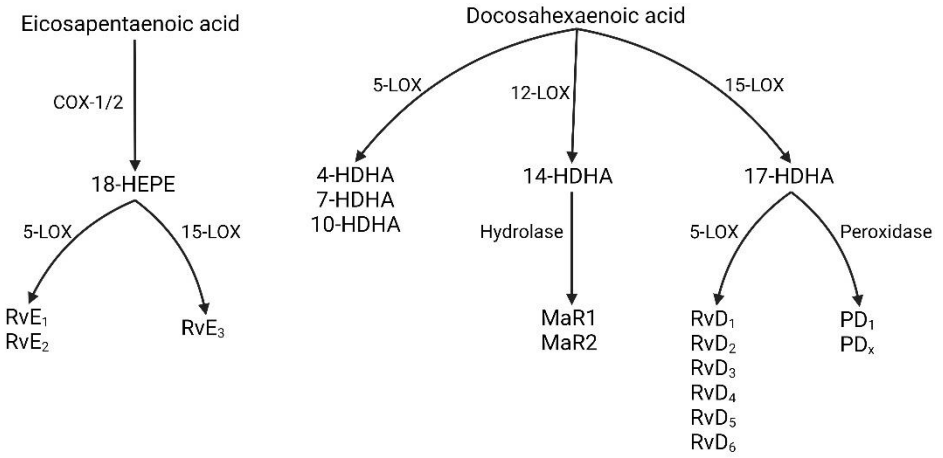

Figure 3. Synthesis of SPMs from eicosapentaenoic acid and docosahexaenoic acid.
Eicosapentaenoic acid undergoes oxygenation by cyclooxygenase (COX)-1/2, resulting in the production of 18-hydroxyeicosapentaenoic acid (HEPE). Subsequent ezymatic actions of 5- and 15-lipoxygenase (LOX) then convert 18-HEPE into E-series resolvins (Rvs). On the other hand, docosahexaenoic acid yields various of hydroxydocosahexaenoic acids (HDHAs) through the enzymatic actions of 5-, 12-, and 15-LOX. Hydrolases catalyze the production of maresins (MaRs) from 14-HDHA, while D-series Rvs and protectins (PDs) are derived from 17-HDHA through the enzymatic actions of 5-LOX and peroxidases. The figure was made using **Biorender.com**.

DHA is oxydized by 15-LOX to produce 17-hydroxydocosahexaenoic acid (HDHA) and subsequently by 5-LOX to yield D-series Rvs, as shown in Figure 3 (2). D-series Rvs are produced by a variety of cell types, including keratinocytes (119), vascular smooth muscle cells (120), conjuctival goblet cells (121), mesenchymal stromal cells (122), and leukocytes, especially neutrophils (113, 123). RvD_1 signal through GPR32 to protect against atherosclerosis (124) and attenuate type 2 diabetes-associated neuroinflammation (125). A critical role of RvD_1 is its enhancement of M2 polarization of macrophages for the resolution of adipose tissue inflammation (126) and limitation of tumor progression (127).

Macrophages are a major source of MaRs derived from DHA by 12-LOX-dependent oxygenation to form 14-HDHA, which is subsequently hydrolyzed to yield the final product (128). MaR1, a well-studied member of this family, alleviates LPS-induced inflammation (129) and dextran sulfide sodium (DSS)-induced colitis by regulating nuclear factor erythroid 2-regulating factor (NRF)2 and NF-κB (130). Additionally, it has potent and long-lasting analgesic effects (131), possibly by promoting nerve regeneration after neuronal damage (132).

PDs are produced in the central nervous system during neuroinflammatory diseases (133, 134). Additionally, endothelial and vascular smooth muscle cells convert DHA to 17-HDHA, by the 15-LOX-mediated oxidation (120), which is then further oxidized by peroxidase to synthetize PD_1 and PD_x. They inhibit macrophage pyroptosis by limiting the assembly of the inflammasome, thereby ameliorating LPS-induced sepsis (135). PD_1 has been shown to mitigate acute kidney injury (136) and attenuate neointimal hyperplasia in rats (137), while PD_x has been shown to exert analgesic functions similar to MaR1 through cAMP-PKA signaling (138). Additionally, PD_x ameliorates hyperoxia-induced lung injury in rats (139) and promotes resolution of inflammation in acute respiratory distress syndrome (140).

1.3 Natural killer cells

NK cells are highly cytotoxic cells capable of inducing cell death in abnormal target cells without prior stimulation (141). They constitute approximately 5-15% of circulating lymphocytes in healthy adults (141, 142). NK cells are not recognized based on traditional lineage markers as T and B cells (142). Rather, they are recognized based on the absence of CD3 expression in combination with organism-specific markers (143). Murine NK cells are commonly recognized based on their expression of NK1.1, NKp46, and CD49b (DX5), while human NK cells are recognized based on their expression of CD56 (143). Regardless of the phenotypic identification, the functions of NK cells are conserved across species, including potent cytotoxicity (141), bridging of the innate and adaptive immune system (144), and secreting high levels of soluble mediators to modulate immune fate (145). NK cells also engage in crosstalk with dendritic cells (146), NK-T cells (147), neutrophils (148), and T cells to fine-tune immune responses depending on the inflammatory microenvironment (142). Recently, a new appreciation for the heterogeneity of circulating NK cells has emerged. Specifically, recent studies have shown a high number of distinct NK cell subpopulations with versatile and dynamic functions in healthy and diseased individuals (149, 150).

1.3.1 Maturation and development

NK cells develop in specialized hematopoietic stem cell niches in the bone marrow of mice (145). Hematopoietic stem cells (HSCs) differentiate into common lymphoid progenitor (CLP) cells, which can give rise to T, B, NK, NK-T, or innate lymphoid cells (ILCs) by upregulated expression of CD127 (145). Subsequently, CLP cells increase their expression of IL-2 receptor β to differentiate into pre-T/NK progenitor cells (145). NK cells then develop following their expression of NK1.1, NKp46, and NKG2D (145). After their release into circulation, NK cells undergo functional maturation either in the bloodstream, secondary lymphoid organs, or at inflammatory sites (145). Murine NK cells are classified into four functional maturation stages depending on their CD11b and CD27 expression, as illustrated in Figure 4A (145, 151). Upon release from the bone marrow, NK cells are initially $CD11b^{-}CD27^{-}$ and considered the most immature NK cell subset in

mice (151). They then upregulate their CD27 expression to become $CD11b^{-}CD27^{+}$ NK cells, which repopulate bone marrow and lymph nodes to produce high levels of cytokines for target cells (151). Subsequently, they upregulate their expression of CD11b to become $CD11b^{+}CD27^{+}$ mature NK cells (145, 151), enabling them to rapidly respond to acute inflammatory signals from complement as CD11b is a part of the complement receptor (CR)3 (152). Finally, NK cells lose their expression of CD27 to become the terminally differentiated subset $CD11b^{+}CD27^{-}$ (151). The two latter NK cell subsets are found in high numbers in circulation and rapidly infiltrate inflamed sites to exert cytotoxic functions and produce pro-inflammatory cytokines (145, 151).

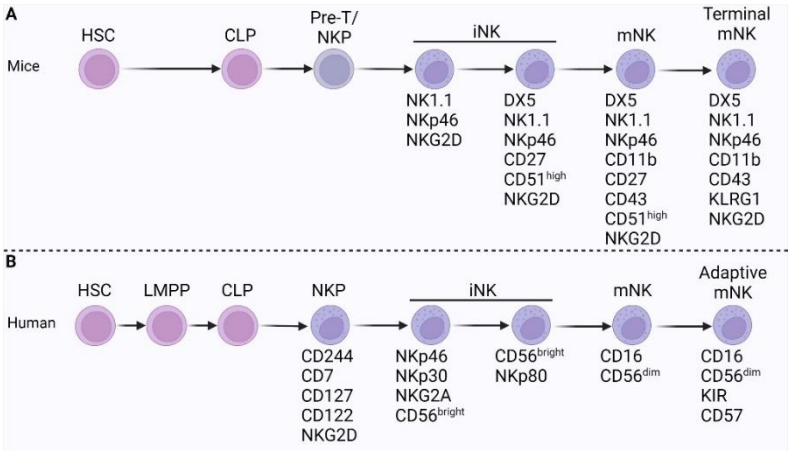

Figure 4. Schematic overview of NK cell development and maturation in mice (**A**) and humans (**B**).

A) In mice, hematopoietic stem cells (HSCs) differentiate into common lymphoid progenitor (CLP) cells in specialized bone marrow niches, and subsequently pre-T/natural killer progenitor (NKP) cells, which give rise to natural killer (NK) cells. They are functionally divided into maturation stages in the circulation based on their CD11b and CD27 expression. NKP cells become NK cells by expressing NK1.1, NKp46, and NKG2D but neither CD11b and CD27. Immature NK (iNK) cells then express CD27 but not CD11b in the final immature state. Following the expression of CD11b with CD27 NK cells are considered mature (mNK). They will rapidly shed their CD27 to only express CD11b and are regarded as terminally differentiated mNK cells. **B**) In humans, HSC differentiate into lymphoid myeloid primed progenitor (LMPP) cells. A subset of these commit to becoming lymphoid cells, developing into CLP cells. The expression of CD122 commits the CLP cells to differentiating into NKP cells. They further progress into iNK by expressing high levels of CD56 but no CD16. Following CD16 expression and a decrease in CD56 they become mNK cells. They then differentiate into adaptive or memory-like NK cells following activation and expression of CD57 and killer immunoglobulin receptors (KIR). Figure modified from (145) and created using **Biorender.com**.

The specific mechanism by which human NK cells develop in bone marrow and secondary lymphoid organs is still under active investigation (145). Regardless of the site, HSCs develop into lymphoid-primed multipotential progenitor cells (145), which later

differentiate into CLP cells. These CLP cells have the potential to become pre-B, pre-T, NK, or innate lymphoid progenitor cells (145). Although the human developmental program of NK cells is less understood than that in mice, a recent study showed that NK cell development in humans consists of six specific stages as seen in Figure 4B (153). NK progenitor cells upregulate their expression of CD56 to become $CD16^{-}CD56^{bright}$ immature cells that repopulate bone marrow and secondary lymphoid organs, similar to their murine counterparts (143, 145). Subsequently, NK cells become functionally mature $CD16^{+}CD56^{dim}$ cells that constitute ~90% of circulating NK cells (145). These cells rapidly respond to inflammatory signals to accumulate at the inflamed site and exert their effector functions as their murine equivalent (143, 145). Eventually, NK cells express CD57 to become adaptive and terminally differentiated NK cells (145). New evidence suggests that NK progenitor cells can become $CD16^{+}CD56^{dim}$ and enter the circulation (154). Ultimately, they then skip the immature $CD16^{-}CD56^{bright}$ maturation state, however, the biological role of this has yet to be determined.

1.3.2 Recruitment axes

In response to chemokine signaling, NK cells accumulate at inflamed sites and in secondary lymphoid organs (155, 156). They also migrate in response to molecules, such as S1P, RvE_1, and chemerin, that do not fall into the chemokine molecule family (118, 156). Depending on the specific chemotactic signal, $CD56^{dim}$ or $CD56^{bright}$ NK cells for humans while murine $CD11b^{-}CD27^{+}$ immature or $CD11b^{+}CD27^{+/-}$ mature NK cells accumulate in distinct target tissues to exert their effector functions (157). The function exerted by the NK cells is determined by the specific recruitment axis to inflamed sites or tissues (155-157). Although NK cells can be recruited in many ways, several integral pathways have been identified in the response to acute inflammation.

Under steady-state conditions, the expression of CCR5 on NK cells regulates their circulation and maturation (158) and ensures retention of human $CD56^{bright}$ NK cells in hepatic sinusses (159). Human dendritic cells can produce CCL5 to recruit NK cells following infectious stimuli via the CCL5-CCR5 axis (160). This recruitment axis plays a crucial role in the accumulation of murine NK cells to limit *Candida albicans* (161) and *Toxoplasma gondii* (162) infections. Furthermore, TLR2-induced CCL5 production and subsequent NK cell recruitment is integral for the amelioration of ischemia-induced reperfusion injury in mice (163). Murine CCR5 expression on NK cells also enhances their recruitment and cytotoxic functions to limit tumor progression (164), and CCR5 has been identified as a novel gene editing target to ensure NK cell infiltration to tumor sites (165). Further, disruption of the CCL5-CCR5 NK cell recruitment axis was implicated in murine hepatitis B infection (166) and its symptoms are exacerbated in CCR5-deficient mice (167, 168). On the other hand, deficiency of CCR5 lowered herpes simplex virus-2 infections in mice due to impaired NK cell recruitment, suggesting a pro-infectious role of NK cells in certain infections (169). Additionally, chemotactic recruitment of human NK cells by CCL5-CCR5 in conjunction with CXCL10-CXCR3 has been implicated in

disease progression of psoriasis (170) and RA (171). Further, poor clinical outcomes in aggressive NK cell leukemia patients was observed after CCR5- and CXCR1-dependent NK cell accumulation in multiple organs (172).

The migration of NK cells to lymph nodes in mice is dependent on CXCL10-CXCR3 signaling (173-175). Both dendritic cells (174, 175) and neutrophils (176, 177) are believed to produce CXCL10 for recruitment of NK cells to lymph nodes or inflamed sites. Dendritic cell-derived CXCL10 promotes the recruitment of murine NK cells to lymph nodes to polarize and mature T cells (175). In contrast, neutrophil-derived CXCL10 promotes pathogenic recruitment of NK cells that is suggested to exacerbate disease progression in mice (176, 177). Murine NK cells recruited through the CXCL10-CXCR3 axis following cytomegalovirus infection exacerbates the neuroinflammatory response (178). On the other hand, NK cells that are recruited in this manner attenuate hepatic fibrosis progression and prevent liver metastasis in mice (179, 180).

The expression of CXCR4 on NK cells enhances their response to the chemokine CXCL12 (181). Murine NK cells recruited to lungs through this axis in late respiratory syncytial virus (RSV) infection contribute to persistent airway inflammation (182). Additionally, a gain-of-function variant of the human CXCR4 gene causes expansion and dysregulation of cytotoxic function of NK cells in human papilloma virus (HPV) infections (183). Human NK cells may be recruited to lungs through either CXCR3 or CXCR4 signaling provide interferon (IFN)-γ and protect against *Mycobacterium tuberculosis* infections and disease progression (184). Moreover, they are recruited to prostate tumor sites through CXCL12-CXCR4 signaling for tumor progression inhibition in humans (185). The expression of chemokine receptors such as CXCR3 and CXCR4 has been found to increase cytotoxic functions of human NK cells in neuroblastoma (186) suggesting non-migratory functions of their signaling. Additionally, human NK cells recruited through the CXCL12-CXCR4 axis have been suggested to exert neuroprotective functions in relapsing remitting MS (187). A similar subset of NK cells are recruited through the CXCL12-CXCR4 axis to the decidua in humans (188) may be integral for successful implantation and pregnancy (189).

1.3.3 NK cells in inflammation

NK cells are stimulated by activatory cytokines, including IL-2, -12, -15, -18 following their accumulation at inflamed sites (190). This stimulation leads to an increase in the production of effector proteins, such as granzyme B, perforin, IFN-γ, TNF-α, and granulocyte macrophage stimulating factor (GM-CSF) thereby licensing them to kill (191, 192). Lipid mediators such as PGs and regulatory mediators such as TGF-β modulate the NK cell response and phenotype by inhibiting the loading of secretory granules (92, 191, 193). An overview of activating and inhibiting receptors in mice and humans can be found in table 1.

Table 1. Certain major receptors expressed on NK cells in mice and humans with resulting functional outcome upon their ligation.

Receptor	Species		Activation/ inhibition
	Mouse	Human	
KIR		X	I/A
Ly49	X		I/A
NKG2A	X	X	I
NKp46	X	X	A
CD16	X	X	A
NKG2C	X	X	A
NKG2D	X	X	A
DNAM-1	X	X	A
2B4	X	X	I (mice)/A (human)

NK cells utilize integrins, such as CD11b and CD18, and DNAX accessory molecule (DNAM)-1 to adhere to target cells and initiate the formation of an immune synapse, as well as migrating to inflammatory sites (191, 194). The immune synapse engages both activating and inhibiting receptors expressed on NK cells (194). Activating receptors include natural cytotoxicity receptors (such as NKp46, members of the C-type lectin-like receptor family (including NKG2C and NKG2D), the signaling lymphocyte activation molecule (SLAM) family receptor (2B4) in humans, and members of the TNF receptor family (such as CD27) (194). Inhibiting receptors, on the other hand, include killer-cell immunoglobulin-like receptors (KIR) in humans and their murine counterparts Ly49, 2B4 in mice, and the C-type lectin like receptor NKG2A (194). Of note, certain KIRs and Ly49s can activate NK cells upon their interaction with their respective ligands (195). Upon ligation of activation receptors, secretory granules containing granzymes and perforin or death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL) and FasL, migrate to the immune synapse (35). These secretory granules then merge with the outer membrane of the NK cell, releasing their cytotoxic contents in a unidirectional manner, or expressing TRAIL and FasL on NK cell surfaces to induce cell death (35). This process is accompanied by transient expression of CD107a, or lysosomal-associated membrane protein (LAMP)-1, on the outer NK cell membrane (196). Inhibitory signals effectively dampen secretory granule loading and fusion to ensure tolerance of healthy cells expressing MHC molecules (35). The release of cytolytic proteins or the ligation of death receptors will induce apoptosis or pyroptosis in target cells, promoting either inflammation or its resolution (35). However, studies have suggested that gap junction formation between the NK cells and their targets is required to induce calcium influx into the target cells and their subsequent apoptosis (197). Additionally, NK cells are capable of degranulating and expressing CD107a without gap junction formation and subsequent apoptosis induction (197).

NK cells and neutrophils are both important players in innate immune responses and engage in crosstalk, thereby forming a regulatory arm of the immune system (148). Human neutrophils downregulate their MHC molecule expression and become more susceptible to NK cell-induced cell death via the ligation of NKp46 and FasL (36, 37, 198). In turn, NK cells modulate the effector functions of neutrophils and increase their expression of CD11b, CD64, CD62L, and CD69 (148, 199). Moreover, both human and mouse neutrophils can affect several aspects of NK cell functionality, such as cytotoxicity, migration, cytokine production, growth factor production, survival, and proliferation through their release of soluble mediators and expression of surface markers (148, 199). It is important to note that human neutrophils can limit NK cell cytotoxicity by cleaving NKp46 from their surfaces (200). In addition, certain cytokine-activated human NK cells provide essential survival cues for neutrophils while preserving their functional capacity *ex vivo* (201, 202). Finally, it is now well-established that binding of antibodies provides a sufficient activatory signal for NK cells to conduct antibody-dependent cellular cytotoxicity (203).

NK cells play a crucial role in the amelioration and progression of several chronic inflammatory diseases due to their ability to produce high levels of cytokines (204). They produce several pro-inflammatory cytokines, including IFN-γ, TNF-α, GM-CSF, and IL-32 (204). Additionally, they secrete chemokines like CCL3-5, which enhance the recruitment of other immune cells (204). However, excessive IFN-γ production has been associated with various inflammatory degenerative diseases, such as MS, RA, SLE, and sepsis (204). Moreover, dysregulated cytotoxic function of NK cells is observed in peripheral blood of patients suffering from chronic inflammatory diseases (204).

NK cells can bridge innate and adaptive immune responses (205). They migrate to lymph nodes in a CCR7- (human), CD62L- (human), or CXCR3-dependent (mice) manner to produce essential cytokines for T and B cells to mount specific adaptive responses (175, 206). IFN-γ produced by murine NK cells in lymph nodes enhances immunoglobulin production by B cells and modulates affinity maturation in germinal centers (206) and polarize T cells to T_H1 cells in draining lymph nodes (175).

Finally, NK cell function is impaired by lipids and their mediators (207). NK cells express lipid scavenging receptors such as macrophage scavenger receptor (MSR)-1, CD36, and CD68, thereby promoting lipid accumulation and dampening their cytotoxic function in mice (207). Metabolic reprogramming of NK cells in patients with obesity impairs the antitumor responses possibly through lipid accumulation in their NK cells (208). Eicosanoids, such as PGE_2, produced by tumor cells effectively dysregulate NK cell responses through EP2 and EP4 signaling, leading to dampened cytokine production and cytotoxic function in mice (92). Although the role of lipid metabolism in NK cells is not fully established, preliminary studies suggests that oxidative phosphorylation and glycolysis are important regulators of their responses (209).

1.3.4 NK cells in inflammation resolution

The precise role of NK cells in resolution of inflammation is not yet well-defined, despite their well-established role in the regulation of acute inflammation. In response to pulmonary inflammation, NK cells are recruited to the lungs by RvE_1 and its binding of chemerin receptor 23 (ChemR23), where they act as resolution effector cells and promote eosinophilic inflammation resolution (118). Similarly, NK cells are recruited by LXA_4 to promote the resolution of eosinophilic inflammation after nasal allergen challenge (210). In patients with severe asthma, dysfunctional NK cells with impaired migratory patterns can have their cytotoxic functions rescued by LXA_4 to ameliorate the clinical symptoms (211). NK cells also play a crucial role in the resolution of murine DSS-induced colitis, by the involvement of annexin A1 and NKG2A (212). Recently, dysregulated NK cell-mediated induction of neutrophil apoptosis prevented resolution of inflammation and stem cell-mediated regeneration following murine volumetric muscle loss (213). In addition, previous research conducted by our research group demonstrated that NK cell depletion impairs resolution of murine antigen-induced peritonitis by causing a continuous influx of neutrophils (214). Collectively, these studies illustrate the integral role of NK cell function in regulating and promoting the resolution of inflammation. However, the precise mechanism by which NK cells exert these functions remains unclear.

2 Aims

The overall goal of this study was to establish the impact of dietary omega-3 PUFAs on inflammation resolution, NK cell recruitment, their phenotype, and immunoregulatory functions in murine inflammation, as well as the effects of PUFAs on human NK cells, their phenotype, crosstalk with neutrophils, and lipid mediator synthesis.

Specific aims:

1. To determine the effects of dietary omega-3 PUFAs on resolution of antigen-induced peritonitis.
2. To investigate the effects of dietary omega-3 PUFAs on NK cell recruitment, phenotype, and function in antigen-induced peritonitis.
3. To establish the effects of omega-3 PUFAs on human NK cell effects on and crosstalk with neutrophils.
4. To ascertain whether NK cells have the capacity to produce SPMs in collaboration with neutrophils.

3 Materials and Methods

3.1 Mice

Female C57Bl6/J mice, aged 6-8 weeks, were obtained from Taconic Europe A/S (Denmark) and housed in the animal facilities at ArcticLAS ehf (Iceland). They were allowed to acclimatize for 7 days prior to initiation of experiments. Mice were provided with diet and water *ad libitum* and were kept in a 12-hour light/dark cycle. Each cage contained a maximum of 10 mice per cage. All mouse experiments were conducted in accordance with the ethical approval granted by the Icelandic Food and Veterinary Authority (MAST, approval #2017-01-04).

3.2 Diets

Following the 7-day acclimatization period, the mice were randomly assigned either a westernized control diet or the same diet enriched with fish oil (Research diets, inc., New Jersey, USA). The fish oil enriched diet contained 2.8% menhaden fish oil (Omega Protein, Virginia, USA), which was added at the expense of safflower oil, resulting in an omega-6:omega-3 ratio of 1.3. To ensure comparable AA content in both diets, AAethyl ester (Nu-Check-Prep, Minnesota, USA) was added to the control diet, which resulted in an omega-6:omega-3 ratio of 11.8. Table 2 provides an overview of the nutritional content of the two diets, while Table 3 shows their respective fatty acid compositions.

Table 2. Composition of the control and fish oil diet.

	Control	Fish oil
Ingredient*	**g/kg**	
Casein	228	229
L-Cystein	3.4	3.4
Corn Starch	274.1	274.2
Maltodextrin 10	85.7	85.7
Sucrose	114.2	114.3
Cellulose	57.1	57.1
Cocoa butter	42.8	42.8
Linseed oil	0	0
Flaxseed oil	5.1	5.1
Palm oil	60	60
Safflower oil	32.5	4.6
Sunflower oil, Trisun	0	0

Sunflower oil, high oleic	30.8	30.9
Arachidonic acid	0.5	0
Menhaden fish oil	0	28
t-BHQ	0.03	0.03
Mineral Mix	11.4	11.4
Di Calcium Phosphate	14.8	14.9
Calcium Carbonate	6.3	6.3
Potassium Citrate	18.8	18.9
Choline Bitatrate	2.3	2.3
Vitamine E Acetate	0.15	0.15

*Information as provided by the manufacturer. Changes in diets shaded with grey.

Table 3. Fatty acid composition of control and fish oil diets.

		Control	Fish oil
Fatty acid*		**g/kg**	
C2	Acetic	0	0
C4	Butyric	0	0
C6	Caproic	0	0
C8	Caprylic	0	0
C10	Capric	0	0
C12	Lauric	0.2	0.2
C14	Myristic	0.5	2.4
C14:1	Myristoleic	0	0
C15		0	0.1
C16	Palmitic	36.8	39.1
C16:1	Palmitoleic	0.1	2.9
C16:2		0	0.4
C16:3		0	0.4
C16:4		0	0.4
C17		0	0.1
C17:1		0	0
C18	Stearic	19.3	19.4
C18:1	Oleic	69.8	69.1
C18:2	Linoleic	37.5	16.1
C18:3	Linoleleic	3.2	3.6
C18:4	Stearidonic	0	1.3
C20	Arachidic	0.7	0.8
C20:1		0	0.4
C20:2		0	0.1
C20:3		0	0.1

C20:4	Arachidonic	0.5	0.6
C20:5	Eicosapentaenoic	0	4
C21:5		0	0.2
C22	Behenic	0	0
C22:1	Erucic	0	0.1
C22:4	Clupanodonic	0	0.1
C22:5	Docosapentaenoic	0	0.8
C22:6	Docosahexaenoic	0	2.9
C24	Lignoceric	0	0.2
C24:1		0	0.1
Total		168.6	165.9
Saturated (g)		57.4	62.3
Monounsaturated (g)		69.9	72.5
Polyunsaturated (g)		41.2	31
Total omega-6 (g)		38	16.9
Total omega-3 (g)		3.2	12.8
P:S ratio		0.7	0.5
Omega-6:omega-3		11.8	1.3

*Information provided by the manufacturer.

The diets were prepared in bags of 60-gram portions, enough for 10 mice for one day. Prior to storage at -20°C, each bag was briefly flushed with nitrogen gas and then sealed to avoid lipid autoxidation. Fresh diet was provided for the mice daily, and any leftover diet was weighed and recorded to monitor their diet consumption (see Appendix I for diet intake). The mice were weighed every other day for the duration of each experiment. There were no differences observed between the dietary groups in the weight of the mice during the experiments (Appendix I).

3.3 Antigen-induced peritonitis

To ensure that dietary lipids were incorporated into cellular membranes, the mice were fed either control or fish oil-enriched diet for one week prior to the first immunization. Mice were immunized subcutaneously (s.c.) with 100 µg methylated bovine serum albumin (mBSA) (Sigma-Aldrich, Germany) emulsified in complete Freund's adjuvant (CFA) (Sigma-Aldrich) in a total volume of 50 µL in their right flank. Two weeks later, a booster immunization was administered s.c. in the opposite flank with 100 µg mBSA emulsified in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich) in a total volume of 50 µL. Three weeks after the first immunization, the mice were challenged with 100 µg mBSA resuspended in saline (total volume 100 µL) via intraperitoneal (i.p.) injection to induce peritonitis. The mice were anesthetized and sacrificed at 0, 1.5, 3, 6, and 12 h post-challenge by isoflurane overdose.

3.3.1 Collection of peritoneal exudate

To collect peritoneal exudate (fluid and cells), 1 mL of ice-cold phosphate buffered saline (PBS) was injected i.p. followed by gentle massage of the abdomen. In most cases, peritoneal cells and fluid were separated by centrifugation. The peritoneal fluid was stored at -80°C until needed for enzyme-linked immunosorbent assays (ELISAs) or Luminex assays. The peritoneal cells were washed twice in PBS, counted using a Countess automated cell counter (Invitrogen, United Kingdom), and resuspended in fluorescence associated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin (BSA), 0.01% NaN_3). For liquid chromatography with tandem mass spectrometry analysis, peritoneal exudate was snap-frozen in liquid nitrogen and stored at -80°C until needed.

3.3.2 Collection of tissues

Mesenteric lymph nodes and spleens were surgically removed post-mortem and placed in ice-cold PBS. To preserve the tissues, lymph nodes were immersed in optimal cutting temperature (OCT) compound (Sakura Finetek Europe B.V, Denmark), snap frozen and stored at -80°C until needed.

3.4 Preparation of fatty acids

Peroxide-free AA, EPA, and DHA in ethanol were obtained from Cayman Chemical (Michigan, USA) in single-use ampules. The fatty acids were dried down under a steady stream of nitrogen gas, resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich), flushed with nitrogen gas, and finally stored in 5 µL aliquots at -80°C. Prior to use, the fatty acids were diluted to a concentration of 2 mM (0.6 µg/µL) in Rosswell Park Memorial Institute (RPMI) medium (Gibco, Thermo Fisher Scientific, United Kingdom) enriched with 10% fetal bovine serum (FBS, Gibco) and Penicilin/Streptavidin (Gibco) (complete RPMI medium) and allowed to incubate at room temperature for one hour to allow binding of fatty acids to albumin.

3.5 Cell isolation and culture

Human blood cells were isolated from either buffy coat obtained at the Icelandic Blood Bank or fresh venous ethylenediaminetetraacetic acid (EDTA) blood from healthy volunteers at the out-patient department at Landspitali – the National University Hospital of Iceland. For neutrophil isolation isolation, fresh venous blood was allowed to stand for one hour at room temperature prior to their isolation.

Informed consent was obtained from all blood donors in accordance with the guidelines for ethical research described by the Directorate-General for Research and Innovation of the European Commission. Permission was granted by the National Bioethics Committee of Iceland, (# 06-068-V1).

3.5.1 Natural killer cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats diluted in PBS and layered over Histopaque-1077 (Sigma-Aldrich). After centrifugation with minimal acceleration and no brake at 400G, the PBMC-rich layer was carefully collected. Next, PBMCs were washed three times in magnet associated cell sorting (MACS) buffer (0.5% BSA, (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich) in PBS), counted by Countess automated cell counter, and resuspended at a cell density of 25 x 10^7 cells/mL.

NK cells were isolated from the PBMC suspension using a negative selection kit following the manufacturer's directions (Miltenyi Biotec, Germany). Briefly, PBMCs were incubated with a biotinylated antibody mix, followed by the addition of streptavidin-coated magnetic beads. Non-NK cells i.e., T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells, were retained from NK cells by passing the bead-cell mixture through a magnetic field of a MACS Separator® (Miltenyi Biotec) using an LS^+ MACS Column® (Miltenyi Biotec). Following negative selection, the purified NK cells were counted using a Countess automated cell counter, pelleted, and resuspended in complete RPMI medium to a cell density of 1 x 10^6 cells/mL for culturing experiments. For protein isolation, NK cells were pelleted and resuspended in ice-cold RIPA buffer containing protease and phosphatase inhibitors (all from Thermo Fisher Scientific) and stored at -80°C. The purity of isolated NK cells was determined by flow cytometry and was ~97% (fig. 6).

3.5.2 Neutrophil isolation

Due to their short half-life of ~4-18 hours *ex vivo* (215), neutrophils were isolated from fresh EDTA venous blood obtained from healthy volunteers, instead of a buffy coat. Histopaque-1077 (Sigma-Aldrich) was carefully layered on Histopaque-1119 (Sigma-Aldrich) and venous blood was layered on top of the two Histopaque solutions. Following centrifugation with minimal acceleration and no brake at 400 G, the granulocyte-rich layer was collected. Any remaining contaminating erythrocytes were pelleted using 3% dextran (Sigma-Aldrich) and lysed using ammonium-chloride-potassium (ACK) lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 M Na_2EDTA in PBS). Granulocytes were then washed twice with Hanks' balanced saline solution (HBSS) without Ca^{2+} or Mg^{2+} (Gibco) and counted manually using a disposable Neubauer improved C-Chip (NanoEnTek, Massachusetts, USA). Neutrophil purity was evaluated by flow cytometry and was determined to be ~98% (fig. 6). Finally, neutrophils were resuspended in complete RPMI medium to a cell density of 2 x 10^6 cells/mL.

3.5.3 Cell culture

NK cells were cultured in either 48-well or 96-well culture plates (Nunc, Thermo Fisher Scientific) or in 24-well Costar culture plates with Transwell® permeable 6.5 mm inserts with a 0.4 µm polyester membrane (Corning Incorporated, Maine, USA), at a cell density

of 1 x 10^6 cells/mL. AA, EPA, or DHA was added at a final concentration of 50 µM or 15 µg/mL (AA-NK cells, EPA-NK cells, or DHA-NK cells). To serve as a solvent control, an equal volume of DMSO was added (C-NK cells), resulting in a final concentration of DMSO of 0.09%. The cultures were incubated for 18 hours at 37°C, 5% CO_2, and 95% humidity to allow incorporation of fatty acids into cellular membranes. Subsequently, the NK cells were stimulated with human recombinant IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) (stimulation cocktail, all from R&D Systems, Bio-Techne, United Kingdom) for up to 24 hours.

To allow for direct cell-cell contact, freshly isolated neutrophils were sometimes added to the NK cell cultures along with the stimulation cocktail at a 1:2 ratio of NK cells to neutrophils. Alternatively, for transwell co-culturing, neutrophils were added in the bottom chamber with the stimulation cocktail, while NK cells were added and pre-incubated in the upper compartment, in the same ratio and cell densities as with co-cultures without transwells. The co-cultures were incubated at 37°C, 5% CO_2, and 95% humidity for up to 24 hours. At different time-points specified throughout the results section, the cells and supernatants were sollected. The cells were stained for surface molecules and their expression evaluated by flow cytometry. Supernatants were collected and stored at -80°C.

To determine the production of lipid mediators by NK cells, alone or in conjunction with neutrophils, co-culturing experiments were performed as described above in 48-well culture plates (Nunc). In short, NK cells were pre-incubated with fatty acids or DMSO as a control for 18 hours at 37°C, 5% CO_2, and 95% humidity. Stimulation cocktail was added to the NK cells either alongside freshly isolated neutrophils at a ratio of 1:2 or without neutrophils. Cell cultures were incubated for an additional 18 hours at 37°C, 5% CO_2, and 95% humidity, before the cells were harvested and immediately flushed with liquid nitrogen and subsequently stored at -80°C. As a control for autoxidation, fatty acids in complete RPMI medium were added to in culture plates without cells and incubated for 18 hours, stimulation cocktail added, and the plates incubated for an additional 18 hours at 37°C, 5% CO_2, and 95% humidity before harvesting the fluid and flushing it with liquid nitrogen and storing the samples at -80°C.

3.6 Flow cytometry

Fc-receptors on murine peritoneal cells were blocked by incubating the cells for 20 minutes with a mixture of normal rat, normal mouse, and normal hamster serum in the ratio of 1:0.5:0.5 (AbD Serotec, BioRad, United Kingdom) at a concentration of 2%. The murine peritoneal cells were subsequently stained with fluorochrome-labelled monoclonal antibodies against a variety of antigens as specified in table 4. Representative gating strategies for murine NK cells and neutrophils are specified in figure 5. Murine NK cells are defined as $CD3^-NK1.1^+CD49b^+$ lymphocytes and murine neutrophils are

defined as CXCR2^{+}Ly6G^{+} granulocytes.

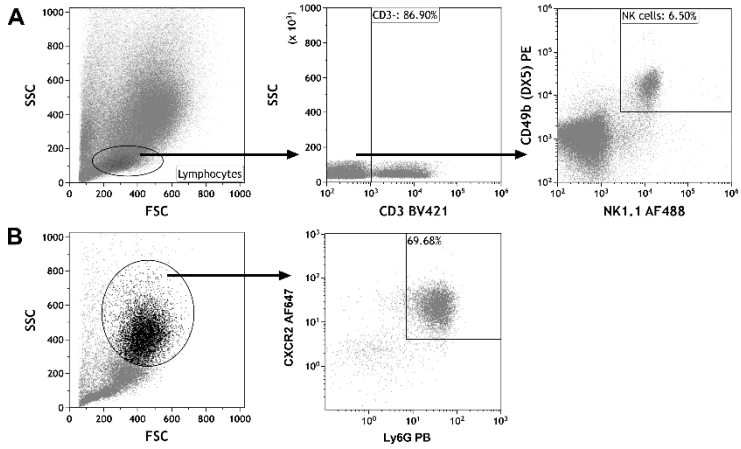

Figure 5. Gating startegy on peritoneal cells for detection of murine NK cells and neutrophils. Mice were fed control or fish oil diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells were isolated 6 h after inflammation induction and stained with monoclonal antibodies against CD3, CD49b, NK1.1, CXCR2, and Ly6G and evaluated by flow cytometry. **A**) NK cells live gated on lympocytes on their side scatter (SSC) and forward scatter (FSC) properties, negatively gated for CD3 brilliant violet 421 (BV421), and finally detected as NK1.1 alexa fluor (AF)488 and CD49b (DX5) phycoerythrin (PE) double-positive cells. **B**) Neutrophils live gated on their SSC and FSC properties and detected as double-positive for CXCR2 AF647 and Ly6G pacific blue (PB).

Table 4. Antibodies against murine antigens used in flow cytometry in mouse experiments.

Antigen	Fluorophore	Clone	Manufacturer
NK1.1	AF488	PK136	*Biolegend*
CD49b	PE	DX5	*Biolegend*
CD3	BV421	17A2	*Biolegend*
CD11b	AF700	M1/70	*Biolegend*
CD27	PE/Cy7	LG.3A10	*Biolegend*
NKG2A	PE/Cy7	16A11	*Biolegend*
CD62L	AF700	MEL-14	*Biolegend*
2B4	AF647	M2B4	*Biolegend*
CD69	PE/Cy7	H1.2F3	*Biolegend*
CXCR2	AF647	SA044G4	*Biolegend*
Ly6G	AF700	1A8	*Biolegend*

CD47	FITC	Miap301	*Biolegend*
TRAIL	AF647	D-3	*Santa Cruz Biotechnology*
CD107a	AF647	H4A3	*Santa Cruz Biotechnology*
LRP6	AF647	C-10	*Santa Cruz Biotechnology*
FasL	PE/Cy7	MFL3	*Invitrogen*
NKp46	AF700	29A1	*Invitrogen*
CCR5	AF700	C6	*R&D Systems*

To limit the background staining of human cells, we first blocked their Fc-receptors were blocked by incubating the cells for 20 minutes with a mixture of normal human and normal mouse serum (AbD Serotec) as well as TruStain FcX™ (Biolegend) at a concentration of 2% and 5%, respectively. Human cells were then stained with fluorochrome-labelled monoclonal antibodies against surface molecules specified in table 5. The gating strategies for identifying human NK cells and neutrophils are illustrated in figure 6. Human NK cells are defined as $CD3^{-}CD56^{+}$ lymphocytes and human neutrophils are defined as $CD49d^{-}CD16a^{+}CD62L^{+}$ granulocytes.

Table 5. Antibodies used for human *in vitro* studies for flow cytometry.

Antigen	Fluorophore	Clone	Manufacturer
CD11b	AF488	M1/70	*Biolegend*
CD62L	AF700	DREG-56	*Biolegend*
NKp46	AF700	9E2	*Biolegend*
CD16a	APC	eBioCD16	*Invitrogen*
CD56	PE	CMSSB	*Invitrogen*
CCR7	PE	G043H7	*Biolegend*
CD3	BV421	OKT3	*Biolegend*
CD49d	BV421	9F10	*Biolegend*
NKG2D	PE/Cy7	1D11	*Biolegend*
CX3CR1	PE	K0124E1	*Biolegend*
NKG2A	PE	DX22	*Biolegend*
CD27	PE/Cy7	O323	*Biolegend*
CD47	PE/Cy7	CC2C6	*Biolegend*
CXCR3	PE/Cy7	G025H7	*Biolegend*
CD16	AF700	3G8	*Bioolegend*
TIGIT	AF647	A15153G	*Biolegend*
CD107a	FITC	H4A3	*Biolegend*

2B4	FITC	C1.7	*Biolegend*
DNAM-1	PE/Cy7	11A8	*Biolegend*

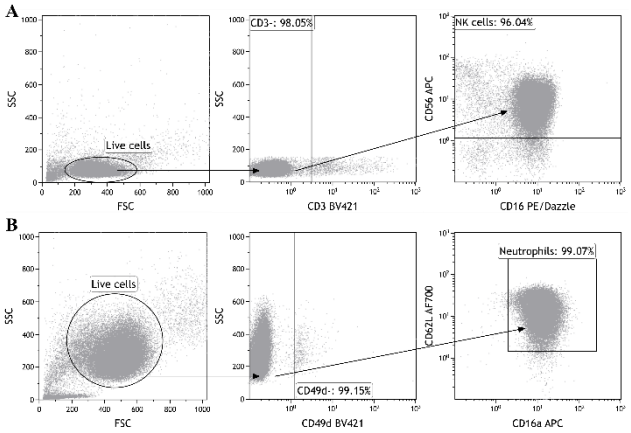

Figure 6. Gating strategy for detection of human NK cells and neutrophils from blood.
Freshly isolated NK cells and neutrophils from buffy coat or EDTA blood, respectively, were stained with monoclonal antibodies against CD3, CD56, CD16, CD49d, CD62L, and CD16a and evaluated by flow cytometry. **A**) NK cells were live gated on their SSC and FSC properties, negatively gated on CD3 BV421, and detected as CD56 APC positive lymphocytes. NK cells were further phenotyped based on their expression of CD16 PE-Dazzle and the level of CD56 APC expression. **B**) Neutrophils were live gated on their SSC and FSC properties, negatively gated on CD49d BV421, and detected as CD16a APC and CD62LAF700 double positive granulocytes.

To evaluate surface marker expression, cells were incubated with fluorochrome-labelled antibodies in FACS buffer (3.8 mM sodium azide, 0.5% BSA, and 2 mM EDTA in PBS) and subsequently pelleted and washed. Murine cells were immediately resuspended in FACS buffer and surface marker expression evaluated by flow cytometry on a Sony SH800S Cell Sorter (Sony Biotechnology, United Kingdom). Human cells were fixed using 2% paraformaldehyde (Sigma-Aldrich) and kept at 4°C for a maximum of 24 hours. Then cells were evaluated by flow cytometry on a Navios EX flow cytometer (Beckman Coulter, Indianapolis, USA) or a Sony SH800S Cell Sorter.

We used two different methods to evaluate neutrophil function in the co-cultures. In some cases, 10 µM dichlorofluorescein diacetate (Abcam, United Kingdom) were added to the co-cultures after 16.5 hours. The cells were then incubated for an additional 90 minutes at 37°C, 5% CO_2, and 95% humidity. In other instances, fluorescein-labelled *E. coli* (Abcam) was added to co-cultures 4 h after initiation and allowed to incubate for another 2 hours at 37°C, 5% CO_2. Cells were harvested, pelleted, and washed before being resuspended in FACS buffer and evaluated on a Sony SH800S Cell Sorter.

Live cells were identified based on their forward and side scatter. For both human and murine samples, a minimum of 50,000 cells were recorded in the NK cell gate. Surface marker expression on cells were evaluated using the Kaluza Analysis Software (Beckman

Coulter). Results are expressed as percentage of positive cells as determined with isotype controls, or as mean fluorescent intensity (MFI), which reflects the level of expression.

3.7 Liquid chromatography with tandem mass spectrometry

Samples were prepared for liquid chromatography with tandem mass spectrometry (LC-MS/MS) by transferring them to a 12-mL glass vial and adding 3 x the sample volume of methanol (LC-MS grade, Sigma Aldrich) to peritoneal lavage or cell cultures. Next, 4 µL internal standard solution (LTB4-d4, 15(S)-HETE-d8, PGE2-d4 and DHA-d5, all 50 ng/mL in methanol, Cayman Chemicals) were added to the samples. They were subsequently vortexed and centrifuged at 4°C, 3,190 x g for 3 min. To repeat the extraction process, 500 µL methanol were added to organic extracts and combined in a 7 mL glass vial. Some of the methanol was evaporated at 40°C for approximately 45 minutes under a gentle stream of nitrogen. Water was subsequently added to the samples to ensure that the methanol content did not exceed 20%. Subsequently, 9 mL water and 20 µl of 6 M formic acid (Sigma-Aldrich) was added to acidify the samples before loading them onto C18 solid phase extraction cartridges (Sep-Pak, Waters, Milford, Massachusetts). The cartridges were washed with 3 mL water and 3 mL n-hexane and eluted with 3 mL methyl formate (Sigma-Aldrich). Extracts were dried down under a gentle stream of nitrogen at 40°C and reconstituted in 200 µL methanol (40%, Sigma Aldrich). Then, the extracts were vortexed and transferred with a micro-insert to a glass auto-sampler. Finally, the samples were then stored at -80°C until analysis.

A QTrap 6500 mass spectrometer operating in negative ESI mode (Sciex, Nieuwerkerk aan den IJssel, The Netherlands) was used for LC-MS/MS analysis, coupled to an LC system employing two LC-30AD pumps, a SIL-30AC autosampler, as well as a CTO-20AC column oven (Shimadzy, 's-Hertogenbosch, The Netherlands). A Kinetex C10 50x2.1 mm column (1.7 µm) protected with a C8 precolumn (Phenomenez, Utrecht, The Netherlands) was used, that was subsequently maintained at 50°C. To create a binary gradient water (A) and methanol (B) containing 0.01% acetic acid was used in the following manner: 30% B at zero minutes, held at one minute, then ramped up to 45% B at 1.1 minute, 53.5% B at two minutes, 55.5% B at four minutes, 90% B at 7 minutes, and 100% B at 7.1 minutes, and held for an additional 1.9 minutes. A volume of 40 µL was injected at a flow rate of 400 µL/minute. Mass transition for each analyte combined with its relative retention time was used for analyte identification. Calibration lines constructed with standard material were used for quantification for each analyte, and peaks with a signal to noise ratio >10 were quantified. Further detail regarding the LC-MS/MS settings can be found in (216).

3.8 Staining of tissue

3.8.1 Tissue preparation

Mesenteric lymph nodes were cryosectioned at 7 µM using a CM1950 cryostat (Leica Biosystems, Germany). The slides were air-dried overnight and kept at -80°C until needed. Prior to staining, the sections were removed from -80°C and allowed to equilibriate to room temperature for 1 minute before staining procedures were initiated.

3.8.2 Immunofluorescent stainings

Cryosections of mesenteric lymph nodes were stained for apoptosis using the "TACS®2 TdT-Fluorescein In Situ Apoptosis Detection Kit" (TUNEL, Trevigen, Bio-Techne, United Kingdom) following the manufacturer's instructions. The cryosections were rehydrated with 100%, 95%, and 70% ethanol and subsequently fixed in 3.7% PBS buffered formaldehyde (Sigma-Aldrich). Following a wash, the sections were covered with Cytonin™ (Trevigen) to permeabilize the cells and incubated at room temperature for 30 minutes. The labeling process was initiated by immersing the sections in 1xTdT labeling buffer (Trevigen) at room temperature for 5 minutes. Next, the sections were then covered with the labeling reaction mix (TdT dNTPs, Co^{2+} cation, and TdT enzyme in 1xTdT labeling buffer, Trevigen). Negative control slides were generated by omitting the TdT enzyme from the labeling reaction mix, while positive controls were generated by treating one sample with TACS-nuclease to induce DNA breaks. The reaction was stopped immersing sections in TdT Stop buffer (Trevigen) for 5 minutes at room temperature. Finally, fluorescein-labelled streptavidin (Trevigen) was added to sections before the slides were mounted with Fluorescent Mounting Medium (Trevigen) and stored at 4°C in the dark until imaged.

3.8.3 Image analysis

The stained sections were imaged using EVOS FL Auto 2 Microscope with 4x, 10x, and 40x magnification. The 4x magnification images of H&E stainings were used to evaluate tissue integrity and cellular architecture.

Fluorescently labeled sections were imaged after adjusting the exposure to minimize background created from the unstained control, unspecific binding by secondary antibody (negative control), and confirming the signal (positive control). For each mouse, three independent lymph node stainings were imaged. The images were evaluated using the ImageJ Software version 1.8.0 (National Institutes of Health, Maryland, United States). $TUNEL^+$ cells were counted using the particle count function in ImageJ after transforming the picture into 8-bit grayscale pictures and correcting for cellular size. To account for anatomical variations in the tissues, an average of the three independent countings was used.

3.9 ELISA and Luminex

To determine peritoneal concentrations of various murine proteins and cytokines, we used the Luminex Customized Assay (R&D Systems) in accordance with the manufacturer's instructions. The analytes included in the assays were adiponectin, angiopoietin-2, B cell-activating factor (BAFF), CCL3, CCL4, CCL5, CCL7, CCL20, chitinase-3-like protein 1 (CHI3-L1), C-reactive protein (CRP), CXCL1, CXCL2, CXCL10, CXCL12, dickkopf-1 (DKK-1), dipeptidyl peptidase (DPP)IV, endoglin, FasL, granulocyte colony stimulating factor (G-CSF), growth/differentiation factor (GDF)-15, GM-CSF, granzyme B, haptoglobin, IFN-γ, IGF-1, IL-1α, IL-1β, IL-5, IL-6, IL-6Rα, IL-10, IL-13, IL-17A, IL-27, IL-33, LPS-induced CXC chemokine (LIX), macrophage colony stimulating factor (M-CSF), metallomatrix proteinase (MMP)-8, MMP-9, receptor for advanced glycosylation endproducts (RAGE), receptor activator of NF-κB ligand (RANKL), resistin, S100A9, syndecan-1, tissue inhibitor of metalloproteinase (TIMP)-1, sTNF RI, sTNF RII, TNF-α, and TNF-like weak inducer of apoptosis (TWEAK).

Concentrations of murine TGF-β and IL-6 in peritoneal fluid were determined using DuoSet ELISA kits (R&D Systems) following the manufacturer's instructions. Concentrations of human TNF-α, IFN-γ, IL-8 (CXCL8), CXCL10, IL-1ra, GM-CSF, and CCL3 in cell culture supernatants were determined using DuoSet ELISA kits (R&D Systems) following the manufacturer's instructions.

3.10 SimpleWestern ImmunoAssay

3.10.1 Protein isolation and concentration determination

NK cells were harvested and resuspended in ice-cold PBS containing protease and phosphatase inhibitors and EDTA (Thermo Fisher Scientific), followed by two repetitions to eliminate carryover of BSA and other proteins from the cell culture. The NK cells were then lysed in ice-cold radio-immunoprecipitation (RIPA) buffer (Thermo Fisher Scientific) containing EDTA, protease and phosphatase inhibitors (all from Thermo Fisher Scientific) and kept on ice for 45 minutes with occasional swirling and sonication. The supernatant containing protein was obtained by centrifugation at 14,000xg for 15 minutes at 4°C and stored at -80°C.

To determine the protein concentrations, samples were thawed on ice and diluted 1:40 due to the high signal created by the RIPA buffer in Pierce™ Coomassie Plus (Bradford) assays (Thermo Fisher Scientific). Standard curves were prepared using 1:40 diluted RIPA buffer and concentrated serum albumin standards (Thermo Fisher Scientific), ranging from 100 – 1500 µg/mL. Ten µl of samples/standards were pipetted into appropriate wells with 300 µl of Coomassie Plus reagent (Thermo Fisher Scientific), mixed on a plate shaker for 30 seconds, and incubated in the dark for 10 minutes at room temperature. Absorbance was measured at 595 nm using a Multiskan™ FC microplate photometer (Thermo Fisher Scientific). Protein concentration was determined

by subtracting blank measurements from the absorbance and imposing the absorbance on the standard curve using the SkanIt 5.0 Software (Thermo Fisher Scientific).

3.10.2 SimpleWestern ImmunoAssay

Simple Western immunoassays (SimpleWes assays) automate Western blotting workflows from protein separation to analysis of data using a capillary-based system. In short, a 12-230 kilodalton (kDa) detection module detection module (ProteinSimple, Bio-Techne, United Kingdom) were used to detect 5-, 12-, and 15-LOX (all from Abcam). Protein was diluted in 0.1xSample Buffer (ProteinSimple) and combined with 5X Fluorescent Master Mix to a final concentration of 0.4 mg/mL. Samples were denatured, vortexed, centrifuged and stored on ice. Primary antibodies were diluted in Antibody Diluent 2 (ProteinSimple) and stored on ice until needed. Secondary horseradish peroxidase (HRP) conjugated antibodies were supplied ready-to-use by ProteinSimple.

In well A1 (fig. 7) of the 12-230 kDa SimpleWes assay plate, 5 µL biotinylated ladder was dispensed, while at least 3 µL diluted, heat-inactivated sample was dispensed into the remaining well in row A (fig. 7). Ten µL of Antibody Diluent 2 were added into row B and well C1 (fig. 7) as a blocking agent. Then, 10 µL of primary antibody mix was dispensed into the remaining wells in row C (fig. 7). To visualize the ladder 10 µL of Streptavidin-HRP were dispensed into well D1 (fig. 7). In the remainder of row D 10 µL secondary antibody were dispensed (fig. 7). All wells in row E contained a 15 µL Luminol-S and Peroxide mix (fig. 7). The assay plate was centrifuged for 5 minutes at 2500 rpm at room temperature and 500 µL wash buffer was added to the top three wash buffer wells (fig. 7).

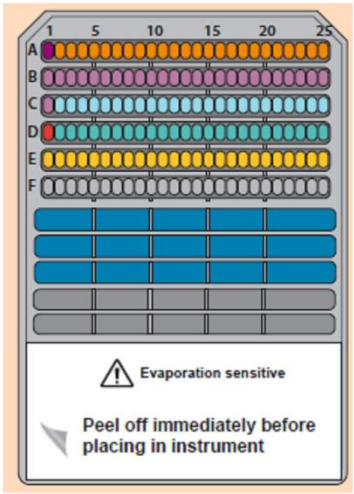

Figure 7. Schematic illustration showing outline of the 25 capillary assay plate used in 12-230 kDa SimpleWes chemiluminescent assays.

The Compass software (ProteinSimple) was used to design the assay and specify the parameters. The capillary cartridge was inserted into the holder (fig. 8) and the evaporation seal of the assay plate was peeled off (fig. 7). Any air bubbles were removed, and the assay was started when the Compass Software indicated readiness. Proper loading of sample protein was visually confirmed with internal standards after 30 minutes. The data was exported and analyzed using the Compass software. The SimpleWes assay data are reported in Western Blot lane views in the present thesis.

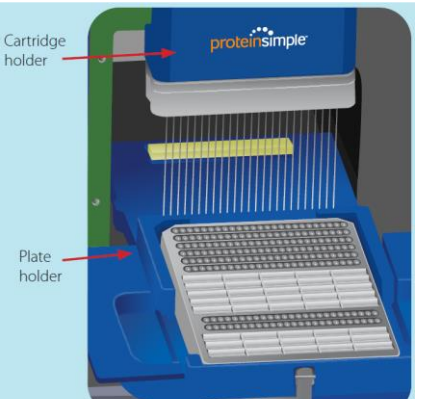

Figure 8. Schematic illustration of the setup of the separation cartridge and the capilaries in the corresponding holders of the Jess®.

3.11 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). All *in vitro* experiments were replicated at least 3 times with samples from a minimum of 2 donors each time. All *in vivo* experiments were replicated at least 2 times with a minimum of 3 mice in each dietary group. Possible outliers were identified using the Grubbs' method with an α of 0.05, omitting one outlier per group, if appropriate.

Resolution interval (R_i) was calculated as previously determined (80). Shortly, it is the difference between the time at which the maximum number of infiltrating neutrophils peak (T_{max}) and the time at which the number of neutrophils has decreased by 50% (T_{50}) ($R_i = T_{max}-T_{50}$).

For *in vitro* studies, groups were compared using one-way or two-way ANOVA. For *in vivo* studies the effect of time in our data was determined using a two-way ANOVA. Comparisons of data between the groups at specific timepoints were determined using the Fisher's LSD test. Comparisons between data for only one timepoint were determined using a Mann-Whitney U-test. Differences were considered statistically significant when the p value was <0.05. Statistical analysis was carried out in GraphPad Prism 9 (GraphPad Software, San Diego, California, USA).

Exploratory statistics, including heatmaps, prcipal component analysis (PCA), loadings, and correlation matrices, were created on log2- or log10-transformed data in R version 4.2.2 (R Core Team (2022). R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. URL https://www.r-project.org/) and RStudio version 2022.7.2.576 (RStudio Team (2022). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/).

4 Results

4.1 Section I: The effects of dietary fish oil on key hallmarks of inflammation resolution (paper II, unpublished data)

Previous work conducted in our laboratory demonstrated that dietary fish oil limits neutrophil infiltration during and promotes resolution of antigen-induced peritonitis (80). However, as the composition of the experimental diet was changed by the manufacturer, it was necessary to replicate previous findings using the modified diet. Results from the latest observations were consistent with the previous ones. Mice fed the control diet exhibited a rapid increase in neutrophil numbers following induction of peritoneal inflammation, peaking at 6 h, and having a R_i of ~4 h (paper I, fig. 4D). In contrast, mice the the new fish oil diet demonstrated an earlier peak in neutrophil numbers (T_{max} = 3 h) and a shorter R_i (~3 h) (paper I, fig. 4D). These results were comparable to previous findings (80), thus, confirming the appropriateness of the modified diets. The new diet enriched with or without fish oil will therefore be called control and fish oil diet henceforth. Additionally, these results indicate that dietary fish oil enhances resolution of inflammation hallmark 1 by limiting neutrophil infiltration of the peritoneum in inflamed mice.

4.1.1 Dietary fish oil enhances peritoneal neutrophil apoptosis and attenuates their CD47 expression (paper I)

Neutrophil apoptosis in the peritoneal cavity was similar in the control and the fish oil groups prior to inflammation induction (paper I, fig. 4E). Following inflammation induction, the levels of neutrophil apoptosis rose in both groups from close to 0% to 5-10% 1.5 and 3 h (paper I, fig. 4E). Dietary fish oil increased neutrophil apoptosis 6 h after induction of inflammation compared to that in mice fed the control diet (paper I, fig. 4B,E). This increase coincided with the decreased peritoneal numbers of neutrophils in inflamed mice fed the fish oil diet compared to that in mice fed the control diet (paper I, fig. 4D).

Studies have shown that CD47 expression inhibit neutrophil apoptosis and subsequent efferocytosis (217, 218). Dietary fish oil attenuated neutrophil surface expression of CD47 compared to that on neutrophils from mice fed the control diet 6 h after inflammation induction (paper I, fig. 4C,F). Prior to the induction of inflammation, few peritoneal neutrophils expressed CD47, regardless of dietary treatment (paper I, fig. 4F). However, the proportion of $CD47^+$ neutrophils in the peritoneum increased to ~10% 1.5 and 3 h after inflammation was induced, regardless of dietary treatment (paper I, fig. 4F).

4.1.2 Dietary fish oil increases the number of apoptotic bodies in mesenteric lymph nodes (paper I)

Dietary fish oil increased the number of apoptotic cells in intrafollicular areas of mesenteric lymph nodes 3 and 6 h following inflammation induction (paper I, fig. 4G,H). The number of apoptotic cells in mesenteric lymph nodes was increased ~1.2-fold in mice fed the fish oil diet compared to that in mice fed the control diet (paper I, fig. 4H). This increase was even more pronounced at 6 h after induction of inflammation, where mesenteric lymph nodes from mice receiving dietary fish oil contained ~1.8 times more apoptotic bodies than those from mice fed the control diet (paper I, fig. 4H).

4.1.3 Dietary fish oil attenuates peritoneal pro-inflammatory cytokine concentrations (paper I, unpublished data)

The concentrations of all measured cytokines, chemokines, and other soluble mediators in the peritoneal fluid during antigen-induced peritonitis are summarized appendix B and Figure 9. Dietary fish oil modified concentrations of several soluble mediators 1.5, 3, and 6 h after induction of inflammation (fig. 9). After 1.5 of inflammation the concentrrations of BAFF, CCL4, CCL5, CXCL1, CXCL2, G-CSF, IL-10, IL-1α, IL-13, IL-6Ra, M-CSF, sTNFRI, sTNFRII, and TNF-α increased (fig. 9). Then, the peritoneal concentrations of CXCL10, GM-CSF, IL-5, and IL-17A increased 3 h following induction of inflammation (fig. 9). Most mediators appeared at the inflammatory peak 6 h after inflammation induction (fig. 9), and several mediators, including angiopoietin-2, CCL4, CCL5, CCL7, CXCL10, CXCL12, DKK-1, DPPIV, G-CSF, granzyme B, IGF-1, IL-1α, IL-1β, IL-5, IL-6Ra, IL-17A, LIX, M-CSF, MMP8, MMP9, sFasL, sTNFRI, sTNFRII, sTWEAK, syndecan-1, and TIMP-1, were identified as potential molecules of interest (fig. 9).

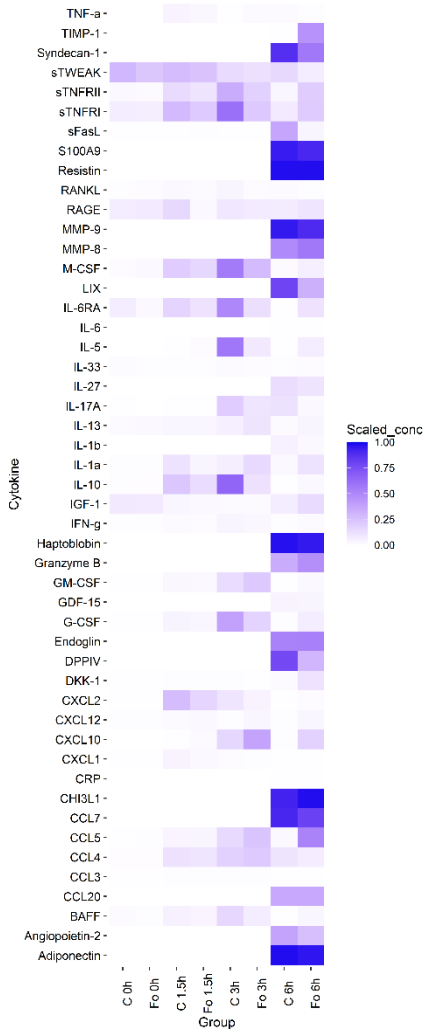

Figure 9. Dietary fish oil modulates concentrations of soluble mediators prior to and during acute inflammation.

Mice were fed control (C) or fish oil (Fo) diets for 5 weeks, immunized twice with mBSA with a two-week interval, and intraperitoneally challenged a week later. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected at 0, 1.5, 3, and 6 hours (h) following induction of inflammation. Concentrations of adiponectin, angriopoietin-2, BAFF, C-C motif chemokine ligand CCL20, -3, -4, -5, -7, CHI3L1, CRP, CXCL1, -10, -12, -2, DKK-1, DDPIV, endoglin, G-CSF, GDF-15, GM-CSF, granzyme B, haptoglobin, IFN-γ, IGF-1, IL-10, -1α, -1β, -13, -17A, -27, -33, -5, -6, -6Ra, LIX, M-CSF, MMP8, -9, RAGE, RANKL, resisitin, S100A9, soluble FasL (sFasL), soluble TNF receptor (sTNFR)I, -II, soluble TWEAK (sTWEAK), syndecan-1, TIMP1, and TNF-α were measured by Luminex. Concentrations were scaled down to a 0-1 range and visualized in a heatmap, n = 6 – 42.

Dietary fish oil attenuated peritoneal concentrations of CXCL1 and CXCL2 1.5 h after induction of inflammation compared to those in mice fed the control diet (paper I, fig.

5A,B). Additionally, the concentrations of CXCL1 in the inflamed peritoneum of mice fed dietary fish oil returned to baseline levels within 3 h following inflammation induction, whereas a slower decline was observed in mice fed the control diet (paper I, fig. 5A).

Peritoneal TNF-α concentration was lower 1.5 h after inflammation induction in mice fed the fish oil compared to that in mice fed the control diet (paper I, fig. 5C). Despite following the same kinetic pattern as TNF-α, the peritoneal concentrations of BAFF, TWEAK and FasL were not affected by dietary fish oil (fig. 10A,B,C). Peritoneal concentrations of soluble TNFRI increased after inflammation was induced in mice regardless of their diet (fig. 10D). Additionally, dietary treatment did not modulate peritoneal RAGE and RANKL concentrations prior to and during acute inflammation (fig. 10E,F).

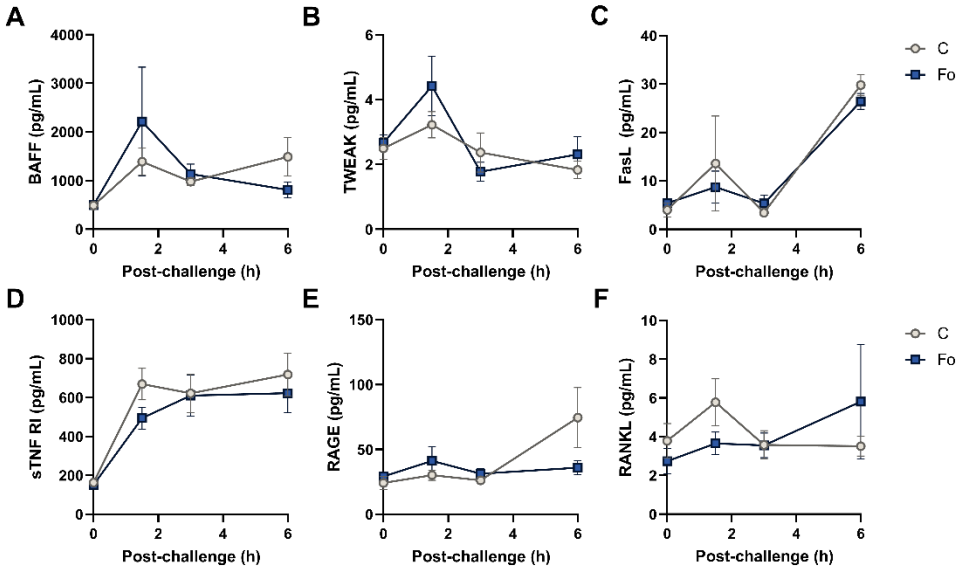

Figure 10. Dietary fish oil does not affects production of several proteins from the tumor necrosis factor family.
Mice were fed control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval and subsequently challenged with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected 0, 1.5, 3, and 6 hours (h) after inflammation induction. Concentrations of BAFF (**A**), TWEAK (**B**), FasL (**C**), sTNF RI (**D**), RAGE (**E**), and RANKL (**F**) were determined by Luminex analysis. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA and multiple comparisons Fisher's LSD test; n = 1 – 11, collected from 4 independent experiments.

Concentration of IL-1β in the inflamed peritoneum was not different in mice 6 h after inflammation induction regardless of dietary treatment (fig. 11A). Due to compatibility issues in later Luminex assays, IL-1β measurements were not included in follow-up studies. Peritoneal concentrations of IL-1α trended higher 3 h after induction of inflammation in mice the fed fish oil diet compared to that in mice fed the control diet ($p = 0.06$, fig. 11B). Dietary fish oil attenuated IL-33 concentrations in the peritoneum 6 h following induction of inflammation compared to that in peritoneal fluid of mice fed the control diet (paper I, fig. 5F).

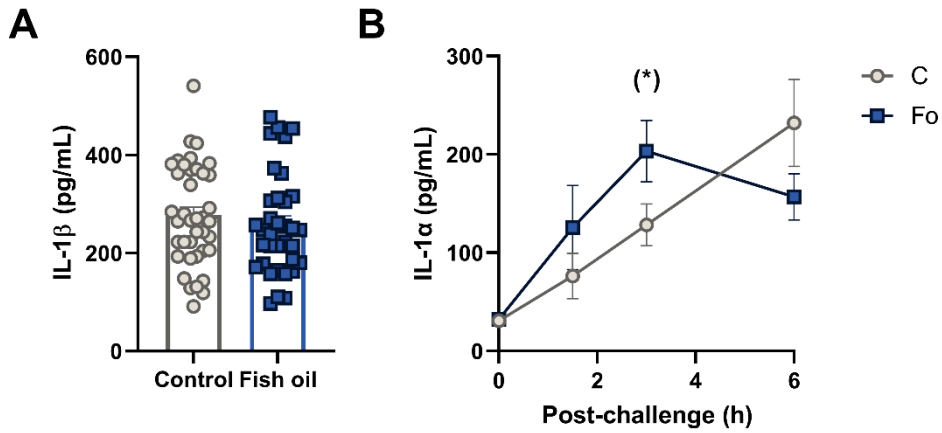

Figure 11. Dietary fish oil does not affect peritoneal interleukin (IL)-1β or IL-1α concentrations in antigen-induced peritonitis.
Mice were fed control (C, beige circles, brown line) or fish oil (Fo, blue squares and line) diets for 5 weeks before being immunized twice with a two-week interval and subsequently challenged with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected 0, 1.5, 3, and 6 h after inflammation induction. Concentrations of IL-1β (**A**) 6 h after and IL-1α (**B**) at the indicated timepoints after inflammation induction were determined by Luminex. Data are expressed as mean ± SEM and differences were determined by Mann-Whitney U-test (IL-1β) or two-way ANOVA and multiple comparisons Fisher's LSD test (IL-1α); (*) $p < 0.1$, $n = 7 – 39$, data obtained from 4 independent experiments.

Peritoneal IL-6 concentration was dampened in mice fed the fish oil diet 6 h after inflammation induction compared to that in mice fed the control diet (paper I, fig. 5E). Dietary fish oil similarly dampened peritoneal concentration of IL-6Rα 1.5 and 6 h following inflammation induction compared to that in mice fed the control diet (paper I, fig. 5F). Dietary fish oil also decreased peritoneal concentration of CCL20, a downstream product of IL-6 signaling (fig. 12).

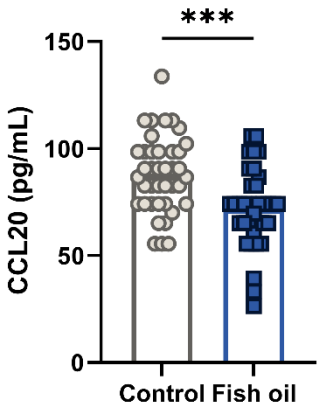

Figure 12. Dietary fish oil decreases CCL20 concentrations 6 h after inflammation induction. Mice were fed control (blue circles) or fish oil (light blue squares) diets for 5 weeks before being immunized twice and subsequently challenged with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected 6 h after inflammation induction. Concentrations of CCL20 was determined by Luminex. Data are expressed as mean ± SEM and differences were determined by Mann-Whitney U testing; *** $p < 0.001$, $n = 36 – 40$, data obtained from 4 independent experiments.

4.1.4 Dietary fish oil increases peritoneal concentrations of IGF-1, TGF-β1, and sTNF RII in antigen-induced peritonitis (paper II)

Surprisingly, dietary fish oil did not enhance peritoneal concentration of IL-10 at any time point during acute inflammation (paper I, fig. 6A). Peritoneal TGF-β concentration was increased in mice fed the fish oil diet 6 h after induction of inflammation compared to that in mice fed the control diet (paper I, fig. 6B). IGF-1 concentration in the peritoneum was higher prior to and 1.5 h after induction of inflammation than at 3 h of in both dietary groups (paper I, fig. 6C). IGF-1 concentrations returned to baseline levels 6 h following induction of inflammation in mice fed the fish oil diet, whereas the levels were still dampened in mice fed the control diet (paper I, fig. 6C). Peritoneal concentration of the lymphotoxin and TNF decoy receptor sTNFRII was slightly increased 1.5 and 3 h following induction of inflammation in both dietary groups (paper I, fig. 6D). Surprisingly, the peritoneal concentrations of sTNFRII continued to rise in inflamed mice fed the fish oil diet 6 h after inflammation induction, whereas it decreased in inflamed mice fed the control diet (paper I, fig. 6D). Thus, the concentration of sTNFRII was ultimately increased by ~5-fold in mice receiving dietary fish oil compared to that in mice receiving the control diet (paper I, fig. 6D).

4.1.5 Dietary fish oil alters the peritoneal lipidomic profile in antigen-induced peritonitis (paper I, unpublished data)

Dietary fish oil modulated peritoneal concentrations of several PUFAs, eicosanoids, SPMs, and their intermediates prior to and during antigen-induced peritonitis (paper I, fig. 7, fig. 13, appendix C). Generally, peritoneal concentrations of omega-3 PUFAs and their derivatives were higher in the peritoneum of mice fed the fish oil diet prior to and during the inflammation compared to that in the peritoneum of mice fed the control diet (fig. 13).

In the present study we detected very low or no SPMs in the peritoneal exudates (paper I, fig. 7, appendix C), highlighting the current challenges in accurately measuring these mediators in biological samples (219). Nonetheless, we were able to detect 5-, 12-, 15-, and 18-HEPE intermediates of EPA-derived SPMs in peritoneal exudates (paper I, fig. 7, appendix C). Dietary fish oil enhanced peritoneal 5- and 18-HEPE concentrations at 6 and 12 h following inflammation induction compared to that in the peritoneum of mice receiving the control diet (paper I, fig. 7). Dietary fish oil also increased the ratio between the omega-3:omega-6-PUFA-derived metabolites during antigen-induced peritonitis (appendix 2). Dietary fish oil likewise enhanced the ratio between HEPEs and HETEs 3, 6, and 12 h after inflammation induction compared to that in the peritoneum of mice fed the control diet (paper I, fig. 7).

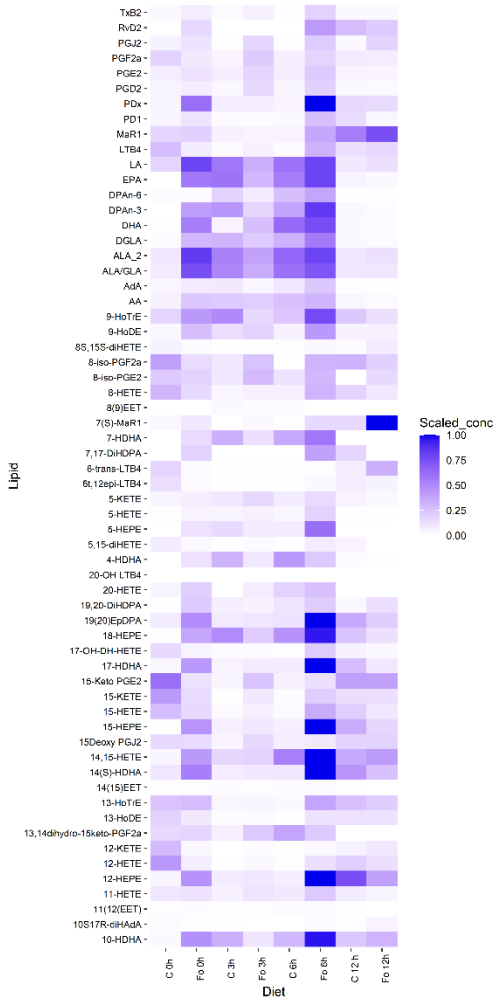

Figure 13. Dietary fish oil modulates peritoneal oxylipidome in antigen-induced peritonitis. Mice were fed control (C) or fish oil enriched (Fo) diets for 5 weeks prior to being immunized twice with a two-week interval against mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal lavage was isolated 0, 3, 6, and 12 hours (h) after inflammation induction. Concentrations of 4-, 7-, 10-HDHA, 10S,17R-diHAdA, 8(9)-, 11(12)-, 14(15)-EET, 5-, 8-, 11-, 12-, 14,15-, 15-, 17-OH-DH-, 20-HETE, 5-, 12-, 15-, 18-HEPE, 5-, 12-, 15-KETE, 13,14dihydro-15keto PGF_{2a}, 9-, 13-HoDE, 9-, 13-HoTrE, 15Deoxy PGJ_2, 15-Keto PGE_2, 19(20)-EpDPA, 19,20-, 7,17-DiHDPA, 20-OH LTB_4, 5,15-, 8S,15S-diHETE, 6-trans-12-epi-LTB_4, 6-trans-LTB_4, 7(S)-MaR1, 8-iso-PGE_2, -PGF_{2a}, AA, AdA, ALA, ALA/GLA, DGLA, DHA, DPAn-3, DPAn-6, EPA, LA, LTB_4, MaR1, PD1, PDx, PGD_2, PGE_2, PGF_{2a}, PGJ_2, RvD_2, and TxB_2 were determined by LC-MS/MS. Data are expressed as mean on a 0 (white) – 1 (blue) range. n = 6, collected from 2 independent experiments.

A correlation analysis of bioactive lipids measured in the peritoneal fluid during acute inflammation was conducted. Correlated lipid concentrations were hierarchically clustered together, revealing four distinct lipid mediator clusters (fig. 14). Cluster 1 and

4 both contained omega-3 PUFAs and omega-3 PUFA-derived lipids (fig. 14). Thus, EPA and DHA were strongly correlated with each other (fig. 14, cluster 1). They clustered together with 18-HEPE, the intermediate for E-series Rvs, and the two hydrolyzed DHA products, 4- and 7-HDHA (fig. 14, cluster 1). DHA and EPA correlated with higher concentrations of 5-, 12-, and 15-HEPE although they did not cluster together (fig. 14). They also correlated with 10-, 14(S)-, and 17-HDHA, as well as PDx as visualized by blue tinted squares (fig. 14, cluster 4). However, DHA and EPA did not correlate with SPM concentrations but the HDHAs and HEPEs did (fig. 14, cluster 4).

AA and its derivatives e.g., HETEs, ketoeicosatetraenoic acids (KETEs), and epoxyeicosatrienoic acids (EETs), were strongly correlated (fig. 14, cluster 2). Likewise, PGs, LTs, and TxB_2 correlated with higher concentrations of AA (fig. 14, cluster 2). Finally, LTs and omega-6 PUFA oxylipins and eicosanoids clustered together (fig. 14, cluster 3).

To summarize, dietary fish oil enhances several key hallmarks of ROI in antigen-induced peritonitis. Specifically, peritoneal neutrophil numbers and CXCL1 and CXCL2 concentrations were lower in the inflamed peritoneum of mice fed the fish oil diet during antigen-induced peritonitis than in the peritoneum of mice fed the control diet. Dietary fish oil increased neutrophil apoptosis while lowering their CD47 surface expression at the peak of inflammation. Likewise, higher numbers of apoptotic bodies were detected in mesenteric lymph nodes draining the inflamed peritoneum in mice fed the fish oil diet than in mice fed the control diet. Dietary fish oil dampened peritoneal concentrations of TNF-α, IL-6, IL-6Rα, and CCL20 during antigen-induced peritonitis. Moreover, higher peritoneal concentrations of the growth factors IGF-1 and TGF-β, as well as the decoy receptor sTNFRII, in inflamed mice fed the fish oil diet than in mice fed the control diet. Finally, dietary fish oil modulated lipidomic profile in the peritoneum with higher concentrations of omega-3 PUFA-derived lipid mediators compared to omega-6 PUFA-derived lipid mediators. All in all, this indicates that dietary fish oil enhances four hallmarks of inflammation resolution, i.e., #1, #2, #3, and #4, in murine antigen-induced peritonitis as per fig. 1.

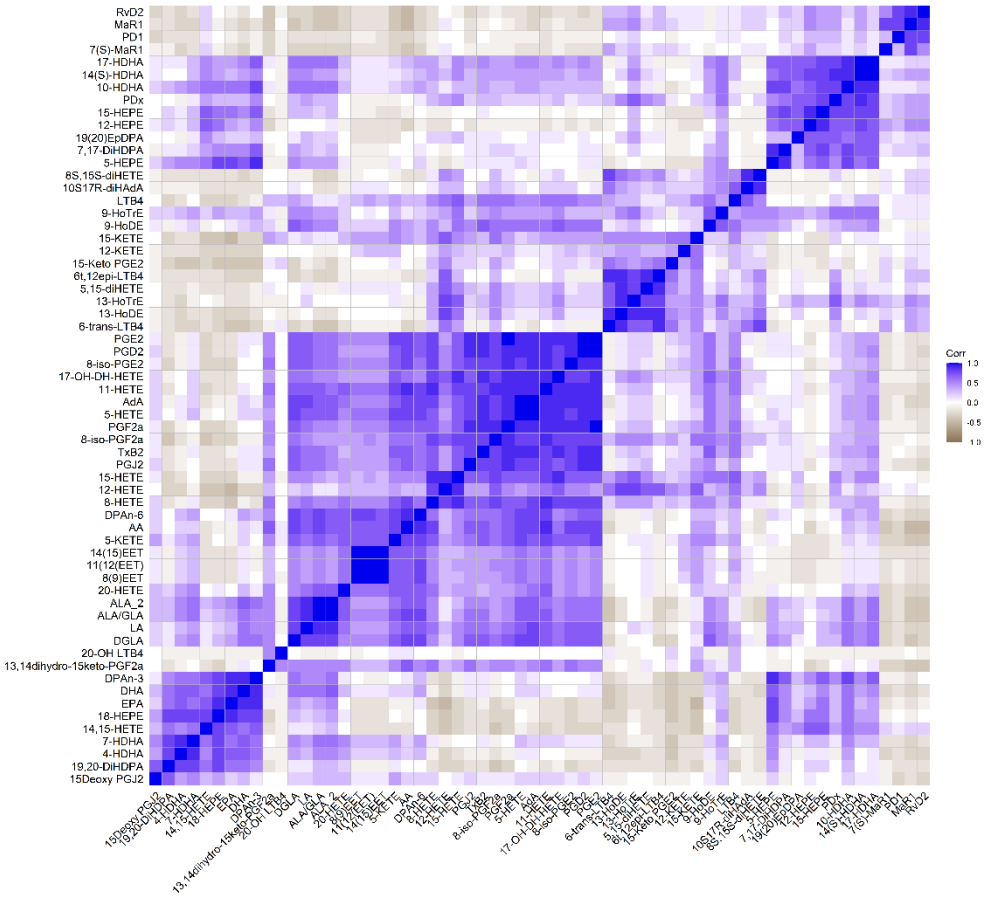

Figure 14. Peritoneal concentrations of lipid mediators and substrate PUFAs correlate in four distinct clusters.

Mice were fed control or fish oil diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal exudate was collected 0, 3, 6, and 12 hours after inflammation induction. Concentrations of 4-, 7-, 10-HDHA, 10S,17R-diHAdA, 8(9)-, 11(12)-, 14(15)-EET, 5-, 8-, 11-, 12-, 14,15-, 15-, 17-OH-DH-, 20-HETE, 5-, 12-, 15-, 18-HEPE, 5-, 12-, 15-KETE, 13,14dihydro-15keto PGF_{2a}, 9-, 13-HoDE, 9-, 13-HoTrE, 15Deoxy PGJ_2, 15-Keto PGE_2, 19(20)-EpDPA, 19,20-, 7,17-DiHDPA, 20-OH LTB_4, 5,15-, 8S,15S-diHETE, 6-trans-12-epi-LTB_4, 6-trans-LTB_4, 7(S)-MaR1, 8-iso-PGE_2, -PGF_{2a}, AA, AdA, ALA, ALA/GLA, DGLA, DHA, DPAn-3, DPAn-6, EPA, LA, LTB_4, MaR1, PD1, PDx, PGD_2, PGE_2, PGF_{2a}, PGJ_2, RvD_2, and TxB_2 were determined by LC-MS/MS. Correlation scores were assigned between all metabolites on a -1 – 1 scale I R and R Studio. Positive correlations (blue) were hierarchically clustered (1-4, omega (ω)-3 or ω-6 polyunsaturated fatty acids) over negative correlations (brown) between lipids; n = 6, collected from 2 independent experiments.

4.2 Section II: The effects of dietary fish oil on NK cell recruitment, phenotype, and function in antigen-induced peritonitis (paper I, unpublished data)

4.2.1 Dietary fish oil increases the accumulation of $CD11b^{+}CD27^{-}$ NK cells to the inflamed peritoneum (paper I, unpublished data)

Similar to previous studies conducted in the laboratory, dietary fish oil led to increased peritoneal NK cell numbers 6 h following inflammation induction compared that in mice fed the control diet (paper I, fig. 1A,C, (80)). By analysing different maturation subsets, we demonstrated that dietary fish oil increase the number of the most mature $CD11b^{+}CD27^{-}$ NK cells as compared that in mice fed the control diet (paper I, fig. 1D).

Prior to the induction of inflammation, peritoneal $CCR5^{+}$ NK cells constituted ~15% and decreased this percentage decreased after inflammation was induced in both dietary groups (fig. 15A). However, mice fed the fish oil diet had a higher percentage of $CCR5^{+}$ peritoneal NK cells 6 h after inflammation induction compared to that in mice fed the control diet (fig. 15A). The number of $CCR5^{+}$ NK cells remained low and comparable in both dietary groups at 0, 1.5, and 3 h following inflammation induction (fig. 15B). However, the number of $CCR5^{+}$ NK cells increased ~4-fold 6 h after the induction of inflammation compared that prior to inflammation in mice fed the fish oil diet (fig. 15B, paper I, fig. 2B). In comparison, the number of $CCR5^{+}$ NK cells did not change in mice fed the control diet (fig. 15B, paper I, fig. 2B). The level of CCR5 expression on peritoneal NK cells (indicated as median fluorescent intensity, MFI) did not change 1.5 and 3 h after inflammation induction in either dietary group (fig. 15C). However, peritoneal NK cell CCR5 expression then decreased in both dietary groups, with NK cells from mice fed the fish oil diet having higher levels of CCR5 expression 6 h following the induction of inflammation compared to that on NK cells from mice fed the control diet (fig. 15C).

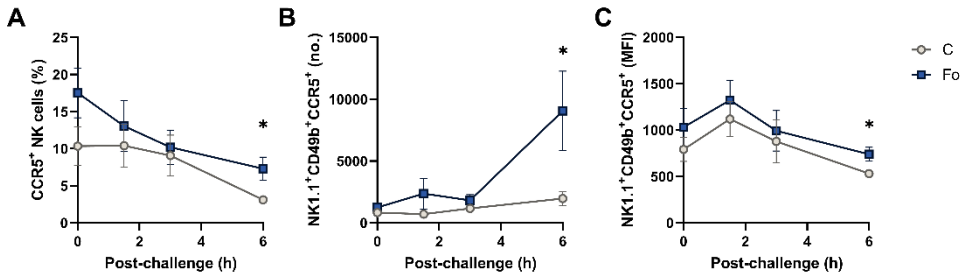

Figure 15. Dietary fish oil increases number and percentages of CCR5$^+$ NK cells and their level of expression 6 h after inflammation induction.
Mice were fed control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells were collected 0, 1.5, 3, and 6 h after inflammation induction. Percentage (**A**), total number (**B**), and median fluorescence intensity (MFI, **C**) of CCR5 on CD3$^-$NK1.1$^+$CD49b$^+$ lymphocytes were determined by flow cytometry. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA and multiple comparisons Fisher's LSD test; *$p < 0.05$, $n = 9 – 14$, data obtained from 4 independent experiments.

Dietary fish oil did not affect peritoneal concentrations of CCL3 or CCL7 6 h after inflammation induction (fig. 16A,C). Peritoneal concentrations of CCL4 increased during antigen-induced peritonitis but dietary fish oil did not affect peritoneal CCL4 concentrations at any timepoint (fig. 16B). Interestingly, peritoneal concentrations of CCL5 and CXCL12 were higher in mice receiving dietary fish oil 6 h after induction of inflammation compared to that in mice receiving the control diet (paper I, fig. 2C). Further, mice fed the fish oil diet also had higher peritoneal concentrations of CXCL10 3 h after induction of inflammation compared to that in mice fed the control diet (fig. 16D).

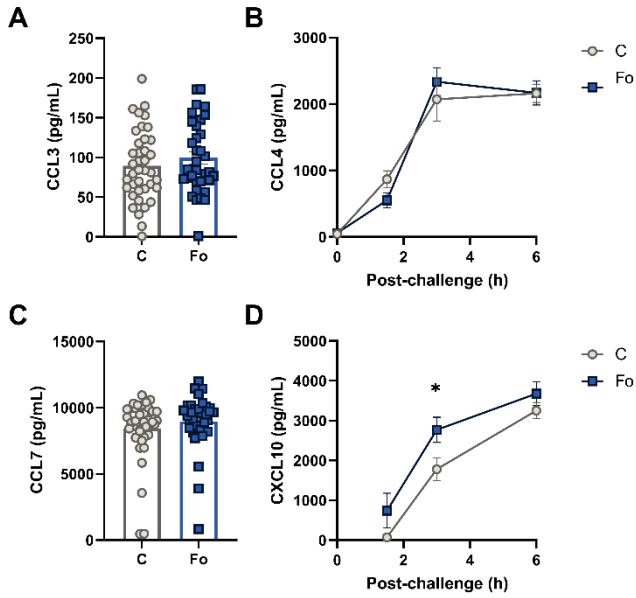

Figure 16. Dietary fish oil enhances CXCL10 3 h after inflammation induction.
Mice were fed control (C, dark blue circles and lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks and immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected 0, 1.5, 3, and 6 h after inflammation induction. Concentrations of CCL3 (**A**), CCL4 (**B**), CCL7 (**C**), and CXCL10 (**D**) at 0, 3, 6, and 12 h after inflammation induction was determined by Luminex. *$p < 0.05$, $n = 10 – 40$, data obtained from 2-4 independent experiments.

4.2.2 Dietary fish oil does not modulate peritoneal granzyme B, IFN-γ, or GM-CSF concentrations (unpublished data)

Dietary fish oil did not affect peritoneal concentrations of Granzyme B 6 h after inflammation induction (fig. 17A). Likewise, peritoneal IFN-γ concentrations did not differ between the dietary groups 6 h following induction of inflammation (fig. 17B). Peritoneal GM-CSF concentrations were low during non-inflamed conditions but increased rapidly after induction of inflammation and remained constant 1.5, 3, and 6 h in both dietary groups (fig. 17C). However, no differences in peritoneal GM-CSF concentrations were detected between the dietary groups (fig. 17C).

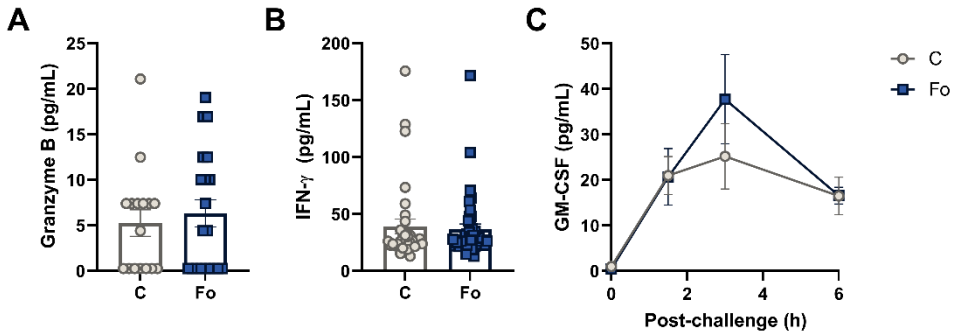

Figure 17. Dietary fish oil does not modulate peritoneal concentrations of granzyme B, IFN-γ, or GM-CSF.

Mice were fed either control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was collected 0, 1.5, 3, and 6 h after inflammation induction. Concentrations of granzyme B (6 h, **A**), IFN-γ (6 h, **B**), and GM-CSF (**C**) were determined by Luminex. Data are expressed as mean ± SEM and differences determined by Mann-Whitney U-test (**A**, **B**) or two-way ANOVA multiple comparisons Fisher's LSD test (**C**); n = 4 – 40, data collected over 2-6 independent experiments.

4.2.3 Dietary fish oil tempers surface expression of CD107a and CD62L on peritoneal NK cells (paper I, unpublished data)

As NK cells are potent cytotoxic cells, the expression of apoptosis inducing molecules TRAIL and FasL on peritoneal NK cells were analyzed (fig. 18). Around half of the NK cells detected in the peritoneum prior to and 1.5 h following induction of inflammation in both dietary groups expressed TRAIL (fig. 18A). The proportion of peritoneal NK cells expressing TRAIL decreased by half and remained stable 3 and 6 h after inflammation induction (fig. 18A). We detected to difference in the proportion of TRAIL$^+$ NK cells at any timepoint between the dietary groups (fig. 18A). The number of TRAIL$^+$ NK cells were increased in mice fed the fish oil diet compared to that in mice fed the control diet 6 h after induction of inflammation, most likely due to the overall increase in peritoneal NK cell numbers (fig. 18B). The expression level of TRAIL on peritoneal NK cells was high prior to and at 1.5 h but lowered 3 and 6 h after inflammation induction (fig. 18C). No differences in the expression level of TRAIL on peritoneal NK cells were observed between the dietary groups (fig. 18C).

The percentage of peritoneal NK cells expressing FasL was between 20-30% prior to inflammation induction and remained stable throughout the inflammatory response apart from a small decrease in FasL expression 3 h after induction of inflammation in both groups (fig. 18D). The number of FasL$^+$ NK cells were increased 6 h following inflammation induction, probably due to increased number of peritoneal NK cells (fig. 18E). FasL expression on peritoneal NK cells decreased steadily after the induction of inflammation for both dietary groups with no differences between the groups (fig. 18F).

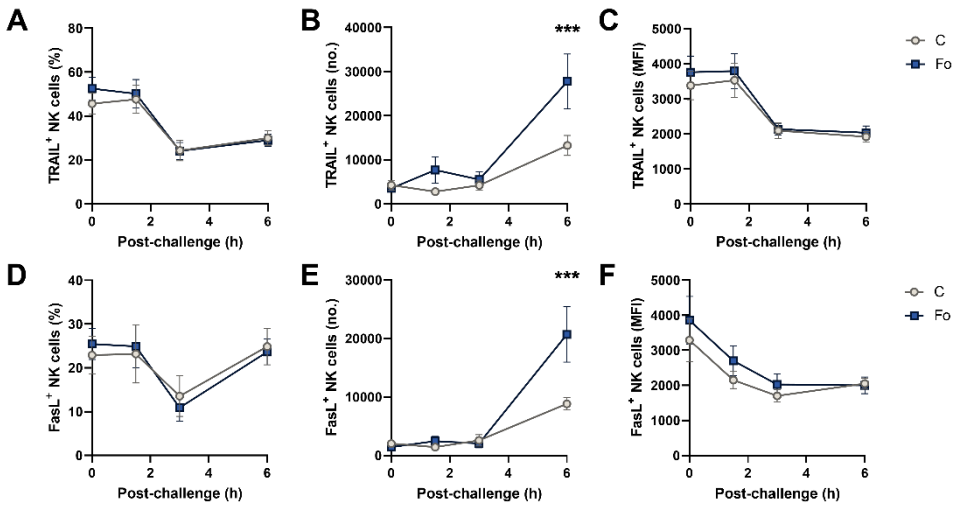

Figure 18. Dietary fish oil increased numbers of TRAIL$^+$ and FasL$^+$ NK cells 6 h after inflammation induction.
Mice were fed with control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells were collected 0, 1.5, 3, 6 h after inflammation induction. Percentage (**A**,**D**), total number (**B**,**E**), and median fluorescent intensity (MFI, **C**,**F**) of TRAIL (**A-C**) and FasL (**D-E**) on CD3$^-$NK1.1$^+$CD49b$^+$ lymphocytes (NK cells) was determined by flow cytometry. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA multiple comparisons Fisher's LSD test; ***$p < 0.001$, n = 6 – 15, data collected over 2 independent experiments.

The percentage of CD107a$^+$ NK cells in mice fed the fish oil diet were stable whereas the percentage in mice fed the control diet fluctuated during inflammation (fig. 19A). This resulted in higher percentage of CD107a$^+$ NK cells in mice fed the fish oil diet 6 h after induction of inflammation compared that that in mice fed the control diet (fig. 19A, paper I, fig. 3C). Dietary fish oil also increased the number of CD107a$^+$ NK cells, this time more than what would be expected from the total increase in peritoneal NK cells (fig. 19B). Likewise, dietary fish oil maintained CD107a expression levels on NK cells 6 h after induction of inflammation compared to that on NK cells in mice fed the control diet (fig. 19C, paper I, fig. 3C).

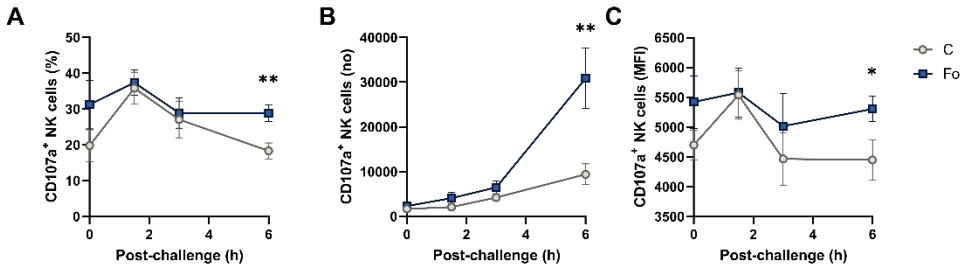

Figure 19. Dietary fish oil enhances the proportion and number of CD107a⁺ NK cells and CD107a expression on NK cells 6 h after inflammation induction.
Mice were fed control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection with mBSA. Peritoneal cells were collected 0, 1.5, 3, and 6 h after inflammation induction. Percentage (**A**), total number (**B**), and median fluorescent intensity (MFI, **C**) of CD107a on $CD3^{-}NK1.1^{+}CD49b^{+}$ lymphocytes (NK cells) was determined by flow cytometry. Data are expressed as mean ± SEM and differences were determine by two-way ANOVA multiple comparisons Fisher's LSD test; *$p < 0.05$, **$p < 0.01$, $n = 8 - 14$, data collected from 3-4 independent experiments.

The percentage of peritoneal NK cells expressing the early activation marker CD69 was low prior to induction of inflammation in mice in both dietary groups (fig. 20A). The percentage in both groups remained low at 1.5 h but increased to ~15% of peritoneal NK cells 3 and 6 h after induction of inflammation (fig. 20A). The number of $CD69^{+}$ NK cells increased in inflamed mice fed the fish oil diet 6 h after induction of inflammation compared to that in inflamed mice fed the control diet, reflecting the increase of total peritoneal NK cells (fig. 20B). The expression level of CD69 on NK cells remained constant prior to and after induction of peritonitis and did not differ between the dietary groups (fig. 20C).

Percentages of peritoneal $NKG2A^{+}$ NK cells remained stable prior to and after inflammation induction in both dietary groups (fig. 20D). Due to the increased number of total NK cells, this resulted in higher numbers of $NKG2A^{+}$ NK cells in the peritoneum of mice fed the fish oil diet 6 h after inflammation induction compared to that in mice fed the control diet (fig. 20E). The level of NKG2A expression on NK cells decreased following induction of inflammation with no differences between the dietary groups at any of the timepoints during inflammation (fig. 20F).

The percentage of peritoneal NK cells expressing 2B4 increased from ~70% prior to inflammation induction to ~90% at 6 h after induction of inflammation (fig. 20G). There were no differences in the percentage of 2B4⁺ NK cells between the dietary groups at any of the timepoints (fig. 20G). Dietary fish oil increased the number of 2B4⁺ NK cells 6 h following induction of inflammation (fig. 20H). This increase is most likely due to the increase in the number of peritoneal NK cells. Expression levels of 2B4 on NK cell increased ~1.4-fold 3 and 6 h compared to that prior to and 1.5 h after inflammation induction with no differences between the dietary groups (fig. 20I).

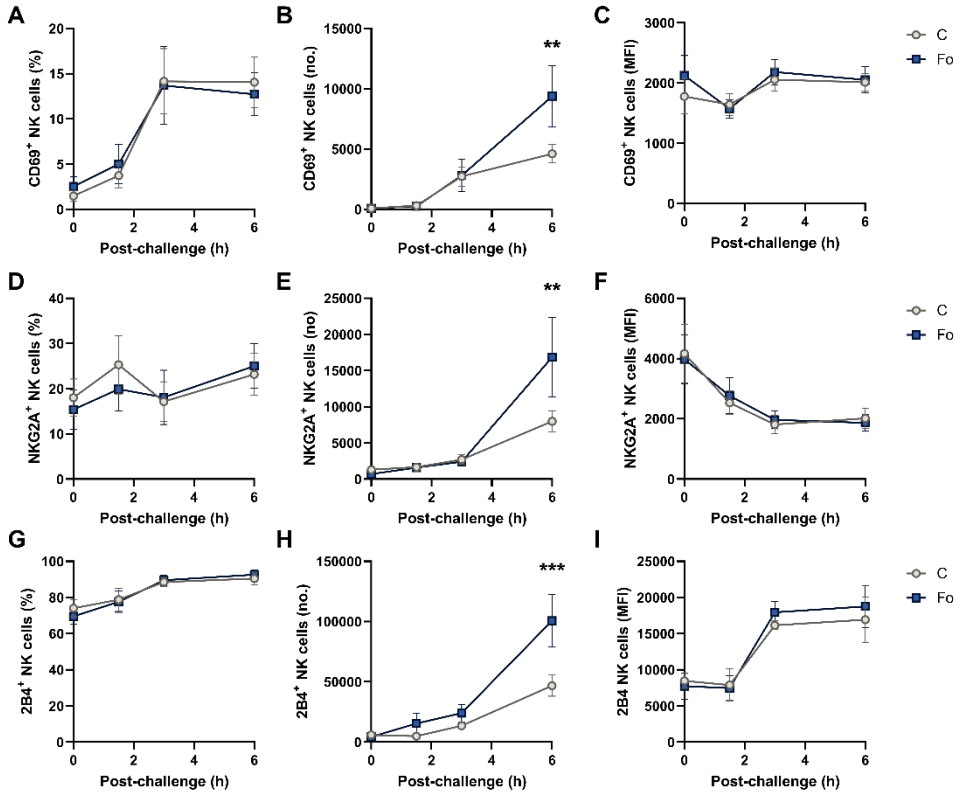

Figure 20. Dietary fish oil increases the number of CD69⁺, NKG2A⁺, and 2B4⁺ NK cells 6 h after induction of inflammation.

Mice were fed control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells were collected 0, 1.5, 3, and 6 h after inflammation induction. Percentage (**A**,**D**,**G**), total number (**B**,**E**,**F**), and median fluorescent intensity (MFI, **C**,**F**,**I**) of CD69 (**A-C**), NKG2A (**D-F**), and 2B4 (**G-I**) on CD3⁻NK1.1⁺CD49b⁺ lymphocytes (NK cells) were determined by flow cytometry. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA multiple comparisons Fisher's LSD test;**p < 0.01, ***p < 0.001, n = 6 – 15, data obtained from 2-3 independent experiments.

Dietary fish oil enhanced the percentage of CD62L$^+$ NK cells in mice fed the fish oil diet prior to and 6 h following induction of inflammation compared to that in mice fed the control diet (fig. 21A, paper I, fig. 3D). The number of CD62L$^+$ NK cells was not different between the two dietary groups prior to, 1.5, and 3 h after inflammation induction (fig. 21B). Dietary fish oil increased the number of CD62L$^+$ NK cells 6 h after induction of inflammation compared to that seen in mice fed the control diet (fig. 21B). The expression level of CD62L on peritoneal NK cells remained stable prior to after induction of inflammation (fig. 21C).

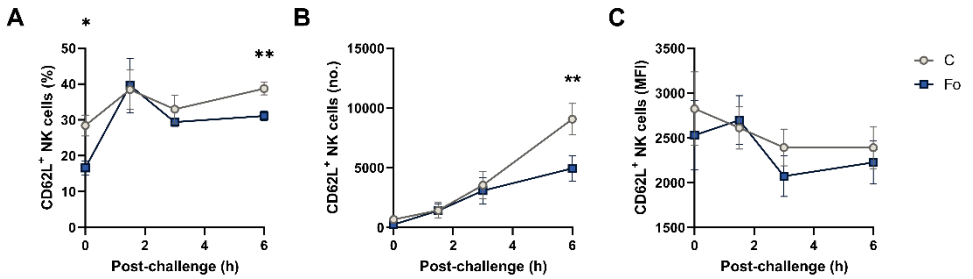

Figure 21. Dietary fish oil decreases the number and proportion of CD62L$^+$ NK cell 6 h after induction of antigen-induced peritonitis.
Mice were fed control (C, beige circles, brown lines) or fish oil (Fo, blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells were collected 0, 1.5, 3, and 6 h after inflammation induction. Percentage (**A**), total number (**B**), and median fluorescent intensity (MFI, **C**) was determined by flow cytometry. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA multiple comparisons Fisher's LSD test; *$p < 0.05$, **$p < 0.01$, $n = 9 – 15$, data collected from 3-4 different experiments.

4.2.4 Dietary fish oil increases peritoneal DKK-1 concentrations (unpublished data)

Prior to and 1.5 h after induction of inflammation ~20-25% of peritoneal NK cells were low-density lipoprotein receptor-related protein (LRP)6$^+$ in both dietary groups (fig. 22A). This percentage had decreased to ~15% 3 and 6 h following inflammation induction with no differences between the two dietary groups (fig. 22A). The number of LRP6$^+$ peritoneal NK cells increased 6 h after induction of inflammation in both dietary groups but no differences were detected between the two dietary groups (fig. 22B). The level of LRP6 expression on peritoneal NK cells increased slightly after inflammation induction with no differences observed between the dietary groups (fig. 22C).

Peritoneal concentrations of DKK-1 were low prior to and early after the induction of inflammation (fig. 22D). Six h after induction of inflammation DKK-1 concentrations had

increased in both dietary groups but more in mice fed the fish oil diet than in mice fed the control diet (fig. 22D).

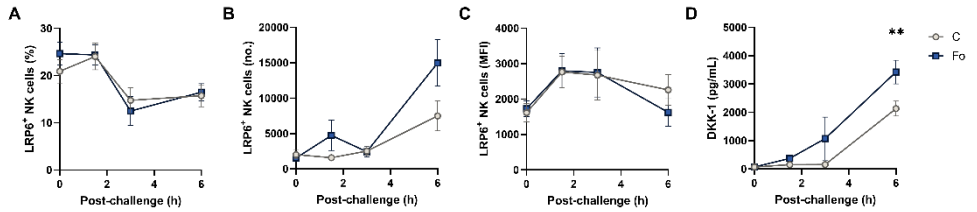

Figure 22. Dietary fish oil enhance peritoneal DKK-1 concentrations 6 h after induction of antigen-induced peritonitis.

Mice were fed either control (beige circles, brown lines) or fish oil (blue squares and lines) diets for 5 weeks before being immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal cells and fluid were isolated 0, 1.5, 3, and 6 hours (h) after inflammation induction. Percentage (**A**), total number (**B**), and median fluorescent intensity (MFI, **C**) of LRP6 on $CD3^{-}NK1.1^{+}CD49b^{+}$ lymphocytes (NK cells) was determined by flow cytometry. Concentration of DKK-1 (**D**) in peritoneal fluid was determined by Luminex. Data are expressed as mean ± SEM and differences were determined by two-way ANOVA multiple comparisons Fisher's LSD test; $**p < 0.01$, n = 9 – 40, data collected from 3-6 independent experiments.

To summarize, dietary fish oil increased the number of peritoneal NK cells 6 h after inflammation induction in mice fed the fish oil diet when compared to that in mice fed the control diet. This increase was mainly caused by higher numbers the most mature subset of NK cells 6 h after inflammation induction in mice fed dietary fish oil. Dietary fish oil also increased percentages and numbers of $CD107a^{+}$ NK cells and expression levels of CD107a on NK cells suggesting increased levels of degranulation of peritoneal NK cells compared to mice fed the control diet. However, this increase did not correlate with observed differences in the levels of the expression of investigated cytotoxic or activation markers on NK cells. Conversely, dietary fish oil decreased the percentages and numbers of $CD62L^{+}$ NK cells and expression levels of CD62L on NK cells 6 h after inflammation induction compared to that in mice fed the control diet. Finally, dietary fish oil increased peritoneal concentration of DKK-1 but not LRP6 expression on NK cells 6 h after induction of inflammation.

4.3 Section III: The effects of omega-3 polyunsaturated fatty acids on human NK cell effects on and crosstalk with neutrophils (paper II)

4.3.1 Docosahexaenoic acid dampens human NK cell induced neutrophil expression of CD11b and CD47 (paper II, unpublished data)

Primary human neutrophils downregulated their expression of CD11b after being cultured alone for 3 h and maintained low expression of CD11b at the remaining timepoints (paper II, fig. 1A). Co-culturing neutrophils with either C-NK or DHA-NK cells increased their expression of CD11b after 18 and 24 h of co-culturing compared to that on neutrophils cultured alone (fig. 23A, paper II, fig. 1A). DHA-NK cells dampened neutrophil CD11b expression 6 and 12 h after initiation of co-cultures compared to that induced by C-NK cells (fig. 23B, paper II, fig. 1A).

Expression levels of CD47 on neutrophils gradually decreased by time and stabilized after 18 h of culture when they were cultured alone (paper II, fig. 1B). Co-culturing neutrophils with C-NK cells increased their CD47 expression after 12 h of co-culture (fig. 23C, paper II, fig. 1B,C). Thereafter, surface expression of CD47 on neutrophils co-cultured with C-NK cells decreased to similar levels as on neutrophils cultured on their own after 24 h of co-culture (paper II, fig. 1B). Neutrophils co-cultured with DHA-NK cells had higher CD47 expression at 12 h than neutrophils cultured alone but lower expression than those cultured with C-NK cells (paper II, fig. 1B,C). Neutrophil expression of CD47 was higher when they were co-cultured with DHA-NK cells than when they were cultured alone and co-cultured with C-NK cells 24 h after the initiation of co-cultures (paper II, fig. 1B). Interestingly, neutrophil expression of CD47 was increased 18 h after co-culturing with EPA-NK cells compared to when they were co-cultured with C-NK (fig. 23D).

Of note, although all neutrophils expressed CD47 12 h after initiation of culture only a proportion of them expressed CD11b (paper II, fig. 1D). However, when the neutrophils were co-cultured with C-NK or DHA-NK cells they all expressed CD11b (paper II, fig. 1D). Interestingly, a distinct $CD47^{high}CD11b^{+}$ neutrophil population was present 12 h of co-culture with C-NK cells (paper II, fig. 1D). This population was smaller when the neutrophils were co-cultured with DHA-NK cells (paper II, fig. 1D) but not when neutrophils were co-cultured with AA-NK or EPA-NK cells (data not shown).

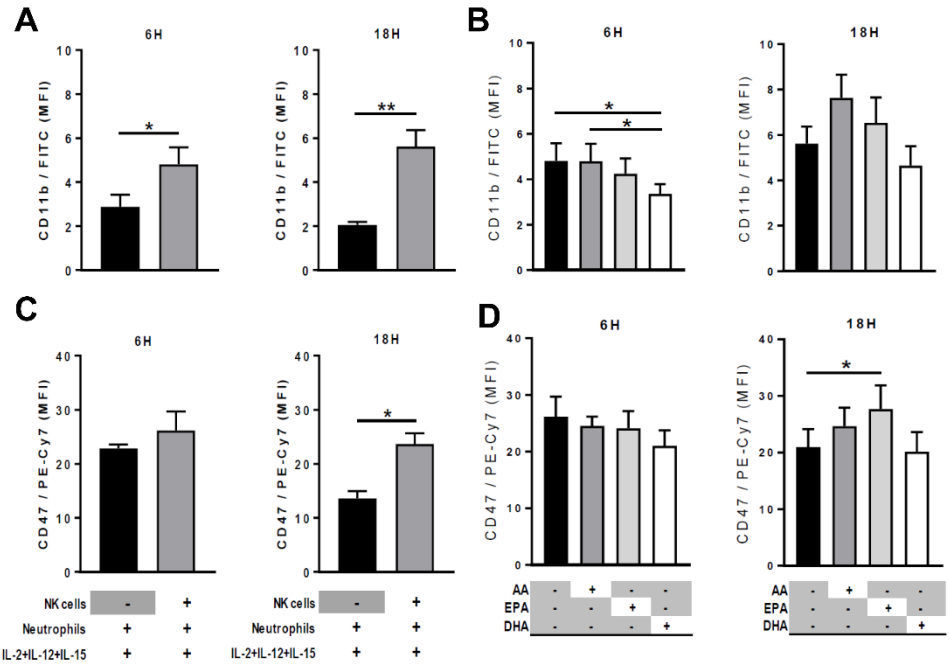

Figure 23. DHA-NK cells attenuate NK cell induced CD11b but not CD47 neutrophil expression. Neutrophils were cultured on their own or added to NK cells that were pre-incubated for 18 hours (h) with 50 μM arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or DMSO as a solvent control with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL). Median fluorescence intensity (MFI) of CD11b or CD47 on neutrophils cultured with or without NK cells (**A**, **C**) or with NK cells pre-incubated with fatty acids (**B**, **D**) for 6 or 18 h was determined with flow cytometry. Data are expressed as means ± SEM and differences between surface expression with or without NK cells were determined with Mann-Whitney U-test and differences between PUFA treatments were determined with two-way ANOVA with Dunn's multiple comparisons; *$p < 0.05$, **$p < 0.01$, $n = 6$, data collected from 3 independent experiments. Graphs reprinted with kind permission from (220).

4.3.2 Human NK cells induce neutrophil production of pro-inflammatory cytokines (paper II, unpublished data)

Neutrophils cultured alone produced low levels of IL-8, IL-1ra, and CXCL10 (fig. 24, paper II, fig. 2). When neutrophils were co-cultured with either C-NK cells or DHA-NK cells they produced higher levels of IL-8 and IL-1ra than when they were cultured alone (fig. 24A,C, paper II, fig. 2A). Neutrophils co-cultured with C-NK cells produced higher levels of CXCL10 than when they were cultured alone (fig. 24B, paper II, fig. 2C). EPA-NK and DHA-NK cells did not attenuate C-NK cell enhanced neutrophil cytokine production (paper II, fig. 2).

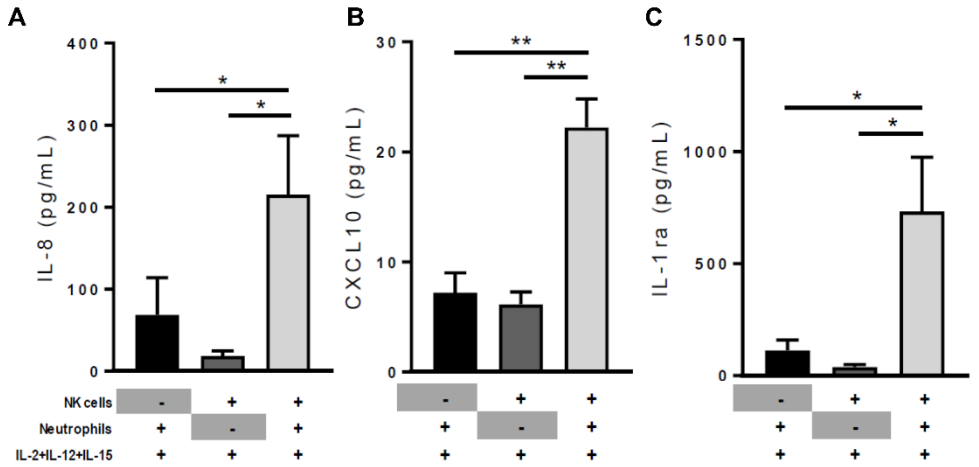

Figure 24. NK cells induce neutrophil production of interleukins and chemokines. Neutrophils were cultured on their own or added to NK cells that were pre-incubated for 18 hours (h) with DMSO and IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL). The cells were cultured together for 18 h. Concentrations of IL-8 (**A**), CXCL10 (**B**), and IL-1ra (**C**) were determined by ELISA. Data are expressed as means ± SEM and differences were determined with Mann-Whitney U-test; *$p < 0.05$, **$p < 0.01$, $n = 6$, data collected from 3 independent experiments. Graphs reprinted with kind permission from (220).

4.3.3 Docosahexaenoic acid modulates human NK cell induction of neutrophil apoptosis (paper II, unpublished data)

Co-culturing neutrophils with C-NK cells or DHA-NK cells enhanced their apoptosis early (6 h) but dampened it later (18 h) after co-culturing (paper II, fig. 3). Co-culturing neutrophils with DHA-NK cells for 18 h dampened their apoptosis more than that seen with C-NK cells (paper II, fig. 3).

Approximately 15% of neutrophils had phagocytosed *E. coli* when cultured alone for 6 h (paper II, fig. 4A). This percentage increased when neutrophils were co-cultured with either C-NK cells or DHA-NK cells to approximately 33% (paper II, fig. 4A). Co-culturing neutrophils with either C-NK cells or DHA-NK cells did not affect their ROS production (paper II, fig. 4B).

4.3.4 Docosahexaenois acid modulates human neutrophil-induced NK cell dampening of NKp46 and CXCR3 expression (paper II, unpublished data)

DHA did not modulate NK cell expression of NKG2D or NKp46 but decreased the percentage of $CXCR3^+$ and $NKG2A^+$ NK cells 24 h after stimulation (data not shown).

Approximately 70% of both C-NK cells and DHA-NK cells were $NKp46^+$ after being stimulated for 6 h (paper II, fig. 5A). When C-NK cells were cultured with neutrophils

this percentage decreased by almost 20% and by almost 50% when DHA-NK cells were cultured with neutrophils (paper II, fig. 5A). Expression levels of NKp46 were similar on C-NK cells and DHA-NK cells when they were cultured alone (paper II, fig. 5A). C-NK and DHA-NK cells expressed lower levels of NKp46 on their surface when cultured with neutrophils than when cultured alone (paper II, fig. 5A).

Neutrophils decreased the percentages of C-NK cells expressing CXCR3 when co-cultured for 6 or 18 h (fig. 25A, paper II, fig. 5B). This decrease was not seen when C-NK cells and neutrophils were cultured without cell-cell contact in a transwell system (fig. 25C). The percentage of DHA-NK cells expressing CXCR3 was lower when they were cultured with neutrophils than when cultured without neutrophils but higher than the percentage of C-NK cells cultured with neutrophils for 6 h (paper II, fig. 5B). Expression levels of CXCR3 on C-NK cells increased 6 h after initiation of co-culture with neutrophils (fig. 25B, paper II, fig. 5B). This increase was dependent on cell-cell contact 6 h after co-culture initiation (fig. 25D). DHA-NK cells expressed higher levels of CXCR3 on their surface 6 h after initiation of co-cultures with neutrophils compared to that of C-NK cells and DHA-NK cells cultured alone or C-NK cells cultured with neutrophils (paper II, fig. 5B).

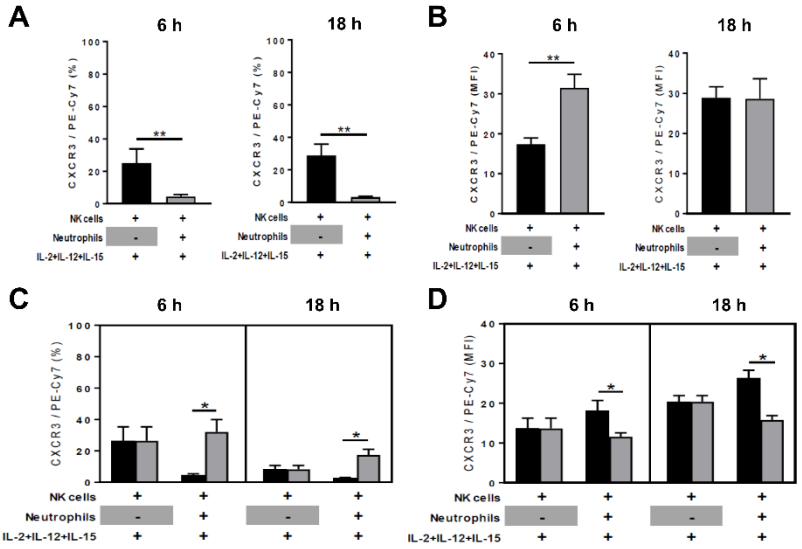

Figure 25. Neutrophils decrease the percentage and expression of CXCR3 on NK cells in a contact-dependent manner.

NK cells were pre-incubated with DMSO for 18 hours (h) before neutrophils were added with or without IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) to either transwell or direct culture systems. The cells were cultured together for 6 or 18 h. Percentage (**A**,**C**) and median fluorescence intesity (MFI, **B**,**D**) of CXCR3 on NK cells in cultures allowing cell-cell contact (**A**,**B**, black bars **C**,**D**) or not (grey bars **C**,**D**). Data are expressed as means ± SEM and differences were determined by Mann-Whitney U-test; *p < 0.05, **p < 0.01, n = 6, data collected from 2-3 independent experiments. Graphs reprinted with the kind permission from (220).

Interestingly, a subset of NK cells with high expression of CXCR3 and NKp46 were detected when they were cultured alone (paper II, fig. 5C). When NK cells were pre-incubated with DHA this subpopulation became smaller but with higher expression of CXCR3 than in C-NK cells (paper II, fig. 5C). This subpopulation of NK cells almost disappeared when the cells were cultured with neutrophils (paper II, fig. 5C).

Pre-incubating NK cells with DHA did not affect expression levels of NKG2D, 2B4, CD107a, DNAM-1, and TIGIT regardless of whether they were cultured with or without neutrophils (data not shown).

4.3.5 Docosahexaenoic acid modified human neutrophil enhanced NK cell cytokine production (paper I, unpublished data)

C-NK cells and DHA-NK cell cultures contained approximately 50 pg/mL of IFN-γ, whereas neutrophil cultures contained very low or undetectable levels of IFN-γ (paper II, fig. 6A). When C-NK cells were co-cultured with neutrophils concentrations of IFN-γ increased to almost 100 pg/mL (fig. 26B, paper II, fig. 6A). IFN-γ concentrations in co-cultures of DHA-NK cells or EPA-NK cells and neutrophils were dampened compared to that of co-cultures of C-NK cells and neutrophils and C-NK cells and DHA-NK cells cultured alone (fig. 26B, paper II, fig. 6A). AA-NK cells co-cultured with neutrophils secreted similar levels of IFN-γ to that of C-NK cells cultured with neutrophils (fig. 26B).

TNF-α concentrations in cell culture supernatants of C-NK cells and DHA-NK cells were ~10 pg/mL and slightly lower than that when neutrophils were cultured alone (paper II, fig. 6B). Co-culturing C-NK cells with neutrophils increased TNF-α concentrations compared to that for C-NK cells cultured alone (paper II, fig. 6B). Concentrations of TNF-α in cell culture supernatants was lower when DHA-NK cells were co-cultured with neutrophils compared to that when C-NK cells were co-cultured with neutrophils (fig. 26A, paper II, fig. 6B).

Cell culture supernatants from C-NK cells and DHA-NK cells cultured alone contained CCL3 at concentrations of 200-250 pg/mL (paper II, fig. 6C). CCL3 was almost undetectable when neutrophils were cultured alone (paper II, fig. 6C). Cell culture supernatant from EPA-NK cells and DHA-NK cells contained lower levels of CCL3 when they were co-cultured with neutrophils compared to that from C-NK cells co-cultured with neutrophils (fig. 26C, paper II, fig. 6C).

GM-CSF concentrations in cell culture supernatants of C-NK cells, DHA-NK cells, or neutrophils cultured alone were ≤ 10 pg/mL (paper II, fig. 6D). When C-NK cells were co-cultured with neutrophils, GM-CSF levels were increased compared to that in cultures with C-NK cells (paper II, fig. 6D). However, this increase did not occur when DHA-NK cells were co-cultured with neutrophils (paper II, fig. 6D).

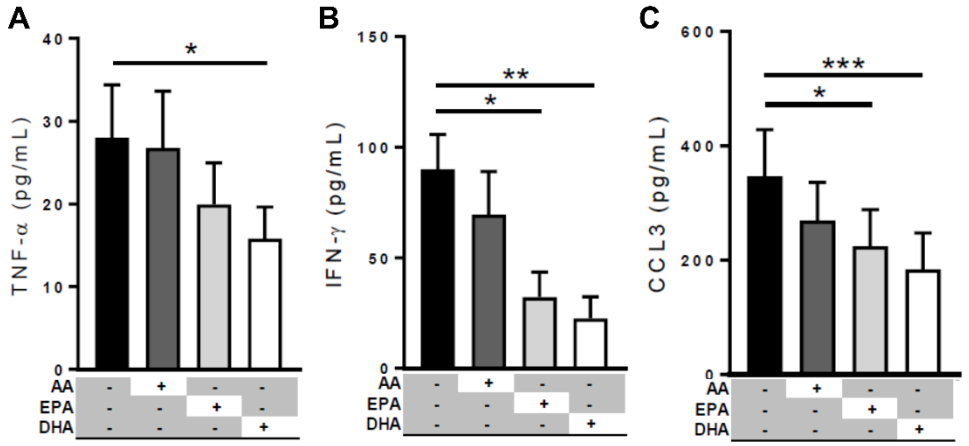

Figure 26. Omega-3 PUFAs attenuate NK cell production of TNF-α, IFN-γ, and CCL3.
NK cells were pre-incubated with arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or DMSO as a solvent control for 18 h before neutrophils were added along with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL). The cells were cultured together for 18 h. Concentrations of TNF-α (**A**), IFN-γ (**B**), and CCL3 (**C**) were determined by ELISA. Data are expressed as means ± SEM and differences were determined by Mann-Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001, n = 6, data obtained from 3-5 independent experiments. Graphs reprinted with kind permission from (220).

In summary, NK cells increased neutrophil expression of CD11b and CD47, leading to the induction of a distinct $CD47^{high}CD11b^{+}$ neutrophil population. NK cells also enhanced neutrophil production of the pro-inflammatory cytokines IL-8, IL-1ra, and CXCL10, increased neutrophil apoptosis early in co-culture, and enhanced their phagocytic capacity. Pre-incubation of the NK cells attenuated their effects on neutrophil CD11b and CD47 expression and induction of the $CD47^{high}CD11b^{+}$ neutrophil subset. Despite this, DHA-NK cells induced neutrophil activation in a similar manner to that of C-NK cells. DHA-NK cells were, on the other hand, more sensitive to neutrophil-induced downregulation of NKp46 on their surfaces and upregulation of CXCR3. Likewise, DHA attenuated neutrophil-induced cytokine production of NK cells when cultured together.

4.4 The effects of polyunsaturated fatty acid pre-incubation of human NK cells on their lipid mediator production (paper III - unpublished)

4.4.1 Human NK cells synthetize specialized pro-resolving mediators from eicosapentaenoic and docosahexaenoic acid (paper III - unpublished)

When pre-incubated with omega-3 or omega-6 PUFAs, these fatty acids and their oxygenated products emerged in NK cell cultures and co-cultures compared to C-NK cell cultures where they were not present (paper III, fig. 1A). Lipid concentrations in NK cell cultures can be found in appendix D. To reduce the weight of highly concentrated lipid mediators and outliers, the data were then scaled to a 0-1 range and centered. Principal component analysis and subsequent clustering analysis revealed four clusters in the lipid dataset (appendix E). Notably, AA-NK, EPA-NK, and DHA-NK cell cultures resulted in distinct clusters from C-NK cell samples located in the center (paper III, fig. 1B). However, neutrophils did not result in the expected separate clusters.

Due to limited bioavailability of SPMs, accurately detecting fully formed SPMs remains a significant challenge in the field (219). To address this, we conducted a correlation analysis of all lipids and oxidated products in our cell culture datasets. We then performed an unsupervised hierarchical clustering analysis to determine lipid mediators commonly detected together. This analysis revealed five distinct clusets, including one containing the supplemented EPA and its oxygenated lipid products and one comprised of DHA and its oxylipid products (paper III, fig. 1C). Interestingly, the EPA-derived SPMs RvE_2 and 18S-RvE_3 was strongly correlated with the addition of EPA to the NK cell cultures (paper III, fig. 1C). Similarly, DHA-derived SPMs, such as PD_1, 7S-MaR1, MaR1, and RvD_1, were likewise strongly correlated with the supplementation of NK cell cultures with DHA (paper III, fig. 1C).

To confirm that the measured lipids were produced via enzymatic reactions and not through autoxidation, we measured the presence of lipids and oxylipid metabolites in a PUFA-enriched cell-free culture system. We considered oxylipids as enzymatically produced when the mean concentration was at least twice as high as the autoxidative levels. All omega-3 PUFA-derived oxylipins measured in the EPA-NK and DHA-NK cell cultures were higher than both autoxidative levels and levels observed in C-NK cell cultures (appendix D, paper III, fig. 2).

The addition of EPA resulted in higher concentrations of 5-, 12-, and 15-HEPE than what was observed in solvent controls (paper III, fig. 2A). Furthermore, the oxylipid 18-HEPE and its downstream product RvE_2 were both found in higher concentrations in EPA-NK cell cultures compared to C-NK cell cultures (paper III, fig. 2A). Interestingly, the addition of neutrophils to the EPA-NK cell cultures decreased the cell culture concentration of 5-

HEPE compared to when EPA-NK cells were cultured alone (paper III, fig. 2A). However, the levels of 12-, 15-, and 18-HEPE, as well as RvE_2 were not modulated by the addition of neutrophils to NK cell cultures (paper III, fig. 2A).

DHA supplementation to the NK cell cultures increased cell culture concentrations of 4-, 7-, 10-, 14-, and 17-HDHA compared to those in C-NK cell cultures (paper III, fig. 2B). The downstream products of 14-HDHA, MaR1 and its stereospecific isomer 7(S)-MaR1, was similarly increased in DHA-NK cell cultures compared to that in C-NK cell cultures (paper III, fig. 2B). Moreover, higher concentrations of RvD_1, the downstream product of 17-HDHA, were detected in DHA-NK cell cultures compared to C-NK cell cultures (paper III, fig. 2B). Generally, co-culturing DHA-NK cells and neutrophil did not affect DHA-derived oxylipid concentrations compared to that when DHA-NK cells were cultured alone (paper III, fig. 2B).

As all EPA- and DHA-derived oxylipids were detected at higher concentrations than the cell-free autoxidation culture controls, we proceeded to investigate whether NK cells expressed LOX proteins. We found that freshly isolated, unstimulated NK cells expressed both 5- and 12-LOX (paper III, fig. 3A,B). They did not express 15-LOX-1, but low levels of the inducible 15-LOX-2 isoform were detected (paper III, fig. 3C). Notably, we observed different levels of protein between the donors for all detected LOXs (paper III, fig. 3C). This variability were not limited to one or two low expressing individuals vut was observed across all donors, which expressed a different LOX protein composition compared to the others (paper III, fig. 3C).

4.4.2 Human NK cells cannot synthetize eicosanoids from arachidonic acid without the aid of neutrophils (paper III – unpublished)

AA and its oxylipid derivatives synthesized through LOX-dependent mechanisms and autoxidative products clustered together in a big cluster (paper III, fig. 1C). Interestingly, prostanoids were clustered in a distinct cluster separate from the other AA-derived oxylipids (paper III, fig. 1C).

Once more, 5-, 12-, and 15-HETE concentrations were increased upon the addition of AA to NK cell cultures compared to their solvent controls (paper III, fig. 5). This synthesis is dependent on LOX-mediated oxidation and were not modulated by the addition of neutrophils to the culturing system (paper III, fig. 5).

Concentrations of PGE_2, PGF_{2a}, and TxB_2 were not modulated in AA-NK cell cultures with autoxidation levels at similar levels, indicating the any presence of these eicosanoids in NK cell cultures are brought about through non-enzymatic production (paper III, fig. 5). However, higher levels of these eicosanoids were detected when AA-NK cells were co-cultured with neutrophils and exceeded the autoxidative levels in cell-free controls indicating enzymatic production (paper III, fig. 5).

To summarize, our findings suggest that PUFA enrichment can modulate lipidomic content in NK cell cultures and neutrophils did not contribute to this. NK cells are capable of producing various lipid mediators derived from AA, EPA, and DHA through LOX-dependent mechanisms when treated with these PUFAs. Specifically, NK cells gave rise to SPMs and emerged as a novel source of Rvs and MaRs. However, NK cells did not produce eiocosanoids without the aid from neutrophils.

5 Discussion

The aim of the present thesis was to investigate the effects of omega-3 PUFAs on the resolution of murine antigen-induced peritonitis and the recruitment, phenotype, and function of NK cells. We also aimed to determine how omega-3 PUFAs affect human NK cell SPM production, as well their effects on, and crosstalk with neutrophils. In addition to confirming that dietary fish oil enhances resolution of inflammation as observed as decreased R_i, our research yielded several interesting findings (table 6). Dietary fish oil attenuated peritoneal concentrations of the chemokines, CXCL1 and -2, that recruit neutrophils to the peritoneal cavity. It also dampened surface expression of CD47 on neutrophils and increased their apoptosis, potentially enhancing their clearance by macrophages. This was supported by the finding that dietary fish oil increased apoptotic bodies in draining lymph nodes. In addition, it increased peritoneal levels of growth factors and the decoy receptor sTNFRII, which may counteract pro-inflammatory signaling. Furthermore, dietary fish oil decreased peritoneal concentrations of prostanoids while increasing peritoneal levels of HEPEs, which may contribute to an anti-inflammatory micro-environment. Taken together, these findings indicate that dietary omega-3 PUFAs facilitate early stages of murine antigen-induced peritonitis resolution.

Dietary fish oil increased the number of the most mature and cytotoxic NK cells, i.e., $CD11b^+CD27^-$ NK cells. These cells were likely attracted to the inflamed site through the CCR5-CCL5, CXCR4-CXCL12, and/or CXCR3-CXCL10 recruitment axes. Dietary fish oil also increased peritoneal NK cell degranulation, as demonstrated by elevated CD107a expression. The increase in $CD107a^+$ NK cells corresponded with increased neutrophil apoptosis, indicating that NK cells may play a role in inducing neutrophil apoptosis for their clearance.

By using cultured human NK cells, we found that DHA tempered NK cell induction of neutrophil expression of CD11b and CD47 on neutrophils. However, it did not affect NK cells' ability to enhance neutrophil apoptosis early in the co-culture, yet it enhanced their ability to improve neutrophil survival after prolonged co-culture. Culturing NK cells with DHA did not affect their ability to induce neutrophils to secrete pro-inflammatory cytokines. Conversely, it made them more sensitive to neutrophil-induced downregulation of their expression of NKp46 but less sensitive to reduction in CXCR3 expression. Finally, NK cells cultured with DHA produced lower levels of IFN-γ, TNF-α, and CCL3 in response to neutrophils compared to NK cells not cultured with DHA.

NK cell cultures did not contain prostanoids unless neutrophils were present. Notably, our study revealed that NK cells contain the enzymes necessary to produce SPMs, i.e., 5-, 12-, and 15-LOX, and that omega-3 PUFAs induce NK cells to produce SPMs without collaborating with neutrophils.

Table 6. Key findings.
Blue arrows indicate an increase; orange arrows indicate a decrease. In murine antigen-induced peritonitis, arrows show differences between fish oil and control diet fed mice. In human cell cultures, arrows show the effect of specified cells on target cell with or without fatty acid treatment of NK cells. The number of arrows indicates relative difference compared to controls, i.e., one arrow is >100% difference, two arrows 100-300%, and three arrows >300%. An upwards facing arrow indicates an increase, whereas a downfacing indicates decrease, and – indicates no change.

Murine antigen-induced peritonitis				
Inflammation resolution				
R_i and neutrophil numbers	↓			
CXCL1, CXCL2	↓			
NP CD47	↓			
NP apoptosis	↑↑			
LN apoptotic bodies	↑↑			
Pro-inflammatory cytokines	↓			
Growth factors and sTNF RII	↑↑			
Prostanoids	↓			
HEPEs	↑↑↑			
NK cells				
CCL5, CXCL10, CXCL12	↑			
Absolute numbers	↑			
$CD11b^{+}CD27^{-}$	↑			
CCR5	↑↑↑			
CD107a	↑			
CD62L	↓			
Human *ex vivo*				
NK cell effects on NPs	**C**		**DHA**	
CD11b, CD47	↑↑		↑	
Apoptosis, 6 h	↑		↑	
Apoptosis, 18 h	↓		↓	
Pro-inflammatory cytokine production	↑↑↑		↑↑↑	
NP effects on NK cells	**C**		**DHA**	
NKp46 (%/MFI)	↓/↓		↓/↓	
CXCR3 (%/MFI)	↓/↑↑		↓/↑↑	
Pro-inflammatory cytokine production	↑		↓	
CCL3	-		↓	
NK cells	**Alone**		**With NP**	
	C	**PUFA**	**C**	**PUFA**
Eicosanoid	-	-	-	↑
SPM	-	↑	-	↑

5.1 Recruitment of degranulating NK cells

Chemotactic signaling may have contributed to the increased number of NK cells accumulated in the peritoneal cavity of inflamed mice fed the fish oil diet. Specifically, CCL5, CXCL10, and CXCL12 concentrations were increased in the peritoneum of inflamed mice fed the fish oil diet at 3 and 6 h after inflammation induction. Correspondingly, the number of $CCR5^+$ NK cells, as well as the percentages of $CCR5^+$ cells of total NK cells and the median fluorescence intensity of CCR5 on NK cells, were higher in mice fed the fish oil diet 6 h after inflammation induction than in mice fed the control diet. These findings suggest that the CCL5- CCR5 recruitment axis is involved in the accumulation of NK cells at the inflamed site and possibly in the resolution of antigen-induced peritonitis. This is consistent with studies using murine poly I:C (158), acute hepatitis B infectious (168), herpes simplex virus type 2 infectious (169), *Toxoplasma Gondii* infectious (162), concanavalin A-induced hepatitis (167), cancer (164, 221), and kidney allografts (222) models, which have demonstrated the importance of the CCL5-CCR5 recruitment axis for NK cells in resolution of inflammation. However, NK cells recruited through the CCL5-CCR5 axis can also contribute to disease progression and worsening of symptoms. For instance, CCR5 knockout mice were protected from developing experimental autoimmune encephalomyelitis, a mouse model of MS, (223) and DSS-induced colitis (224). Although these models, like ours, involve sterile and not infectious inflammation, our data indicate that CCL5-CCR5-dependent NK cell recruitment may promote inflammation resolution in antigen-induced peritonitis.

Several studies indicate that there may be a relationship between NK cell chemokine receptor expression, the axis through which they are recruited, and their cytotoxic function. We demonstrated that dietary fish oil increased peritoneal CCL5 concentrations and NK cell expression of CCR5, but also increased NK cell expression of CD107a. This finding corresponds with our observation that the most mature and cytotoxic NK cell subset, the $CD11b^+CD27^-$ subset, accumulated in the inflamed peritoneum in higher numbers in mice fed the fish oil diet. As CD107a is transiently expressed on NK cells following their degranulation (225) these findings indicate that the NK cells recruited to the inflamed peritoneum may degranulate more. For example, a recent study demonstrated that NK cells of human immunodeficiency virus (HIV) viremic patients expressed higher levels of CCR5 and CXCR4 as well as CD107a following anti-retroviral therapy (226). Comparable findings have been reported in MS, experimental autoimmune encephalomyelitis (187), and RA (204) suggesting that chemotactic receptor expression and cytotoxic function is tightly linked in viral infections and autoimmunity. In the present study we did not investigate whether the NK cells created gap junctions, thus, the increased CD107a expression may not be exclusively cytotoxic. Collectively, these findings indicate a functional link between chemokine receptor expression and cytotoxic functions of NK cells to limit the inflammatory response and possibly promote its resolution.

The accumulated peritoneal NK cells observed in the present study may also have been recruited by the CXCL12-CXCR4 axis as dietary fish oil increased peritoneal concentrations of CXCL12 conjointly with CCL5. This recruitment axis have been implicated in the recruitment of immunoregulatory NK cells to ischemic stroke lesions in mice (227) and the decidua to ensure successful implantation during pregnancy (228, 229). NK cells in viremic HIV patients expressed higher levels of CXCR4 conjointly with CD107a as specified above (226). Further, NK cells engineered to express high levels of CXCR4 were more effective in controlling tumor progression in a murine neuroblastoma model (230). This strengthens the notion that the mechanism by which NK cells are recruited to tissues determine their effector functions. On the other hand, CXCR4-dependent accumulation of NK cells promoted disease progression of late RSV infection-induced lung inflammation (182).

All in all, our data indicates that dietary fish oil may modulate the recruitment of degranulating and possibly cytotoxic NK cells through chemotactic recruitment to promote resolution of antigen-induced peritonitis.

5.2 Reduction in neutrophil numbers

The decreased peritoneal concentrations of CXCL1 and CXCL2 in mice fed the fish oil diet may have contributed to the reduction in the number of neutrophils recruited to the peritoneum following inflammation induction. A study using the same antigen-induced peritonitis model we used in the present study demonstrated that CXCL1 and CXCL5 are essential for recruitment of neutrophils to the inflamed peritoneum (231). However, they did not detect a role for CXCL2 in the recruitment of neutrophils to the inflamed peritoneum in that study (231) although the recruitment of neutrophils by both CXCL1 and CXCL2 has been implicated in inflammatory neutrophil recruitment in several other disease models (232-238). In an infectious mouse model, LTB_4 induced CXCL1 and CXCL2 production to ensure the recruitment of inflammatory neutrophils to combat infection (238). Yet, inflammatory neutrophils recruited through the same pathway are known to exacerbate autoimmune disease and tumor progression (239, 240), which may be because of the non-infectious nature of this inflammation. Recent findings suggest that the EPA metabolite 12-HEPE downregulates keratinocyte production of CXCL1 and CXCL2, thereby limiting neutrophil recruitment and ameliorating contact hypersensitivy dermatitis (241). This is in accordance with our hypothesis that by suppressing CXCL1 and CXCL2 dietary fish oil limits neutrophil recruitment to the inflamed site and promotes inflammation resolution.

The increased neutrophil apoptosis in the peritoneum of inflamed mice fed the fish oil diet may be brought about by the increase in the number of degranulating NK cells. As mentioned previously, NK cells are reported to alleviate severe pulmonary murine allergic inflammation (242), DSS-induced colitis (212, 243), asthma (118), and antigen-induced peritonitis (214) by inducing apoptosis of inflammatory granulocytes. For instance, NK

cells accumulated in the lungs in asthmatic pulmonary inflammation via RvE_1-ChemR23 signaling to limite the number of inflammatory eosinophils, and depletion of NK cells resulted in an extended R_i (118). Our research group later demonstrated that resolution of antigen-induced peritonitis may also be NK cell-dependent (214). Yet, this NK cell depletion study conducted in our research group suggested that the immature $CD11b^-CD27^+$ subset was most affected and potentially responsible for resolving inflammation (214). Conversely, Haworth et. al suggested that it is the mature and cytotoxic $CD11b^+CD27^+$ NK cell subet that was responsible for the resolution of asthmatic pulmonary inflammation (118). In the present study, however, we found that the NK cell subset most enhanced and, potentially, most effective in promoting resolution of inflammation was the most mature and cytotoxic $CD11b^+CD27^-$ subset. These differences may be explained by differences in the inflammatory responses of different mouse strains, such as BALB/cj mice and C57Bl/6 mice (244, 245). The discrepancies in NK cell subsets most affected in the studies from our research group and the one of Haworth et al. may be due to different functional maturation of NK cells. The immature NK cells depleted in the previous study in our group may indicate a reduced potential for NK cells to accumulate at the inflamed site and mature on-site to exert their cytotoxic functions. Therefore, dietary fish oil may enhance functional maturation of NK cells in the peritoneum of inflamed mice to promote inflammation resolution by exerting their cytotoxic effector functions to enhancing neutrophil apoptosis.

The fish oil diet dampened neutrophil expression of CD47 and increased their level of apoptosis, consistent with previous research (218). This decrease may have enhanced the removal of the accumulated neutrophils from the peritoneal cavity, as reduced CD47 expression has been shown to enhance macrophage engulfment of apoptotic neutrophils (218). Our study supports this hypothesis, as inflamed mice fed the fish oil diet had increased numbers of apoptotic bodies in their mesenteric lymph nodes. Interestingly, another study suggested that CD47 expression is essential for neutrophil transmigration (246), which could have contributed to the decreased neutrophil accumulation in the inflamed peritoneum of mice fed the fish oil diet. Surprisingly, our *in vitro* experiments showed that human NK cell can induce neutrophil expression of CD47 and a $CD11b^{high}CD47^+$ neutrophil subset. A recent publication revealed that CD47 and CD11b may be co-expressed to mediate neutrophil transmigration to inflamed sites (247). The induction of neutrophil CD47 expression in co-cultures with human NK cells in our study was tempered when the NK cells had been pre-incubated with DHA. This induction of neutrophil expression of CD47 is in accordance with the dampened neutrophil expression of CD47 in mice fed the fish oil diet, which was concurrent with increased numbers of cytotoxic NK cells. Thus, omega-3 PUFAs may modify NK cells' induction of neutrophil CD47 expression to promote inflammation resolution.

Taken together, omega-3 PUFAs can reduce the number of neutrophils accumulated in the inflamed peritoneum in antigen-induced peritonitis. This effect may be attributed to the dampened production of neutrophilic-chemotactic molecules and the enhanced levels

of neutrophil apoptosis due to the increased number of mature NK cells in the inflamed peritoneum of mice fed the fish oil diet. Additionally, the reduced expression of CD47 on neutrophils surfaces may have contributed to a lower recruitment rate, whilst enhancing efferocytosis of apoptotic neutrophils for their removal to draining lymph nodes. Ultimately, these processes may have caused the dramatic limitation of neutrophil numbers and the shortened R_i we observed in our study.

5.3 Regulation of cytokine production

Dietary fish oil surprisingly led to a dramatic increase in peritoneal concentration of sTNFRII during antigen-induced peritonitis. sTNFRII acts as a decoy receptor that impairs TNF-α signaling (22, 248) yet, peritoneal TNF-α concentration were only decreased in mice fed the fish oil diet at in early time-point when there was no difference in sTNFRII concentrations between the two groups. It has been shown that while recombinant sTNFRII does not decrease TNF-α concentrations, it dampens inflammatory TNF-α and downstream cytokine products of TNF-α (249). It has been reported in different disease models that TNF-α induces expression of other pro-inflammatory cytokines, such as IL-6, IL-6Rα, IL-12, and IL-23 (250, 251) possibly through NF-κB signaling (252). IL-6, in turn, induces the expression of CCL20 in target cells by additional NF-κB signaling (253). In the present study the increased peritoneal concentration of sTNFRII in mice fed the fish oil diet may have dampened the effect of TNF-α to induce the production of the pro-inflammatory cytokines and thereby led the the lower peritoneal concentrations of IL-6, IL-6Rα, and CCL20. In paper II, we reported that the CCL20 concentration in peritoneal fluid of inflamed mice were not changed due to differences in the statistical testing. However, following reanalysis, we discovered that the peritoneal concentrations of CCL20 was dampened. Thus, although dietary fish oil did not affect peritoneal concentration of TNF-α at the time-point when it increased concentrations of sTNFRII, the modulation of its downstream products indicating that this signaling is modulated by the increased sTNFRII concentration. This is of importance as CCL20 is suggested to drive the pathogenesis of autoimmune diseases such as inflammatory bowel disease (254) and RA (255), both diseases that, as our inflammation model, are marked by sterile inflammatory responses. In our co-culture system of human NK cells and neutrophils, DHA tempered NK cell production of TNF-α and other pro-inflammatory cytokines, such as IFN-γ and GM-CSF. Thus, omega-3 PUFAs may directly modulate cytokine production of NK cells to enhance their immunoregulatory functions. Treatment of chronic inflammation-associated degenerative diseases with sTNFRII is routinely used to manage clinical symptoms through a downregulation of pro-inflammatory cytokine signaling and production (256). For example, recombinant sTNFRII attenuates murine psoriasis (251), COVID-19 (257), influenza pneumonia (258), sepsis-induced liver injury (259), and murine RA (260) by attenuating the pro-inflammatory signaling and subsequent production of downstream mediators. Interestingly, NF-κB is also an important inducer of COX that catalyzes the production of prostanoids, such as PGE_2, PGF_{2a}, and TxB_2

(261). Omega-3 PUFAs have been suggested to dampen NF-κB-induced COX expression and subsequent prostanoid production during inflammation and tumor progression (262-264). Thus, the dampened concentrations of prostanoids during antigen-induced peritonitis in mice fed the fish oil diet may have been caused by suppressed NF-κB signaling brough about the enhanced concentration of sTNFRII.

Dietary fish oil also enhanced peritoneal concentration of TGF-β1 in inflamed mice. The increase in TGF-β1 in our study confirms previous data from our group using the same murine model (80) as well as that of Rosa et al. (265) that showed that dietary fish oil increased TGF-β in rats with precancerous colon lesions. TGF-β1 has been shown to promote the resolution of influenza A infection-induced (32) and monohydrate crystal-induced (33) inflammation in mice by reducing the production of pro-inflammatory cytokines. Omega-3 PUFAs have been shown to increase TGF-β1 secreted by PBMCs from healthy adults (266) and TGF-β1 levels in the dermis of murine atopic dermatitis to ameliorate clinical symptoms (267). However, some studies showed that EPA-enriched phospholipids and DHA dampened TGF-β1 levels and signaling in renal interstitial fibrosis in rats (268) and breast cancer cell lines (269), respectively, and led to higher pro-inflammatory cytokine levels. These studies used high concentrations of PUFAs in the diet and the cell culture, respectively, and the rat study also failed to control for the differing fat contents between the groups. Thus, these confounding factors may have led to the discrepancies between the results in our and their studies. Additionally, we found that dietary fish oil promoted the return of IGF-1 to baseline levels in the peritoneum of inflamed mice after the drop in its concentration having decreased upon induction of inflammation. As IGF-1 has been shown to attenuate pro-inflammatory cytokine responses and ameliorate inflammation in myocardial infarction (26), hyperoxia-induced lung inflammation (27), endotoxemia (28), and acute ischemic stroke (270), the higher concentration of IGF-1 in mice fed the oil diet may have contributed to the reduced peak inflammation. Moreover, IGF-1 has been reported to promote the M2-polarization of macrophages and may have further enhanced efferocytosis of apototic neutrophils and their removal from the peritoneum to draining lymph nodes in mice fed the fish oil diet (271, 272). Interestingly, MaR1, a DHA metabolite, was recently shown to normalize IGF-1 levels in murine liver following injury (273) similarly to what we observed in the peritoneum of inflamed mice fed the fish oil diet. Thus, the observed increase in peritoneal IGF-1 to baseline levels in the present study may have been induced by an increase in MaR1, but this hypothesis would have to be investigated further. Thus, TGF-β1 and IGF-1 may be enhanced at peak inflammation in antigen-induced peritonitis in mice fed the fish oil diet to promote its resolution.

5.4 Production of lipid mediators

In mice fed the fish oil diet, the increase in peritoneal prostanoid concentrations generated by COX-dependent mechanisms were dampened upon induction of inflammation. These results are consistent with findings by others for tumor progression,

non-alcoholic liver disease, as well as hypoxia- and aipouch-induced inflammation (274-277). Interestingly, the enhanced degranulation of NK cells, shown by enhanced CD107a expression on their surfaces, may have been mediated by the lowered production of PGE_2 in inflamed mice. This is supported by the fact that PGE_2 inhibits NK cell effector functions through ligation of EP2 and EP4 on NK cell surfaces (91, 92, 278). The metabolism of AA is modulated by the presence of EPA due to their competitive relationship for enzymatic conversion (279). Additionally, it has been reported that DHA also modulates prostanoid synthesis by dampening the expression of PG synthase (280). Thus, the modulation of PGs and Tx during antigen-induced peritonitis may be due to the lowered AA metabolism both due to EPA enzyme competition and omega-3 PUFA mediated decrease of PG synthase. This hypothesis is further supported by the improvement in the HEPE:HETE ratio in the peritoneum of mice fed the fish oil diet, which is consistent with findings in fish oil fed prediabetic rats (281). In line with the observations for prostanoids, we found lower concentrations of 5-, 12-, and 15-HETE at peak inflammation in mice fed the fish oil diet. As HETEs have been suggested to drive chronic inflammatory pathologies, this decrease may have contributed to the promotion of resolution of inflammation (97-100). Along these lines, HEPEs and HDHAs have been reported to possess pro-resolving functions of their own (282-284), thus, increased synthesis of HEPEs and HDHAs may have supported resolution of inflammation processes in the inflamed mice fed the fish oil diet in the present study.

We confirmed that human NK cells express the LOX enzymes, 5-, 12-, and 15-LOX-2, necessary for PUFA-derived SPM synthesis, as first described in a murine model of type I diabetes (285). Contrary to the consensus in the field that neutrophils are a cellular source of SPMs, we did not observe any SPM production by human neutrophils when they were added to PUFA-enriched NK cell cultures (63, 128, 286, 287). However, we demonstrated that human NK cells, on their own, produced SPMs and their intermediates, most likely through LOX-dependent mechanisms. The fact that neutrophils did not contribute to the production of SPMs in our culture system may be due to incomplete activation of neutrophils required for this, as described in a recent study (288). In human NK cell cultures enriched with EPA or DHA we detected the fully formed SPMs MaR1 and its stereoisomer 7(S)-MaR1, RvD_1, and RvE_2. However, no, or very low levels, of fully formed SPMs were detected in peritoneal exudate from inflamed mice fed the fish oil diet. As there are field-wide difficulties in detecting SPMs in biological samples, we conducted two correlation analyses; bioactive lipid mediators measured in human NK cell cultures and in peritoneal exudates of inflamed mice. We found that the presence of fully formed SPMs correlated with the presence of their substrate PUFA in the cell cultures but could not confirm this for our biological samples. However, we did see a correlation of EPA- and DHA-derived LOX-dependent intermediates of SPMs in the peritoneal exudates of inflamed mice. Thus, when EPA and DHA were found in high concentrations in the peritoneal fluid, the presence of HEPEs and HDHAs were strongly correlated, suggesting that the LOX-dependent metabolism of omega-3 PUFAs may be enhanced in

inflamed mice. However, our finding should be confirmed with the use of inhibitors of LOXs and *in vivo* confirmations in inflamed NK cell-depleted and fish oil fed mice to confirm that they are a novel cellular source of SPMs.

All in all, these findings have further implicated NK cells as resolution effector cells, which is an effector function that has not been described.

6 Conclusions

In the present study, we confirm that dietary fish oil enhances resolution of antigen-induced peritonitis as shown previously by our group (80). The mechanism by which dietary fish oil promotes resolution of antigen-induced peritonitis is summarized in Figure 27. Shortly, dietary fish oil reduced the number of inflammatory neutrophils that accumulated in the inflamed peritoneum of mice possibly by dampening the production of CXCL1 and CXCL2. Dietary fish oil also led to increased accumulation of highly cytotoxic $CD11b^{+}CD27^{-}$ NK cells that may have led to neutrophil apoptosis, thereby

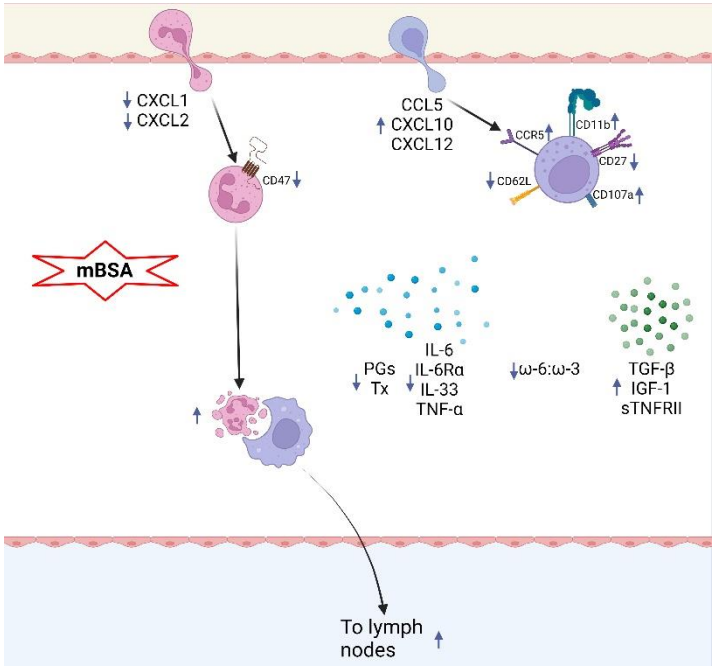

Figure 27. Proposed mechanism of the effect of dietary fish oils on resolution of inflammation. Upon induction of inflammation, dietary fish oil dampens the production of neutrophil-chemotactic molecules (CXCL1 and CXCL2) and neutrophil expression of CD47. Dietary fish oil increases the production of NK cell-recruiting chemokines (CCL5, CXCL10, CXCL12) and the accumulation of $CD11b^{+}CD27^{-}$ NK cells expressing high levels of CCR5 and CD107a, but lower expression of CD62L than that in mice fed the control diet. Dietary fish oil decreased peritoneal ratio of omega (ω)-6:ω-3 fatty acids, as well as concentrations of IL-6, IL-6Rα, IL-33, TNF-α, PGs, and Tx while it inreased peritoneal concentrations of TGF-β, IGF-1, and sTNFRII. Finally, dietary fish oil increased neutrophil apoptosis and their removal to draining lymph nodes. Blue arrows indicate differences in concentrations observed in inflamed mice fed the fish oil diet compared to that in mice fed control diet. Figure created using **Biorender.com**.

contributing to the decreased numbers of NK cells. Induction of neutrophil apoptosis causes neutrophils to downregulate their expression of CD47, thereby marking them for engulfment by macrophages and subsequent removal to draining lymph nodes. Further, dietary fish oil may promote more efficient resolution of antigen-induced peritonitis by dampening the production of pro-inflammatory cytokines, possibly through dampened NF-κB signaling due to increased peritoneal concentrations of sTNFRII, TGF-β1, and IGF-1. Dietary fish oil also directly decreased the peritoneal omega-6:omega-3 PUFA ratio and dampened the synthesis of prostanoids. All in all, dietary fish oil enhances resolution of inflammation by promoting several early mechanisms to ensure complete resolution of murine antigen-induced peritonitis.

The effects of omega-3 PUFAs on human NK cells and their interactions with neutrophils are summarized in Figure 28. We showed that DHA modulated NK cell effects on and crosstalk with neutrophils by tempering NK cell-mediated induction of neutrophil CD47 and CD11b expression. DHA also rendered NK cells more sensitive to neutrophil-associated cleavage of NKp46 while dampening neutrophil-induced production of pro-inflammatory cytokines. Surprisingly, human NK cells were shown to express LOX and to potentially synthesize omega-3 PUFA-derived bioactive lipid mediators. These findings indicate that NK cells may enhance certain hallmarks of inflammation resolution that is enhanced by dietary fish oil.

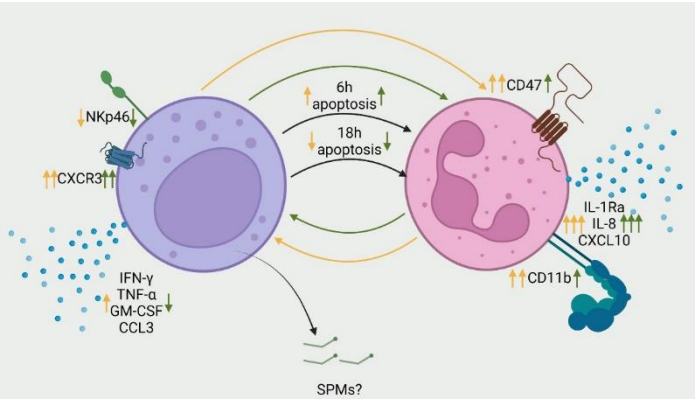

Figure 28. Proposed regulation of crosstalk between human NK cells (left) and neutrophils (right) by DHA.

NK cells modulate neutrophil survival, CD47 and CD11b expression, and production of IL-1Rα, IL-8, and CXCL10. Neutrophils, in turn, modulate NK cell expression of NKp46 and CXCR3, and production of IFN-γ, TNF-α, and GM-CSF. These effects are indicated by yellow arrows. DHA (green arrows) temper NK cell effects on neutrophils while it modulates the sensitivity of NK cells to neutrophil associated modifications. Finally, NK cells may produce SPMs from omega-3 PUFAs to further promote pro-resolving functions and crosstalk. Figure created using **Biorender.com**.

6.1 Limitations

In the present study, we determined the effects of dietary fish oil on resolution of antigen-induced peritonitis, as well as on NK cell recruitment and phenotype. These studies were decriptive studies and did not allow us to draw conclusions on the function of NK cells or the mechanism by which they may affect the resolution of murine antigen-induced peritonitis. The data obtained *in vitro* to demonstrate NK cell effects and crosstalk with neutrophils are also decriptive. As *in vitro* investigations are artificial inflammatory micro-environments, NK cell and neutrophil co-cultures may have lacked one or more additional cell types to provide integral cues for their pro-resolving functions.

It is uncertain whether the concentration of PUFAs used in the cell cultures (50 µM) is close to that at the inflamed site *in vivo*. As PUFAs have been reported to have potential toxicity when used in high concentrations (150 µM) in cell culture (289), we evaluated the viability of NK cells and found no effect of PUFAs up to 50 µM.

In our investigations we determine the presence of SPMs in NK cell cultures with or without neutrophils. These studies did not allow us to conclude that NK cells are capable of synthesizing SPMs via LOXs, as we did not include functional studies due to time constraints in the present project. As reviewed by Schebb and colleagues (219), when measuring SPMs, it is necessary to adhere to high scientific rigor, as SPMs can be produced by autoxidation. This is especially true in cell cultures, as the cells and PUFAs in the culture system are directly exposed to oxygen and oxygenated compounds. We determined the level of autoxidative lipid mediator production in our cell culture system by measuring the levels of PUFA-derived bioactive lipid mediators in cell-free cultures. In addition, further studies using LOX inhibitors will be conducted to determine whether NK cells produce SPMs enzymatically.

In the present study, we used an antigen-induced peritonitis model as opposed to the more commonly used zymosan-induced peritonitis model (40, 124, 290-293). As illustrated in a previous study conducted by our research group (294), antigen-induced peritonitis exhibits a more rapid resolution phase with dampened neutrophil influx than observed in the zymosan-induced peritonitis model (292). However, zymosan-induced inflammation is almost exclusively innate with limited instruction of the adaptive immune defense (295). As we have shown that NK cells are integral for the resolution of antigen-induced peritonitis (214) we decided to use this model in the present project.

C57Bl/6 mice were chosen by the research group when it began to explore the effects on omega-3 PUFAs on antigen-induced peritonitis since their immune response is skewed towards a T_H1-prone immune response (296) and several human autoimmune diseases are driven by T_H1 responses. However, there is evidence that NK cell responses in C57Bl/6 mice are less effective in antitumor effector functions compared to other mouse strains, such as 129/J and CBA/J mice (296). Therefore, the NK cell responses in antigen-induced peritonitis in C57Bl/6 mice may have been less effective than that in 129/J mice.

Further, laboratory mice are inbred and show less variations than outbred mice and what is expected in the human population. There have been mixed results regarding the use of C57Bl/6 mice to explore treatment of human diseases, as it has yielded an effective type I IFN treatment of MS (297) but also treatments that are not effective or even harmful in humans, as seen with specific T cell depletions in MS patients (297).

The effects of dietary fish oil on resolution of inflammation in the mice (79, 80) may be more pronounced than they would be in humans, as rodent cells are more sensitive to the incorporation of omega-3 PUFAs than human cells are (298). The Canadian Health Ministry recommends a minimum daily intake of 0.5% omega-3 PUFAs (299). As for the amount of the omega-3 PUFAs given to the mice, it did not exceed what could be consumed by humans if calculated bases on the percentage of energy. EPA and DHA provided 0.8% and 0.65% of the daily calories consumed by the mice, which would equal a daily consumption of 1.8 g of EPA and 1.4 g DHA in a human diet of 2,000 calories. Thus, the total amount of EPA and DHA would equal ~3.2 g per day which is considered safe (300). Although vitamin A and D are commonly present in fish oil the supplemented fish oil used in the present study did not contain these vitamins and therefore we did not need to take their effects on inflammatory responses in both mice and humans into consideration (301, 302).

6.2 Future perspectives

To verify that NK cells are a novel cellular source of SPMs derived from LOX-dependent oxidation of omega-3 PUFAs, several studies should be conducted. Firstly, it should be verified that the production of lipid mediators from omega-3 PUFAs are enzymatically produced by inhibitation of 5-, 12-, and 15-LOX-2 in NK cells. NK cells with the LOX proteins knocked down or inhibited and adoptively transferred to inflamed mice may provide a mechanistic insight into the biological function of their SPM production. It is also of importance to investigate how NK cells regulate LOX expression, especially the inducible 15-LOX-2 enzyme, to contribute to the SPM production during inflammation resolution. Further, it should be investigated whether NK cells express other SPMs that are not derived from omega-3 PUFAs, such as Annexin A1.

Depleting NK cells in mice fed the fish oil diet and inducing antigen-induced peritonitis may provide valuable biological insights into the pro-resolving functions of NK cells *in vivo*. A study like this would also allow us to distinguish the effect of NK cells on the resolution of inflammation and not the effect of dietary fish oil. Based on the data presented in the present study it would be beneficial to investigate how NK cells are recruited to the inflamed site to promote resolution of inflammation. Thus, inhibiting chemotactic NK cell recruitment axes, such as the CCL5-CCR5, CXCL10-CXCR3, and CXCL12-CXCR4 axis, would reveal which is integral for the pro-resolving accumulation of NK cells in murine antigen-induced peritonitis.

Several molecules of interest were identified as potential pro-resolving mediators in the present study. The role of sTNFRII in the promotion of resolution of antigen-induced peritonitis should be investigated. Further studies utilizing recombinant sTNFRII, knockout mouse models, and sTNFRII inhibition, to determine its role in inflammation and its resolution would be benificial. By utilizing our approach to delineate the hallmarks of inflammation resolution, we can identify which specific mechanisms sTNFRII enhance to promote resolution of inflammation. Similar experimental approaches could be used to establish the role of TGF-β1 and IGF-1 in the resolution of inflammation.

Finally, the role of CD47 in inflammation resolution should be determined. Further, verifying that neutrophil expression of CD47 can be modulated by both NK cells and omega-3 PUFAs in the diet may shed light on a novel immune checkpoint in resolution responses.

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ORIGINAL RESEARCH

Dietary Fish Oil Increases the Number of CD11b^{+}CD27^{-} NK Cells at the Inflammatory Site and Enhances Key Hallmarks of Resolution of Murine Antigen-Induced Peritonitis

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Purpose: To determine the effects of dietary omega-3 polyunsaturated fatty acids (PUFAs) on recruitment of natural killer (NK) cells and resolution responses in antigen-induced peritonitis in mice.

Methods: Mice were fed fish oil-enriched or control diets, immunized twice and challenged intraperitoneally with methylated bovine serum albumin. Prior to and at different time-points following inflammation induction, expression of surface molecules on peritoneal cells was determined by flow cytometry, concentration of soluble mediators in peritoneal fluid by ELISA or Luminex, and of lipid mediators by LC-MS/MS, and number of apoptotic cells in mesenteric lymph nodes by TUNEL staining.

Results: Mice fed the fish oil diet had higher number of CD11b^{+}CD27^{-} NK cells as well as a higher proportion of CD107a^{+} NK cells in their peritoneum 6 h after inflammation induction than mice fed the control diet. They also had higher numbers of CCR5^{+} NK cells and higher concentrations of CCL5 and CXCL12. Additionally, a higher fraction of apoptotic neutrophils but lower fraction of CD47^{+} neutrophils were present in the peritoneum of mice fed the fish oil diet 6 h after inflammation induction and the fish oil fed mice had a shorter resolution interval. They also had lower concentrations of pro-inflammatory mediators but higher concentrations of the anti-inflammatory/pro-resolution mediators TGF-β, IGF-1, and soluble TNF RII, as well as higher ratios of hydroxyeicosapentaenoic acid (HEPE) to hydroxyeicosatetraenoic acid (HETE) than mice fed the control diet.

Conclusion: The results demonstrate that dietary fish oil increases the number of mature NK cells at the inflamed site in antigen-induced peritonitis and enhances several key hallmarks of resolution of inflammation, casting light on the potential mechanisms involved.

Keywords: natural killer cells, neutrophils, apoptosis, lipid mediators

Introduction

Acute inflammation is a protective response initiated upon tissue injury or pathogenic infections. It is characterized by rapid influx of innate immune cells, eg, neutrophils, and the production and release of pro-inflammatory cytokines, chemokines, and lipid mediators primarily derived from arachidonic acid (AA).[1,2] Due to the destructive nature of prolonged inflammation, these processes are effectively limited through a lipid mediator switch from production of pro-inflammatory mediators to anti-inflammatory and pro-resolution mediators in a tightly controlled cascade of events characterizing resolution of

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the acute inflammation.[3] The omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been suggested to dampen production of pro-inflammatory mediators, eg, interleukin (IL)-6, IL-33, tumor necrosis factor (TNF)-α, as well as the AA-derived prostaglandins (PGs) and thromboxane B_2 (TXB_2).[6,7] Additionally, EPA and DHA serve as substrates for lipoxygenase (LOX) enzymes to produce specialized pro-resolution mediators (SPMs), such as the E- and D-series resolvins (Rvs), protectin Ds (PDs), and maresins (MaR).[1,3–5] These lipid mediators and their intermediates induce resolution of acute inflammation, indicating that omega-3 PUFAs and their derivatives act as resolvents and not exclusively as anti-inflammatory compounds. In the resolution process, pro-inflammatory signaling is dampened and neutrophil recruitment is halted through cessation of production of neutrophil-recruiting chemokines, including C-X-C motif chemokine ligand (CXCL)1 and CXCL2.[8] In addition, enhanced tissue regeneration occurs through increased production of growth factors.[1] Lowered expression of the eat-me-not marker CD47 on neutrophils has been associated with enhanced spontaneous apoptosis and neutrophil efferocytosis[9,10] and is implicated in resolution of inflammation.[11] If acute inflammation is improperly resolved, it may develop into chronic inflammation, an underlying pathology of several prominent degenerative diseases, including cancer, diabetes, and autoimmune disorders.[5,12,13]

Natural killer (NK) cells are innate lymphocytes exerting potent anti-viral and anti-tumor functions that can induce apoptosis in aberrant cells without prior stimulation.[14] NK cells in circulation are commonly divided into four functional maturation groups depending on their expression of CD11b and CD27.[15,16] NK cells are matured in specialized bone marrow niches and released into circulation as immature $CD11b^-CD27^-$ NK cells whereafter they undergo further maturation.[17] The intermediately mature $CD11b^-CD27^+$ NK cells are regarded as the main cytokine producers, whereas the fully differentiated $CD11b^+CD27^-$ NK cells are potent cytotoxic cells.[15] Although the mechanism of NK cell recruitment to virally infected and inflamed sites is still debated, it has been shown that NK cells can migrate in response to a wide array of chemokines, including C-C motif chemokine ligand (CCL)2, CCL3, CCL4, CCL5, CCL19, and CCL20 through the expression of their respective receptors.[18] Studies have suggested that NK cells play a role in resolution of inflammation as their effector functions are pivotal for proper resolution.[19–21] Interestingly, NK cells have recently been shown to express high levels of 12/15-LOX, the murine ortholog of human 15-LOX, in a murine model of type I diabetes.[22] However, it remains unclear whether NK cells exert pro-resolution functions through the production of SPMs or their precursors.

Dietary consumption of omega-3 PUFAs has been shown to alleviate clinical symptoms of rheumatoid arthritis[23] and inflammatory bowel disease.[24] Dietary supplementation of fish oil in mouse studies has similarly shown anti-inflammatory and pro-resolution effects in murine peritonitis,[25] chemically induced colitis,[24] and experimental autoimmune encephalomyelitis.[26] Our previous results support a role for NK cells in resolution of inflammation as dietary fish oil induced an early recruitment of NK cells and enhanced resolution of inflammation in antigen-induced peritonitis in mice.[25] Additionally, we showed that depletion of NK cells severely abrogated proper resolution of the antigen-induced inflammation.[21] Furthermore, we have shown that the omega-3 PUFA DHA modulates human NK cell effects on and crosstalk with neutrophils in vitro by ameliorating neutrophil expression of CD11b and CD47.[27] The aim of the current study was to determine the effects of dietary fish oil on NK cell recruitment, maturation status, and functional phenotype, as well as on several hallmarks of resolution of inflammation in antigen-induced peritonitis.

Materials and Methods

Mice and Diets

Female (6–8-week-old) C57Bl6/J mice were obtained from Taconic Europe A/S (Denmark) and maintained in the experimental animal facilities at ArcticLAS ehf. Colonies of 10 mice per cage were kept in a 12-hour light/dark cycle. Mice were acclimatized for 7 days where they had access to regular chow and water ad libitum, whereafter they were randomly assigned either a Westernized diet (Research Diets, Inc., New Jersey, USA) (control diet) or a Westernized diet enriched with fish oil (fish oil diet). The fish oil diet contained 2.8% menhaden fish oil (Omega Protein, Virginia, USA) comprised of 15.6% of EPA and 11.3% of DHA, resulting in 4.0 g EPA and 2.9 g DHA per kg diet. The fish oil was added to the Westernized diet at the expense of safflower oil. AA ethyl ester (Nu-Check-Prep, Minnesota, USA) was added to the control diet to adjust for the AA content in the fish oil diet. The omega-6/omega-3 ratios of the control and fish oil diets were 11.8 and 1.3, respectively. All mice

were provided with fresh food daily and had free access to food and water. Diet consumption and weight development of both dietary groups were monitored with no difference observed between the groups (Supplementary Figure 1). All experiments were conducted in accordance with the NRC's Guide for the Care and Use of Laboratory Animals with approval by the Icelandic Food and Veterinary Authority (MAST, # 2017-01-04).

Antigen-Induced Peritonitis

Mice received experimental diets for 1 week prior to the first immunization to ensure incorporation of dietary lipids into cells.[28,29] Mice were immunized twice subcutaneously in opposite flanks with methylated bovine serum albumin (mBSA, Sigma-Aldrich, Germany) emulsified in complete Freund's adjuvant (Sigma-Aldrich) the first time and in incomplete Freund's adjuvant (Sigma-Aldrich) the second time with a 2-week interval. One week later, peritonitis was induced intraperitoneally with mBSA resuspended in saline. Mice were sacrificed by isoflurane overdose prior to (0 h) and at 1.5, 3, 6, and 12 h after peritonitis induction.

Collection of Peritoneal Lavage and Mesenteric Lymph Nodes

Peritoneal exudate (fluid and cells) was obtained by injecting 1 mL PBS into the peritoneal cavity, massaging gently, and collecting the exudate. In most cases, cells and fluid were separated by centrifugation and the fluid kept at −80°C until used in ELISA or Luminex assays for analysis of soluble molecules. Peritoneal cells were washed twice in ice-cold PBS, counted by Countess® automated cell counter (Invitrogen, Thermo Fisher Scientific, UK), and resuspended in PBS containing 1% BSA (Sigma Aldrich) and 0.01% NaN_3 (FACS buffer) for flow cytometric staining. For lipidomic analysis, peritoneal exudate, containing both cells and fluid, was snap frozen in liquid nitrogen, flushed with N_2 and stored at −80°C until run by liquid chromatography with tandem mass spectrometry (LC-MS /MS). Mesenteric lymph nodes were removed, placed in ice-cold PBS, immersed in O.C.T. compound (Sakura Finetek Europe B.V., Denmark), snap frozen, and stored at −80°C until cryosectioned.

Flow Cytometry

Peritoneal cells resuspended in FACS buffer ($1x10^6$ cells/100 µL/tube) were incubated with 2% normal rat:normal mouse: normal hamster serum (AbD Serotec, BioRad, UK), to block Fc receptors. The cells were subsequently stained with fluorochrome-labeled monoclonal antibodies against NK1.1 (PK136), CD49b (DX5), CD3 (17A2), CD11b (M1/70), CD27 (LG.3A10), CD62L (MEL-14), C-X-C motif chemokine receptor (CXCR)2 (SA044G4), Ly6G (1A8), CD47 (miap301) (BioLegend, Nordic Biosite, Sweden), CD107a (H4A3) (Santa Cruz Biotechnology, Texas, USA), or C-C motif chemokine receptor (CCR)5 (R&D Systems, Bio-Techne, UK). Excess antibodies were removed from the tubes with three subsequent washes and resuspension in cold FACS buffer for flow cytometric analysis. At least 100,000 events were collected using Sony SH800S flow cytometer (Sony Biotechnologies, UK) and data analyzed using Kaluza software (Beckman Coulter, California, USA). Appropriate isotypic antibodies were used to evaluate background staining. NK cells were defined as $CD3^-NK1.1^+CD49b^+$ lymphocytes and neutrophils were defined as $CXCR2^+Ly6G^+$ granulocytes. Maturation of NK cells was determined by CD11b and CD27 expression as previously reported.[15] Apoptosis of neutrophils was determined by further staining with FITC-labeled Annexin V (BioLegend) and analyzing as described above.

Immunohistochemical Staining

Cryosections of mesenteric lymph nodes were transferred onto glass slides, air-dried, fixed in acetone, and stored at −80°C. Apoptotic cells were detected by TUNEL staining using the "TACS®2 TdT-Fluor In Situ Apoptosis Detection Kit" (Trevigen®, Bio-Techne) according to the manufacturer's instructions. The sections were imaged using an Evos FL Auto 2 microscope (Thermo Fisher Scientific) and evaluated blindly using the ImageJ software (National Institutes of Health, Maryland, USA).

LC-MS/MS

Samples were prepared for LC-MS/MS by adding 3 × sample volume of methanol (LC-MS grade, Sigma-Aldrich) to peritoneal lavage or cell cultures after transfer to a 12-mL glass vial. Then, 4 µL internal standard solution consisting of LTB4-d4, 15(S)-hydroxyeicosatetraenoic acid (HETE)-d8, PGE2-d4, DHA-d5 and DHA (all 50 ng/mL, except DHA 500 ng/mL in methanol, Cayman Chemicals) were added to peritoneal exudate or cell cultures, vortexed and subsequently spun at 4°C, 3190 × g for 3 min. Extraction was repeated with 500 µL methanol, and the organic extracts were combined in a 7-mL glass vial. Some of the methanol was evaporated under a gentle stream of nitrogen (40°C, ± 45 min) and water was added to reach a final methanol concentration not exceeding 20%. The

samples were acidified to pH = 3 using formic acid and loaded onto C18 solid-phase extraction cartridges (Sep-Pak, Waters, Milford, Massachusetts, USA). The cartridges were washed with 3 mL water and 3 mL n-hexane prior to elution with 3 mL methyl formate (Sigma-Aldrich). The extract was dried down under a gentle stream of nitrogen at 40°C, reconstituted in 200 µL 40% methanol, vortexed, and transferred to a glass autosampler vial with a micro-insert. The samples were then stored at −80°C until analysis. LC-MS/MS analysis was performed on a QTrap 6500 mass spectrometer operating in negative ESI mode (Sciex, Nieuwerkerk aan den IJssel, The Netherlands) coupled to an LC system employing two LC-30AD pumps, a SIL-30AC autosampler, and a CTO-20AC column oven (Shimadzu,'s-Hertogenbosch, The Netherlands) as specified previously.[30] A 1.7 µm Kinetex C18 50×2.1 mm column protected with a C8 precolumn (Phenomenex, Utrecht, The Netherlands) was used and maintained at 50°C. A binary gradient of water (A) and methanol (B) containing 0.01% acetic acid was generated as follows: 30% B at 0 min, held for 1 min, then ramped to 45% B at 1.1 min, 53.5% B at 2 min, 55.5% B at 4 min, 90% B at 7 min, 100% B at 7.1 min, and held for 1.9 min. The injection volume was 40 µL, and the flow rate was 400 µL/min. For analyte identification, the mass transition used for each analyte was combined with its relative retention time (RRT). The calibration lines constructed with standard material for each analyte were used for quantification, and only peaks with a signal to noise (S/N) ratio > 10 were quantified. More detailed LC-MS/MS settings can be found elsewhere.[30]

Luminex

Peritoneal concentrations of CXCL1, CXCL2, TNF-α, IL-33, IL-6 receptor (IL-6R)α, IL-10, insulin-like growth factor (IGF)-1, CCL5, soluble TNF RII (sTNF RII) and CXCL12 were determined using a customized Luminex Multiplex Immunoassay (R&D Systems) following the manufacturer's instructions and evaluated using BioPlex 200 System (BioRad, California, USA).

ELISA

Peritoneal concentrations of IL-6 and transforming growth factor (TGF)-β were determined using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Latent TGF-β was activated by 1 N HCl for 10 minutes at room temperature, followed by neutralization by 1.2 N NaOH/0.5 mM HEPES before being measured.

Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). The data were collected from at least two independent experiments with at least three mice in each group, where n refers to the number of mice per group but not technical replicates. Groups were compared using two-way ANOVA, and time-points were compared with multiple comparisons using an uncorrected Fisher's LSD test. All statistical analyses were carried out using GraphPad Prism 9 (GraphPad Software, California, USA).

Results

Dietary Fish Oil Enhances Recruitment of $CD11b^+CD27^-$ NK Cells to the Peritoneum of Mice with Antigen-Induced Peritonitis

Mice fed the fish oil diet had 55% higher numbers of total peritoneal NK cells than mice fed the control diet ($p<0.001$, Figure 1A and C) 6 h after induction of inflammation. This increase was entirely comprised of higher numbers of the most mature $CD11b^+CD27^-$ NK cells ($p=0.01$) with no difference in the number of less mature NK cells (Figure 1B and D). These findings indicate that dietary fish oil enhances recruitment of the most mature subtype of NK cells or enhances the on-site maturation of recruited NK cells during inflammation.

Dietary Fish Oil Enhances Peritoneal Concentrations of the NK Cell Recruiting Chemokines CCL5 and CXCL12 as Well as NK Cell Expression of CCR5

Four- to six-fold higher numbers of NK cells expressing the chemokine receptor CCR5 were present in the peritoneum of mice receiving dietary fish oil than in mice receiving the control diet ($p<0.001$, Figure 2A and B) 6 h after inflammation induction. Additionally, higher percentages of $CCR5^+$ NK cells (4.2%, $p=0.003$) with higher CCR5 expression level (46%, $p=0.02$) were observed in the fish oil fed mice compared to the control diet fed mice 6 h after inflammation induction (Supplementary Figure 2). To evaluate which chemokines might be affecting the infiltration of NK cells, multiplexing analysis of several NK cell recruiting chemokines was conducted. Mice fed dietary fish oil had higher peritoneal concentrations of CCL5 (46%, $p<0.001$) and CXCL12 (88%, $p<0.001$) 6 h after induction of inflammation than mice

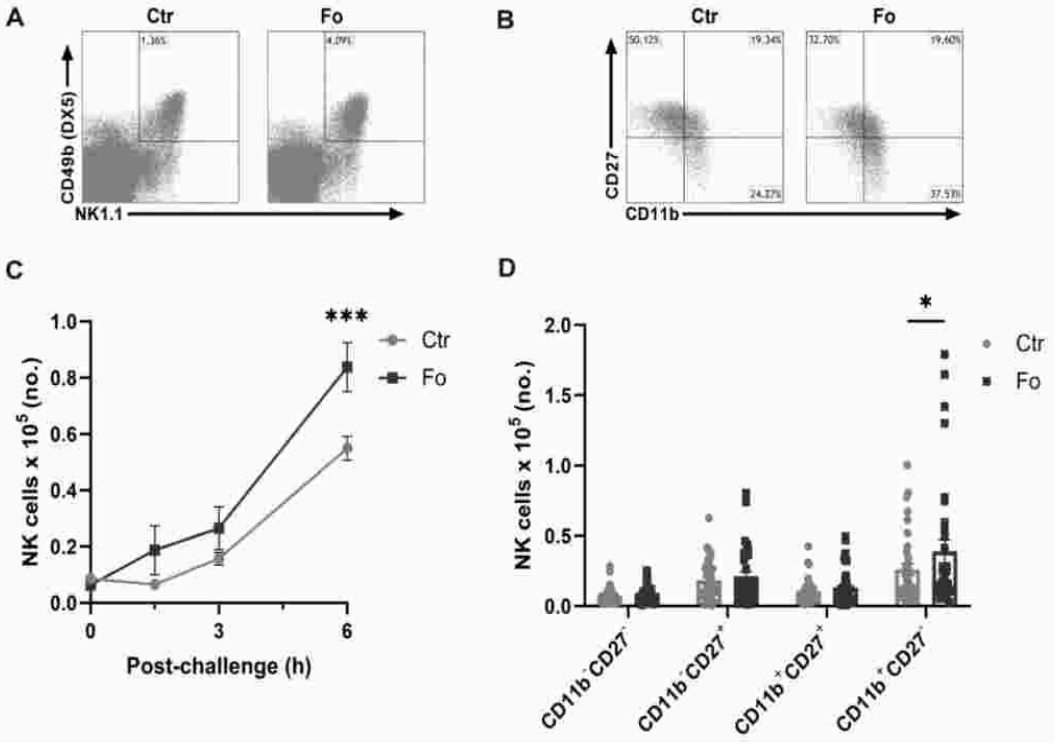

Figure 1 Dietary fish oil increases the number of $CD11b^+CD27^-$ NK cells in peritoneum of mice 6 h after inflammation induction. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 0, 1.5, 3, and 6 h following challenge and peritoneal cells collected. Peritoneal cells were counted with a Countess automated cell counter, stained with monoclonal antibodies against CD3, NK1.1, CD49b (DX5), CD11b and CD27 and evaluated by flow cytometry. Representative gating strategy on $CD3^-$ lymphocytes to identify NK cells based on their NK1.1 and CD49b (DX5) expression (**A**) and separation of NK cells into 4 distinct subtypes based on their CD27 and CD11b expression (**B**) 6 h after inflammation induction in mice fed either Ctr or Fo diets. Number of $CD3^-NK1.1^+CD49b\ (DX5)^+$ lymphocytes at different timepoints following induction of inflammation (**C**) and of those the number of $CD11b^-CD27^-$, $CD11b^-CD27^+$, $CD11b^+CD27^+$, and $CD11b^+CD27^-$ cells at 6 h after inflammation induction (**D**). $^*p < 0.05$, $^{***}p < 0.001$. n = 9–12 for the 0 and 3 h time-points and 35–48 for 6 h post-challenge. Results are shown as mean ± standard error of the mean from data collected from at least four independent experiments.

receiving the control diet (Figure 2C). Dietary fish oil did not affect the concentrations of other NK cell recruiting chemokines, such as CCL2, CCL3, CCL4, CCL19, and CCL20 (data not shown). These results indicate that in mice fed the fish oil diet higher numbers of $CD11b^+CD27^-$ NK cells may be recruited to the inflamed peritoneum by higher concentrations of CCL5 and CXCL12, possibly through the CCR5/CCL5 and CXCR4/CXCL12 axes.

Dietary Fish Oil Enhances Surface Expression of CD107a on Infiltrating NK Cells

Dietary fish oil increased expression levels of CD107a on peritoneal NK cells 6 h following inflammation induction compared to NK cells from mice receiving the control diet (21%, p=0.02, Figure 3A and C). Additionally, a larger proportion of the peritoneal NK cells was $CD107a^+$ in the fish oil fed mice compared to that in the control diet fed mice 6 h after induction of inflammation (10%, p=0.002, Figure 3A and C). Dietary fish oil did not affect the expression levels of the lymph node homing receptor CD62L on peritoneal NK cells (Figure 3B and D). However, a lower fraction of NK cells was $CD62L^+$ in mice fed the fish oil diet compared to that in mice receiving the control diet 6 h following induction of inflammation (8%, p=0.003, Figure 3B and D). These results suggest that dietary fish oil may enhance NK cell degranulation and thereby their cytotoxic function but may reduce their ability to home to draining lymph nodes to mount adaptive immune responses.[31]

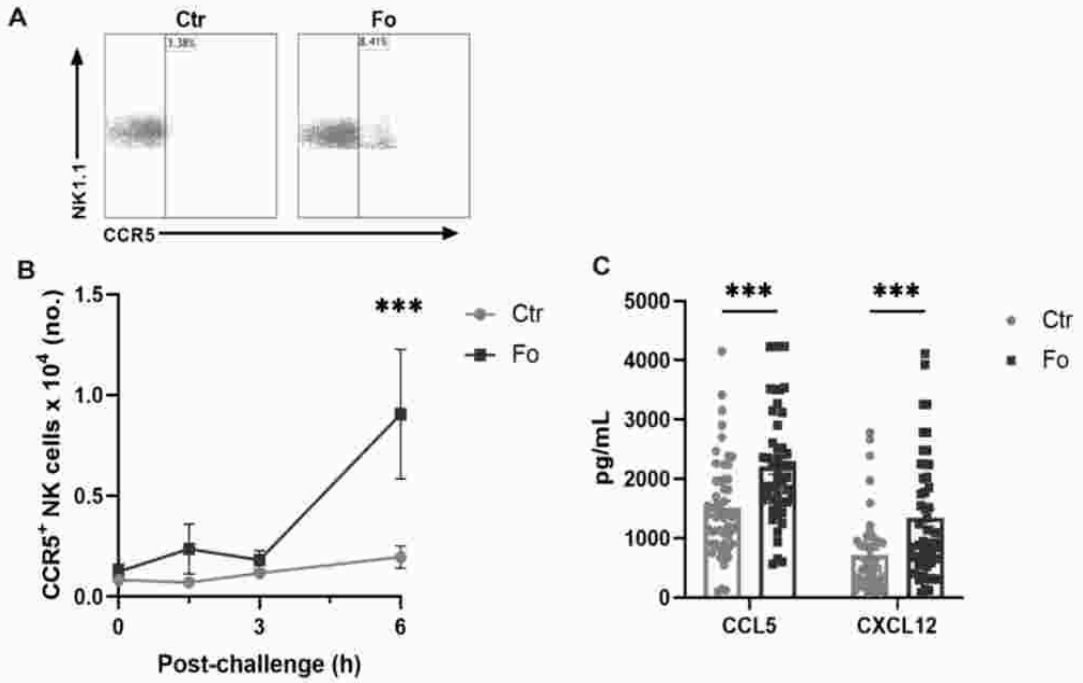

Figure 2 Dietary fish oil increases the number of CCR5$^+$ NK cells and enhances CCL5 and CXCL12 concentrations in peritoneum 6 h after induction of inflammation. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 0, 1.5, 3, and 6 h following challenge and peritoneal lavage collected. Peritoneal cells were counted with a Countess automated cell counter, stained with monoclonal antibodies against CD3, NK1.1, CD49b (DX5), and CCR5 and analyzed by flow cytometry. Representative dot plots of NK cell expression of CCR5 in mice receiving Ctr and Fo diets 6 h after inflammation induction (**A**). Number of CCR5$^+$ NK cells at 0, 1.5, 3, and 6 h (**B**) and peritoneal concentrations of CCL5 and CXCL12 6 h (**C**) following inflammation induction in mice receiving Ctr and Fo diets. ***$p < 0.001$, n = 8–15 (**B**) and n = 47–50 (**C**). Results are shown as mean ± standard error of the mean from data collected from at least four independent experiments.

Dietary Fish Oil Enhances Neutrophil Apoptosis and Apoptotic Cell Numbers in Draining Lymph Nodes of Mice with Antigen-Induced Peritonitis

In mice fed the fish oil diet, neutrophil numbers peaked earlier (3 h) after inflammation induction but were much lower than in mice fed the control diet at peak of inflammation (6 h) (51%, p<0.001, Figure 4A and D). In addition, the resolution interval was 1 hour shorter in inflamed mice fed the fish oil diet compared to that in mice receiving the control diet (Figure 4D). The percentages of apoptotic neutrophils in the peritoneum were higher 6 h after induction of inflammation in mice fed the fish oil diet than in mice fed the control diet (17%, p<0.001, Figure 4B and E) with lower percentages of the neutrophils from mice fed the fish oil diet expressing CD47 (27%, p=0.002, Figure 4C and F). Furthermore, a higher number of TUNEL$^+$ cells was present in mesenteric lymph nodes at 3 (~2.2-fold, p=0.002), and 6 h (~2.9-fold, p<0.001) after induction of inflammation in mice fed the fish oil diet compared to that in mice receiving the control diet (Figure 4G and H). These results indicate that dietary fish oil enhances the resolution of inflammation by increasing neutrophil apoptosis and their egress from the inflamed site, possibly by decreasing neutrophil expression of the eat-me-not molecule CD47 to aid their efferocytosis.

Dietary Fish Oil Dampens Production of Pro-Inflammatory Mediators While Enhancing Production of Anti-Inflammatory Ones

Peritoneal concentrations of the neutrophil recruiting chemokines CXCL1 and CXCL2 peaked at 1.5 h after inflammation was induced (Figure 5A and B). In mice fed the fish oil diet, peak concentrations of CXCL1 and CXCL2 were lower than that in mice fed the control diet (45%, p<0.001 and 55%, p<0.001, respectively, Figure 5A and B). TNF-α and IL-6Rα concentrations similarly peaked at 1.5 h after inflammation induction in mice receiving both diets, but

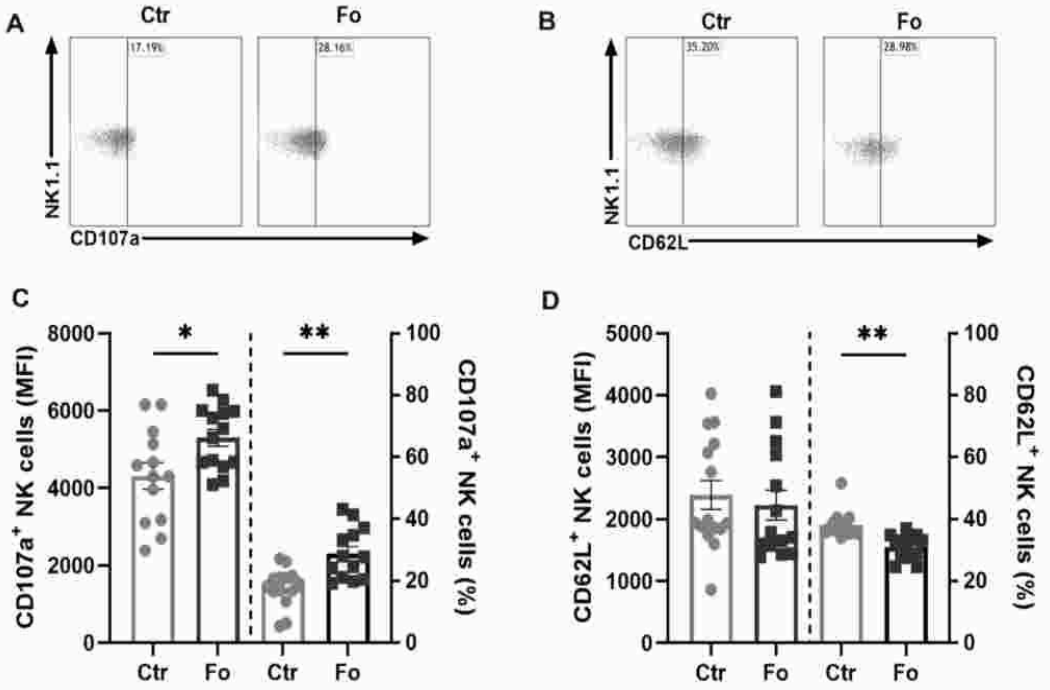

Figure 3 Dietary fish oil increases NK cell expression of CD107a and the proportion of NK cells that express CD107a but decreases the proportion of NK cells expressing CD62L. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 6 h following challenge and peritoneal cells collected, counted with a Countess automated cell counter, stained with monoclonal antibodies against CD3, NK1.1, CD49b (DX5), CD107a, and CD62L, and evaluated by flow cytometry. Representative dot plots of CD107a (**A**) and CD62L (**B**) expression on NK cells in mice receiving Fo and Ctr diets 6 h after inflammation induction. Expression levels (MFI) and percentages of $CD107a^+$ (**C**) and $CD62L^+$ (**D**) NK cells 6 h following induction of inflammation. *p < 0.05, **p < 0.01; n = 13–14. Results are shown as mean ± standard error of the mean from data collected from at least four independent experiments.

dietary fish oil dampened peak concentrations of both mediators (41%, p=0.02 and 46% p<0.001, respectively, Figure 5C and D). Peritoneal concentrations of IL-6 and the IL-1 family member IL-33 were highest 6 h after inflammation induction with lower concentrations in mice fed the fish oil diet than in mice fed the control diet (24%, p=0.004 and 64%, p=0.02, respectively, Figure 5E and F). Dietary fish oil did not affect peritoneal concentrations of key NK cell–derived cytokines, such as interferon (IFN)-γ and granzyme B, in inflamed mice (data not shown). Interestingly, IL-10 concentrations were comparable throughout the inflammatory response regardless of dietary treatment (Figure 6A). Mice fed the fish oil diet had higher peritoneal concentrations of the growth factors TGF-β and IGF-1 6 h following inflammation induction compared to those receiving the control diet (~2.2-fold, p=0.005 and ~2-fold, p=0.001, respectively, Figure 6B and C). Surprisingly, dietary fish oil increased peritoneal concentrations of sTNF RII ~5-fold at 6 h following inflammation induction compared to that in mice fed the control diet (p<0.001, Figure 6D). Taken together, these data suggest that dietary fish oil attenuates pro-inflammatory cytokine production while enhancing regeneration and anti-inflammatory cues through increased production of growth factors and shedding of TNF RII.

Dietary Fish Oil Dampens Production of Pro-Inflammatory Lipid Mediators and Increases the Production of Anti-Inflammatory HEPEs

Peritoneal concentrations of PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB_2 (Figure 7A–D), as well as their precursor AA (Supplementary Figure 3A), increased with time in mice fed the control diet but not in mice fed the fish oil diet. The concentrations of PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB_2 were lower in mice fed the fish oil diet 12 h after induction of inflammation than in mice fed the control diet (70%, p=0.04; 64%, p=0.02; 64%, p=0.01; and 74%, p=0.009, respectively) with AA concentration being higher at 6 h only (54%, p=0.009). Peritoneal concentrations of 5- and 18-

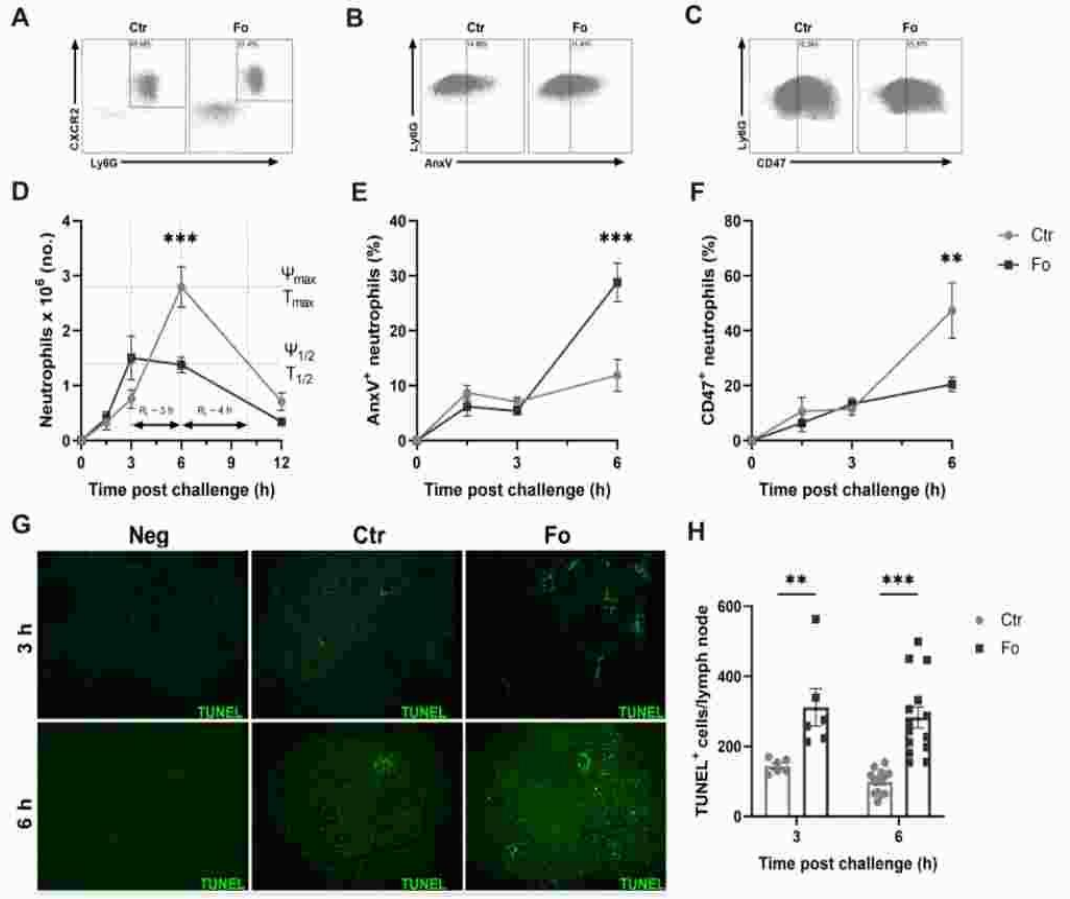

Figure 4 Dietary fish oil shortens the resolution interval, enhances neutrophil apoptosis and egress to draining lymph nodes but decreases neutrophil CD47 expression. Mice were fed control (Ctr, grey lines with grey circles) or fish oil (Fo, black lines with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and challenged intraperitoneally. Mice were sacrificed at 0, 1.5, 3, 6, and 12 h post-challenge and peritoneal cells and mesenteric lymph nodes harvested. Peritoneal cells were stained with monoclonal antibodies against CXCR2, Ly6G, and CD47. Apoptotic neutrophils were stained with FITC-labeled annexin V (AnxV). Neutrophils were defined as $CXCR2^+Ly6G^+$ granulocytes. Representative dot plots of neutrophil gating strategy (**A**), $AnxV^+$ neutrophils (**B**) and $CD47^+$ neutrophils (**C**) 6 h after inflammation induction in mice fed either Ctr or Fo diets. Neutrophil numbers with time after inflammation induction in mice fed Ctr and Fo diets determining the infiltration peak (Ψ_{max}), peak infiltration time (T_{max}), half the peak neutrophil number ($\Psi_{1/2}$) and the time when neutrophil numbers reach half Ψ_{max} ($T_{1/2}$) to determine the resolution interval (R_i), n = 6–12 for 0, 3 and 12 h and 47–50 for the 6 h time-point (**D**). Percent neutrophils stained with AnxV (**E**) or CD47 (**F**) 6 h after inflammation induction in mice fed Ctr and Fo diets, n = 6 for all time-points. Representative TUNEL staining of mesenteric lymph nodes from mice fed Ctr or Fo diets at 3 and 6 h following inflammation induction (**G**). $TUNEL^+$ cells in all stained lymph nodes from mice fed Ctr and Fo diets, n = 6 for the 3 h time-point and 14 for 6 h post-challenge (**H**). $^{**}p < 0.01$, $^{***}p < 0.001$, n = 6–40. Results are shown as mean ± standard error of the mean from data collected from at least two independent experiments.

hydroxyeicosapentaenoic acids (HEPEs) were higher in mice fed the fish oil diet 6 h (8.4-fold, p=0.04 and 3.8-fold, p=0.002, respectively) and 12 h (~7.1-fold, p=0.04 and ~2.9-fold, p=0.06, respectively) following induction of inflammation than those in mice fed the control diet (Figure 7E and H), whereas peritoneal concentrations of 12- and 15-HEPEs only had a tendency towards being higher 6 h after inflammation induction (p=0.09 and 0.08, respectively, Figure 7F and G). Peritoneal concentration of EPA increased with time after induction of inflammation in mice fed the fish oil diet and was higher at 3 h (13-fold, p<0.001), 6 h (9.8-fold, p<0.001), and 12 h (6.4-fold, p=0.007) following induction of inflammation than that in mice receiving the control diet (Supplementary Figure 3B). However, peritoneal concentration of DHA was only higher 12 h after inflammation induction in fish oil fed mice (3.5-fold, p=0.02, Supplementary Figure 3C). The formation of HEPEs through 5-, 12-, and 15-LOX was preferred over the formation of HETEs at several time-points after induction of inflammation in mice receiving the fish oil diet (Figure 7I–K). However, the presence of E-series resolvins could not be detected in the peritoneum of mice fed the fish oil diet (data not shown).

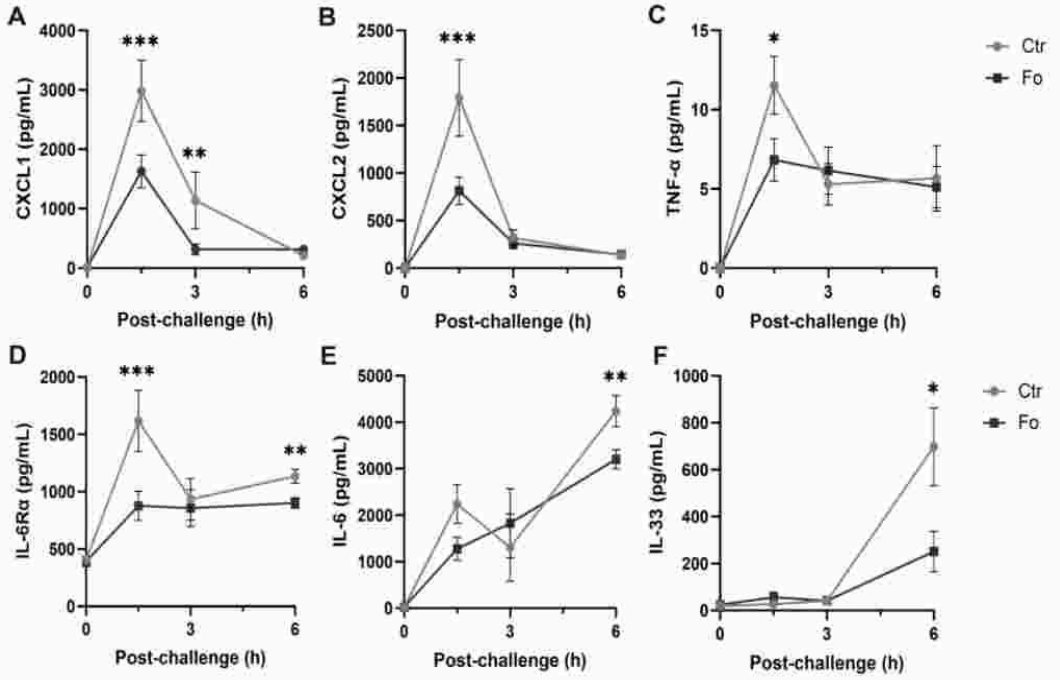

Figure 5 Dietary fish oil decreases peritoneal concentrations of pro-inflammatory chemokines and cytokines. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 0, 1.5, 3, and 6 h following challenge, peritoneal fluid collected and concentrations of CXCL1 (**A**), CXCL2 (**B**), TNF-α (**C**), IL-33 (**D**), and IL-6Rα (**F**) measured by Luminex and that of IL-6 (**E**) by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001; n = 12 for 0 and 3 h and 40 for the 6 h post-challenge. Results are shown as mean ± standard error of the mean from collected from at least four independent experiments.

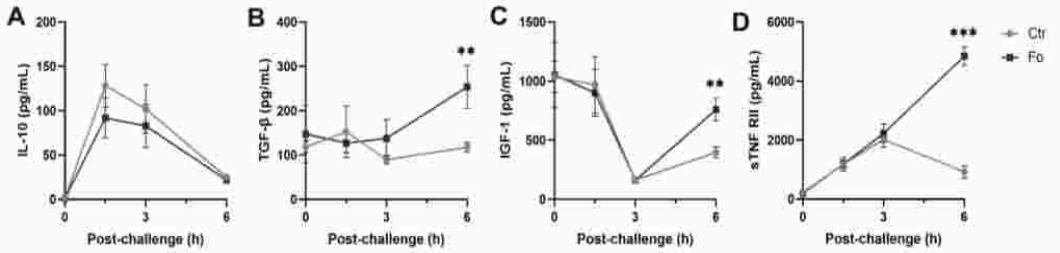

Figure 6 Dietary fish oil enhances peritoneal concentrations of anti-inflammatory cytokines and growth factors. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 0, 1.5, 3, and 6 h following challenge and peritoneal fluid collected. Concentrations of IL-10 (**A**), IGF-1 (**C**), and sTNF RII (**D**) were determined by Luminex and TGF-β (**B**) by ELISA. **p < 0.01, ***p < 0.001, n = 8 for 0 and 3 h, 40 for 6 h post-challenge (**A, C**, and **D**) and 3–8 for all time-points (**B**). Results are shown as mean ± standard error of the mean from data collected over at least two independent experiments.

These results suggest that dietary fish oil dampens production of pro-inflammatory lipid mediators and increases production of anti-inflammatory HEPEs.

Discussion

In the present study, we follow up on our previous results that showed that dietary fish oil enhanced resolution of inflammation and induced an early peak of peritoneal NK cells in antigen-induced peritonitis[25] along with our results showing that depletion of NK cells severely disrupted resolution of the inflammation.[21] The present study demonstrates that the NK cells increasing the most in mice fed the fish oil diet are the functionally mature $CD11b^{+}CD27^{-}$ NK cells. The fish oil diet increases peritoneal concentrations of CCL5 and CXCL12 as well as NK cell expression of CCR5, the receptor for CCL5. Furthermore, dietary fish oil enhances neutrophil apoptosis and efferocytosis and shortens the resolution interval. We

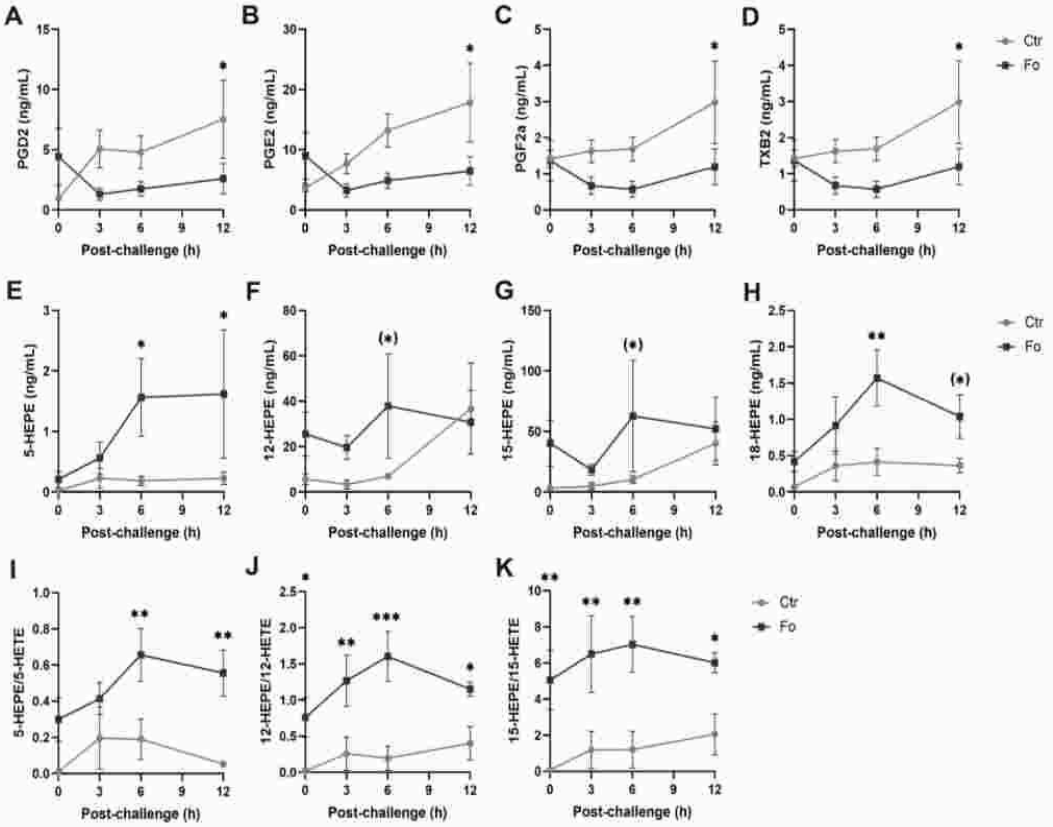

Figure 7 Dietary fish oil dampens pro-inflammatory lipid mediator concentrations, increases anti-inflammatory HEPEs concentrations and increases the ratio of HEPEs to HETEs. Mice were fed control (Ctr, grey line with grey circles) or fish oil (Fo, black line with black squares) diets for 5 weeks. They were immunized twice with mBSA with a 2-week interval and subsequently challenged intraperitoneally. Mice were sacrificed at 0, 3, 6, and 12 h following challenge and peritoneal lavage collected. Peritoneal concentrations of PGD_2 (**A**), PGE_2 (**B**), $PGF_{2\alpha}$ (**C**), TXB_2 (**D**), 5-HEPE (**E**), 12-HEPE (**F**), 15-HEPE (**G**), 18-HEPE (**H**) and 5-HETE, 12-HETE, and 15-HETE were determined by tandem LC-MS/MS and 5-HEPE/5-HETE (**I**), 12-HEPE/12-HETE (**J**), and 15-HEPE/15-HETE (**K**) calculated for each mouse at the indicated timepoints. (*)$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 6 for all time-points. Results are shown as mean ± standard error of the mean from data collected from at least two independent experiments.

also show that dietary fish oil enhances production of the pro-resolution mediators TGF-β, IGF-1, and SPM pathway markers but dampens concentrations of pro-inflammatory cytokines, neutrophil chemokines, and lipid mediators.

The increase in the number of NK cells in the peritoneum of mice fed the fish oil diet in the present study may be due to increased recruitment from the circulation caused by higher peritoneal concentrations of the chemokines CCL5 and CXCL12. The higher expression of CCR5, the receptor for CCL5, on the surface of NK cells from mice fed the fish oil diet may also have contributed to their increased recruitment.

The $CD11b^+CD27^-$ NK cells, which were increased in the peritoneum of mice fed the fish oil diet, are primarily reported to exert cytotoxic function rather than to produce and secrete cytokines.[15] The NK cells in the peritoneum of mice fed the fish oil diet had higher expression of CD107a than NK cells from mice fed the control diet indicating their enhanced degranulation and substantiating their cytotoxic potential.[32] The enhanced neutrophil apoptosis in the peritoneum of mice fed the fish oil diet observed at the same time-point as the higher number of degranulated NK cells may indicate involvement of the $CD11b^+CD27^-$ NK cells in inducing neutrophil apoptosis in the resolution phase of the inflammation.

Lower proportions of the peritoneal neutrophils from mice fed the fish oil diets expressed CD47 on their surface than those of neutrophils from mice fed the control diet. As neutrophil expression of CD47 has been linked to decreased spontaneous apoptosis and decreased efferocytosis of apoptotic neutrophils,[9,10] the decreased proportion of peritoneal neutrophils expressing CD47 in mice fed the fish oil diet may be responsible for the increase in neutrophil apoptosis and subsequent removal from the

peritoneum. In addition, CD47 interaction with toll-like receptor 2 is thought to aid neutrophil transmigration to inflamed sites,[33,34] therefore the decrease in $CD47^+$ neutrophils in mice fed the fish oil diet may have led to fewer neutrophils being recruited to the inflamed site. Lower concentrations of the neutrophil recruiting chemokines CXCL1 and CXCL2[35] in mice fed the fish oil diet may also have been partly responsible for decreased recruitment of neutrophils to their peritoneum.

The decreased proportion of neutrophils expressing CD47 in mice fed the fish oil diet in the present study is in concordance with our previous results showing that human neutrophils co-cultured with DHA-primed NK cells had lower expression of CD47 than neutrophils co-cultured with control NK cells.[27] This indicates that the lower proportion of neutrophils expressing CD47 in mice fed the fish oil diet in the present study may be caused by interaction with murine NK cells that have been in contact with or have incorporated omega-3 PUFAs into their membranes.

The lower peritoneal concentrations of the pro-inflammatory cytokines IL-6, IL-6Rα, TNF-α, and IL-33 in mice fed the fish oil diet than in mice fed the control diet are in line with what has been shown previously by our and other groups in mice and humans.[6,25,36] Dampening of pro-inflammatory cytokine production by omega-3 PUFAs may be caused by downregulation and dampening of both NFAT and NF-κB signaling,[37,38] possibly through activation of AMPK.[39–41]

In contrast, dietary fish oil enhanced peritoneal concentrations of the anti-inflammatory/pro-resolution molecules TGF-β and IGF-1, which is in concordance with what we and others have shown previously for TGF-β in mice and rats.[25,42] IGF-1 may polarize macrophages to the pro-resolution M2-like phenotype,[43,44] which enhances efferocytosis of apoptotic neutrophils.[45] Therefore, the increased concentrations of IGF-1 in mice fed the fish oil diet may have led to development of M2-like macrophages participating in efferocytosis, explaining the higher number of apoptotic cells in mesenteric lymph nodes of mice fed the fish oil diet than in the mice fed the control diet. The sharp increase and the large difference in peritoneal concentration of sTNF RII 6 h after inflammation induction was interesting as sTNF RII, by acting as a decoy receptor to neutralize TNF and lymphotoxin signaling,[46] may contribute to the resolution response. This may be of importance as sTNF RII is currently being used as a clinical treatment of many chronic inflammatory diseases under the name Etanercept.[47]

Similar to what has been shown by others,[48,49] the fish oil diet prevented the increase in peritoneal concentrations of the pro-inflammatory cyclo-oxygenase generated lipid mediators, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2 that was seen in mice fed the control diet following induction of inflammation. Additionally, dietary fish oil led to higher ratios of HEPEs to HETEs generated by 5-, 12- and 15-LOX. These results are in line with recent findings in prediabetic rats showing that dietary supplementation of omega-3 PUFAs enhanced ratios of HEPEs to HETEs.[50] Higher concentrations of the EPA-derived HEPEs in mice fed the fish oil diet may well have contributed to the enhanced resolution response as HEPEs have been suggested to have anti-inflammatory and pro-resolving activities.[51,52] The lower concentrations of the AA-derived PGs, TBx_2 and HETEs could also contribute to a less pro-inflammatory environment in the fish oil fed mice to ensure proper resolution of the antigen-induced peritonitis.[53] NK cells have been shown to express high levels of 12/15-LOX in a murine model of type I diabetes[22] and could, therefore, be the source of the 12- and 15-HEPEs in the present study. Very low concentrations of the SPMs, maresin 1, protectin D_1, protectin D_x, and resolvin D_2 were detected based on retention time matching and a characteristic mass transition in peritoneal lavage of mice fed both diets (data not shown). Whether other SPMs were produced during the inflammatory response but below their detection limits or whether the SPMs were rapidly utilized during the resolution response can only be speculated about.

Conclusion

Dietary fish oil increased the number of mature NK cells at the inflamed site and enhanced several key hallmarks of resolution of inflammation. The fish oil diet increased peritoneal concentrations of CCL5 and CXCL12 that may have led to enhanced recruitment of the mature NK cells, possibly aided by an increase in NK cell expression of CCR5. The increase in mature, cytotoxic NK cells may have enhanced neutrophil apoptosis, possibly facilitated by decreased neutrophil expression of CD47. Higher peritoneal concentration of IGF-1 may have led to enhanced M2 polarization of macrophages and in turn increased efferocytosis of apoptotic neutrophils and their drainage to mesenteric lymph nodes. Finally, decreased concentrations of pro-inflammatory lipid mediators and increased concentrations of anti-inflammatory and pro-resolving mediators in mice fed the dietary fish oil may have facilitated the enhanced resolution of inflammation. Overall, these results

cast further light on the potential mechanisms by which dietary fish oil enhances the resolution of acute inflammation.

Abbreviations

AA, arachidonic acid; BSA, bovine serum albumin; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; IGF, insulin-like growth factor; IL, interleukin; IL-6R, IL-6 receptor; LOX, lipoxygenase; mBSA, methylated bovine serum albumin; NK, natural killer; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SEM, standard error of the mean; SPM, specialized pro-resolution mediator; sTNF RII, soluble TNF receptor II; TGF, transforming growth factor; TNF, tumor necrosis factor; TX, thromboxane.

Data Sharing Statement

The raw data supporting the conclusions drawn in the current paper will be made available upon request, without undue reservations.

Ethics Approval

All experiments were conducted in accordance with the ethical approval of the Icelandic Food and Veterinary authority (MAST, approval number #2017-01-04).

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Disclosure

The authors report no conflicts of interest in this work.

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Paper II

frontiers in Immunology

ORIGINAL RESEARCH
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Docosahexaenoic Acid Modulates NK Cell Effects on Neutrophils and Their Crosstalk

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Natural killer (NK) cells and neutrophils engage in crosstalk that is important in inflammation and likely also for resolution of inflammation. NK cells activate neutrophils and induce their infiltration to the inflamed sites but may also influence their apoptosis and their subsequent efferocytosis by macrophages. Several studies indicate that docosahexaenoic acid (DHA) can inhibit NK cell cytotoxicity but the effects of DHA on the ability of NK cells to engage in crosstalk with neutrophils and affect their functions have not been described. This study explored the kinetics of the effects of NK cells and NK cells pre-treated with DHA on neutrophil surface molecule expression and apoptosis, as well as the ability of NK cells to affect other neutrophil functions. In addition, the study explored the effects of neutrophils on NK cell phenotype and function. Primary NK cells were pre-incubated with or without DHA, then stimulated and co-cultured with freshly isolated neutrophils. When co-cultured with NK cells, neutrophils had higher expression levels of CD11b and CD47; secreted more IL-8, IL-1ra, and CXCL10; had increased phagocytic ability; and their apoptosis was increased early after initiation of the co-culture while dampened at a later time-point. Pre-incubation of NK cells with DHA attenuated NK cell-induced upregulation of CD11b and CD47 on neutrophils, had minor effects on NK cell induction of cytokine/chemokine secretion or their phagocytic ability. Neutrophils also affected the function of NK cells, lowering the frequency of NKp46$^+$ and CXCR3$^+$ NK cells and increasing the concentrations of IFN-γ, TNF-α, and GM-CSF in the co-cultures. Pre-incubation of NK cells with DHA further decreased the frequency of NKp46$^+$ NK cells in the co-culture with neutrophils and decreased the concentrations of IFN-γ, CCL3 and GM-CSF. These findings indicate that NK cells have mostly pro-inflammatory effects on neutrophils and that DHA can attenuate some of these pro-inflammatory effects. Neutrophils had both anti- and pro-inflammatory effects on NK cells. When NK cells had been pre-treated with DHA, the anti-inflammatory effects were increased and some of the pro-inflammatory effects attenuated. Overall, the results suggest that DHA may lead to a more anti-inflammatory microenvironment for NK cell and neutrophil crosstalk.

Keywords: natural killer cells, neutrophils, docosahexaenoic acid, apoptosis, CD47, NKp46, phagocytosis

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INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes best known for their ability to target aberrant cells without prior sensitization (1). They are potent producers of cytokines and chemokines, such as IFN-γ, GM-CSF, TNF-α, and CCL3 (2). Neutrophils are innate cells that readily infiltrate inflamed sites and exert their functions through phagocytosis, cytotoxicity, extracellular traps, and secretion of a wide array of anti-microbial compounds, cytokines, and chemokines, such as IL-8, CXCL10, and IL-1ra (3–5).

NK cells and neutrophils engage in crosstalk and can modulate activation, infiltration, and survival of each other (6). One indication of NK cells being able to activate neutrophils is that they induce neutrophil expression of the integrin CD11b. This has been suggested to occur through NK cell production of GM-CSF (7, 8). That GM-CSF produced by NK cells has been shown to potentiate several chronic inflammatory disorders (9) may indicate involvement of NK cell-neutrophil crosstalk in these diseases. NK cells recruit neutrophils to inflammatory sites through several mechanisms, including the CCL3-CCR5 signaling axis (2, 10). Expression of CD47 on neutrophils is also implicated in inducing their transmigration in both bacterial and fungal infections (11–13). Furthermore, low CD47 expression on neutrophils has been associated with enhanced phagocytosis of anergic and apoptotic cells by macrophages and hence is regarded as being anti-inflammatory (14).

NK cells induce neutrophil apoptosis in fungal infections (15) through NKp46- and/or Fas-dependent mechanisms (16) and upregulation of MHC class I expression on neutrophils is associated with higher susceptibility to NK cell-induced apoptosis (17). On the contrary, two independent studies have shown that NK cells inhibit neutrophil apoptosis *in vitro* (7, 8). NK cells also play a role in modulating neutrophil reactive oxygen species (ROS) production, enhancing ROS production only when the neutrophils receive a low-grade stimulation (7, 8). NK cell ability to enhance neutrophil phagocytosis is thought to occur through a cell-to-cell mediated mechanism (7). However, their induction of neutrophil phagocytosis of *Candida albicans* as well as their ability to enhance fungicidal activity of neutrophils is through a mechanism yet to be described (18). Not only can NK cells affect neutrophil function, but neutrophils can also affect NK cell function. Neutrophils can act as a cellular source of IL-18 that in collaboration with IL-12 activates NK cells (19) and stimulates NK cell production of IFN-γ, TNF-α and GM-CSF. Neutrophil production of ROS induces NK cell apoptosis, primarily in the $CD56^{low}$ subset (20, 21) and lowers their expression of NKp46 and thereby inhibits their cytotoxic function (22).

Omega-3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory effects and affect both NK cells and neutrophils. Their effects on inflammation are partly because they are incorporated into cellular membranes at the expense of the omega-6 PUFA arachidonic acid (23, 24). Arachidonic acid is the substrate for pro-inflammatory lipid mediators, such as prostaglandins, thromboxane, leukotrienes, and lipoxins (25). On the other hand, the omega-3 PUFAs eicosapentaenoic acid and docosahexaenoic acid (DHA) are substrates for specialized pro-resolution mediators (SPMs), such as resolvins, protectins, and maresins, that drive resolution of inflammation (26, 27). Dietary omega-3 PUFAs inhibit NK cell cytotoxicity (28, 29) and thereby impair resistance to influenza in mice by suppressing NK cell cytotoxicity (30). In addition, the SPM Resolvin E1 enhances NK cell infiltration into inflamed tissues through their receptor ChemR23 (31), leading to the suggestion that NK cells actively contribute to resolution of inflammation (32). Our group showed that dietary fish oil enhanced the resolution phase of inflammation in antigen-induced peritonitis and led to an early peak in NK cell numbers compared to that in mice fed a control diet (33). We subsequently showed that depletion of NK cells in this model resulted in an increase in neutrophil infiltration to the inflamed site with the inflammation remaining unresolved for at least 24 h (34). These findings suggest that NK cells are pivotal players in limiting neutrophil infiltration to inflammatory sites and inducing resolution of inflammation. In the current study, we hypothesized that NK cells modulate neutrophil function, phenotype, and survival, that neutrophils might also affect NK cell phenotype and function, and that this crosstalk could be modulated by DHA. The results provide an insight into the kinetics of NK cell and neutrophil crosstalk, confirming that NK cells have mostly pro-inflammatory effects on neutrophils and that neutrophils affect NK cell phenotype and function. It also demonstrates that pre-incubating NK cells with DHA modulates the effects of NK cells on neutrophils on the NK cells in an anti-inflammatory manner.

MATERIALS AND METHODS

Preparation of Docosahexaenoic Acid

Docosahexaenoic acid (DHA) was obtained from Cayman Chemical (Michigan, USA) as peroxidase free, in single-use ampules. DHA was dried down under nitrogen and resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) and flushed with nitrogen. Aliquots of DHA were stored at −80°C until needed. Before use, DHA was resuspended in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Massachusetts, USA), enriched with 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (Pen/Strep, Gibco) (complete RPMI medium) to a concentration of 2 mM and incubated at room temperature for 1 h to allow binding of DHA to albumin.

NK Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers at the Icelandic Blood Bank (permission # 06-068-V1). Buffy coats were diluted in phosphate buffered saline (PBS), layered over Histopaque-1077 (Sigma-Aldrich), centrifuged and the PBMC layer collected. NK cells were negatively isolated from PBMCs using an NK cell isolation kit (Miltenyi Biotec, Germany) following the manufacturer's directions. The purity of the NK cells was determined by flow cytometry and was ~93%. NK cells were cultured in 48-well culture plates (Nunc, Thermo Fisher Scientific) in complete RPMI medium at a density of 1×10^6

cells/ml. DHA was added at a final concentration of 50 μM (DHA-NK cells). Equal volume of DMSO was added to the cultures as a control (C-NK cells) with the final concentration of DMSO being 0.06%. The plates were incubated for 18 h at 37°C, 5% CO_2 and 95% humidity to allow incorporation of DHA into the cell membranes, before the cells were stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) (all from R&D Systems, Bio-Techne, United Kingdom).

Neutrophil Isolation and Co-Culture With NK Cells

Neutrophils were isolated from fresh EDTA venous blood of healthy volunteers (permission # 06-068-V1) and incubated for 30 min at room temperature. Histopaque-1077 (Sigma-Aldrich) was carefully layered onto Histopaque-1119 (Sigma-Aldrich). EDTA venous blood was layered onto the Histopaques, centrifuged and the granulocyte-rich layer collected. Remaining erythrocytes were pelleted with 3% dextran (Sigma-Aldrich) and lysed in ACK lysis buffer. The purity of the neutrophils was determined by flow cytometry and was ~98%. Neutrophils were resuspended to a density of 2×10^6 cells/ml in complete RPMI medium with IL-2, IL-12, and IL-15 and added to the NK cell cultures at a 1:2 ratio of NK cells and neutrophils. The cytokines added did not affect the phenotype or function of the neutrophils when cultured alone in control studies. The co-cultures were incubated at 37°C, 5% CO_2, and 95% humidity for up to 24 h before the cells were harvested, pelleted, stained for surface molecules, and evaluated by flow cytometry. Supernatants were collected, aliquoted and kept at −80°C until cytokine and chemokine concentrations were measured by ELISA.

Reactive Oxygen Species Production

Following co-culture of NK cells and neutrophils for 16.5 h, 10 μM 2′,7′-dichloroflourescein diacetate (DCFDA, Abcam, United Kingdom) was added to the wells. The cells were incubated for further 90 min at 37°C, 5% CO_2 and 95% humidity. Cells were harvested, washed and ROS production was determined by flow cytometry using a Sony SH800 flow cytometer (Sony Biotechnology, United Kingdom). Results are presented as percent positive cells and cells without added DCFDA served as a negative staining control.

Phagocytosis Assay

After co-culturing NK cells and neutrophils for 4 h, heat-inactivated, FITC-labelled *E. coli* (Abcam) (5 μl) were added to the wells. Cells were incubated for additional 2 h at 37°C, 5% CO_2 and 95% humidity before being harvested, washed, and evaluated by flow cytometry using a Sony SH800 flow cytometer. Neutrophils not receiving FITC-labelled *E. coli* served as a negative control. Results are presented as percent positive cells compared to the negative control.

ELISA

Concentrations of TNF-α, IFN-γ, IL-8 (CXCL8), CXCL10, IL-1ra, GM-CSF, and CCL3 in cell culture supernatants were determined using DuoSet ELISA kits (R&D Systems).

Flow Cytometry

Cells were harvested after 3, 6, 12, 18, or 24 h of co-culture, pelleted and washed. Prior to staining, Fc-receptors were blocked by incubating the cells with 2% heat-inactivated mix of normal human serum and normal mouse serum (AbD Serotec, Bio-Rad, United Kingdom) and 5% TruStain FcX™ (BioLegend, California, USA) and stained for 20 min on ice. NK cells were stained with monoclonal fluorochrome-labeled antibodies against CD3 (OKT3, BioLegend), CD56 (CMSSB, eBioscience, Thermo Fisher Scientific), CD16 (3G8, BioLegend), CXCR3 (G025H7, BioLegend), and NKp46 (9E2, BioLegend. Neutrophils were stained with monoclonal fluorochrome-labeled antibodies against CD11b (M1/70, BioLegend), CD16a (CB16, BioLegend), CD47 (miap301, BioLegend), and CD62L (DREG-56, BioLegend). Appropriate isotype controls were used to determine positive staining. Apoptotic cells were stained using a FITC Annexin V apoptosis detection kit with propidium iodide (BioLegend) following the manufacturer's instructions. Following washing, cells were fixed in 2% paraformaldehyde and kept at 4°C until they were evaluated on a Navios EX flow cytometer (Beckman Coulter, Indianapolis, USA) or a Sony SH800 flow cytometer. Live cells were gated based on their forward and side scatter. NK cells were defined as $CD3^-CD56^+$ lymphocytes and neutrophils were defined as $CD16a^+CD62L^+CD49d^-$ granulocytes (see **Supplementary Figure 1**). Cells were analyzed using the Kaluza Analysis Software (Beckman Coulter).

Statistical Analysis

Results are presented as means standard error of the mean (SEM). The n indicated in each figure legend refers to the number of independent cell donors, not technical replicates. All data presented are an average from a minimum of three independent experiments. Outliers were identified using the Grubbs' method with an α of 0.05, omitting only one outlier per group, if appropriate. Groups were compared using one-way or two-way ANOVA. Difference between groups was regarded as significant when the p-value < 0.05. All statistical analysis was carried out in GraphPad Prism 8 (GraphPad Software, California, USA).

RESULTS

DHA Attenuates NK Cell Induction of CD47 and CD11b Expression on Neutrophils

Expression of CD47 and CD11b on neutrophils is pivotal for neutrophil transmigration and defense against both bacterial and fungal infections (11, 35). Previous studies suggest that NK cells upregulate CD11b expression on neutrophils through their GM-CSF production (7). **Figure 1A** shows that NK cells cultured without DHA (C-NK cells) rapidly upregulate CD11b expression on neutrophils after 6 h in co-culture. The CD11b upregulation was maintained throughout the 24 h co-culture period with the highest expression observed at 12 h (**Figures 1A, C**). Co-culturing neutrophils with C-NK cells also enhanced their expression levels of CD47 at 12 and 18 h with the highest

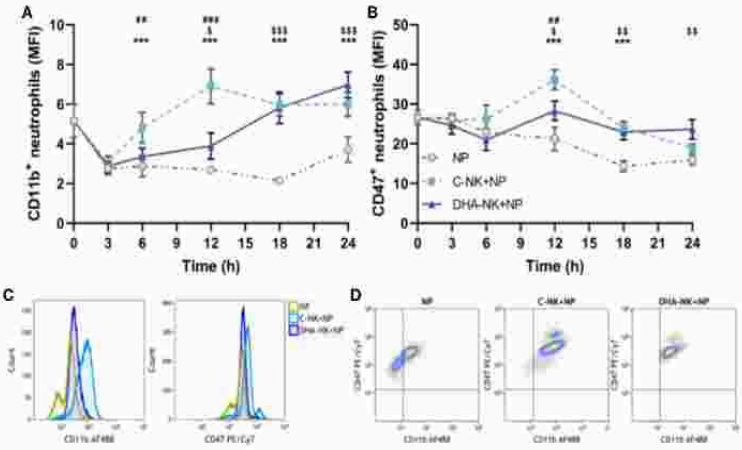

FIGURE 1 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil expression of CD11b **(A)** and CD47 **(B)**. Neutrophils were culture alone (NP) or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM of docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 0, 3, 6, 12, 18, and 24 h. Expression levels were determined by flow cytometry and are presented as mean fluorescence intensity (MFI). Representative overlay histograms of CD11b and CD47 expression levels at 12 h **(C)**. Representative dot plots of expression levels of CD11b and CD47 at 12 h **(D)**. Positive gating was determined with appropriate isotype controls. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, $ difference between DHA-NK+NP and NP, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. $n = 6$ (independent donors), collected in three independent experiments.

expression observed at 12 h (**Figures 1B, C**). Interestingly, co-culturing neutrophils with NK cells pre-incubated with DHA (DHA-NK cells) delayed their upregulation of CD11b and expression levels of CD11b did not reach the same levels as when the neutrophils were co-cultured with C-NK cells until at 18 h (**Figures 1A, C**). Co-culturing neutrophils with DHA-NK cells also attenuated neutrophil upregulation of CD47 at 12 h (**Figures 1B, C**) but enhanced it in a similar manner as co-culturing neutrophils with C-NK cells at 18 h (**Figure 1B**). All neutrophils expressed CD47 but the proportion of neutrophils expressing CD11b increased when co-cultured with C-NK cells or DHA-NK cells (**Figure 1D**). A small population of neutrophils expressing higher levels of CD47 appeared when they were co-cultured with either C-NK cells or DHA-NK cells (**Figure 1D**).

DHA Does Not Affect NK Cell Induction of Neutrophil Production of IL-8, IL-1ra, or CXCL10

Neutrophils produce high levels of IL-8 and CXCL10 to potentiate inflammatory responses (4, 5) but mediate anti-inflammatory responses by producing IL-1ra (3). Higher levels of IL-8, IL-1ra, and CXCL10 were present in supernatants when neutrophils and C-NK cells were cultured together compared with that when either cell type was cultured alone (**Figures 2A–C**). Co-culturing neutrophils with DHA-NK cells showed a slightly higher average concentration of IL-8 (**Figure 2A**) and slightly lower concentrations of IL-1ra and CXCL10 (**Figures 2A, B**) than when co-culturing them with C-NK cells, but the differences were not statistically significant and it is doubtful that they have biological significance.

DHA Dampens Further NK Cell-Induced Lowering of Neutrophil Apoptosis

NK cells have been shown to modulate neutrophil survival by either inhibiting (7, 8) or inducing (15, 16) their apoptosis. Additionally, NK cells enhance neutrophil phagocytosis and ROS production through undetermined mechanisms (7, 8). In the present study, addition of C-NK cells to neutrophils led to a slight increase in their apoptosis after 6 h of co-culture (**Figures 3A, B**). However, when prolonging the co-culture to 18 h, C-NK cells seemed to delay their apoptosis as it had reached similar levels as in the control group at 24 h (**Figures 3A, B**). DHA-NK cells did not affect neutrophil apoptosis differently from C-NK cells at 6 h but had more of a dampening effect on neutrophil apoptosis at 18 h (**Figures 3A, B**). Neutrophil phagocytosis was enhanced when they were co-cultured with C-NK cells for 6 h (**Figure 4A**) but their production of ROS was not affected by co-culturing them with C-NK cells (**Figure 4B**), contrary to previous findings (8). Pre-incubating the NK cells with DHA did not alter their enhancement of neutrophil phagocytosis nor their lack of effect on neutrophil ROS production (**Figures 4A, B**).

Neutrophils Modulate NKp46 Expression on NK Cells Pre-Incubated With DHA

NK cells induce neutrophil apoptosis through an NKp46- and/or Fas-dependent mechanism (16). CXCR3 mediates NK cell

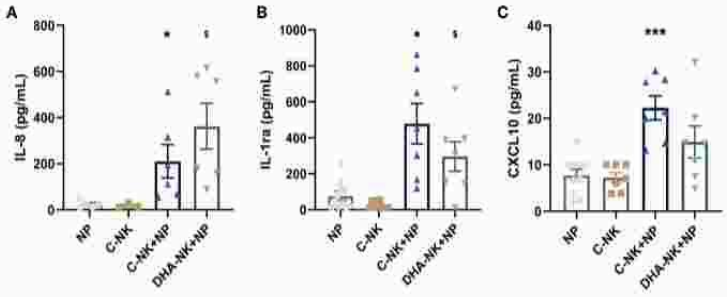

FIGURE 2 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil production of IL-8 **(A)**, IL-1ra **(B)**, and CXCL10 **(C)**. Neutrophils (NP) and NK cells (C-NK) were cultured alone or together after the NK cells had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK +NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 18 h. Cytokine concentrations in supernatants were determined by ELISA and are presented as pg/ml. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, and $ difference between DHA-NK+NP and NP. One symbol indicates $p < 0.05$ and three symbols $p < 0.001$. One outlier was removed from the NP group in A and B and one outlier in the C-NK group in C. n = 5 - 11 (except for C-NK in A, n = 3), collected in 4 independent experiments. In **Supplementary Figure 2** all outliers are included.

migration to draining lymph nodes during inflammation (2). In this study, when co-cultured with neutrophils a lower proportion of C-NK cells expressed NKp46 and expression levels of NKp46 were also lower than when the NK cells were cultured alone (**Figure 5A**). When DHA-NK cells were co-cultured with neutrophils the proportion of the NK cells expressing NKp46 and expression levels of NKp46 were still lower than when C-NK cells were co-cultured with neutrophils (**Figure 5A**). The proportion of NK cells expressing CXCR3 was also lower when the NK cells were co-cultured with neutrophils as compared with when the NK cells were cultured alone, regardless of whether the NK cells had been pre-incubated with DHA or not (**Figure 5B**). A small population of NK cells expressing CXCR3 and high levels of NKp46 ($NKp46^{hi}CXCR3^{+/-}$) was present on NK cells cultured alone but mostly disappeared when the NK cells were co-cultured with neutrophils (**Figure 5C**). The tiny population of $NKp46^{+}CXCR3^{hi}$ NK cells present following co-culture with neutrophils (**Figure 5C**) resulted in the higher expression levels of CXCR3 in the co-cultures with neutrophils (**Figure 5B**). When NK cells pre-treated with DHA were cultured alone their expression of NKp46 and CXCR3 was not different from that of untreated NK cells (**Figures 5A–C**).

Pre-Incubation of NK Cells With DHA Reduces Neutrophil-Induced NK Cell Production of IFN-γ and GM-CSF

Previous studied have shown that neutrophils can enhance NK cell production of IFN-γ, TNF-α and GM-CSF (6, 19) and that NK cells can modulate neutrophil function and migration through their expression of CCL3 (6). Culturing C-NK cells with neutrophils increased their secretion of IFN-γ, TNF-α and GM-CSF compared with that when the C-NK cells were cultured alone (**Figures 6A, B, D**). The concentration of IFN-γ was lower in co-cultures of DHA-NK cells and neutrophils than in co-cultures of C-NK cells and neutrophils and had a tendency towards being lower than when C-NK cells were cultured alone (**Figure 6A**). In addition, lower concentrations of CCL3 and GM-CSF were present in co-cultures of DHA-NK cells and neutrophils compared with that in co-cultures of C-NK cells and neutrophils (**Figures 6C, D**). Pre-treatment of NK cells with DHA did not affect their secretion of the cytokines (**Figures 6A–D**). Neutrophils cultured alone produced very low levels of IFN-γ, TNF-α and CCL3 (**Figures 6A–C**) but produced GM-CSF at similar levels as untreated NK cells cultured alone (**Figure 6D**).

DISCUSSION

Omega-3 PUFAs affect inflammation and its resolution (26, 27). Whether they affect the crosstalk between NK cells and neutrophils, a crosstalk important for inflammation and its resolution, has not been previously described. In the present study, DHA attenuated the effects of NK cells to enhance neutrophil expression of the pro-inflammatory surface molecules CD11b and CD47. DHA did not affect NK cell induction of neutrophil phagocytosis nor their ROS production. On the other hand, DHA enhanced the pro-survival (anti-apoptotic) effect NK cells have on neutrophils late in their co-culture. Pre-incubation of NK cells with DHA also modulated the effects neutrophils had on the NK cells, enhancing their ability to decrease NK cell expression of NKp46 and CXCR3, but decreasing their secretion of IFN-γ, CCL-3, and GM-CSF. Hence, the results indicate that DHA has mostly anti-inflammatory effects on the crosstalk between NK cells and neutrophils.

NK cells enhanced neutrophil expression of CD11b and CD47 and their phagocytosis, similar to what has been shown by Costantini et al. and Bhatnagar et al. (7, 8). Neutrophil expression of CD11b and CD47 is important for their transmigration to inflamed sites (12, 35), therefore, NK cell induction of neutrophil expression of these surface molecules may render them more capable of transmigrating to the inflamed

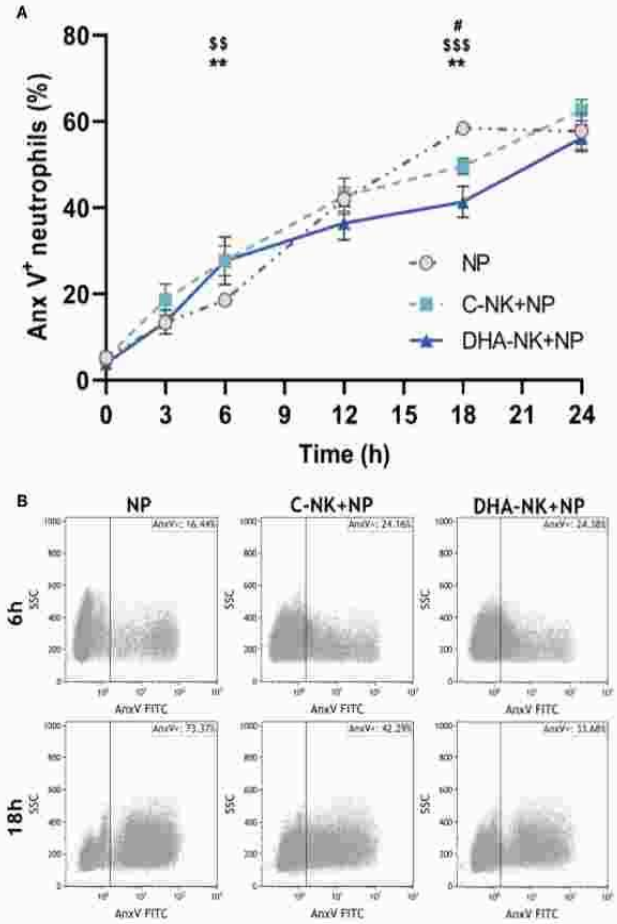

FIGURE 3 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil apoptosis. Neutrophils were cultured alone (NP), or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 0, 3, 6, 12, 18, and 24 h. Apoptosis was determined by flow cytometric analysis of annexin V (Anx V) binding to neutrophils and are presented as Anx V$^+$ neutrophils **(A)**. Representative dot plots of Anx V binding to neutrophils after 6 and 18 h of co-culture **(B)**. Positive gating was determined with an unstained control. Data are shown as mean ± SEM; * indicates difference between C-NK+NP and NP, $ difference between DHA-NK+NP and NP, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. $n = 8$ (independent donors), collected in 4 independent experiments.

sites. NK cell production of CCL3 may also promote extravasation (36). Pre-incubation of NK cells with DHA, in the present study, reduced the ability of the NK cells to induce neutrophil expression of both CD11b and CD47 and decreased their production of CCL3 in co-cultures with neutrophils, thereby possibly tempering NK cell ability to induce migration of neutrophils to inflamed sites. The lowered expression of CD47 caused by pre-incubating NK cells with DHA may also induce neutrophil efferocytosis by macrophages as low levels of CD47 have been shown to promote this way of removal of apoptotic neutrophils (14). Neutrophil expression of CD11b and CD47 has also been indicated to be important for neutrophil phagocytosis of pathogens (12, 35) and NK cell induction of neutrophil expression of CD11b and CD47 may be involved in increased neutrophil phagocytosis seen when co-culturing C-NK cells with neutrophils in the present study. However, decreased CD11b and CD47 expression on neutrophils co-cultured with NK cells pre-incubated with DHA was not accompanied by a reduction in the ability of NK cells to induce neutrophil phagocytosis, which was maintained as high as when the neutrophils were incubated with NK cells cultured in the absence of DHA.

NK cells produce cytokines that activate neutrophils, including IFN-γ and TNF-α (6), and neutrophils produce a wide array of cytokines and chemokines, including IL-8, that can induce chemotaxis of several innate immune cells (37). In the present study, TNF-α concentration was higher in co-cultures of

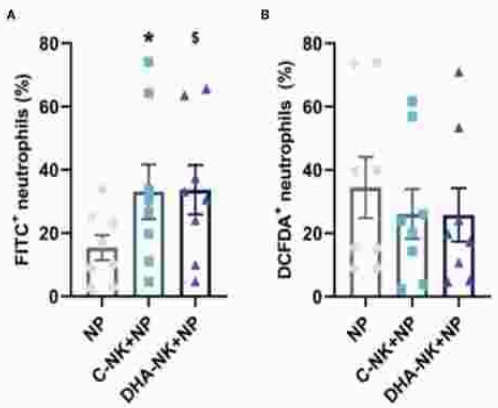

FIGURE 4 | The effects of NK cells and NK cells pre-incubated with DHA on neutrophil phagocytosis **(A)** and reactive oxygen species production **(B)**. Neutrophils were cultured alone (NP) or with NK cells that had been pre-incubated for 18 h without (C-NK+NP) or with 50 μM docosahexaenoic acid (DHA-NK+NP) and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml). The cells were cultured together for 6 h. Phagocytosis was determined by flow cytometric analysis of engulfed FITC-labeled *E. coli* and presented as percent of the total granulocyte population **(A)**. Reactive oxygen species production was measured by DCFDA conversion to the highly fluorescent 2',7'-dichlorofluorescein, determined by flow cytometry and presented as percent of the total neutrophil population **(B)**. Positive gating was determined by appropriate untreated controls. Data are shown as mean SEM; * indicates difference between C-NK+NP and NP and $ difference between DHA-NK+NP and NP. One symbol indicates $p < 0.05$. n = 8 (independent donors), collected in three independent experiments.

NK cells and neutrophils than when the cells were cultured alone, which could have led to the increase in IL-8 production by the neutrophils when cultured with NK cells. Furthermore, neutrophil production of the anti-inflammatory IL-1ra was increased when the neutrophils were co-cultured with NK cells, possibly through increased NK cell TNF-α and GM-CSF production (38). Pre-incubation of NK cells with DHA suppressed their production of IFN-γ when co-cultured with neutrophils. This diminished production of IFN-γ by DHA pre-treatment of NK cells did not lead to a decrease in IL-8 production, suggesting that IL-8 production was induced by another mechanism.

NK cells induce and inhibit neutrophil apoptosis depending on the stimulus and timing as described in several studies. NK cells have been shown to induce neutrophil apoptosis through NKp46 and/or Fas-signaling or by diminishing MHC class I expression on neutrophils (16, 17). In addition, NK cells are suggested to either inhibit or increase neutrophil apoptosis through a GM-CSF-mediated mechanism (7, 8, 17). In the present study, we examined the kinetics of the effects of NK cells on neutrophil apoptosis, to shed light on these contradicting results. NK cells induced neutrophil apoptosis after 6 h in co-culture, similar to that seen in the studies by Bernson et al. and Thorén et al. also investigating apoptosis at early time-points (16, 17). By contrast, prolonged co-culture (18 h) of NK cells and neutrophils suppressed neutrophil apoptosis, comparable to that shown by Bhatnagar et al. and Costantini et al. (7, 8). NK cell induction of neutrophil apoptosis at 6 h, in the present study, is not likely to be mediated by NK cell expression of NKp46 as suggested by Thorén et al. (16) as co-culture with neutrophils

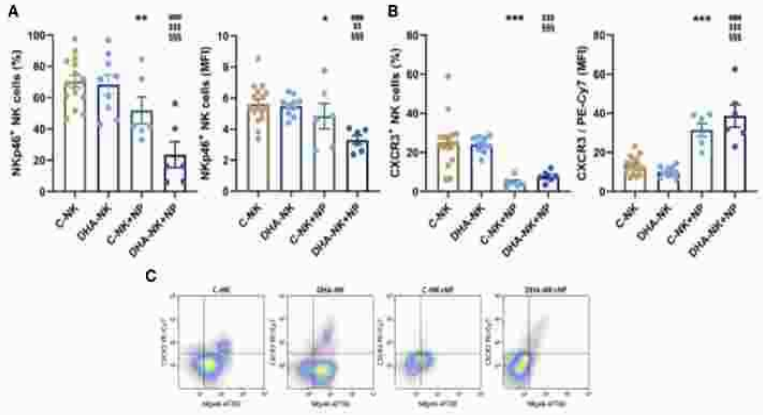

FIGURE 5 | Effects of neutrophils on expression of NKp46 **(A, C)** and CXCR3 **(B, C)** on NK cells pre-incubated with DHA or not. NK cells were pre-incubated for 18 h in the absence (C-NK) or presence (DHA-NK) of 50 μM docosahexaenoic acid and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) and cultured further for 6 h alone or with neutrophils (C-NK+NP and DHA-NK+NP). Expression levels were determined by flow cytometry and are presented either as percent positive cells of the total NK cell population or as mean fluorescent intensity (MFI) of the positive cell population **(A, B)**. Positive gating was determined with appropriate isotype controls. Data are shown as mean SEM; * indicates difference between C-NK+NP and C-NK, § difference between DHA-NK and DHA-NK +NP, $ difference between DHA-NK+NP and C-NK, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. n = 8–15 (independent donors), collected in at least three independent experiments.

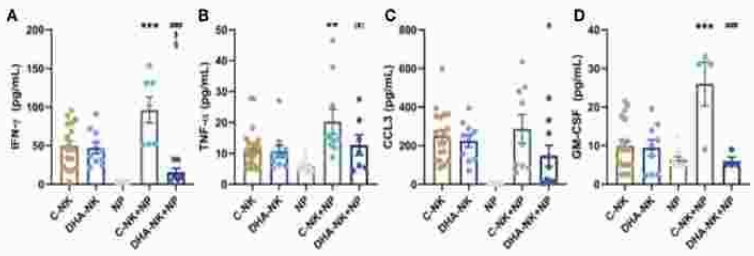

FIGURE 6 | The effects of neutrophils on production of IFN-γ **(A)**, TNF-α **(B)**, CCL3 **(C)**, and GM-CSF **(D)** in co-cultures with NK cells pre-incubated with DHA or not. NK cells were pre-incubated for 18 h in the absence (C-NK) or presence (DHA-NK) of 50 μM docosahexaenoic acid and then stimulated with IL-2 (2 ng/ml), IL-12 (2 ng/ml), and IL-15 (10 ng/ml) and cultured with or without neutrophils for further 18 h (C-NK+NP and DHA-NK+NP). Neutrophils were also cultured alone for 18 h (NP). Cytokine concentrations were determined by ELISA and are presented as pg/ml. Data are presented as means ± SEM: * indicates difference between C-NK+NP and C-NK, § difference between DHA-NK and DHA-NK+NP, $ difference between DHA-NK+NP and C-NK, and # difference between DHA-NK+NP and C-NK+NP. One symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. Symbol in parenthesis indicates $p = 0.05$–0.1. One outlier was removed in the NP group in A and B, one outlier was removed in the C-NK+NP group in B and D and one outlier was removed in the DHA-NK+NP group in A. n = 4–21 (independent donors), collected in at least three independent experiments. In **Supplementary Figure 3** all outliers are included.

decreased NKp46 expression and pre-incubation of NK cells with DHA decreased NKp46 expression even more. Neither is it likely that the effects of NK cells on neutrophil apoptosis in the present study were mediated by a GM-CSF-mediated mechanism as co-culturing NK cells with neutrophils increased their GM-CSF production but when the NK cells had been pre-treated with DHA their GM-CSF production was reduced to even lower than that when they were cultured alone. Surprisingly, pre-incubating NK cells with DHA enhanced NK cell-induced suppression of apoptosis at 18 h leading to increased survival of the neutrophils. These results indicate not only the potential of NK cells to prevent resolution of inflammation but also that pre-incubation with DHA could enhance this anti-resolution function of the NK cells.

According to Mair et al. $NKp46^{hi}CXCR3^{+}$ cells are potent responders in inflammation (39). However, in the present study, when NK cells were co-cultured with neutrophils the $NKp46^{hi}CXCR3^{+}$ population that was present when NK cells were cultured alone more or less disappeared, suggesting that these NK cells were less likely to migrate to draining lymph nodes, as described by Martin-Fontecha et al. (40), and subsequently to induce an inflammatory response.

In summary, the results from this study show that pre-treatment of NK cells with DHA attenuated NK cell ability to induce upregulation of CD11b and CD47 expression on neutrophils. This may indicate that DHA can diminish NK cell ability to promote neutrophil migration to inflamed sites. However, pre-treatment with DHA enhanced the pro-survival effect of NK cells on neutrophils, late in the co-culture, indicating that DHA could increase NK cell ability to hamper neutrophil removal from the inflamed site. Pre-treatment of NK cells with DHA also diminished NK cell expression of activation molecules and production of several pro-inflammatory cytokines in co-cultures with neutrophils. Our findings indicate that DHA may attenuate some of the pro-inflammatory effects NK cells have on neutrophils, as well as increase the anti-inflammatory and attenuate some of the pro-inflammatory effects neutrophils have on NK cells. Overall, the results suggest that DHA may lead to a more anti-inflammatory microenvironment for NK cell and neutrophil crosstalk.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The National Bioethics Committee, Iceland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KJ, IH, and JF designed the research. KJ, SO, and MR conducted the research and obtained the data. KJ, SO, MR, IH, and JF analyzed the data. IH and JF supervised the study. KJ, IH, and JF drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.570380/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with two of the authors, JF and IH.

Paper III

Natural Killer Cells are a Novel Cellular Source of Specialized Pro-Resolving Mediators

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Abstract. Chronic inflammation contributes to approximately half of all disease-associated deaths worldwide. It is caused by impaired resolution of inflammation (ROI). Lipid-derived specialized pro-resolving mediators (SPMs) are produced through the actions of 5-, 12-, and 15-lipoxygenase (LOX). Neutrophil, macrophages, and platelets contribute to ROI in part through their production of SPMs. We and others have shown that natural killer (NK) cells are indispensable for successful ROI. The aim of this study was to determine whether NK cells could promote ROI by providing lipid intermediates for neutrophil SPM production. Surprisingly, NK cells produced EPA- and DHA-derived SPMs without the aid of neutrophils. Oxylipins in synthesis pathways of SPM production were enriched in NK cell cultures. Finally, NK cells express the LOX proteins required for production of SPMs. Neutrophils only aided in the production of eicosanoids in NK cell cultures. The present study identifies NK cells as a novel cellular source of SPMs.

Keywords: Specialized pro-resolving mediators, natural killer cells, resolution of inflammation, bioactive lipids

Abbreviations: Arachidonic acid (AA), cyclooxygenase (COX), dimethyl sulfide (DMSO), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), hydroxydocosahexaenoic acid (HDHA), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxytetraenoic acid (HETE), leukotriene (LT), lipoxin (LX), lipoxygenase (LOX), maresin (MaR), natural killer cells (NK cells), P450 cytochrome epoxygenase (CYP450), peripheral blood mononuclear cells (PBMCs), polyunsaturated fatty acid (PUFA), prostaglandin (PG), protectin (PD), resolution of inflammation (ROI), resolvin (Rv), specialized pro-resolving mediator (SPM)

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1 Introduction

Resolution of inflammation (ROI) is a tightly regulated process dictating the temporal progression of acute inflammation to homeostasis [1]. Impaired ROI can cause chronic inflammation which is an underlying pathology for many degenerative diseases, including cancer and autoimmunity [1]. Integral to successful ROI is the lipid mediator class switching from pro-inflammatory eicosanoids to specialized pro-resolving mediators (SPMs) [2]. SPMs are a large group of compounds derived from omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) including arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [3]. AA, EPA, and DHA give rise to several classes of SPMs, i.e., lipoxins (LXs), resolvins (Rvs), maresins (MaRs), and protectins (PDs) [4, 5]. They are synthetized by the enzymatic actions of cyclooxygenases (COXs), lipoxygenases (LOXs), and P450 cytochrome epoxygenases (CYP450) [5]. Collectively, SPMs promote several cellular and molecular events to enhance ROI, such as enhancing neutrophil apoptosis and their removal from inflamed sites, promoting non-phlogistic immune cell recruitment, and macrophage polarization towards an M2-like phenotype [6].

The biosynthesis of SPMs is carried out by a number of cells, either on their own or in collaboration between different cell types [1]. Human macrophages, as well as dendritic cells, express high levels of 12-LOX and produce MaRs derived from DHA [7]. Human neutrophils express 5-, 12-, and 15-LOX required for the synthesis of SPMs and are widely reported as the main source of SPMs [8, 9]. Likewise, eosinophils have been shown to produce resolving lipids during nasal inflammation and acute peritonitis [10, 11]. Finally, leukocytes, endothelial cells, and platelets interact for the formation of SPMs, such as aspirin-triggered lipoxins, to promote ROI [12].

Natural killer (NK) cells are innate lymphocytes that induce apoptosis in aberrant target cells without prior stimulation [13]. During inflammation, they infiltrate the inflammatory site to induce apoptosis in transformed or damaged cells [13]. Additionally, NK cells regulate the inflammatory fate through their production of pro- and anti-inflammatory cytokines [14]. Thus, the cues provided for NK cells determine their function during inflammatory responses. NK cell function can be affected by eicosanoids both in cancer and inflammation progression. A recent study demonstrated that PGE_2 modulates infiltrating NK cell immunoregulatory function, thereby dictating tumor fate [15]. This modulation is mediated by the ligation of PGE_2 to its receptors, EP2 and EP4, to suppress NK cell cytotoxicity in the inflammatory tumor

microenvironment [16-19]. Interestingly, NK cells have been shown to enhance inflammation in a murine type I diabetes model through the enzymatic actions of 12/15-LOX and subsequent lipid mediator production [20]. These studies suggests that eicosanoids and their production modulate NK cell function in tumors and chronic inflammation.

NK cells are suggested to play a role in ROI through their cytotoxic effector functions [21-23]. We have previously shown that NK cells are indispensable for ROI in antigen-induced inflammation for the induction of neutrophil apoptosis and their removal from the inflamed site [24, 25]. They were established as integral effector cells for resolution of murine asthmatic inflammation [21], nasal inflammation [23], and DSS-colitis [22]. However, it has not been established whether NK cells contribute to ROI through other mechanisms than cytotoxicity.

In the present study, we demonstrate that NK cells can produce SPMs. They did not produce pro-inflammatory eicosanoids without the aid of neutrophils. We show that NK cells possess the enzymes required for SPM synthesis and that LOX inhibitor attenuates this function. Thus, we identify NK cells as a novel cellular source of SPMs identifying another mechanism by which they may ensure successful ROI.

2 Materials and methods

2.1 Preparation of fatty acids

AA, EPA, and DHA (Cayman Chemical, Michigan, USA) were dried down under a gentle stream of liquid nitrogen and resuspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany). PUFAs and DMSO aliquots as controls were flushed with liquid nitrogen and stored at -80°C to avoid autoxidation. Prior to use, PUFAs and DMSO were resuspended in complete RPMI medium (RPMI 1640 medium (Gibco, Thermo Fisher Scientific, United Kingdom) containing 10% heat-inactivated fetal bovine serum (Gibco) as well as penicillin/streptavidin (Gibco)) and incubated at room temperature for 1 hour (h) to allow binding to serum albumin.

2.2 Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers (permission # 06-068-V1) at the Icelandic Blood Bank using Histopaque-1077 (Sigma-Aldrich). NK cells were enriched from PBMCs by negative selection using an NK cell isolation kit (Miltenyi Biotec, Germany). Fresh venous EDTA blood from healthy volunteers (permission # 06-068-V1) was used to isolate primary neutrophils with a density gradient of layered Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich). Erythrocytes were pelleted with 3% dextran (Sigma-Aldrich) and remaining red cells lysed using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 10 µM Na_2EDTA, all from Sigma Aldrich). The purity of the NK cells and neutrophils was determined by flow cytometry to be ~97% and ~98%, respectively.

NK cells were cultured in 48-well culture plates (Nunc, Thermo Fisher Scientific) at a cell density of 1 x 10^6 cells/mL in complete RPMI medium. They were incubated with 50 µM AA, EPA, or DHA (AA-NK, EPA-NK, or DHA-NK, respectively) for 18 h before being stimulated with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) (all from R&D Systems, Bio-Techne, United Kingdom). NK cells cultured with DMSO served as a solvent control (C-NK). For co-culture experiments, freshly isolated neutrophils were added to the NK cell cultures at a cell density of 2 x 10^6 cells/mL along with the stimulation cocktail. For inhibitor assays, Zileuton (5-LOX inhibitor, Tocris Bioscience, Bio-Techne) and/or ML351 (12/15-LOX inhibitor, Tocris Bioscience) were added to the NK cells with the stimulation cocktail at their reported IC_{50} concentrations of 6 µM or 200 nM, respectively. Cells were cultured at 37°C, 5% CO2 and 95% humidity for an additional 18 h before being harvested.

For LC-MS/MS analysis, cell cultures were immediately resuspended in 3 x sample volume of methanol (LC-MS/MS grade, Sigma Aldrich) and stored at -80°C. For purity checks, cells were pelleted and stained for surface markers and evaluated by flow cytometry. For protein isolation, cells were pelleted, washed in ice-cold PBS with protease and phosphatase inhibitors (Thermo Fisher Scientific), and resuspended in RIPA buffer (Thermo Fisher Scientific) with EDTA, protease, and phosphatase inhibitors.

2.3 Tandem Liquid Chromatography Mass Spectrometry (LC-MS/MS)

Internal standard solution containing leukotriene (LT)B_4-d4, 15(S)-hydroxyeicosatetraenoic acid (HETE)-d8, prostaglandin (PG)E_2-d4, and DHA-d5 (Cayman Chemicals) in methanol were added to the samples before vortexing, spinning, and adding methanol for lipid extraction. After evaporating excess methanol, water and formic acid (Sigma-Aldrich) were added to the samples before loading them onto a C18 solid phase extraction cartridge (Sep-Pak, Waters, Milford, Massachusetts, USA). The cartridges were washed with water and n-hexane and subsequently eluted with methyl formate (Sigma Aldrich). The extracts were dried under a steady stream of nitrogen and reconstituted in methanol. The extraction process has been described in more detail in [26].

Samples were run on a QTrap 6500 mass spectrometer (Sciex, Nieuwerkerk aan den IJssel, The Netherlands). The exact settings and parameters used are specified in [26].

2.4 Protein extraction and SimpleWes assays

Total protein was extracted using RIPA buffer supplemented with EDTA, and protease and phosphatase inhibitors according to the manufacturers' protocol. Concentration of total protein was determined using a Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) according to the manufacturers' directions.

Protein was diluted to a working concentration of 0.2-0.4 mg/mL for SimpleWestern assays. SimpleWestern assays were performed according to the manufacturers' protocols using the 12-230 kDa (ProteinSimple, Bio-Techne) separation kit and the chemiluminescence rabbit detection kit (ProteinSimple). Monoclonal antibodies to

detect 5-LOX (clone EP6072(2), Abcam) and 15-LOX-1 (clone EPR22136, Abcam), and polyclonal antibodies against 12-LOX (Abcam) and 15-LOX-2 (Abcam), were used as primary antibodies.

2.5 Flow cytometry

Cells were pelleted and Fc receptors blocked with a 2% mix of normal human and normal mouse serum and 5% TruStain FcX™ (Biolegend, California, USA). Cells were incubated with monoclonal antibodies against CD3 (clone OKT3, Biolegend), CD16a (clone CB16, Biolegend), CD56 (clone CMSSB, eBioscience, Thermo Fisher Scientific), CD49d (clone 9F10, Biolegend), and CD62L (clone DREG-56, Biolegend). NK cells were defined as $CD3^{-}CD56^{+}$ lymphocytes and neutrophils were defined as $CD49d^{-}CD16a^{+}CD62L^{+}$ granulocytes. Cells were evaluated on a Sony SH800S flow cytometer and gating strategies were established using isotype and unstained controls. Data were analyzed using the Kaluza Analysis Software (Beckman Coulter, Indianapolis, USA).

2.6 Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Reported n-values indicate individual cell donors. Heatmapping, partial least square discriminant analysis, and loading analysis were carried out on scaled LC-MS/MS data in R version 4.2.2 (R Core Team (2022). R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. URL https://www.r-project.org/) and RStudio version 2022.7.2.576 (RStudio Team (2022). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). Correlation scorings and matrices were likewise created using R and RStudio on scaled data. Groups were compared using one-way repeated measures ANOVA when comparing three or more groups for key lipid mediators. Differences were regarded as significant when the corrected p-value < 0.05. Statistical analyses of key lipid mediators were carried out in GraphPad Prism 9 (GraphPad Software, California, USA).

3 Results

3.1 Natural killer cells synthetize specialized pro-resolution mediators

NK cells have been shown to be instrumental for resolution of inflammation but their ability to produce oxylipins remains undetermined [21, 24]. Following pre-incubation of NK cells with AA, EPA, or DHA and stimulation with the presence or absence of co-cultured neutrophils, we measured cell culture oxylipin concentrations by LC-MS/MS.

A wide array of oxidized lipids was detected in the NK cell cultures when they were pre-incubated with PUFAs (fig. 1A). To evaluate underlying differences in the lipidome

of the cell cultures, we scaled and centered the data and conducted principal component analysis on the dataset. Clustering analysis revealed that 4 clusters were evident in our dataset (data not shown). PCA and clustering analysis revealed distinct cell culture clusters due to the PUFA enrichment on a three-dimensional plane (fig. 1B). Neutrophils did not contribute to the clusters and were interspersed in the PUFA clusters (data not shown).

A correlation analysis of all oxylipins detected in our NK cell cultures revealed that EPA and DHA metabolites, including the SPMs, are significantly correlated in two distinct clusters (fig. 1C). Prostanoids derived from AA clustered together whereas LOX- and other COX-derived AA metabolites, AA, and autoxidative products caused a distinct cluster (fig. 1C). Finally, a distinct cluster of lipids that were not associated with the enzymatic oxygenation of AA, EPA, and DHA could be observed (fig. 1C).

Taken together, these data illustrates that NK cells provided with PUFAs have distinct oxylipidomic profiles and neutrophils do not modulate these.

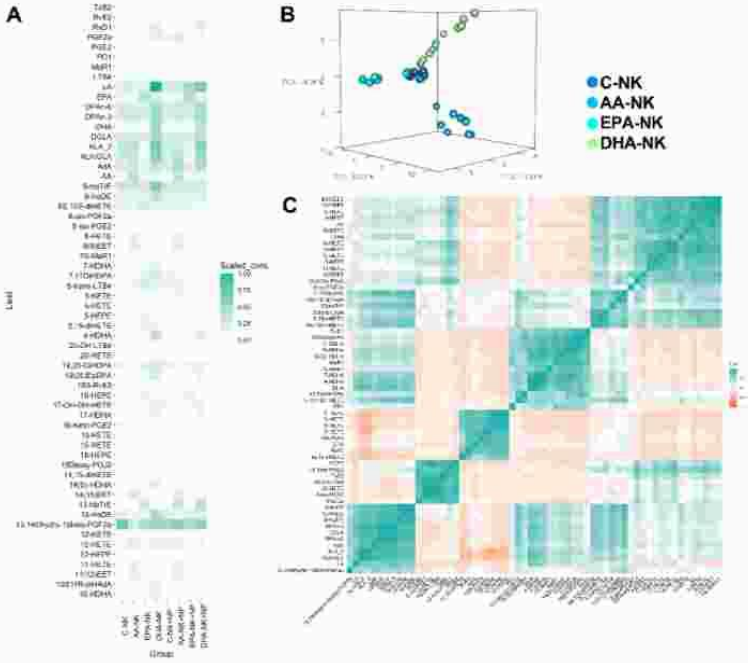

Figure 1. Neutrophils does not modify the oxylipid profile in PUFA-treated NK cell cultures. Natural killer cells were pre-incubated with 50 µM arachidonic acid (AA-NK), eicosapentaenoic acid (EPA-NK), docosahexaenoic acid (DHA-NK), or DMSO (C-NK) for 18 h. They were stimulated with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) with or without the addition of freshly isolated neutrophils (NP, unfilled symbols) and incubated for an additional 18 h. Oxylipin concentrations in cell cultures were determined by LC-MS/MS and results given as ng/mL or area ratios if concentrations could not be accurately determined. All

measurements were scaled to a 0-1 range, centered, and heatmapping of all measurements conducted (**A**). **B**) Principal component analysis of data revealed four distinct clusters based on fatty acid treatment with no additional separation by neutrophils (C-NK = dark blue, AA-NK = blue, EPA-NK = light blue, DHA-NK = green). **C**) Correlation of lipids in NK cell cultures were hierarchically clustered based on positive (green) and negative (orange) clustering scores. n = 6-12

3.2 Synthesis pathways for specialized pro-resolution mediators are enhanced by NK cells pre-incubated with polyunsaturated fatty acids

SPMs are synthetized through several oxidation steps from their substrate PUFA catalyzed by various oxygenases {Philip, 2020 #296}. Concentrations of 5-, 12-, and 15-HEPEs were increased in EPA-NK cell cultures compared C-NK cultures (fig. 2A). Importantly, concentration of the SPM intermediate 18-HEPE was increased in EPA-NK cell cultures compared to that in C-NK cell cultures (fig. 2A). This was in line with the increase in RvE_2 detected in EPA-NK cell cultures, as previously described (fig. 2A, supp. fig. 1). The addition of neutrophils in EPA-NK cell cultures decreased 5-HEPE concentrations compared with EPA-NK cells alone (fig. 2A, after dotted line). All other concentrations of detected EPA-derived oxylipins were unaffected by the addition of the neutrophils (fig. 2A, after dotted lines).

DHA-NK cell cultures contained higher concentrations of DHA compared with the other PUFA groups (fig. 2B). Concentrations of 4-, 7-, and 10-HDHA were all heightened in DHA-NK cell cultures (fig. 2B). Higher concentrations of the 14-HDHA isomer and its oxidative products MaR1 and the stereoisomer 7(S)-MaR1, as well as 17-HDHA and RvD_1, were also detected in DHA-NK cell cultures as compared with that of other cultures (fig. 2B). All products detected in the DHA oxylipin synthesis pathways were well above autoxidative control levels and, therefore, regarded as enzymatically produced (fig. 2B). The addition of neutrophils to the NK cell cultures did not affect the production of any oxidative derivatives of DHA (fig. 2B, after dotted lines).

Taken together, our data indicate that the NK cell production of SPMs are conducted through known enzymatic pathways. To further evaluate whether NK cells can produce SPMs through enzymatic pathways we proceeded to evaluate their expression of LOX enzymes.

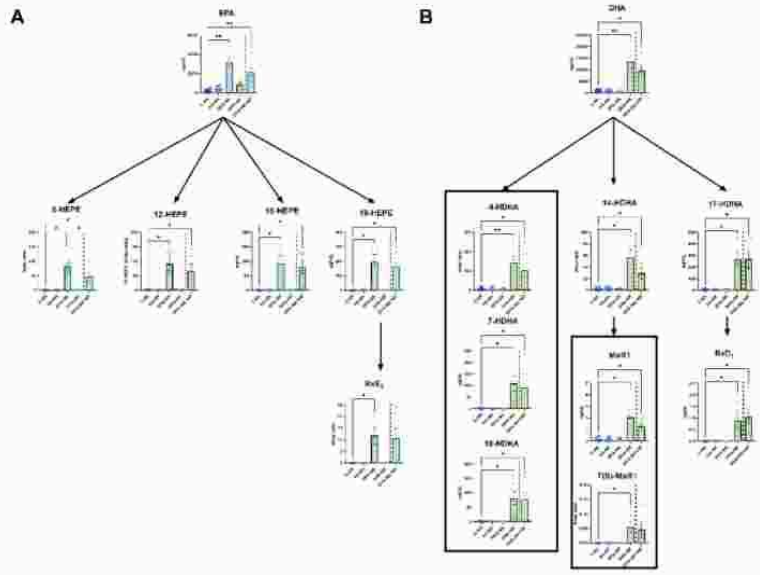

Figure 2. Natural killer cells synthetize specialized pro-resolution mediators and their intermediates from eicosapentaenoic and docosahexaenoic acid. NK cells were pre-incubated with 50 µM arachidonic acid (AA-NK, blue filled squares), eicosapentaenoic acid (EPA-NK, light blue filled upward triangles), docosahexaenoic acid (DHA-NK, green filled downward triangles), or DMSO (C-NK, dark blue filled circles) for 18 h. They were stimulated with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) with or without the addition of freshly isolated neutrophils and incubated for an additional 18 h. Oxylipin concentrations in cell cultures were determined by LC-MS/MS and results given as ng/mL, except if concentrations could not be accurately determined, area ratio estimates were used. Cell-free cultures enriched with fatty acids served as an autoxidation control (red dotted line). EPA and its metabolites 5-, 12-, 15-, 18-hydroxyeicosapentaenoic acid (HEPE), and resolvin $(Rv)E_2$ are shown in (**A**). DHA and synthesis pathways for its metabolites 4-, 7-, 10-, 14(S)-, 17-hydroxydocosahexaenoic acid (HDHA), maresin (MaR)1, 7(S)-MaR1, and RvD_1 are shown in (**B**). Oxylipin metabolites synthetized through the same pathway are surrounded by a black box. Statistical analysis was performed using one-way repeated measures ANOVA; $^{**}p < 0.01$, $^{*}p < 0.05$, n = 6.

3.3 Natural killer cells express 5-, 12-, and 15-lipoxygenase-2

LOXs are required for the enzymatic oxygenation of EPA and DHA {Chiang, 2020 #239}. Freshly isolated NK cells expressed 5- and 12-LOX at high levels without any stimulation (fig. 4A,B). Expression of 15-LOX-1 in freshly isolated NK cells and in cultured and stimulated NK cells was not observed (data not shown). In contrast, the inducible form of 15-LOX-2 were detected in freshly isolated NK cells (fig. 4C). The levels of 15-LOX-2 did not increase in NK cells following culture and stimulation (data not shown). Interestingly, the expression of the LOXs varied considerably between

individual NK cell donors, and there was no individual correlation between the levels of the different LOXs (fig. 4). Thus, NK cells contain all necessary enzymes for SPM production.

Figure 3. Natural killer (NK) cells express 5-, 12-, and 15-lipoxygenases (LOXs). NK cells were isolated from peripheral blood mononuclear cells (PBMCs). Cells were lysed, protein concentration measured, and loaded into SimpleWestern cassettes at 0.2 mg/mL containing internal loading standards. 5-LOX (**A**), 12-LOX (**B**), and 15-LOX-2 (**C**) were detected using antibodies, 12-230 kDa separation modules, and rabbit detection modules. Data are visualized in lane view like traditional western blots and separate lanes are individual cell donors. n = 6-7.

3.4 Natural killer cells cannot synthetize eicosanoids without the aid of neutrophils

AA can be oxygenated through COX- and LOX- dependent pathways to give rise to eicosanoids {Philip, 2020 #296}. As indicated in figure 1A, higher concentrations of LOX-derived oxylipins from AA, such as 5-,12-, and 15-HETE were observed (fig. 5). Furthermore, addition of neutrophils to the NK cell cultures did not affect the concentrations of 5-, 12-, or 15-HETE (fig. 5).

The concentrations of PGE_2, $PGF_{2\alpha}$, and TxB_2 did not exceed the autoxidative control levels in AA-NK cell cultures (fig. 5). However, when neutrophils were added to the NK cell cultures (AA-NK+NP), higher concentrations of PGE_2 and $PGF_{2\alpha}$ were observed as compared with AA-NK cells alone (fig. 5). AA-NK cell culture concentrations of TxB_2 trended higher with rather than without the presence of neutrophils (fig. 5).

Taken together, these results illustrate that neutrophil must be present for the formation of eicosanoids.

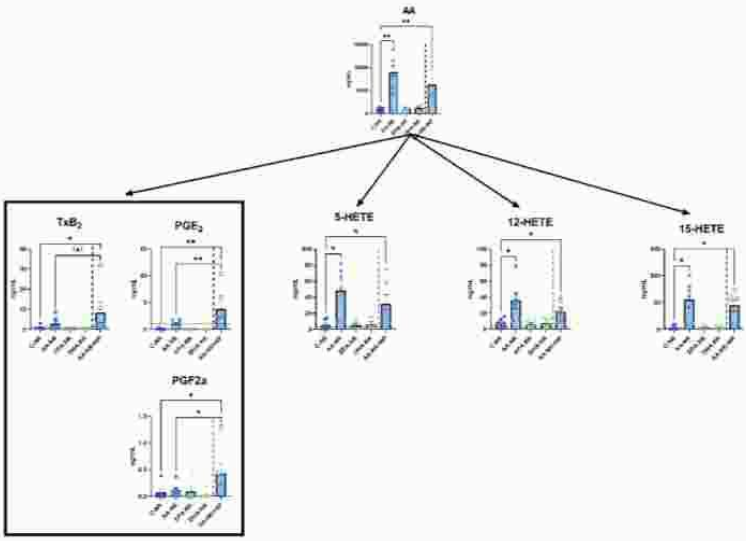

Figure 4. Neutrophils are required for the synthesis of prostaglandin (PG)E_2, PGF_{2a}, and thromboxane (Tx)B_2. Natural killer (NK) cells were pre-incubated with 50 µM arachidonic acid (AA-NK, blue filled squares), eicosapentaenoic acid (EPA-NK, light blue filled upward triangles), docosahexaenoic acid (DHA-NK, light green filled downward triangles), or DMSO (C-NK, dark blue filled circles) for 18 h. They were stimulated with IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL) with or without the addition of freshly isolated neutrophils and incubated for an additional 18 h. Cell culture concentrations of AA, TxB_2, PGE_2, PGF_{2a}, 5-, 8-, 11-, 12-, 15-hydroxyeicosatetraenoic acid (HETE) were determined using LC-MS/MS and cell-free cultures enriched with fatty acids served as an autoxidation control (red dotted line). Lipoxin $(LX)A_4$ cell culture concentrations were determined by ELISA on cell culture supernatants. Statistical analysis was performed using one-way repeated measures ANOVA: (*)$p < 0.1$, *$p < 0.05$, **$p < 0.01$, $n = 6$.

4 Discussion

In the present study we showed that PUFA treatment of NK cells modulate their oxylipin profile and neutrophil presence does not significantly contribute to this. NK cells were able to produce RvE_2, RvD_1, MaR1, and 7S-MaR1 on their own without the aid of neutrophils. This was supported by a general increase in intermediates for SPMs and a correlation between the substrate PUFA and subsequent oxylipin products in NK cell cultures. Importantly, we showed that NK cells express 5-, 12-, and 15-LOX without stimulation. Neutrophils only contributed to the production of PGE_2, PGF_{2a}, and TxB_2. Taken together, we have identified NK cells as a novel source of SPMs.

These findings may explain one of the mechanisms by which NK cells can promote ROI.

To date, cellular sources of SPMs have included neutrophils, eosinophils, monocytes, macrophages, endothelial cells, and platelets [5, 11, 27]. This production has been thought to be in unison of two or more cell types or on their own [8, 11, 12, 27]. To our surprise, neutrophils did not contribute to SPM production in our culture system when added to cultures enriched with PUFAs. A recent paper showed that neutrophils produce lipid mediators in response to a wide array of stimuli but not in resting conditions [8]. Inhibiting 15-LOX activity efficiently modulated human eosinophil and neutrophil synthesis of SPMs indicating enzymatic involvement in their production [28]. The discrepancies between their and our findings may be explained by different experimental procedures. Along those lines, neutrophils were provided with fatty acids and intermediates for oxylipin production in this study, whereas we provide PUFAs to NK cells prior to the addition of neutrophils to cultures. We did not establish whether NK cells release the oxylipins produced to cell culture supernatants for neutrophils to take up. The authors state that neutrophils prefer taking up AA rather than DHA, but preferred DHA-derived oxylipins rather than their AA-derived counterparts [8]. Their findings may provide an explanation of our observation that neutrophils contribute to PG and TxB_2 synthesis but not to SPM production from omega-3 PUFAs.

NK cells are described to be cytotoxic cells infiltrating inflamed tissues thereby limiting neutrophil and eosinophil numbers to contribute to ROI [21-23, 30]. Along these lines, we have shown that NK cell depletion leads to impaired ROI in antigen-induced peritonitis [24]. However, it has not been investigated if NK cells contribute to ROI in other manners than through their cytolytic functions. Considering recent studies revealing several novel NK cell subsets, we speculated if they could produce oxylipins for neutrophils to promote ROI. To our surprise, NK cells alone produced SPMs and oxylipins derived from AA, EPA, and DHA, but not PGs and TxB_2. These concentrations were higher than autoxidative levels from cell-free cultures enriched with PUFAs. Thus, we concluded that NK cells produce oxylipin derivatives from PUFAs, possibly through LOX-mediated oxidations. We detected high levels of 5- and 12-LOX in circulating NK cells from peripheral blood. Additionally, these NK cells also contained detectable levels of 15-LOX-2 but not 15-LOX-1. These findings are in line with a study in mice showing that NK cells contribute to murine type I diabetes through the enzymatic actions of 12/15-LOX [20].

We hypothesized that NK cells could provide neutrophils with the precursors required for SPM synthesis. We observed that NK cells contain the enzymes required for SPM production and that they can synthetize SPM without the aid of neutrophils. As we have shown that NK cells are required for proper ROI this provides possible mechanism aside from cytolytic activity explaining our observations [24]. We have not established how NK cells take up PUFAs and release them for SPM production. Additionally, the recruitment of a potential pro-resolving NK cell subset should be investigated *in vivo*. More studies regarding the induction of 15-LOX-2 in NK cells, as well as how resolving functions in NK cells are regulated remains to be determined.

Nevertheless, we have shown that NK cells constitute a novel cellular source of SPMs with the ability to promote successful ROI.

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6 Statements and Declarations

6.1 Funding

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6.2 Competing Interests

The authors declare no competing interests.

6.3 Author contributions

KJ, MG, IH, and JF designed the research. KJ, SO, and MH conducted the research and obtained the data. KJ, MG, IH, and JF analyzed the data. IH and JF supervised the study. KJ, MG, IH, and JF drafted the manuscript. All authors contributed to the article and approved of the submitted version.

6.4 Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

6.5 Ethics approval

The studies involving human participants were reviewed and approved by the National Bioethics Committee, Iceland.

6.6 Consent to participate

The participants provided their written informed consent to participate in this study.

7 Supplementary Tables and figures

Supplementary table 1. Mass-to-charge ratios and mass spectrums of oxylipins measured in NK cell cultures and co-cultures.

Name	Lipid Maps ID	Q1 Mass (Da)	Q3 Mass (Da)
LXA4	LMFA03040001	351.1	114.8
5-HETE	LMFA03060002	319.1	115.0
8-HETE	LMFA03060006	319.1	154.9
11-HETE	LMFA03060003	319.1	167.0
12-HETE	LMFA03060007	319.1	179.0
15-HETE	LMFA03060001	319.1	219.1
15-HEPE	LMFA03070009	317.1	219.0
18-HEPE	LMFA03070038	317.1	259.0
LXB4	LMFA03040002	351.1	220.9
LTB4	LMFA03020001	335.1	195.0
17-HDHA	LMFA04000072	343.1	245.0
14,15-diHETE	LMFA03060077	335.1	207.0
19,20-DiHDPA	LMFA04000043	361.1	273.0
RvE1	LMFA03140003	349.1	195.0
RvE2	LMFA03140011	333.1	114.9
18S-RvE3	LMFA03140007	333.1	245.2
18R-RvE3	LMFA03140006	333.1	245.0
TxB2	LMFA03030002	369.1	169.0
6-trans-LTB4	LMFA03020013	335.1	194.9
20-OH-LTB4	LMFA03020018	351.1	195.0
PGD2	LMFA03010004	351.1	233.0
PDX	LMFA04040003	359.1	153.0
PD1	LMFA04040001	359.1	153.0
MaR1	LMFA04050001	359.2	250.2
LTE4	LMFA03020002	438.1	333.1
8S,15S-diHETE	LMFA03060050	335.1	207.9
LTD4	LMFA03020006	495.1	177.0
LTB4-d4	LMFA03020030	339.1	196.9
15-HETE-d8	LMFA03060080	327.2	226.0
PGE2-d4	LMFA03010008	355.1	193.0
7,17-DiHDPA	N/A	361.1	198.9

RvD1	LMFA04030011	375.1	215.0
RvD2	LMFA04030001	375.1	277.1
6-trans-12-epi-LTB4	LMFA03020014	335.1	194.9
PGF2alpha	LMFA03010002	353.1	193.0
PGE2	LMFA03010003	351.2	271.1
13-HoTrE	LMFA02000051	293.0	195.0
13-HoDE	LMFA02000228	295.0	194.9
7(S)-MaR1	LMFA04050003	359.1	249.9
15-Keto-PGE2	LMFA03010030	349.0	234.9
13,14-dihydro-15-keto-PGF2alpha	LMFA03010027	353.1	195.0
8-iso-PGE2	LMFA03110003	351.1	271.0
8-iso-PGF2alpha	LMFA03110001	353.1	193.0
9-HoTrE	LMFA02000024	293.0	170.9
9-HoDE	LMFA02000188	295.0	171.0
AA	LMFA01030001	303.0	205.1
DHA	LMFA01030185	327.1	229.2
EPA	LMFA01030759	301.0	202.9
AdA	LMFA01030178	331.1	233.0
DPAn-3	LMFA04000044	329.1	231.1
DHA-d5	LMFA01030762	332.0	288.1
LA	LMFA01030120	279.0	261.0
ALA/GLA	LMFA01030152, LMFA01030141	277.0	233.0
PGJ2	LMFA03010019	333.0	271.0
5,15-diHETE	LMFA03060010	335.0	173.1
10-HDHA	LMFA04000027	343.1	153.0
7-HDHA	LMFA04000025	343.1	141.1
14(15)-EET	LMFA03080005	319.0	218.9
11(12)-EET	LMFA03080004	318.9	166.9
8(9)-EET	LMFA03080003	319.0	154.9
15-deoxy-PGJ2	LMFA03010021	315.0	203.0
20-HETE	LMFA03060009	319.0	289.1
4-HDHA	LMFA04000058	343.1	101.0
14(S)-HDHA	LMFA04000058	343.1	204.9
5-HEPE	LMFA03070027	317.0	114.9
12-HEPE	LMFA03070031	317.0	179.0

5-KETE	LMFA03060011	317.0	203.1
12-KETE	LMFA03060019	317.0	153.0
15-KETE	LMFA03060051	317.0	113.0
19(20)-EpDPA	LMFA04000038	343.1	281.1
DGLA	LMFA01030158	305.1	261.2
8-iso-PGF2alpha-d4	N/A	357.3	197.0
14(15)-EET-d11	N/A	330.2	219.0
RvD5	LMFA04030003	359.2	198.9
12-oxo-LTB4	LMFA03020024	333.2	179.0
13,14-dihydro-15-keto-PGE2	LMFA03010031	351.1	235.0
12-HHTrE	LMFA03050002	279.2	179.1
DPAn-6	LMFA01030182	329.1	231.1

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Appendix A: Mouse weights and dietary intake

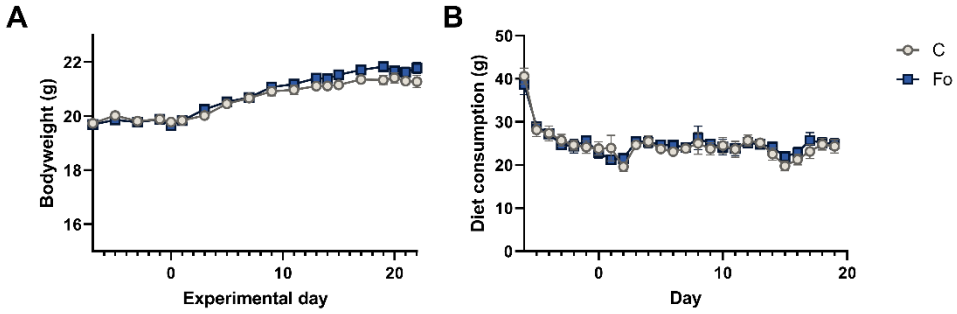

Figure 29. Development of mouse weights and dietary intake did not differ between control (C) or fish oil (Fo) enriched diets.
Mice were fed C (beige circles with brown borders) or Fo (blue squares) diets for 5 weeks and immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Mice were weighed every other day and on immunization days to determine the effect of C and Fo diet on mouse bodyweights in grams (g, **A**). Diets were changed every day and remaining diet was weighed to determine the dietary intake (**B**). Data are expressed as mean ± SEM and differences was determined using two-way ANOVA with Fisher's LSD multiple comparisons test; n = 102.

Appendix B: Peritoneal concentrations of soluble mediators in mice

Table 7. Concentrations of soluble mediators measured in peritoneal fluid during antigen-induced peritonitis.

Mice were fed control (C) or fish oil (Fo) enriched diets for 5 weeks and immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal fluid was isolated at 0, 1.5, 3, and 6 h after inflammation induction. Concentrations of angioponectin, angiopoietin-2, BAFF, CCL20, -3, -4, -5, -7, CHI3L1, CRP, CXCL1, -10, -12, -2, DKK-1, DPPIV, endoglin, G-CSF, GDF-15, GM-CSF, granzyme B, haptoglobin, IFN-γ, IGF-1, IL-10, -13, -17A, -1α, -1β, -27, -33, -5, -6, -6Ra, LIX, MMP8, MMP9, RAGE, RANKL, Resistin, S100A9, sFasL, sTNF RI, sTNF RII, sTWEAK, syndecan-1, TGF-β, TIMP-1, TNF-α were determined by Luminex or ELISA. Data are expressed as mean (SEM) and differences were determined by two-way ANOVA and Fisher's LSD multiple comparisons test; significantly different data are highlighted in bold, n = 6-49

	0 h		1.5 h		3 h		6 h	
Mediator	C	Fo	C	Fo	C	Fo	C	Fo
Adiponectin							**44901.6 (3445.8)**	**41487.5 (3664.2)**
Angiopoietin-2							4461.5 (532.4)	4510.9 (520.0)
BAFF	324.5 (71.0)	330.2 (72.1)	926.6 (268.4)	1845.6 (955.4)	702.8 (192.5)	514.1 (154.9)	286.4 (109.6)	108.9 (43.4)
CCL20							**88.0 (2.8)**	**71.9 (3.1)**
CCL3	0.0 (0.0)	0.0 (0.0)	**80.1 (8.3)**	**51.8 (7.2)**	80.0 (13.1)	105.4 (10.5)	43.4 (8.4)	53.6 (9.2)
CCL4	44.2 (15.1)	59.3 (0.0)	869.0 (128.3)	551.7 (111.5)	2072.7 (326.2)	2336.2 (208.4)	2160.1 (139.8)	2168.6 (181.4)
CCL5	53.5 (8.6)	48.4 (3.8)	139.1 (26.1)	478.0 (260.0)	715.0 (155.0)	694.7 (61.7)	**1118.7 (161.3)**	**2163.1 (374.4)**
CCL7							8430.5 (364.0)	8958,5 (325.5)
CHI3L1							1337.0 (113.2)	1262.2 (117.3)
CRP							71047 (5121)	134625 (30677)
CXCL1	10.2 (1.5)	10.8 (1.2)	**2984.7 (517.2)**	**1630.0 (277.2)**	1139.4 (478.9)	315.3 (89.2)	211.4 (24.6)	316.4 (42.0)

CXCL10	0.000 (0.000)	0.000 (0.000)	74.8 (26.5)	739.7 (436.3)	**1779.9 (287.6)**	**2767.1 (317.4)**	3252.1 (204.9)	3678.3 (299.3)
CXCL12	101.6 (3.4)	108.2 (17.8)	110.7 (15.5)	285.3 (130.7)	144.7 (39.2)	156.8 (30.5)	**382.2 (109.3)**	**1298.1 (357.1)**
CXCL2	2.2 (0.1)	1.9 (0.3)	**1790.2 (401.8)**	**813.5 (143.9)**	318.8 (84.5)	261.5 (56.6)	132.3 (13.8)	143.4 (11.6)
DKK-1	70.3 (14.5)	70.0 (18.8)	153.8 (43.2)	374.3 (117.7)	156.2 (48.9)	1072.5 (760.3)	**2132.2 (261.4)**	**3421.9 (415.8)**
DPPIV							16495.9 (1355.1)	14985.5 (1428.9)
Endoglin							137.5 (13.2)	121.3 (12.9)
G-CSF	19.6 (2.4)	18.8 (3.7)	302.0 (39.2)	614.1 (351.6)	2595.9 (309.1)	2680.7 (497.4)	4218.2 (576.2)	2928.6 (754.6)
GDF-15							13.7 (0.8)	13.3 (0.8)
GM-CSF	1.0 (0.3)	0.4 (0.2)	21.0 (4.2)	20.7 (6.2)	25.2 (7.2)	37.7 (9.8)	16.5 (4.1)	16.5 (1.8)
Granzyme B							1.6 (0.6)	2.4 (0.7)
Haptoblobin							154099 (118386)	1425623 (129253)
IFN-g	3.2 (0.7)	3.1 (0.7)	8.2 (3.9)	66.3 (62.2)	17.3 (1.9)	20.8 (3.7)	30.7 (4.6)	35.0 (4.1)
IGF-1	1035.9 (133.4)	1051.3 (275.3)	968.5 (237.8)	901.6 (197.8)	143.6 (12.7)	162.3 (21.5)	**397.4 (46.2)**	**759.8 (96.2)**
IL-10	1.2 (0.3)	0.9 (0.2)	129.2 (25.8)	77.3 (21.0)	101.1 (25.3)	65.5 (22.7)	24.6 (2.1)	20.2 (3.0)
IL-13	5.6 (1.3)	4.7 (1.1)	8.5 (2.0)	48.7 (26.2)	24.0 (8.6)	26.0 (7.1)	5.4 (2.1)	3.6 (1.4)
IL-17A	0.1 (0.1)	0.0 (0.0)	10.1 (2.9)	12.1 (4.5)	68.4 (15.7)	93.2 (48.0)	28.4 (15.7)	8.1 (3.1)
IL-1a	20.5 (5.1)	18.8 (5.2)	57.3 (19.7)	104.7 (38.0)	118.2 (20.3)	192.0 (35.9)	361.4 (29.4)	436.0 (50.8)
IL-1b							207.9 (20.8)	303.0 (127.6)
IL-27	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	44.8 (42.0)	5.4 (2.1)	10.6 (4.6)	68.5 (8.1)	70.2 (11.8)
IL-33	19.8 (4.2)	23.4 (7.2)	27.2 (7.8)	56.1 (21.9)	41.3 (5.8)	41.6 (7.5)	**697.7 (165.9)**	**251.3 (85.8)**
IL-5	0.0 (0.0)	0.0 (0.0)	10.3 (2.7)	28.3 (12.2)	148.3 (42.8)	75.1 (20.3)	44.6 (17.6)	33.5 (13.3)
IL-6							**4238.6 (333.2)**	**3346.8 (199.2)**

IL-6RA	410.0 (35.1)	393.3 (46.3)	**1617.3 (267.0)**	**877.3 (127.2)**	934.2 (182.0)	857.9 (161.0)	**1136.3 (60.7)**	**903.0 (45.4)**
LIX							203.9 (24.9)	224.4 (28.4)
M-CSF	0.3 (0.1)	0.3 (0.1)	3.5 (0.7)	5.0 (2.5)	9.4 (1.3)	7.5 (1.2)	6.1 (0.6)	6.2 (0.7)
MMP-8							33.8 (2.7)	32.5 (2.8)
MMP-9							4495.3 (383.5)	4305.1 (399.4)
RAGE	12.1 (4.4)	19.5 (4.7)	51.8 (24.6)	37.8 (10.7)	20.4 (3.5)	28.6 (4.8)	40.2 (4.6)	26.1 (2.2)
RANKL	2.5 (0.8)	1.8 (0.6)	4.8 (1.2)	14.7 (11.7)	3.4 (0.7)	3.1 (0.7)	0.7 (0.2)	1.2 (0.7)
Resistin							**253700.2 (19477.2)**	**234963.0 (21110.4)**
S100A9							3410.0 (280.8)	3258.4 (299.5)
sFasL	1.3 (0.7)	0.4 (0.4)	5.7 (4.3)	238.2 (235.8)	1.3 (0.5)	1.0 (0.7)	92.0 (31.8)	51.2 (19.4)
sTNFRI	108.7 (24.0)	101.0 (22.9)	614.1 (92.9)	566.6 (132.1)	613.1 (104.5)	508.2 (106.3)	535.8 (49.9)	558.0 (29.9)
sTNFRII	227.5 (14.1)	198.0 (12.2)	1182.6 (233.3)	1186.5 (142.0)	2022.1 (248.9)	2228.7 (315.1)	**924.2 (206.7)**	**4841.3 (309.3)**
sTWEAK	1.7 (0.4)	1.8 (0.4)	2.7 (0.5)	3.7 (0.9)	2.0 (0.6)	1.5 (0.3)	2.8 (0.2)	3.0 (0.3)
Syndecan-1							828.1 (100.4)	1001.4 (83.3)
TGF-β	118.7 (13.2)	147.7 (64.9)	152.4 (58.0)	127.3 (21.3)	89.7 (9.6)	137.9 (41.8)	**117.4 (11.0)**	**254.3 (48.1)**
TIMP-1							39308.7 (5302.3)	49041.2 (4490.0)
TNF-a	0.0 (0.0)	0.0 (0.0)	7.7 (2.0)	40.3 (35.3)	4.1 (1.2)	4.5 (1.4)	0.9 (0.4)	0.8 (0.3)

Appendix C: Peritoneal concentrations of lipid mediators in mice

Table 8. Peritoneal concentrations of lipid mediators in mice fed Westernized control (C) or fish oil (Fo) enriched diet in antigen-induced peritonitis.

Mice were fed C or Fo diets for 5 weeks and immunized twice with a two-week interval with mBSA. A week later inflammation was induced by intraperitoneal injection of mBSA. Peritoneal exudate was isolated 0, 3, 6, and 12 h after inflammation induction. Concentrations of 4-, 7-, 10- HDHA, 10S,17R- diHAdA, 8(9)-, 11(12)-, 14(15)EET, 5-, 8-, 11-, 12-, 14,15-, 15-, 17-OH-DH-, 20-HETE, 5-, 12-, 15-, 18-HEPE, 5-, 12-, 15-KETE, 13,14dihydro-15keto PGF_{2a}, 9-, 13-HoDE, 9-, 13-HoTrE, 15Deoxy PGJ_2, 15-Keto PGE_2, 19(20)-EpDPA, 19,20-, 7,17-diHDPA, 20-OH LTB_4, 5,15-, 8S,15S-diHETE, 6-trans-12-epi-LTB_4, 6-trans-LTB_4, 7(S)-MaR1, 8-iso-PGE_2, -PGF_{2a}, AA, AdA, ALA, ALA/GLA, DGLA, DHA, DPA_{n-3}, DPA_{n-6}, EPA, LA, LTB_4, MaR1, PD_1, PD_x, PGD_2, PGE_2, PGF_{2a}, PGJ_2, RvD_2, and TxB_2 were determined by LC-MS/MS. Data are expressed as mean (SEM), n = 6.

	0 h		3 h		6 h		12 h	
Lipid	C	Fo	C	Fo	C	Fo	C	Fo
10-HDHA	1.4 (0.6)	3.6 (1.1)	1.7 (0.8)	2.7 (0.7)	3.6 (1.0)	4.4 (2.1)	3.4 (0.7)	4.3 (2.3)
10S17R-diHAdA	0.4 (0.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)
11(12(EET)	0.3 (0.1)	0.3 (0.1)	1.3 (0.5)	0.3 (0.1)	2.4 (1.4)	0.3 (0.1)	1.6 (0.7)	0.2 (0.1)
11-HETE	5.7 (1.0)	7.0 (3.2)	6.1 (1.2)	2.8 (0.4)	9.2 (2.6)	4.3 (0.8)	10.3 (3.3)	4.1 (1.3)
12-HEPE	5.6 (2.4)	25.6 (9.8)	3.4 (2.0)	19.6 (5.3)	6.9 (1.5)	37.9 (23.0)	36.8 (20.2)	30.8 (14.0)
12-HETE	216.0 (50.6)	95.6 (42.3)	48.6 (14.1)	13.3 (2.2)	165.1 (47.4)	27.0 (10.6)	124.9 (22.5)	44.7 (16.4)
12-KETE	1.2 (0.3)	0.4 (0.1)	0.3 (0.1)	0.1 (0.0)	0.7 (0.3)	0.1 (0.0)	1.1 (0.9)	0.3 (0.1)
13,14dihydro-15keto-PGF2a	0.2 (0.1)	0.2 (0.1)	0.2 (0.0)	0.2 (0.1)	0.3 (0.1)	0.1 (0.1)	0.2 (0.1)	0.3 (0.1)
13-HoDE	689.6 (254.3)	210.6 (44.1)	81.3 (23.4)	42.2 (10.4)	257.4 (75.7)	88.7 (32.8)	298.2 (49.9)	111.2 (36.2)
13-HoTrE	51.4 (17.9)	29.5 (6.2)	5.6 (1.6)	4.6 (1.4)	23.5 (8.3)	13.1 (8.2)	40.8 (7.5)	14.1 (5.9)
14(15)EET	0.2 (0.1)	0.2 (0.1)	1.1 (0.4)	0.2 (0.1)	1.9 (1.1)	0.2 (0.1)	1.1 (0.5)	0.2 (0.0)
14(S)-HDHA	45.6	91.7 (19.3)	18.6 (4.7)	28.9 (5.1)	70.9 (21.8)	70.8 (44.6)	90.2 (16.9)	69.4

	(11.2)							(34.3)
14,15-HETE	0.4 (0.1)	1.5 (0.4)	0.6 (0.2)	3.1 (0.8)	1.4 (0.7)	3.3 (1.1)	1.4 (0.6)	2.1 (0.6)
15Deoxy PGJ2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
15-HEPE	3.1 (0.9)	40.3 (18.9)	4.8 (3.0)	21.5 (3.2)	10.3 (3.2)	62.9 (46.1)	40.2 (17.8)	49.0 (27.2)
15-HETE	30.7 (7.0)	21.6 (9.7)	10.2 (3.7)	3.3 (0.7)	31.7 (10.9)	9.2 (4.5)	32.4 (8.8)	9.0 (3.1)
15-KETE	2.5 (0.4)	1.2 (0.1)	1.2 (0.3)	0.4 (0.1)	2.1 (0.8)	0.9 (0.2)	1.6 (0.4)	1.1 (0.5)
15-Keto PGE2	0.6 (0.2)	0.2 (0.0)	0.3 (0.1)	0.1 (0.0)	0.4 (0.1)	0.2 (0.1)	0.4 (0.0)	0.3 (0.1)
17-HDHA	38.6 (18.3)	133.7 (52.4)	25.6 (7.4)	38.0 (7.9)	110.7 (40.8)	146.8 (105.8)	148.5 (35.0)	120.4 (79.8)
17-OH-DH-HETE	0.5 (0.1)	0.6 (0.3)	0.2 (0.0)	0.1 (0.1)	0.6 (0.2)	0.2 (0.1)	1.0 (0.4)	0.2 (0.1)
18-HEPE	0.1 (0.0)	0.4 (0.1)	0.4 (0.2)	1.1 (0.4)	0.4 (0.2)	1.6 (0.4)	0.4 (0.1)	0.8 (0.3)
19(20)EpDPA	68.5 (34.4)	140.7 (68.6)	29.1 (15.7)	43.6 (17.4)	87.9 (53.6)	160.6 (131.0)	93.3 (56.9)	55.8 (27.9)
19,20-DiHDPA	2.6 (1.1)	6.3 (3.4)	4.0 (0.9)	31.3 (14.4)	7.3 (1.8)	27.6 (7.6)	3.7 (0.6)	10.9 (2.7)
20-HETE	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	0.1 (0.0)	0.0 (0.0)
20-OH LTB4	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
4-HDHA	0.1 (0.0)	0.3 (0.1)	0.5 (0.2)	1.0 (0.4)	0.8 (0.3)	1.4 (0.6)	0.3 (0.1)	0.7 (0.3)
5,15-diHETE	1.5 (0.9)	0.3 (0.1)	0.2 (0.1)	0.1 (0.0)	0.5 (0.1)	0.2 (0.1)	0.7 (0.2)	0.2 (0.1)
5-HEPE	0.0 (0.0)	0.2 (0.1)	0.2 (0.2)	0.7 (0.2)	0.2 (0.1)	1.6 (0.6)	0.2 (0.1)	1.5 (1.1)
5-HETE	1.2 (0.8)	3.4 (2.0)	2.2 (0.4)	1.4 (0.5)	3.8 (1.5)	2.1 (0.5)	7.3 (3.3)	2.0 (1.0)
5-KETE	0.2 (0.0)	0.2 (0.1)	0.4 (0.1)	0.2 (0.1)	0.5 (0.2)	0.3 (0.1)	0.5 (0.2)	0.2 (0.1)
6t,12epi-LTB4	6.0 (2.7)	0.7 (0.1)	0.3 (0.1)	0.1 (0.0)	1.2 (0.4)	0.2 (0.1)	0.9 (0.2)	0.7 (0.3)
6-trans-LTB4	8.6 (2.7)	0.9 (0.1)	0.4 (0.2)	0.1 (0.1)	1.7 (0.6)	0.2 (0.1)	1.2 (0.2)	1.9 (1.1)
7,17-DiHDPA	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.1 (0.0)	0.2 (0.2)

7-HDHA	0.0 (0.0)	0.3 (0.2)	0.3 (0.2)	1.1 (0.6)	0.8 (0.4)	1.4 (0.5)	0.5 (0.2)	0.4 (0.2)
7(S)-MaR1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)
8(9)EET	0.0 (0.0)	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)	0.3 (0.2)	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)
8-HETE	2.8 (0.8)	2.5 (1.2)	1.6 (0.4)	0.7 (0.2)	4.1 (0.9)	1.2 (0.4)	3.3 (0.8)	1.2 (0.3)
8-iso-PGE2	0.7 (0.3)	1.0 (0.4)	0.7 (0.1)	0.3 (0.1)	1.1 (0.3)	0.5 (0.2)	1.4 (0.5)	0.6 (0.2)
8-iso-PGF2a	0.4 (0.1)	0.2 (0.1)	0.3 (0.1)	0.1 (0.0)	0.3 (0.1)	0.2 (0.0)	0.4 (0.1)	0.2 (0.1)
8S,15S-diHETE	10.8 (5.3)	0.9 (0.2)	0.7 (0.4)	0.1 (0.1)	1.6 (0.6)	0.3 (0.2)	1.1 (0.2)	1.0 (0.5)
9-HoDE	185.0 (84.9)	188.5 (72.7)	122.6 (31.5)	75.9 (18.5)	166.2 (44.4)	151.2 (42.5)	270.8 (84.4)	270.8 (84.4)
9-HoTrE	47.4 (15.8)	37.4 (9.7)	29.0 (9.0)	25.3 (8.5)	32.9 (7.2)	40.0 (11.3)	47.3 (8.7)	35.1 (13.0)
AA	286.8 (162.0)	601.3 (290.0)	930.7 (107.8)	460.8 (50.9)	1084.7 (271.1)	586.3 (55.8)	903.2 (286.6)	528.2 (133.3)
AdA	45.7 (29.1)	136.3 (75.3)	80.3 (12.9)	32.1 (5.8)	154.5 (41.4)	68.4 (18.6)	227.5 (95.6)	72.6 (27.8)
ALA/GLA	329.6 (89.8)	1060.6 (346.4)	985.1 (98.5)	942.0 (140.1)	1477.1 (311.3)	1213.7 (176.3)	1171.0 (312.5)	926.0 (240.7)
ALA_2	0.1 (0.0)	0.4 (0.1)	0.3 (0.0)	0.3 (0.0)	0.5 (0.1)	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)
DGLA	0.2 (0.1)	0.3 (0.1)	0.4 (0.0)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.6 (0.2)	0.5 (0.2)
DHA	21.5 (2.3)	817.4 (414.7)	581.6 (89.4)	2009.5 (382.7)	1532.6 (367.6)	2466.3 (477.0)	778.1 (264.8)	1686.5 (685.3)
DPAn-3	17.5 (17.0)	227.8 (134.5)	222.0 (144.2)	493.8 (96.7)	268.9 (119.2)	759.6 (236.9)	208.7 (74.3)	656.4 (310.9)
DPAn-6	0.4 (0.3)	0.4 (0.3)	1.1 (0.1)	0.6 (0.1)	2.0 (0.6)	1.1 (0.2)	1.7 (0.7)	0.8 (0.3)
EPA	7.9 (4.2)	137.8 (100.9)	140.7 (104.8)	484.3 (81.4)	151.1 (94.4)	558.5 (116.7)	61.0 (13.1)	390.4 (166.5)
LA	2480.9 (149.0)	3578.6 (914.0)	3289.9 (331.1)	2952.8 (405.1)	4582.6 (737.8)	3765.5 (458.8)	3864.9 (815.2)	3300.3 (668.9)
LTB4	4.0 (1.2)	1.7 (0.4)	0.7 (0.2)	0.3 (0.1)	2.7 (0.8)	0.7 (0.5)	2.7 (1.1)	1.7 (0.9)

MaR1	1.2 (0.3)	1.8 (0.7)	0.1 (0.1)	0.2 (0.0)	0.4 (0.1)	0.4 (0.3)	1.1 (0.5)	0.9 (0.5)
PD1	1.5 (0.5)	6.0 (2.4)	0.2 (0.1)	0.3 (0.1)	0.8 (0.3)	1.0 (0.6)	1.5 (0.5)	0.9 (0.5)
PDx	3.4 (1.7)	3.7 (1.4)	0.7 (0.4)	0.9 (0.4)	2.3 (1.2)	3.3 (2.7)	3.0 (0.7)	3.0 (1.8)
PGD2	2.4 (1.5)	4.4 (2.4)	4.3 (1.5)	1.3 (0.5)	4.8 (1.4)	1.7 (0.6)	7.5 (3.3)	2.6 (1.3)
PGE2	6.0 (2.4)	9.0 (3.9)	7.7 (1.7)	3.3 (1.1)	11.5 (2.9)	4.9 (1.3)	17.9 (6.6)	6.5 (2.4)
PGF2a	1.4 (0.2)	1.4 (0.6)	1.4 (0.3)	0.7 (0.2)	1.7 (0.3)	0.6 (0.2)	3.0 (1.1)	1.2 (0.5)
PGJ2	0.3 (0.1)	0.4 (0.2)	0.5 (0.2)	0.2 (0.1)	0.5 (0.1)	0.3 (0.1)	0.6 (0.3)	0.4 (0.1)
RvD2	0.2 (0.1)	0.3 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)
TxB2	6.4 (2.2)	11.3 (5.4)	6.4 (1.6)	2.0 (0.6)	11.4 (4.0)	4.7 (2.1)	26.6 (11.8)	7.0 (3.6)

Appendix D: Cell culture concentrations of lipids

Table 9. NK cell culture and co-culture with neutrophils concentrations of oxylipins.
NK cells were pre-incubated with 50 µM arachidonic acid (AA-NK), eicosapentaenoic acid (EPA-NK), docosahexaenoic acid (DHA-NK), or DMSO as a solvent control (C-NK) for 18 hours (h). Neutrophils were added to the cultures or not alongside IL-2 (2 ng/mL), IL-12 (2 ng/mL), and IL-15 (10 ng/mL). Cells were cultured together for 18 h. Concentrations of 4-, 7-, 10-, 14(S)-, 17-HDHA, 10S,17R-diHAdA, 8(9)-, 11(12)-, 14(15)EET, 5-, 5,15-di-, 8-, 8S,15S-di-, 11-, 12-, 14,15-, 15-, 17-OH-DH-, 20-HETE, 5-, 12-, 15-, 18-HEPE, 5-, 12-, 15-KETE, 9-, 13-HoDE, 9-, 13-HoTrE, 13,14dihydro-15-keto-, 8-iso-, PGF_{2a}, 15Deoxy-PGJ_2, 15-Keto-, 8-iso-, PGE_2, 18S-RvE_3, 19(20)-EpDPA, 7,17-, 19,20-DiHDPA, 20-OH-, 6-trans-, LTB_4, 7S-MaR1, AA, AdA, ALA, ALA/GLA, DGLA, DHA, DPA_{n-3}, DPA_{n-6}, EPA, LA, MaR1, PD_1, RvD_1, RvE_2, and TxB_2 were determined by LC-MS/MS. Data are expressed as mean (SEM) and differences were determined using two-way ANOVA and Fisher's LSD multiple comparisons test; significant differences from the proper control is highlighted in bold, n = 6.

Lipid	C-NK	AA-NK	EPA-NK	DHA-NK	C-NK+NP	AA-NK+NP	EPA-NK+NP	DHA-NK+NP
10-HDHA	1.4 (0.7)	1.3 (0.7)	1.2 (0.6)	**80.3 (21.3)**	1.5 (0.8)	1.4 (0.7)	1.1 (0.6)	**77.7 (23.0)**
10S17R-diHAdA	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)
11(12)EET	0.3 (0.1)	**7.4 (2.0)**	0.4 (0.2)	0.4 (0.1)	0.3 (0.1)	**5.6 (1.8)**	0.3 (0.1)	0.4 (0.2)
11-HETE	2.0 (1.0)	**19.0 (5.0)**	2.0 (1.0)	1.7 (0.8)	2.0 (1.1)	**13.3 (3.9)**	1.7 (0.8)	1.9 (0.9)
12-HEPE	1.6 (0.5)	1.5 (0.5)	**91.2 (25.9)**	2.1 (0.6)	1.5 (0.5)	1.9 (0.8)	**67.6 (23.5)**	2.0 (0.7)
12-HETE	8.3 (2.2)	**36.1 (10.0)**	6.6 (2.0)	7.5 (2.4)	7.5 (2.5)	**22.2 (5.7)**	5.5 (1.8)	7.4 (2.4)
12-KETE	0.0 (0.0)	0.7 (0.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.2 (0.1)**	0.0 (0.0)	0.0 (0.0)
13,14dihydro-15keto-PGF2a	0.2 (0.1)	0.1 (0.1)	0.0 (0.0)	0.1 (0.1)	0.2 (0.1)	0.3 (0.1)	0.2 (0.1)	0.3 (0.1)
13-HoDE	3.4 (1.2)	3.5 (1.2)	2.3 (0.7)	**5.6 (1.8)**	4.2 (1.3)	3.8 (1.2)	2.5 (0.7)	5.8 (2.1)
13-HoTrE	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	0.1 (0.0)	0.0 (0.0)	0.1 (0.0)
14(15)EET	0.1 (0.0)	**1.5 (0.3)**	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	**1.5 (0.4)**	0.1 (0.0)	0.1 (0.0)
14(S)-HDHA	3.0 (1.1)	2.9 (1.0)	2.6 (1.0)	**56.5 (14.4)**	2.4 (0.9)	2.1 (0.8)	1.8 (0.6)	**29.1 (10.3)**

14,15-diHETE	0.3 (0.2)	0.1 (0.1)	**6.4 (1.7)**	0.1 (0.1)	0.1 (0.1)	0.4 (0.4)	**11.7 (4.1)**	0.1 (0.1)
15Deoxy PGJ2	0.1 (0.0)	**0.6 (0.2)**	0.1 (0.0)	0.1 (0.1)	0.1 (0.0)	1.2 (0.5)	0.2 (0.1)	0.2 (0.1)
15-HEPE	0.6 (0.3)	0.6 (0.2)	**92.2 (27.0)**	1.0 (0.3)	0.5 (0.2)	0.6 (0.2)	**80.3 (24.3)**	1.8 (0.5)
15-HETE	3.5 (1.7)	**55.8 (15.4)**	3.8 (1.5)	3.1 (1.4)	3.6 (1.8)	**44.4 (11.0)**	3.4 (1.3)	3.5 (1.5)
15-KETE	0.9 (0.5)	**10.1 (2.9)**	1.4 (0.6)	1.3 (0.6)	0.4 (0.2)	**2.6 (1.0)**	0.9 (0.5)	0.7 (0.3)
15-Keto-PGE2	0.1 (0.1)	**0.5 (0.1)**	0.1 (0.1)	0.1 (0.0)	0.1 (0.0)	0.7 (0.3)	0.1 (0.0)	0.1 (0.0)
17-HDHA	6.6 (3.0)	5.6 (2.5)	5.2 (2.5)	**268.3 (75.8)**	6.2 (3.0)	5.4 (2.5)	4.2 (2.1)	**266.4 (77.1)**
17-OH-DH-HETE	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.5 (0.2)
18-HEPE	0.9 (0.4)	0.8 (0.3)	**196.3 (52.3)**	1.4 (0.4)	0.9 (0.4)	1.4 (0.4)	**164.9 (45.8)**	2.3 (0.6)
18S-RvE3	0.0 (0.0)	0.0 (0.0)	**1.2 (0.5)**	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.7 (0.5)	0.0 (0.0)
19(20)EpDPA	18.7 (7.8)	18.2 (8.3)	16.3 (7.9)	**437.8 (90.9)**	15.2 (6.7)	20.2 (8.1)	13.8 (5.0)	**485.2 (132.9)**
19,20-DiHDPA	1.4 (0.5)	1.2 (0.5)	1.5 (0.5)	**3.6 (0.6)**	1.1 (0.4)	2.6 (1.3)	1.3 (0.5)	**8.1 (2.3)**
20-HETE	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.2 (0.1)	23.2 (12.1)	0.5 (0.1)	0.4 (0.1)
20-OH LTB4	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	3.1 (2.2)	0.1 (0.0)	0.2 (0.0)
4-HDHA	7.3 (3.6)	7.0 (3.5)	6.9 (3.6)	**143.7 (31.3)**	5.6 (2.8)	7.3 (3.9)	5.0 (2.6)	**103.5 (38.3)**
5,15-diHETE	0.7 (0.4)	1.6 (0.5)	0.6 (0.3)	0.8 (0.4)	0.5 (0.2)	1.4 (1.0)	0.3 (0.2)	0.9 (0.5)
5-HEPE	0.6 (0.3)	0.6 (0.3)	**82.1 (23.3)**	1.0 (0.3)	0.5 (0.3)	0.6 (0.3)	**47.3 (14.6)**	1.0 (0.4)
5-HETE	5.3 (2.6)	**48.3 (12.9)**	5.4 (2.3)	6.6 (2.8)	4.2 (2.1)	**31.6 (11.6)**	3.8 (1.7)	6.2 (3.1)
5-KETE	0.5 (0.2)	**11.2 (4.1)**	0.7 (0.3)	0.9 (0.4)	0.8 (0.4)	**8.2 (2.5)**	1.0 (0.4)	1.8 (0.9)
6-trans-LTB4	0.4 (0.2)	0.9 (0.3)	0.4 (0.2)	0.3 (0.2)	0.2 (0.1)	0.8 (0.3)	0.2 (0.1)	0.3 (0.2)
7,17DiHDPA	0.7 (0.3)	0.7 (0.4)	0.9 (0.4)	0.9 (0.4)	0.6 (0.3)	1.5 (0.8)	0.7 (0.3)	1.4 (0.8)

7-HDHA	1.7 (0.8)	1.6 (0.9)	1.4 (0.8)	**109.1 (31.9)**	1.6 (0.9)	1.6 (0.9)	1.2 (0.7)	**91.2 (35.0)**
7S-MaR1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.1 (0.0)**	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)
8(9)EET	0.1 (0.1)	0.8 (0.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.8 (0.3)**	0.0 (0.0)	0.0 (0.0)
8-HETE	1.4 (0.7)	**15.8 (4.4)**	1.5 (0.7)	1.2 (0.6)	1.5 (0.8)	**14.6 (4.1)**	1.4 (0.6)	1.4 (0.7)
8-iso-PGE2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.7 (0.5)	0.0 (0.0)	0.0 (0.0)
8-iso-PGF2a	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
8S,15S-diHETE	0.4 (0.2)	0.9 (0.3)	0.6 (0.2)	0.5 (0.2)	0.3 (0.1)	0.8 (0.4)	0.3 (0.1)	0.6 (0.3)
9-HoDE	10.5 (3.6)	11.0 (3.9)	7.0 (2.1)	**15.8 (4.9)**	11.2 (4.1)	12.2 (4.1)	8.0 (2.2)	17.4 (6.8)
9-HoTrE	1.6 (0.5)	1.9 (0.6)	1.3 (0.4)	**2.7 (0.7)**	1.9 (0.7)	1.8 (0.6)	1.4 (0.5)	2.6 (1.0)
AA	901.8 (228.2)	**8953.3 (1509.8)**	1067.3 (209.6)	1163.9 (289.0)	772.8 (240.8)	**6234.4 (1366.2)**	1012.0 (248.8)	1024.0 (283.6)
AdA	52.3 (14.7)	**98.3 (29.5)**	45.8 (13.5)	**112.8 (32.0)**	59.2 (17.3)	144.2 (45.9)	54.2 (16.6)	**167.2 (60.2)**
ALA/GLA	146.8 (45.0)	**209.7 (55.3)**	**25.3 (8.4)**	**389.7 (100.9)**	144.0 (39.7)	**246.0 (53.4)**	**28.1 (11.5)**	345.5 (120.8)
ALA_2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.1 (0.0)**	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.1 (0.0)**
DGLA	1.3 (0.4)	2.1 (0.7)	1.4 (0.4)	**3.6 (1.2)**	1.0 (0.3)	2.0 (0.7)	1.2 (0.4)	3.2 (1.2)
DHA	1068.6 (298.7)	966.8 (230.0)	962.8 (301.4)	**13475.9 (2240.5)**	921.5 (294.0)	921.9 (287.9)	885.9 (266.7)	**9605.2 (2968.0)**
DPAn-3	519.3 (169.1)	680.5 (237.5)	539.2 (172.6)	**1330.3 (462.7)**	426.1 (142.3)	689.9 (241.9)	493.7 (162.0)	1271.6 (480.4)
DPAn-6	0.5 (0.1)	0.6 (0.2)	0.4 (0.1)	**1.1 (0.3)**	0.4 (0.1)	0.7 (0.2)	0.4 (0.1)	1.1 (0.4)
EPA	272.2 (90.6)	**407.2 (107.7)**	**3166.7 (460.7)**	**887.0 (196.8)**	215.1 (71.1)	**364.7 (115.6)**	**2202.8 (418.0)**	**659.1 (223.0)**
LA	1010.3 (221.7)	**702.6 (137.3)**	950.9 (128.4)	**2350.9 (578.3)**	1067.5 (189.1)	**732.8 (172.6)**	1033.4 (331.2)	1975.5 (542.5)
LTB4	0.4 (0.4)	**2.4 (0.9)**	0.4 (0.4)	0.1 (0.1)	0.4 (0.2)	1.6 (0.9)	0.4 (0.3)	0.5 (0.2)

MaR1	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	**2.0 (0.7)**	0.1 (0.0)	0.1 (0.1)	0.1 (0.0)	**1.3 (0.3)**
PD1	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	5.7 (4.2)	0.1 (0.0)	0.2 (0.1)	0.1 (0.1)	5.8 (3.9)
PGE2	0.1 (0.1)	**1.0 (0.3)**	0.0 (0.0)	0.0 (0.0)	0.2 (0.1)	**3.8 (1.6)**	0.0 (0.0)	0.1 (0.1)
PGF2a	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)	0.1 (0.0)	**0.4 (0.2)**	0.1 (0.0)	0.0 (0.0)
RvD1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**0.9 (0.3)**	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**1.0 (0.3)**
RvE2	0.0 (0.0)	0.0 (0.0)	**11.8 (3.5)**	0.0 (0.0)	0.1 (0.0)	0.2 (0.1)	**10.7 (4.5)**	0.1 (0.1)
TxB2	0.9 (0.4)	2.8 (1.4)	0.3 (0.1)	0.3 (0.1)	0.8 (0.4)	8.4 (5.0)	0.3 (0.1)	0.3 (0.1)

Appendix E: Optimal number of clusters in PCA

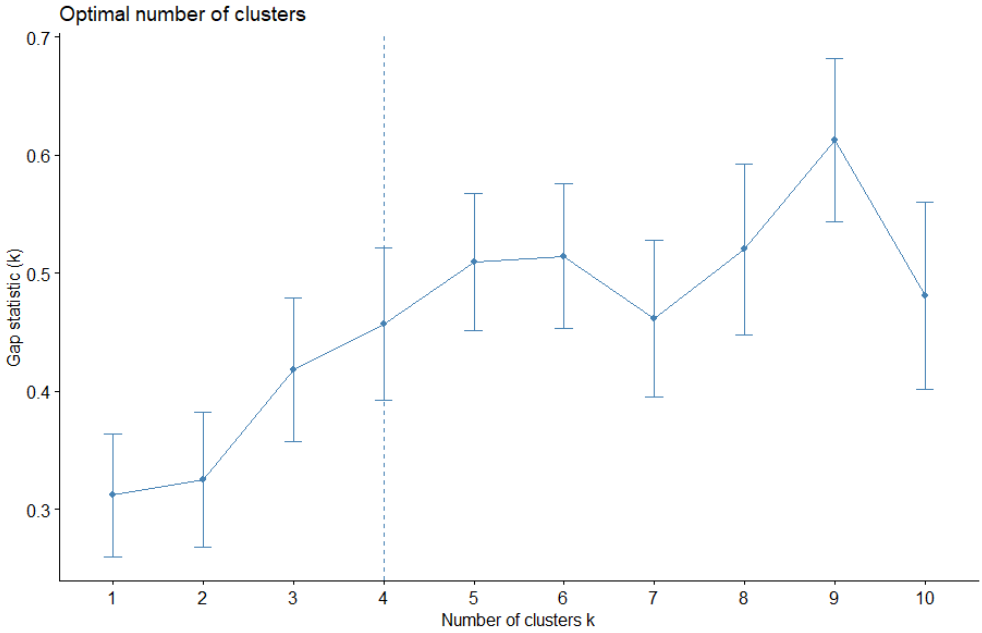

Figure 30. K-mean analysis revealed four underlying clusters in the NK cell cultures.
NK cells were pre-incubated for 18 h with 50 µM arachidnoic, eicosapentaenoic, or docosahexaenoic acid, or DMSO as a solvent control. Freshly isolated neutrophils were then added along with IL-2 (2 ng/mL), IL-12 (2 ng.mL), and IL-15 (10 ng/mL) or not. The cells were cultured together for an additional 18 h. Concentrations of lipids and their oxygenated metabolites were determined by LC-MS/MS. Data were scaled down to a 0-1 range and centered to limit the weight of outliers and highly concentrated mediators. Data received z-scores for principal component analysis and a k means analysis was conducted to reveal the optimal number of clusters. The dotted line specifies the optimal number of clusters, n = 6.