The effects of dietary fish oil on the induction and resolution of antigen-induced inflammation

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Áhrif fiskolíu í fæði á byrjunar- og lausnarfasa vakamiðlaðrar bólgu

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Ágrip

Á síðustu áratugum hefur tíðni ýmissra langvinnra sjúkdóma aukist og komið í ljós að bólga er undirliggjandi í mörgum þessum sjúkdómum (t.d. liðagigt, tannholdsbólgu, hjarta- og æðasjúkdómum, krabbameini og alzheimer súkdómnum). Mikil áhersla hefur verið lögð á rannsóknir á byrjunarfasa bólgu en nú er vitað að hjöðnun bólgu er virkt ferli sem er nauðsynlegt til að binda enda á bólgusvarið og stuðla að því að fyrra jafnvægi náist. Talið er mögulegt að ónæg bólguhjöðnun geti leitt til viðvarandi bólgu sem er einmitt einkennandi fyrir marga langvinna sjúkdóma. Breytingar á mataræði geta hugsanlega útskýrt að hluta þá aukningu sem orðið hefur á langvinnum bólgutengdum sjúkdómum í hinum vestræna heimi á síðustu áratugum. Minni neysla á n-3 fjölómettuðum fitusýrum (FÓFS), sem eru einmitt byggingarefni fyrir hjöðnunarboðefni, getur verið áhrifaþáttur í þessum breytingum. Sýnt hefur verið að n-3 FÓFS dempa byrjunarfasa bólgu en áhrif þeirra á hjöðnun bólgu hafa ekki verið skoðuð. Auk þess er lítið vitað um áhrif n-3 FÓFS á sérhæfða ónæmiskerið í bólguferlinum.

Markmið rannsóknarinnar var að ákvarða áhrif fiskolíu í fæði músa á byrjunar- og hjöðnunarfasa bólgu og á sérhæfða ónæmiskerfið í vakamiðlaðri bólgu. Bólgunni í þessu módeli var komið af stað með sérhæfðu ónæmissvari sem líkist því sem sést í byrjun sjálfsofnæmissvars. Þetta bólgumódel hentar því vel til þess að rannsaka áhrif fiskolíu í fæði ekki einungis á byrjunar- og hjöðnunarfasa bólgu heldur einnig á eitilfrumusvarið og vakasérhæfðu mótefnaframleiðsluna.

Kvenkyns C57BL/6 mýs fengu fóður með samsetningu líka því sem er í vestrænu fæði með eða án 2,8% fiskolíu (FO) í 4-6 vikur. Mýsnar voru bólusettar tvisvar undir húð með metýleruðu nautgripaalbúmíni (mBSA) með tveggja vikna millibili. Einni viku eftir seinni bólusetninguna var kviðarholsbólga mynduð með því að sprauta mBSA í kviðarhol músanna. Músunum var fórnað fyrir myndun kviðarholsbólgu og á nokkrum tímapunktum eftir myndun hennar (3, 6, 12, 24 og 48 klst, 5 dögum (d) og 10 d). Blóði, kviðarholsvökva og miltum var safnað. Frumur voru taldar og greindar út frá stærð, kyrningu og tjáningu á yfirborðssameindum með frumusjárlitun. Styrkur á flakkboðum, frumuboðum og leysanlegum boðefnaviðtökum í kviðarholsvökva og mBSA-sértækum mótefnum í sermi var ákvarðaður með ELISA aðferð. Stærð kímstöðva og fjöldi IgM^+ og IgG^+ frumna í milta metinn með ónæmisvefjalitun.

Eftir sprautun með mBSA sáust tveir hópar neutrófíla í kviðarholi. Þeir voru mismunandi að stærð, kyrningu og tjáningu á yfirborðssameindum. Fjöldi neutrófíla náði hámarki 6 klst eftir sprautun með mBSA en þeir voru horfnir eftir 48 klst. Staðbundnir kviðarholsmakrófagar hurfu úr kviðarholinu eftir sprautun

með mBSA en birtust aftur í hjöðnunarfasanum eftir að hafa umbreyst úr aðkomnum mónócýtum. Makrófagarnir skiptust í tvo hópa sem höfðu mismunandi tjáningu á F4/80. $F4/80^{++}$ makrófagar tjáðu ódæmigerða flakkboðaviðtakann D6 og CCR7 og $F4/80^{+}$ makrófagar tjáðu CD11c og CD138. Eósínófílar voru til staðar í kviðarholi 3 klst eftir sprautun með mBSA og náðu hámarki í fjölda á sama tíma og $F4/80^{++}$ makrófagarnir, þ.e. eftir 48 klst. Á þeim tíma höfðu þeir aukið tjáningu sína á CCR3 en minnkað tjáningu á CD11b. $F4/80^{+}$ makrófagar náðu hámarki í fjölda í síðbúnum hjöðnunarfasa 5 d eftir sprautun með mBSA. NK og T frumur náðu hámarksfjölda 48 klst eftir sprautun með mBSA á meðan B frumur náðu hámarki á d 5 og voru B1 frumur meirihluti þeirra. Styrkur bólgumyndandi frumuboðefnanna IL-1β og IL-6 og flakkboðanna CCL2 og CCL3 náði hámarki 3 klst eftir sprautun með mBSA og styrkur G-CSF, CXCL1 og CCL11 eftir 6 klst. Á hinn bóginn var styrkur flestra bólguhamlandi boðefna í hámarki seinna, styrkur IL-1ra eftir 6 klst, sTNF-R eftir 24 klst og sIL-6R ogTGF-β eftir 48 klst.

Þegar áhrif fiskolíu í fæði músanna voru skoðuð sýndu niðurstöðurnar að mýs sem fengu FO fóður höfðu minna bólgusvar og betri hjöðnun en mýs sem fengu C fóður. Minna bólgusvar kom fram í færri neutrófílum í músum sem fengu FO fóður samanborið við mýs sem fengu C fóður. Auk þess höfðu mýs sem fengu FO fóður lægri styrk af bólgmyndandi boðefnunum G-CSF, CXCL1, IL-6 og CCL11 en mýs sem fengu C fóður. Betri hjöðnun hjá músum sem fengu FO fóður samanborið við hjá músum sem fengu C fóður kom fram í styttra hjöðnunarbili (R_i), en það er notað sem mælieining á lengd hjöðnunarfasans. Önnur vísbending um betri hjöðnun í músum sem fengu FO fóður var að kviðarholsmakrófagar þeirra tjáðu meira af D6 og CCR7, sameindum sem tengjast hjöðnun, en kviðarholsmakrófagar úr músum sem fengu C fóður. Auk þess kom fram snemmbúið bólguhamlandi svar hjá músum sem fengu FO fóður sem ekki var til staðar, eða í minna mæli, í músum sem fengu C fóður. Þetta bólguhamlandi svar kom fram í auknum fjölda NK frumna í kviðarholi og aukinni tjáningu á F4/80 á kviðarholsmakrófögum sem og í auknum styrk bólguhamlandi boðefnanna TGF-β og sIL-6R. Einnig bendir meiri fjöldi eósínófíla og $CD138^{+}$ makrófaga í kviðarholi músa sem fengu FO fóðursem fengu C fóður til að fiskolía hafi bætt síðbúinn hjöðnunarfasa.

Fiskolía í fæði hafði einnig áhrif sérhæfða ónæmissvarið og sýndu niðurstöðurnar að í músum sem fengu FO fóður varð meiri aukning á T eitilfrumum eftir sprautun með mBSA en hjá músum sem fengu C fóður. Mýs sem fengu FO fóður höfðu einnig fleiri $CD4^{+}$ og $CD8^{+}$ T frumur en mýs sem fengu C fóður 2 d eftir sprautun með mBSA. Þá varð meira B1 frumusvar hjá músum sem fengu FO fóður samanborið við mýs sem fengu C fóður eftir sprautum með mBSA sem kom fram í auknum fjölda B1 frumna 24 klst og 5 d

eftir sprautun með mBSA, fleiri IgM^+ frumum í rauðu kviku milta eftir 5 og 10 d og hærri styrk BSA sértækra IgM mótefna í sermi eftir 5 d. Fiskolía í fæði hafði engin áhrif á fjölda B2 frumna í kviðarholi né á styrk BSA sértækra IgG mótefna í sermi.

Niðurstöður verkefnisins benda til þess að fiskolía í fæði hafi dempandi áhrif á bólgusvar í vakamiðlaðri bólgu, hraði byrjun bólgunnar og auki virkni hjöðnunarfasans. Að auki benda niðurstöðurnar til þess að fiskolía auki B1 frumusvarið og geti því mögulega aukið vörn gegn endurteknum sýkingum.

Lykilorð:

Vakamiðluð bólga, mBSA, fiskolía, byrjunarfasi bólgu, hjöðnun bólgu

Abstract

Over the last decades the prevalence of numerous chronic diseases has increased and inflammation has emerged as a central component in many of these diseases (i.e. arthritis, periodontal disease, cardiovascular diseases, cancer and Alzheimer's disease). Studying inflammatory mechanisms, much emphasis has been put on understanding the inductive phase of inflammation but recently the resolution phase of inflammation has been recognized to be an active endogenous process that may be of major importance in terminating inflammatory reactions, allowing the organism to return to homeostasis. It has been suggested that inadequate resolution of inflammation may lead to the persistent inflammation that characterizes many chronic diseases today. The increased prevalence of these inflammation-related chronic diseases in Western societies may in part be explained by dietary changes. These include, in particular, the decreased consumption of n-3 polyunsaturated fatty acids (PUFA) that are precursors for many pro-resolution mediators. Although n-3 PUFA have been shown to influence the inductive phase of inflammation and suggested to have beneficial effects in inflammatory conditions their effects on resolution of inflammation have not been studied in any detail. Furthermore, their effects on the adaptive immune response in the resolution phase of inflammation are largely unknown.

The objective of this study was to examine the effects of dietary fish oil, rich in n-3 PUFA, on the inductive phase, resolution phase and the adaptive immune response in antigen-induced peritonitis. The inflammation in this model is induced by the adaptive immune response and mimics the inflammation observed during a flare-up in autoimmune diseases and provides a model that is well suited for investigating, not only the role of dietary fish oil on the induction and resolution phases of inflammation, but also its effects on the lymphocyte response, including antigen-specific antibody production.

Female C57BL/6 mice were fed a Western diet with or without 2.8% fish oil (FO) for 4-6 weeks. The mice were immunized twice subcutaneously with methylated bovine serum albumin (mBSA) with two weeks interval. Three weeks after the initial immunization peritonitis was induced by injecting mBSA intraperitoneally (i.p.). Mice were sacrificed prior to and at several time points after peritonitis induction (3 hours (h), 6 h, 12 h, 24 h, 48 h, 5 days (d) and 10 d) and blood, peritoneal fluid, and spleen collected. The cells were counted and analyzed for size and granulation as well as expression of surface molecules. Concentration of chemokines, cytokines, soluble cytokine receptors in peritoneal fluid and mBSA-specific antibodies in serum were determined by ELISA. The

levels of germinal center B cells and IgM^+, IgG^+ cells in spleen were evaluated by immunoenzyme staining.

In mice receiving the control (C) diet, two neutrophil populations infiltrated the peritoneal cavity following administration of mBSA. They differed in both size and granularity and slightly in expression of surface molecules, peaked at 6 h but had disappeared at 48 h. Resident peritoneal macrophages disappeared from the peritoneal cavity after mBSA administration but appeared again in the resolution phase having differentiated from recruited monocytes. They also constituted two different populations differing in expression of F4/80. The $F4/80^{high}$ macrophages expressed the atypical chemokine receptor D6 as well as CCR7, while the $F4/80^{low}$ macrophages expressed CD11c and CD138. Eosinophils appeared in the peritoneal cavity 3 h following administration of mBSA and peaked simultaneously with the $F4/80^{high}$ macrophages at 48 h. At that time-point they had increased their expression levels of CCR3 but decreased their expression levels of CD11b. $F4/80^{low}$ macrophages peaked in the post resolution phase at d 5. NK cells and T cells peaked at 48 h, whereas B cells peaked at 5 d, with the majority of B cells being B1 cells. Peritoneal concentrations of the pro-inflammatory cytokines IL-β and IL-6 and the chemokines CCL2 and CCL3 peaked at 3 h and G-CSF, CXCL1 and CCL11 at 6 h. However, concentrations of most of the anti-inflammatory mediators peaked later, with IL-1ra peaking at 6 h, sTNF-R at 24 h and sIL-6R and TGF-β at 48 h.

When the effects of dietary FO were studied in the mBSA model the results showed that mice fed the FO diet had reduced inflammatory response and enhanced resolution activity when compared with mice fed the C diet. The reduction in the initial inflammatory response was manifested by lower and earlier peak number of infiltrated peritoneal neutrophils in mice fed the FO diet when compared with mice fed the C diet. In addition, mice fed the FO diet had lower peak concentrations of the pro-inflammatory mediators G-CSF, CXCL1, IL-6 and CCL11 than mice fed the C diet. The enhanced resolution activity was evident by the shorter resolution interval (R_i), which measures the kinetics of the resolution phase, in mice fed the FO diet compared with mice fed the C diet. Enhanced resolution was also indicated by higher expression of molecules linked to resolution, D6 and CCR7, on peritoneal macrophages in mice fed the FO diet than in mice fed the C diet. In addition, mice fed the FO diet showed an earlier anti-inflammatory response when compared with mice fed the C diet. An early peak was seen in the number of peritoneal NK cells and in the expression levels of F4/80 on peritoneal macrophages in mice fed the FO diet but not in mice fed the C diet. In mice fed the FO diet there was also an early peak in the peritoneal concentration of the anti-inflammatory mediators TGF-β and sIL-6R that was not seen in the mice fed the C diet. Furthermore, mice fed the FO diet

may have had an improved late-resolution response as they had a higher number of peritoneal eosinophils and $CD138^+$ macrophages than mice fed the control diet.

When the effects of dietary FO on the adaptive response were studied in the mBSA model the results showed that mice fed the FO diet had increased number of total peritoneal B and T lymphocytes following mBSA administration when compared to mice fed the C diet. Mice fed the FO diet had increased number of both $CD4^+$ and $CD8^+$ T cells 2 d following mBSA administration when compared with mice fed the C diet. Mice fed the FO diet had increased B1 response when compared with mice fed the C diet, seen in higher number of peritoneal B1 cell at 24 h and 5 d, higher levels of splenic IgM^+ cells in the red pulp of the spleen at d 5 and 10 and higher serum levels of BSA-specific IgM antibodies at d 5. Dietary FO did not affect the number of peritoneal B2 cells or serum levels of BSA-specific IgG antibodies in the mBSA model.

The results in this thesis indicate that dietary FO may dampen the inflammatory response, accelerate the onset and increase the activity of the resolution phase. In addition, the results indicate that dietary FO may enhance the B1 cell response and, thereby, possibly improve protection against secondary infections.

Keywords:

Antigen-induced inflammation, mBSA, fish oil, induction of inflammation, resolution of inflammation

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Contents

List of abbreviations

AA	Arachidonic acid
ALA	α-linolenic acid
APC	Antigen presenting cells
AU	Arbitrary units
BCR	B cell receptor
C	Control
CFA	Complete Freund's adjuvant
COX	Cyclooxygenases
CRP	C reactive protein
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular patterns
DARC	Duffy antigen receptor for chemokines
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FLAP	Nuclear envelope 5-LO activating protein
FO	Fish oil
FPRL	Formylpeptide receptor-like ligand
GC	Germinal centre
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDHA	Hydroxydosahexaenoic acid
HPETE	Hhydroperoxyeicosatetraenoic acid
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
I.p.	Intraperitoneally
IBD	Inflammatory bowel disease
IFA	Incomplete Freund‘s adjuvant
IFN	interferon
Igs	Immunoglobulins
IL	Interleukin
LA	Linoleic acid

LEC cell	lymphatic endothelial cell
LO	Lipoxygenases
LOX-1	Lectin-like oxidized LDL receptor-1
LPM	Large peritoneal macrophages
LPS	Lipopolysaccharide
LRP	LDL-receptor-related protein
LT	Leukotriene
LX	Lipoxin
MaR	Maresin
mBSA	Methylated bovine serum albumin
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MZ	Marginal zone
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NO	Nitric oxide
NPD1	Neuroprotectin-1
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular patterns
PC	Plasma cell
PD	Protectin
PD1	PGD_2 receptor
PG	Prostaglandin
PGI	Prostacyclin
PLA_2	Phospholipase A_2
PNA	Peanut agglutinin
PPAR-γ	Peroxisome proliferator activated receptor-γ
PRR	Pattern recognition receptors
PSR	Phosphatidylserine receptor
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
R_i	Resolution interval

ROS	Reactive oxygen species
Rv	Resolvin
S.c.	Subcutaneously
SEM	Standard error of the mean
SPM	Small peritoneal macrophages
SR-B1	Scavenger receptor class B type I
TCR	T cell receptor
Tfh cell	T follicular helper cell
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptors
TM	Thiglycolate medium
TNF	Tumour necrosis factor
T_{REG} cell	Regulatory T cell
TX	Thromboxane
UC	Ulcerative colitis
VCAM	Vascular cell adhesion protein
VLA	Very late antigen
ψmax	maximum number

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List of papers

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

I. Tomasdottir, V., Vikingsson, A., Hardardottir, I.* and Freysdottir, J.* Murine antigen-induced inflammation - a model for studying induction, resolution and the adaptive phase of inflammation. Manuscript submitted for publication.

II. Tomasdottir, V., Vikingsson, A., Freysdottir, J. and Hardardottir, I. Dietary fish oil reduces the acute inflammatory response and enhances resolution of antigen-induced peritonitis. Journal of Nutritional Biochemistry 2013; 24(10):1758-1765.

III. Tomasdottir, V., Thorleifsdottir, S., Vikingsson, A., Hardardottir, I. and Freysdottir, J. Dietary omega-3 fatty acids enhance the B1 but not the B2 cell immune response in mice with antigen-induced peritonitis. Journal of Nutritional Biochemistry 2014; 25(2):111-117.

*Joint last authorship

Declaration of contribution

Paper I: In this paper we describe the cellular and molecular kinetics in the induction phase and the resolution phase of inflammation as well as the adaptive response in murine antigen-induced peritonitis, a model for subacute/chronic inflammation. I participated in designing the hypothesis of this study with my mentors Dr. Freysdottir, Dr. Hardardottir and Dr. Vikingsson. I took part in taking care of the laboratory animals in collaboration with Hildur Hrönn Arnardóttir who was a doctoral student at that time. I took part in immunizing the mice, euthanizing them and obtaining the cells with help from Dr. Arnardottir and Dr. Freysdottir. I performed all the assays (ELISA and flow cytometry) and wrote the paper in collaboration with my mentors.

Paper II: In this paper we describe the effects of dietary fish oil on the induction and resolution of inflammation in murine antigen-induced peritonitis. I participated in designing the hypothesis of this study with my mentors Dr. Freysdottir, Dr. Hardardottir and Dr. Vikingsson. I took part in taking care of the laboratory animals in collaboration with Hildur Hrönn Arnardottir and changed the diets daily throughout the dietary experiments (4-6 weeks). I took part in immunizing the mice, euthanizing them and obtaining the cells with help from Dr. Arnardottir and Dr. Freysdottir. I performed all the assays (ELISA and flow cytometry) and wrote the paper in collaboration with my mentors.

Paper III: In this paper we describe the effects of dietary fish oil on the adaptive response in murine antigen-induced peritonitis. I participated in designing the hypothesis of this study with my mentors Dr. Freysdottir, Dr. Hardardottir and Dr. Vikingsson. I took part in taking care of the laboratory animals in collaboration with Hildur Hrönn Arnardottir and changed the diets daily throughout the dietary experiments (4-6 weeks). I took part in immunizing the mice, euthanizing them and obtaining the cells and organs with help from Dr. Arnardottir and Dr. Freysdottir. The immunohistochemistry studies were all performed by Sigrún Þorleifsdóttir MSc student. I carried out all other assays (ELISA and flow cytometry) and wrote the paper in collaboration with my mentors.

1 Introduction

Induction and resolution of inflammation are intertwined and closely controlled processes. Efficient induction of inflammation is vital for protecting the host from invading microorganisms and tissue injury but timely termination of the inflammatory response, i.e. resolution of inflammation, is also of paramount importance to minimize inflammation-related collateral damage and to promote tissue healing. Over the last decades the incidence of chronic inflammatory and/or degenerative conditions has markedly increased [1-3]. Also pathogenic role for persistent low grade inflammation in some of these disorders has been suggested by studies showing that healthy individuals with high baseline values of blood inflammatory markers, such as C reactive protein (CRP), are at increased future risk for coronary artery disease, malignancy, and Alzheimer's dementia [4, 5].The exact cause of this low grade inflammation is not known; partly it may be due to persistent immune activation [6] but it could also be caused by inadequate resolution of inflammation [7].

To help understand how the immune response can be better regulated and the risk of it evolving into chronic inflammation minimized, better information on the factors that regulate the induction and resolution phases of inflammation is needed. Environmental causes for the increase in many chronic conditions are without doubt a big factor, especially the modern Western diet. Diet with a high ratio of n-6 to n-3 polyunsaturated fatty acids (PUFA) has been postulated to promote the pathogenesis of numerous diseases. Over the past 100-150 years the consumption of food rich in n-3 PUFA has declined and there has been a dramatic increase in the consumption of food containing n-6 PUFA. It has been estimated that the ratio of n-6 to n-3 PUFA in the diet has changed from being around 1:1 in the diet when human beings evolved to around 10:1 – 25:1 in Western diet today [8-10]. This could indicate deficiency in n-3 PUFA in Western diet compared with the diet when humans evolved on when their genetic patterns were established.

The purpose of this study was to determine the effects of dietary fish oil, or n-3 PUFA, on the induction and resolution phases of inflammation and the adaptive response in a murine antigen-induced peritonitis model. These results should contribute to a better understanding of n-3 PUFA and the mechanisms by which they affect the immune system, both the innate and the adaptive in chronic diseases.

1.1 The immune system

Our survival is greatly dependent on a properly functioning immune system, a system that is made up of a network of cells, tissues and organs that cooperate in protecting the body. The immune response is aimed at identifying, attacking and eliminating organisms and substances that invade our body and cause diseases. The immune system is tightly regulated and discriminates between self and non-self molecules as well as between harmful and non-harmful non-self molecules and assures tolerance of non-harmful and elimination of harmful antigens. The cells of the immune system arise from pluripotent hematopoietic stem cells in the bone marrow and are stored throughout the body in several locations, for example the thymus, spleen, lymph nodes and lymphoid tissues associated with various organs like the gut. When in the bloodstream, the immune cells are commonly termed white blood cells or leukocytes. They circulate through the body between organs and lymph nodes through both bloodstream and lymphatic vessels.

1.1.1 The innate immune system

The cells of the innate immune system provide the first line of defence against invading pathogens and protect the host from damaged or malignant cells. The innate immunity is thought to represent an evolutionary ancient defence strategy that has no memory and is not influenced by previous exposure to infective agents. This system provides a physiological barrier (skin and mucosa) and detects and eliminates damaged cells via damage-associated molecular patterns (DAMPs) and pathogens via non-specific pattern recognition receptors (PRR), like Toll-like receptors (TLR), that recognize pathogen-associated molecular patterns (PAMPs) [11, 12]. The innate response is based on the following steps: 1) Recruitment of immune cells to the sites of infection and inflammation by inflammatory mediators such as cytokines and chemokines; 2) Activation of the complement cascade to identify bacteria, activate cells and promote clearance of dead cells and antibody complexes; 3) Identification and removal of foreign substances present in tissues, organs, blood and lymph by specialized phagocytosis; 4) Activation of the adaptive immune system through antigen presentation. The innate immune system involves mast cells, neutrophils, monocytes, macrophages, dendritic cells (DCs), eosinophils, and natural killer (NK) cells.

Mast cells, best known for their orchestrating role in allergy, are key players in the immune surveillance and activation. They are located at the interface between the host and the environment like in the gastrointestinal mucosa, respiratory mucosa and skin but also in most other tissues including the

peritoneal cavity [13, 14]. Their location makes them ideal to detect foreign substances or inflammatory signals, upon which they are stimulated to rapidly release pro- and anti-inflammatory mediators selectively from their preformed granules and to express surface molecules that are important in co-stimulation in both the adaptive and the innate immune response [15, 16].

Neutrophils are short-lived circulating blood cells with an immense inflammatory inducing potential. They are the main phagocytic cell type in inflammation and their accumulation at the inflamed site is essential for the destruction and removal of invading microorganisms. Recent studies indicate that neutrophils are heterogeneous and that there may be subpopulations of neutrophils that can have immunosuppressive properties; including being able to secrete high levels of interleukin (IL)-10 and suppressing T cell responses [17-19]. Human neutrophils can talk with B cells and T cells and can be induced by T cells to express major histocompatibility complex (MHC) class II molecules *in vitro* and consequently to promote the differentiation of antigen-specific type 1 T helper (Th) cells as well as Th17 cells [20]. Recent evidence also suggests that neutrophils can migrate to lymph nodes following antigen capture [21, 22]. Neutrophils are thought to be involved in the induction of resolution. Late in the acute inflammatory response neutrophils switch their eicosanoid biosynthesis from leukotriene (LT)B_4 to lipoxin (LX)A_4, which can inhibit neutrophil recruitment through its interaction with its G protein coupled receptor LXA_4R [23]. Neutrophils are also thought to contribute to the biosynthesis of the n-3 derived pro-resolving mediators resolvins (Rv) and protectins (PD)1, that inhibit the migration of neutrophils into inflamed tissues [23, 24]. Human neutrophils express the chemokine receptors CXCR1 and CXCR2 that bind to the chemoattractant CXCL8 (IL-8). In mice the equivalent receptor-ligand interaction is represented by CXCR2, (but not CXCR1) that binds with high affinity to CXCL1 (KC) and CXCL2 (MIP-2) [25].

Monocytes migrate from the circulation and extravasate through the endothelium and differentiate into macrophages or DCs in response to differentiation factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF or CSF-1). The primary role of monocytes is to replenish the pool of tissue-resident macrophages and DCs in steady state and in response to inflammation.

Macrophages are resident in almost every tissue, including liver, lung, brain and the peritoneal cavity. Macrophages have been classified as either classically (M1) or alternatively (M2) activated, depending on the microenvironment. M1 macrophages are pro-inflammatory and appear in Th1 cytokine environment and upon recognition of PAMPs [26, 27]. M2 macrophages are more heterogeneous

and are thought to play a role in Th2 responses, resolving inflammation and promoting tissue repair and remodelling [28, 29]. This classification of macrophages into M1 and M2 is based on treatment of monocytes *in vitro* and their development into macrophages. Recent studies indicate that this classification does not correlate well with macrophage phenotypes *in vivo* and that macrophage phenotypes *in vivo* may depend on the tissue type, the stimulus, the phase of inflammation as well as the cytokine environment [30, 31]. These studies identified a population of macrophages that possessed characteristics of both M1 and M2 macrophages but were more regulatory and modulatory in nature and identified them as resolution phase macrophages (rM) [30, 31]. The macrophages that initially respond to injury or infection usually exhibit an inflammatory phenotype and secrete pro-inflammatory mediators, such as tumour necrosis factor (TNF)-α, nitric oxide (NO) and IL-1β. They participate in the activation of various antimicrobial mechanisms including oxidative processes that contribute to the killing of invading organisms [32]. At inflamed sites, macrophages evolve from monocytes that are rapidly recruited upon inflammation. They phagocytose apoptotic neutrophils in a nonphlogistic process, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines, such as transforming growth factor (TGF)-β. Subsequently, they need to be cleared for resolution to occur and some emigrate by the lymphatics to the draining lymph nodes while others seem to die locally [33-35].

Eosinophils reside within mucosal tissues interfacing with the environment and within primary and secondary lymphoid tissue but are found in low numbers in blood. Their location at mucosal surfaces is ideal for their role in identifying and responding rapidly to pathogens and within secondary lymphoid tissues they are well poised for bridging innate and adaptive immunity. Eosinophils are best recognized for their cytotoxic effector functions, causing damage to parasitic pathogens in helminth infections and to host tissues in allergic diseases. However, in the last decade eosinophil studies have indicated that they can bridge innate and adaptive immunity, take part in tissue remodelling and have a role in regulating inflammation [36]. Many of these functions are related to the fact that eosinophils store a plethora of cytokines, chemokines and growth factors, available for very rapid stimulus-dependent release. They are also the major source of the immunosuppressive cytokine TGF-β [37] and a recent study identified eosinophils as cells that take part in the resolution of inflammation and as major producers of the pro-resolving n-3 derived mediator PD1 [38].

NK cells are granular innate lymphocytes that are a first line of defence against virally infected cells and cancer [39, 40] and are able to kill aberrant cells without prior sensitization [41]. They are found in bone marrow, spleen and blood but also in other tissues, such as lung, liver and peritoneal cavity [42, 43].

Resident NK cell in these tissues have distinctive phenotypes and potentially different local function. NK cells contribute to and enhance the immune response by triggering activation of other innate immune cells and by inducing pro-inflammatory responses [44]. They play an important role in adaptive immune response through optimal T cell activation via direct interaction with DCs and may induce selective killing of macrophages, DCs and/or T cells [45]. In recent years NK cell subsets that accumulate at sites of inflammation, such as the skin lesions in psoriatic patients and the synovial membrane in rheumatoid arthritis (RA) patients, have been shown to display immunoregulatory function [46, 47]. Recent studies also indicate that NK cells may accelerate neutrophil apoptosis in an NKp46- and Fas-mediated fashion, be involved in resolution of acute inflammation and can serve as targets for pro-resolving mediators in the resolution of adaptive immunity [48, 49].

1.1.2 The adaptive immune system

Activation of the innate immune system drives activation of the adaptive immune system [50]. Adaptive immune responses involve T and B lymphocyte production of cytokines and immunoglobulins (Igs). All stages of the adaptive immune response are specific to the unique antigen, from the time the antigen is recognized by antibody producing B cells (humoral) or T cells (cell mediated), through lymphocyte activation, to effector function (elimination of antigen) and the development of immunologic memory.

B cells secrete antibodies that can neutralize viruses and toxins, activate the complement system or opsonize pathogens for phagocytosis and are therefore important in the protection against extracellular pathogens. Conventional B cells (also called B2 cells) become activated when they recognize their target antigen, that binds to the B cell receptor (BCR), and migrate to the border between the T and B cell zones in the lymph nodes or spleen [51, 52]. There the majority of the cells migrate to the B cell follicles where they form germinal centres (GCs), while some migrate to the medulla of the lymph nodes or red pulp of the spleen and become short-lived plasmablasts. During the GC reaction, B cells undergo proliferation, class switch recombination, somatic hypermutation and affinity maturation and differentiate into either plasma cells (PCs) or memory B cells. In the class switch recombination (or isotype switching), B cells stop making IgM or IgD and switch to IgG, IgA or IgE producing cells. Apart from the B2 cells, B1 cells and marginal zone (MZ) B cells have also been identified [53-55]. The MZ B cells are sometimes grouped with the B2 cells, mainly because of their location within the spleen [56]. However, they are most often grouped with the B1 cells as innate-like B cells with restricted BCR repertoires, in which antibody generation occurs without T cell help and somatic hypermutation, affinity

maturation and class switching is limited [53]. B1 cells reside mainly in the peritoneal and pleural cavities, as well as in the spleen. They are mainly T cell-independent and produce the majority of the IgM antibodies found in the body. These antibodies recognize both self and foreign antigens [56]. Peritoneal B1 cells have been shown to participate in protection of the gut barrier by producing secretory IgA against commensal bacteria [57]. B1 cells can be further divided into B1a and B1b cells. B1a cells spontaneously produce natural IgM antibodies, including most of the serum IgM being produced during homeostasis [58]. B1b cells also contribute to natural IgM antibody production but in addition they can be induced to isotype switching to IgG3 or IgA [59]. The newly discovered regulatory B10 cells, defined by their ability to secrete IL-10, have been found amongst the B1a cells [55].

T cells express the T cell receptor (TCR) and its associated molecule CD3. The majority of the T lymphocytes express the TCR-associated molecules CD4 or CD8. CD4 acts as a co-receptor for TCR binding to MHC class II on antigen presenting cells (APCs). Activated $CD4^+$ cells play a major role in mediating immune responses by secreting specific cytokines and are mainly involved in activating conventional B cells and macrophages [60]. Effector $CD4^+$ cells are further divided into Th1, Th2, Th17, T follicular helper (Tfh) and regulatory T (T_{REG}) cells [61]. Th1 cells mainly secrete cytokines that activate cell mediated immune responses, like interferon (IFN)-γ and are important in activating macrophages [62]. Th2 cells are important in mediating immune response against various bacteria and parasites by secreting cytokines, such as IL-4, IL-5 and IL-13. Both Th1 and Th2 cells secrete cytokines that activate B cells to isotype switching and Ig secretion [63, 64]. The newly defined Tfh cells are located close to the B cell areas in lymphoid tissues where they provide B cell help [65], whereas Th17 cells play an important role in elimination of extracellular pathogens by secreting IL-17 that induces secretion of neutrophil chemoattractants [66]. CD8 acts as a co-receptor for the TCR binding to MHC class I on APCs. $CD8^+$ cells are also known as cytotoxic T lymphocytes (CTL) as they are primarily involved in killing of virus infected cells and tumour cells [67].

1.1.3 Cytokines and chemokines

Cytokines are small (10-25 kDa) signalling proteins that mediate communication between cells [68]. They are a diverse group of proteins with various functions that participate in virtually all physical processes. Cytokines are among others regulators of host responses to infection, inflammation, and trauma. Cytokines are grouped by structure into different families. These families include CSFs, ILs, TNFs, IFNs, TGFs and chemokines (discussed below). Some cytokines induce inflammation and are called pro-inflammatory cytokines, whereas others

serve to reduce inflammation and promote healing and are called anti-inflammatory [69]. The pro-inflammatory cytokines include TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-17, IL-18, IL-21, IL-22, IL-23, IL-33 and IFN-γ and the anti-inflammatory ones include IL-4, IL-10, IL-13, IL-27, IL-35, IL-37 and TGF-β [70]. These cytokines are secreted by various cells of the immune system.

Chemokines are small (8-10 kDa) secreted proteins that are traditionally classified according to the position of the N-terminal cysteine residues into C chemokines (XCL), CC chemokines (CCL), CXC chemokines (CXCL) and CX3C chemokines (CX3CL) [71]. The major role of chemokines is to act as chemoattractants to promote accumulation of leukocytes at the source of chemokine production. Some chemokines are produced constitutively in discrete tissue locations and are involved in controlling the migration of cells during normal processes of tissue maintenance or development (homeostatic chemokines) [72]. In contrast, inflammatory chemokines are produced in high amounts in response to injury or infection in many different tissues and cells types. They are induced by inflammatory cytokines and TLR ligands and recruit cells of the immune system to sites of infection or injury [68, 73-76]. When mediating their effects, chemokines interact with target cells by binding to seven-transmembrane spanning receptors, which are usually coupled to G-proteins. Each chemokine receptor can interact with several chemokines and each chemokine is able to bind to more than one receptor [73]. The receptors can be categorized, as the chemokines, as being constitutive or inflammatory and they have been grouped into a C chemokine receptor (XCR), CC chemokine receptors (CCRs), CXC chemokine receptors (CXCRs) and a CX3C chemokine receptor (CX3CR) [77, 78].

1.1.4 The spleen

The spleen is the body's largest filter of blood, located behind the stomach just beneath the diaphragm. It collects antigens from the blood and is involved in immune responses to blood borne pathogens. Lymphocytes enter and leave the spleen via blood vessels and the spleen also collects and disposes of senescent red blood cells. The spleen is divided into two distinct regions that are, based on their appearance, called the red pulp and the white pulp (Fig. 1). The bulk of the spleen is composed of the red pulp which has an important function in filtering the blood and recycling iron. Scattered around the red pulp are lymphocytes that surround the arterioles running through the spleen, forming isolated areas of white pulp. The white pulp has an organization that closely resembles the lymph nodes and is subdivided into several regions. The periarteriolar lymphoid sheath (PALS), known as the T cell zone, is where the T cells interact with DCs and passing B cells (Fig. 1). Also located in the white pulp are the B cell containing

follicles, where clonal expansion, isotype switching and somatic hypermutation takes place [79]. The marginal zone surrounds the white pulp and separates the white pulp from the red pulp. It is rich in macrophages and non-circulating population of MZ B cells but few T cells. Most of the cells entering the spleen must migrate through the marginal zone and blood borne microbes, soluble antigens and antigen-antibody complexes are filtered from the body by macrophages and immature DCs within the marginal zone. The spleen, operating in a similar way as lymph nodes and mucosa-associated lymphoid tissue, traps antigens and APCs from sites of infection and enables them to present antigen to migratory small lymphocytes, thus inducing adaptive immune responses. It also provides sustaining signals to lymphocytes that do not encounter their specific antigen immediately, so that they survive and continue to recirculate [68].

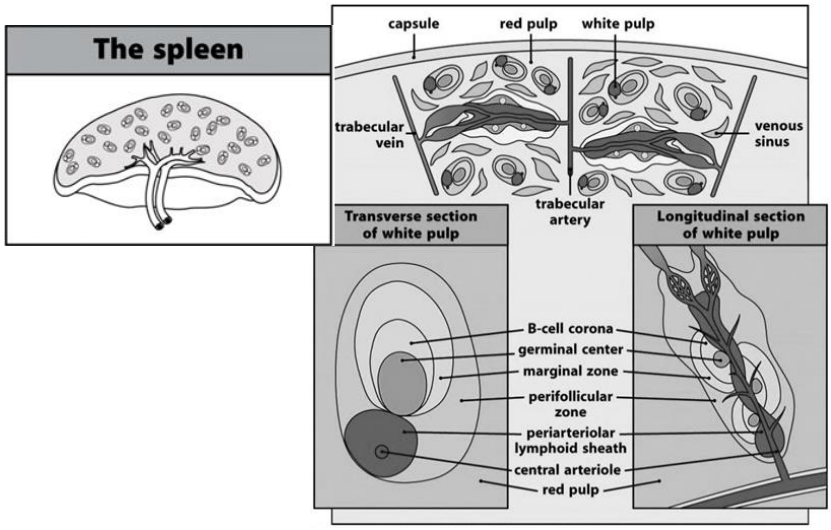

Figure 1. Organization of the lymphoid tissues of the spleen.

Figure obtained from Janeways immunobiology [68], reproduced by permission of Garland Science/Taylor & Francis LLC.

1.2 Inflammation

In general, the inflammatory response initiates within minutes (min) and, in its purest form, will resolve within hours, with many cells participating in the induction and resolution processes (Fig. 2). Inflammation is characterized by the following events: Vasodilation resulting in increased blood flow that causes the redness (rubor) and increased heat (calor), increased permeability of the blood vessels that results in a leakage of plasma proteins and fluid and recruitment of leukocytes (mainly neutrophils) into the infected or injured tissue that causes

swelling (tumour), pain (dolor) and loss of function. The inflammatory response is essential to protect the host and involves interactions of many cell types and production of various chemical mediators. The mediators produced are damaging to pathogens but can also cause damage to the host tissue and therefore the inflammation must be well regulated. Failure in the regulatory processes can lead to chronic inflammation that may last for years and involves simultaneous destruction (on-going inflammatory process) and healing.

1.2.1 Induction of inflammation

Acute inflammation is a short-term response, defined by an initial recruitment of neutrophils to the injured site followed by monocytes, which differentiate locally into macrophages (Fig. 2). The initial response is triggered by tissue resident macrophages and mast cells. Their degranulation and activation leads to the sequential release of pro-inflammatory mediators, such as cytokines, chemokines, and lipid mediators, which drive leukocyte recruitment and activation [80]. The pro-inflammatory cytokines include TNF-α, a potent inducer of local inflammation and endothelial activation; IL-1β, which activates T cells as well as macrophages; IL-6, which induces fever; and G-CSF, the major regulator of neutrophil production that promotes neutrophil release from the bone marrow [81, 82]. The pro-inflammatory chemokines include CXCL1, which is the mouse homologue of human IL-8 and attracts and activates neutrophils; CCL2 (MCP-1), which is a monocyte attractant; and CCL3 (MIP-1α), which attracts neutrophils and macrophages [83]. The pro-inflammatory lipid mediators include prostaglandin (PG)E_2, which is involved in the early increase of blood flow and vasodilation needed for leukocytes to undergo firm adhesion and diapedesis; LTB_4, a potent chemoattractant involved in the recruitment of additional neutrophils and leukocytes to the initial area of insult; and thromboxane (TX)A_2, which is thought to facilitate cytokine synthesis, in addition to its effect on platelet aggregation and vasoconstriction [83, 84]. The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes [83]. At the afflicted tissue site, neutrophils are activated, through the actions of cytokines secreted by tissue-resident cells or by direct contact with the pathogen. Activated neutrophils then release toxic contents of their granules, including reactive oxygen species (ROS), proteinase 3, cathepsin G, elastase and neutrophil extracellular traps (NETs) in their attempt to kill the invading agents [85, 86].

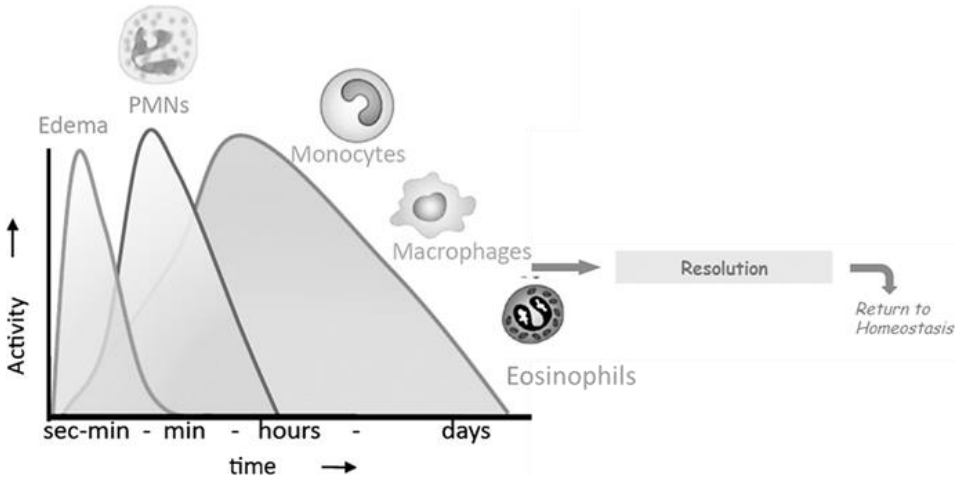

Figure 2. Cellular events in acute inflammation and resolution of inflammation.

Figure adapted from Serhan and Petasis [87].

1.2.2 Resolution of inflammation

Although the inflammatory response is essential to protect the host, its resolution is also pivotal to prevent unnecessary tissue damage as well as preventing the immune response from progressing into a chronic inflammation. Compared to knowledge about the cells, receptors and signalling pathways that initiate and promote the inflammatory response, less is known about the factors that mediate its resolution. In the resolution phase the concentration of pro-inflammatory mediators, such as cytokines, chemokines, and inflammatory lipid mediators, decreases whilst the concentration of anti-inflammatory cytokines, atypical chemokine receptors, and anti-inflammatory lipid mediators increases. On a cellular level, the influx of neutrophils halts, they undergo apoptosis and are rapidly cleared by macrophages [88, 89]. The resolution interval (Ri), the time it takes to reduce the maximal neutrophil numbers by 50% has been used to measure the kinetics of the resolution process [90].

The removal of pro-inflammatory mediators is necessary to halt the influx of inflammatory cells to the area. This may be achieved by decreased production and/or increased clearance of these mediators [91]. Pro-inflammatory cytokines can be neutralized by soluble cytokine receptors, such as sIL-1Ra, sIL-6R and sTNF-αR, which bind and inactivate the respective cytokines. These receptors are thought to be important in resolution of inflammation and for instance an increase in sIL-6R has been shown to be a prerequisite for reduction in neutrophil accumulation during peritoneal inflammation [92].

Chemokines can be neutralized or inactivated by atypical chemokine receptors or decoy receptors, such as Duffy antigen receptor for chemokines (DARC), D6, and CCX-CKR [93]. Decoy receptors lack the conserved DRY motif that is present in the intracellular loop of regular chemokine receptors and is involved in coupling to G-proteins. Receptors lacking the DRY motif bind to chemokines without launching a signalling cascade leading to cell migration [94]. D6 is the major decoy receptor expressed on lymphatic endothelial cells (LECs) and is expressed on macrophages [95] among other cells. D6 can bind to at least 12 chemokines of the CC subfamily that are all inflammatory. Exaggerated inflammatory responses have been observed in mice deficient in D6 [93, 96-100]. In addition to the atypical chemokine receptors, some classical chemokine receptors, such as CCR5 on apoptotic neutrophils, can assist in neutralizing chemokines [101]. Inactivation of the pro-inflammatory lipid mediators PGs and LTs involved in acute inflammation occurs by chemical reactions involving oxidation and reduction reactions [102].

After engulfing pathogens, the neutrophils undergo apoptosis, a process that occurs within hours [103], and need to be removed without provoking an additional inflammatory response [104]. The removal of apoptotic neutrophils via efferocytosis of macrophages depends on macrophages expressing receptors for apoptotic cells, including the vitronectin receptor αVβ3, scavenger receptors, such as CD36, CD68 (oxidized LDL receptor), scavenger receptor class B type I (SR-B1), lectin-like oxidized LDL receptor-1 (LOX-1), phosphatidylserine receptor (PSR), LDL-receptor-related protein (LRP), formylpeptide receptor-like ligand (FPRL)-1, LXA_4R and the receptor tyrosine kinase Mer. These receptors bind apoptotic neutrophils that are taken up by the macrophages and removed from the inflamed site [105, 106].

Anti-inflammatory mediators include the anti-inflammatory cytokines IL-4, IL-10, IL-13 and TGF-β that have been shown to be released in response to phagocytosis of apoptotic cells [107-109]. IL-4 plays a central role in the control and regulation of the immune and inflammatory system, IL-10 is a potent suppressant of macrophage functions, IL-13 inhibits macrophage inflammatory cytokine production, and TGF-β controls the initiation and resolution of inflammatory responses through regulation of chemotaxis, activation, and survival of lymphocytes, NK cells, DCs, macrophages, mast cells, and granulocytes [110]. These cytokines appear at the inflamed site later than the pro-inflammatory cytokines and peak only after the pro-inflammatory cytokines start to decline [111]. The anti-inflammatory n-6 PUFA-derived lipid mediators LXA_4 and LXB_4 and the n-3 PUFA-derived pro-resolution mediators Rvs, PDs and maresins (MaRs) (discussed in detail below) are generated during the resolution of acute inflammation and have been shown to dampen inflammation and mediate resolution of inflammation [112, 113].

1.2.3 Macrophages and eosinophils in the resolution phase

Macrophages have been shown to play an important role in resolution of inflammation. Upon engulfment of apoptotic neutrophils they turn off their production of pro-inflammatory cytokines, chemokines and lipid mediators and start to release IL-10 and TGF-β [114]. The main features of murine resolution phase macrophages were revealed by transcriptomic analyses [31]. These resolution phase macrophages are enriched in molecules important for antigen processing and presentation, express MHC class II, CD74, CD86, PSR, TIM4 and TGF-β and secrete chemokines that attract B and T lymphocytes [31]. Functional characterization has shown the resolving macrophages to have lower expression levels of CD11b and enhanced capacity to engulf apoptotic neutrophils. They also showed reduced responsiveness to TLR4 ligands, indicating that they are satiated. The macrophages then leave the resolving inflammation site and emigrate by the lymphatics to the draining lymph nodes [34], although a new study using thioglycollate medium (TM)-induced peritonitis indicates that part of the macrophages die locally [35]. In peritonitis, macrophages move directly across the encompassing peritoneal mesothelial lining and this movement is regulated by adhesion molecules like vascular cell adhesion protein (VCAM)-1 expressed both on the vascular endothelium and on mesothelial cells [115]. In the resolution phase, macrophages adhere specifically to the mesothelium overlying draining lymphatics and this is partly mediated by macrophage expression of very late antigen (VLA)-4 and VLA-5 [116]. In zymosan-induced peritonitis cells containing zymosan accumulate in draining lymph nodes already four h after induction of peritonitis [24]. The distribution of emigrated inflammatory macrophages within the draining lymph nodes and the fact that zymosan-containing cells can be observed within four h of induction in the draining lymph nodes and within 24 h in the spleen, is suggestive of antigen presentation to T cells and induction of adaptive immune response [24, 34].

A recent study indicates that mouse eosinophils take part in the resolution of zymosan induced peritonitis [38]. Eosinophils are recruited to the site of inflammation during the resolution phase and *in vivo* depletion of eosinophils by anti-IL-5 antibody delayed resolution in the zymosan model, while adoptive transfer of eosinophils rescued the resolution. The eosinophils in the zymosan model that appeared in the resolution phase were identified as the major producers of the pro-resolving n-3 derived lipid mediator PD1 and by applying PD1 intraperitoneally (i.p.) resolution in eosinophil depleted mice could be restored [38].

1.2.4 Participation of the adaptive immune response in resolution

Little is known about the role of lymphocytes in resolution of inflammation. Resident T and B lymphocytes in naive mouse peritoneal cavity have been shown to regulate the severity of the early phase of inflammation by producing anti-inflammatory cytokines and dampening neutrophil influx [117]. In the zymosan model resident lymphocytes disappeared when neutrophils started to accumulate in the peritoneal cavity [117]. However, lymphocytes have been shown to repopulate the site of injury as inflammation decreases [117-120]. In zymosan-induced peritonitis, the majority of peritoneal lymphocytes are B1 cells, with the remaining cells being B2 cells, T cells and NK cells [117]. In this model, the lymphocytes were shown to be dispensable for resolution to occur, as inflammation in wild type and lymphocyte-deficient $RAG1^{-/-}$ mice resolved equally [117]. However, the same study showed that repopulation of the peritoneal cavity with lymphocytes was critical for modulating responses to secondary infection. In mBSA-induced peritonitis (discussed in detail below), B1 cells were also prominent, however, the number of peritoneal lymphocytes was not reduced during the inflammatory phase like in the zymosan-induced peritonitis [121]. Resolution-phase macrophages are rich in molecules important for antigen processing and presentation and secrete T cell and B cell chemoattractants (XCL1, CCL5 and CXCL13) [31]. The lymphocytes that repopulate sites of resolving inflammation comprise T cells, B1 cells, B2 cells and NK cells, which might exert a protective effect in the post-resolution phase [117].

1.3 Murine models of peritoneal inflammation

Animal models have long been used for the research of human diseases, allowing investigations that would be impossible in humans. The mouse peritoneal cavity is an ideal site in which to study the development of an inflammatory reaction, because it is a sterile compartment containing high number of cells, which can easily be isolated and monitored. Injection of an irritant into the peritoneal cavity induces inflammation with pain, leukocyte infiltration and synthesis of inflammatory mediators. Both TM and zymosan have been widely used in inflammation research. I.p. injection of TM induces a sterile inflammatory peritonitis and a subsequent leukocyte migration and influx into the peritoneal cavity [122]. This model has especially been used for elicitation of macrophages from mice and rats [123-125]. Zymosan is a yeast cell wall particle containing mainly polysaccharides, of which β-glucan and mannan are the major constituents [126]. I.p. injection of zymosan induces all the hallmarks of acute

inflammation, with leukocyte infiltration and synthesis of inflammatory mediators [127]. Zymosan-induced peritonitis has been widely used as a self-resolving model of inflammation in mice and most of the current knowledge about the resolution of peritoneal inflammation has come from studies using this model [128].

I.p injection of zymosan results in a rapid and robust inflammatory response that peaks within 12 h and resolves within 48-72 h [129]. In the TM- and zymosan-induced inflammatory models, an acute inflammatory response is mounted against a foreign intruder and the host response in these inflammatory models may not reflect the inflammatory response seen in many diseases, in which chronic inflammation may be an underlying condition. Chronic inflammation may, however, be induced by inflammation induced by antigens and experimental models of antigen-induced immune inflammation may reproduce some of the features of chronic inflammation [130-132].

Andrew D. Cook and colleagues designed a peritonitis model [121] based on the antigen-induced arthritis model, where methylated bovine serum albumin (mBSA) is used as an antigen [133]. In this peritonitis model, mice were sensitized with mBSA in complete Freund's adjuvant (CFA), boosted 14 days later with the same and 7 d later i.p. injection of mBSA resulted in an inflammatory response. The mBSA-induced inflammatory response was comparable to that induced in the TM model in terms of the number of inflammatory cells in the exudates. However, the macrophages in the mBSA-induced inflammation were smaller and less granular, expressed more MHC class II and were able to stimulate allogeneic T lymphocytes. Therefore, an inflammatory response elicited by mBSA may be more relevant for studying the inflammatory responses in many diseases, such as those of autoimmune origin and those involving an adaptive immune response.

1.4 PUFA

Lipids are important in our diet, both as a source of energy and to provide essential fatty acids, i.e. fatty acids that cannot be synthesized *de novo* in animal cells and must, therefore, be obtained from the diet. Fatty acids are hydrocarbon chains, varying in length, with a methyl group on one end (the omega end) and a reactive carboxyl group on the other end (the alpha end). PUFA have two or more double bonds and they are divided into two major families based on the position of the first double bond from the methyl end. In n-3 PUFA (also called omega-3 PUFA) the first double bond is on the third carbon from the methyl end, whereas it is on the sixth carbon from the methyl end in n-6 PUFA (Fig. 3). There are two essential fatty acids, the n-6 fatty acid linoleic acid (LA, 18:2 n-6)

and the n-3 fatty acid α-linolenic acid (ALA, 18:3 n-3). The desaturase enzyme necessary to insert double bonds between the ninth carbon and the methyl end is missing in mammalian cells and, therefore, they cannot synthesize LA or ALA [134].

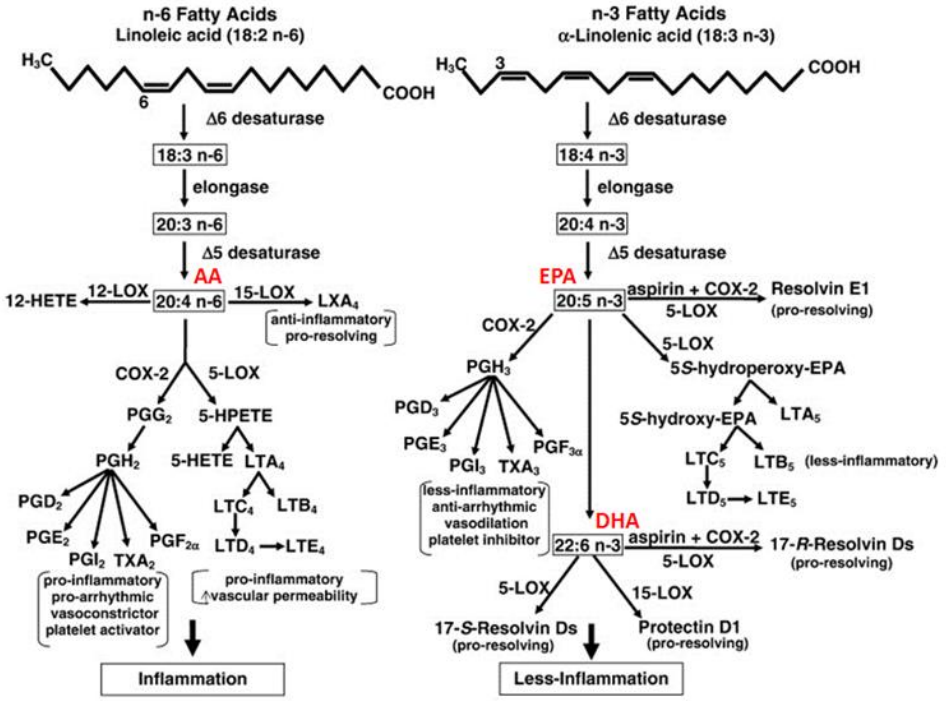

Figure 3. The metabolism of n-3 and n-6 PUFA and the subsequent biosynthesis of eicosanoids and proresolving mediators.

Figure adapted from from Adkins and Kelley [135].

LA is found in high concentrations in grains, many seeds, meat and most vegetable oils, e.g. corn oil and safflower oil. ALA is found in high concentrations in plants, seeds, green vegetable, legumes, nuts and some vegetable oils like rapeseed, flaxseed and soybean oils. In animal cells LA and ALA can be further elongated and desaturated, forming longer and more unsaturated fatty acids of the n-6 and n-3 families. This metabolism of the n-6 and the n-3 PUFA is competitive, since both pathways use the same set of enzymes for the elongation and desaturation. Arachidonic acid (AA, 20:4, n-6) is a product of the n-6 pathway and eicosapentaenoic acid (EPA, 20:5, n-3) and subsequently

docosahexaenoic acid (DHA, 20:6 n-3) are products of the n-3 pathway (Fig. 3) [136]. The conversion of ALA to EPA and DHA is limited [137]; therefore, these fatty acids may have to be obtained directly from the diet [138]. The major sources of EPA and DHA are oily fish, like salmon, mackerel and herring or fish oils.

Fatty acids have various roles in immune cells; they are a source of energy, components of membrane phospholipids that affect membrane viscosity and lipid raft structure, substrates for synthesis of lipid mediators, act as ligands for nuclear receptors and influence intracellular signalling and gene expression [139]. After consumption, PUFA are absorbed in the intestines and transported, in lipoproteins, to virtually all cell types where they are incorporated into cell membranes and have various effects on cell function [140]. Increased consumption of EPA and DHA leads to increased availability and increased incorporation of these long chain PUFA into cell membranes [141]. This increase in n-3 PUFA is usually at the expense of n-6 PUFA, especially AA. Time-course studies indicate that incorporation of EPA and DHA into human immune cells reaches a plateau within four weeks [142]. Increased amount of n-3 PUFA in the cell membrane is expected to influence the function of immune cells, e.g. by modulating membrane integrity, influencing intracellular signalling and gene expression and affecting biosynthesis of lipid mediators, like eicosanoids, and lipid raft structure [139]. Lipid rafts are subdomains of the plasma membrane that contain high concentration of cholesterol, glycosphingolipids and saturated phospholipids and are resistant to detergents. Though they appear small in size they are thought to constitute a large part of the plasma membrane [143]. Lipid rafts are thought to serve as “signalling platforms” for signal transduction into the cells and transmembrane proteins are found in lipid rafts, where two acyl moieties seem to target proteins to lipid rafts [144].

1.4.1 Eicosanoids

Eicosanoids are signalling molecules derived from the 20 carbon PUFA, dihomo-γ-linolenic acid (DGLA, 20:3 n-6), AA and EPA. They elicit their biochemical effects through G-protein-linked receptors. They are important mediators in both inflammation and homeostasis in the body and consist of the LTs and prostanoids. Prostanoids are further divided into PGs, prostacyclins (PGIs) and TXs. The enzymes that catalyze the oxidation of PUFA into eicosanoids are the cyclooxygenases (COX) that generate the prostanoids and the lipoxygenases (LOX) that generate the LTs (Fig.3). AA is typically the major precursor for the eicosanoids, since it is in relatively high amount in the immune cell membranes phospholipids. Biosynthesis of most eicosanoids starts when AA is released from the cell membranes through activation of phospholipase A_2 (PLA_2) which is

activated by inflammatory stimuli. The availability of DGLA, AA and EPA influences what family of eicosanoids is formed, since they compete as substrates for the same metabolic enzymes [145]. DGLA is the precursor for prostanoids of the 1-series and LTs of the 3-series. AA is the precursor for prostanoids of the 2-series and LTs of the 4-series and EPA is the precursor for prostanoids of the 3-series and LTs of the 5-series [139]. The AA-derived 2-series prostanoids and 4-series LTs are considered more inflammatory than the 1-series and 3-series prostanoids and the 3-series and 5-series LTs [146]. EPA and DGLA partially inhibit AA metabolism by the COX and LOX enzymes [139]. Incorporation of increased amounts of EPA and DHA into cell membranes results in decreased amounts of AA-derived eicosanoids. Increased proportion of EPA and DHA in cell membranes is believed to lead to less inflammatory immune response or even anti-inflammatory response [142].

At present there are three COX isoenzymes known: COX-1, COX-2 and COX-1b (COX-3). COX-1 is produced constitutively and COX-2 is induced by stimuli like cytokines and mitogens, whereas COX-3 is not believed to exert cyclooxygenase activity and does therefore most likely not have a role in inflammation and fever in humans [147-149]. After PLA_2 releases AA from the cell membrane, COX metabolizes the free fatty acids to the unstable intermediate PGH_2, the precursor of the 2-series prostanoids. In the synthesis of prostanoids this is the rate limiting step. PGH_2 is further converted into the bioactive prostanoids, PGF_2, PGI_2, PGE_2, PGD_2 and TXA_2 by cell specific prostanoid synthases [147] (Fig. 3).

In humans there are three main LOX enzymes, 5-, 12- and 15-LOX. Expression of LOX in mice is different as they do not express 15-LOX but express among others 5-, 12- and 12S-LOX [150, 151]. The free AA, released from the cell membrane by the action of PLA_2 and sequestered at the nuclear membrane, is brought in contact with 5-LOX by its helper protein the nuclear envelope 5-LOX activating protein (FLAP) [152]. 5-LOX catalyzes AA to 5-hydroperoxyeicosatetraenoic acid (HPETE) and subsequently to LTA_4 that is converted to LTB_4 or LTC_4. LTC_4 can then be further converted to the biologically active metabolites LTD_4 and LTE_4 [153, 154] (Fig. 3). The 12- and 15-LOX convert AA to 12- and 15-HPETE, respectively. The LOX enzymes catalyze the first steps in the synthesis of the anti-inflammatory or pro-resolving lipid mediators LXs, Rvs, PDs and MaRs [155, 156] (Fig. 3).

1.4.2 Pro-resolving lipid mediators

Resolution that was in the past thought to be a passive process has in recent years been shown to be an active process coordinated by a group of pro-

resolving lipid mediators. In the resolution phase ,the eicosanoids undergo a class switch, where the production of LTs and PGs ceases and production of pro-resolving lipid mediators is initiated (Fig. 4) [157]. These novel oxygenated products generated from PUFA exert immunoregulatory actions at the nano- and picomolar range [23]. As stated above, LXs are derived from the n-6 PUFA AA, while Rvs, PDs and MaRs are derived from the n-3 PUFA EPA and DHA (Fig. 4).

LXs are the only pro-resolving lipid mediators that are synthesized from AA (Fig. 4). They were identified in purified fractions of leukocyte suspension by Serhan and colleagues 30 years ago [158]. Though the main function of LXs is not fully understood, it seems to be to counter-regulate inflammation by having inhibitory actions on neutrophils, eosinophils and NK cells while stimulating monocytes and macrophages. The LXs have been shown to down-regulate neutrophil recruitment, adhesion and transmigration, promote non-inflammatory recruitment of monocytes and enhance macrophage engulfment of apoptotic neutrophils [159].

Rvs (resolution phase interaction products) were originally identified in spontaneous self-limited murine inflammatory exudates *in vivo* [160, 161]. The E series Rvs (RvE) are derived from EPA and the D series Rvs (RvD) from DHA [162] (Fig. 4). RvE1 reduces neutrophil infiltration in mice *in vivo* and blocks transmigration of human neutrophils *in vitro* [160]. It also reduces production of pro-inflammatory cytokines and chemokines and stimulates clearance of microbes *in vivo* [24, 163]. RvD1 reduces neutrophil infiltration in murine peritonitis, blocks transendothelial migration of human neutrophils and enhances phagocytosis by human macrophages *in vitro* [161, 164, 165]. RvE1 can bind to and activate the LTB_4 receptor, BLT1 on neutrophils and ChemR23 that is expressed in macrophages and neutrophils [163, 166, 167]. RvE1 and RvD1 have, in addition, been shown to help reduce arthritis pain [168, 169].

PDs are only synthesized from DHA (Fig. 4) and they have a conjugated triene double bond in their structure. These compounds were named protectins because of their potent tissue protective actions in brain, immune and retinal cells. In neural tissue, PD1 is termed neuroprotectin (NPD1) to reflect its site of biosynthesis [170]. PD1 along with the D series Rvs was identified in murine exudates, murine brain cells, human microglial cells and human blood [161, 171]. PDs reduce infiltration of neutrophils, stimulate macrophage uptake of apoptotic neutrophils, promote wound healing and they also demonstrate neuroprotective actions *in vivo* [172-174].

MaRs are the most recently discovered pro-resolving lipid mediators. They are produced by human and mouse macrophages and are only synthesized

from DHA [156] (Fig. 4). Synthesis of MaR1 is catalyzed by 12/15-LOX and this biosynthetic pathway is activated in mouse and human macrophages during phagocytosis [156]. MaRs are potent in reducing neutrophil infiltration in peritonitis as well as in enhancing human macrophage uptake of apoptotic neutrophils [156].

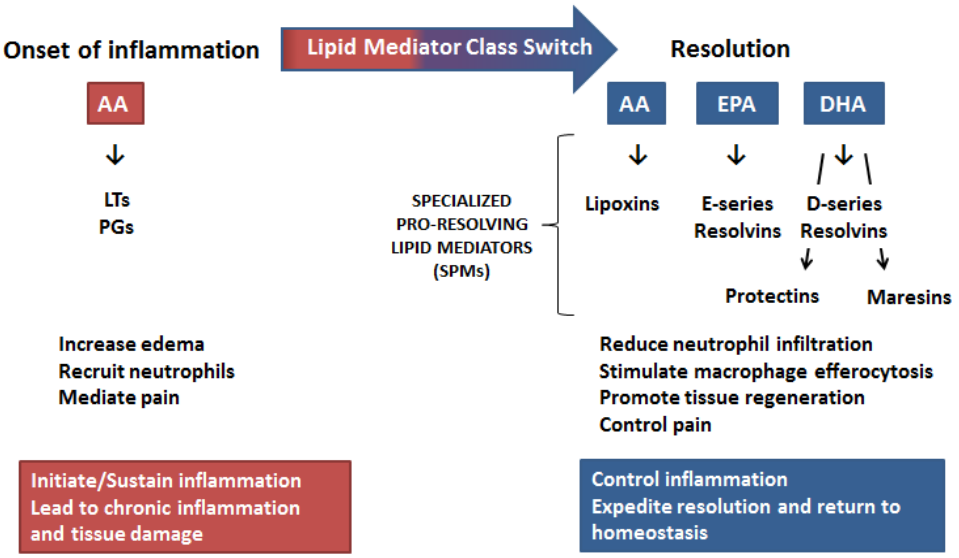

Figure 4. Lipid mediators and their role in acute inflammation and resolution of inflammation.

Figure regenerated from Recchiuti and Serhan [157].

1.5 The effects of n-3 PUFA on the immune function

That n-3 PUFA could have beneficial effects on health was first brought to light when two Danish researchers investigated, in the 1970s, why Inuits, despite consuming a diet high in fat, had low death rates from cardiovascular diseases. They discovered that the Inuits had favourable blood lipids and found that the fatty acids EPA, DPA and DHA were in higher concentration in the Inuits than in Danes [141, 175]. From that time much research has been carried out to investigate whether n-3 PUFA can have beneficial effects on other diseases, including chronic inflammatory diseases. Several studies have suggested that increased consumption of n-3 PUFA may beneficially influence numerous diseases, including cardiovascular [176, 177], neoplastic [178] and Alzheimer's disease [179, 180], all diseases that have chronic inflammation involved in their progression.

Although the n-3 PUFA are generally considered to have beneficial effects on inflammation, studies on the effects of n-3 PUFA on the immune system have provided inconsistent outcomes. It has been suggested that the contrasting results may be because of different experimental setup, like for example, usage of different species or different strains within species, usage of different cells from different tissues, studies being *in vivo*, *ex vivo* or *in vitro* and the time and amount of n-3 PUFA used in the studies. In the following sections the effects of n-3 PUFA on different aspects of the immune system and response will be discussed in more detail.

1.5.1 The effects of n-3 PUFA on host response and inflammatory conditions

In studies on host response, dietary n-3 PUFA have both been shown to improve and impair host resistance to a number of pathogens [181]. For example, fish oil enriched diet improved survival of mice following infection with *Klebsiella pneumoniae* [182] but reduced survival of mice following *Listeria monocytogenes* infection [183]. In the latter study the fish oil diet also led to reduced bacterial clearance and diminished IFN-γ signalling in macrophages [183]. In addition, mice fed a fish oil diet had impaired resistance to influenza infection, with higher lung viral load 7 d post infection and reduced survival when compared with mice fed a control diet [184].

The effects of n-3 PUFA on chronic diseases like RA and inflammatory bowel disease (IBD) have been extensively studied and while n-3 PUFA have been shown to have beneficial effects on RA, the beneficial effects on IBD are not as clear [185]. Results from a systematic review that included 23 studies supported the previously held view that dietary marine n-3 PUFA were effective in reducing symptoms in RA as well as the need for anti-rheumatic medication [186]. Studies of the effect of n-3 PUFA in murine RA models suggest that these fatty acids may have a role in both decreasing the risk of developing RA and help in decreasing the severity of the disease [186]. For example, mice fed a fish oil diet showed delayed onset and reduced incidence and severity of collagen-induced arthritis when compared with mice fed a diet containing vegetable oil, an effect that was linked to decreased production of PGs of the 2-series [187]. Furthermore, a recent study showed that mice fed diets containing krill oil or fish oil had significantly reduced arthritis scores and hind paw swelling when compared with mice fed a control diet [188]. Results from murine studies examining the effects of dietary n-3 PUFA on IBD are inconsistent with results seen in IBD patients. Rats with a chemically induced ulcerative colitis (UC), which is one of the two main subtypes of IBD, had reduced colonic damage and inflammation when fed a diet supplemented with cod liver oil compared with that

in rats fed a diet rich in sunflower oil [189]. In the same model, fish oil supplementation resulted in less colonic inflammation and decreased LTB_4 in the colon tissue compared with that in rats fed a diet enriched in cotton and sunflower oil [190]. In addition, systematic treatment with the n-3 PUFA-derived RvD1 in two mouse models of colitis reduced colonic damage and decreased neutrophil infiltration [191]. Although n-3 PUFA have appeared to have beneficial effects on IBD, meta-analyses have failed to confirm this [192]. In a meta-analysis that reviewed the efficacy and safety of n-3 PUFA in IBD, incidence of diarrhoea and symptoms from the gastric intestinal tract were higher in the n-3 PUFA group than in control groups [193]. Therefore, although n-3 PUFA seem to have beneficial effects in animal models of IBD, in particular UC, the same has not been shown to be the case for IBD patients.

Although n-3 PUFA seem to have beneficial effects on inflammatory diseases, it is currently being speculated whether overconsumption may lead to too much immune suppression. The dampening effect that n-3 PUFA intake has on immune function has been suggested to make the host more susceptible to infections and to reduce survival, whereas the dampening effect may be beneficial in chronic inflammatory conditions and autoimmune diseases [194].

1.5.2 The mechanism by which n-3 PUFA affect inflammation

The mechanisms behind the anti-inflammatory effects of n-3 PUFA have been widely studied. Increased consumption of EPA and DHA results in increased proportion of these fatty acids in inflammatory cell membrane phospholipids, partly at the expense of AA. This leads to less substrate being available for production of the more potent inflammatory eicosanoids derived from AA. Several studies have shown that supplementing human or animal diets with fish oil decreases *ex vivo* inflammatory cell production of PGE_2, TXB_2, LTB_4, 5-HETE and LTE_4 [195] and higher levels of EPA in immune cell phospholipids result in increased production of PGE_3 and LTB_5 that are less inflammatory than the AA-derived mediators [185]. In addition, EPA and DHA in the immune cell phospholipids give rise to production of the pro-resolving lipid mediators Rvs, PDs and MaRs [185] that were discussed in chapter 1.4.2.

Incorporation of n-3 PUFA into cell membranes has also been shown to influence lipid rafts, altering their size, as well as its composition and organization, leading to altered cell signalling and function [196]. Studies with $CD4^+$ T cells, macrophages and splenocytes indicate that disruption of rafts with n-3 PUFA generally suppresses cellular function [196-199], although a recent *ex vivo* study showed that disruption of lipid rafts by n-3 PUFA enhanced the innate B cell response [200].

A number of *in vitro* studies have shown that n-3 PUFA influence activation of nuclear factor kappa B (NF-κB), the main transcription factor involved in up-regulation of genes encoding inflammatory cytokines, adhesion molecules and COX-2. Both EPA and DHA decreased endotoxin-induced activation of NF-κB in human monocytes [201-203], cultured macrophages [204] and DCs [205] and may thereby decrease inflammatory gene expression. In addition, the nuclear receptor peroxisome proliferator activated receptor (PPAR)-γ, which is thought to be an anti-inflammatory transcription factor [206], has been suggested to be activated by n-3 PUFA [207, 208]. Several of the membrane bound G-protein coupled receptors, like GRP120 that is expressed at high levels in macrophages, can bind long chain unsaturated fatty acids, including EPA and DHA [209, 210]. DHA inhibited TNF-α- and lipopolysaccharide (LPS)- induced cytokine production in the RAW 264.7 macrophage cell line, an effect that was not seen in GPR120 knockdown cells, suggesting a role for GPR120 in mediating the anti-inflammatory effects of n-3 PUFA [211]. Both PPAR-γ and GPR120 seem to inhibit pro-inflammatory gene transcription by inhibiting the activation of NF-κB [185]. N-3 PUFA have also been suggested to influence inflammasomes [212], cytosolic protein complexes that are central regulators of the innate immunity and inflammation and are assembled in response to infection or injury [213]. Inflammasomes aid the maturation and release of some pro-inflammatory cytokines and are involved in several inflammatory disorders [213]. A recent study indicates that n-3 fatty acids may inhibit NLRP3 inflammasome activation via GPR40 and GPR120 and can thereby decrease inflammation and prevent inflammation-driven diseases [212].

1.5.3 The effects of n-3 PUFA on the inductive phase of inflammation

Most studies examining the effects of n-3 PUFA on inflammation have focused on the inductive phase of inflammation that is characterised by increased secretion of pro-inflammatory mediators and infiltration of neutrophils to the inflamed site. Murine studies have shown that dietary fish oil or n-3 PUFA decrease circulating levels of TNF-α, IL-1β and IL-6 following injection with endotoxin [214] and decrease *ex vivo* production of TNF-α, IL-1β and IL-6 by endotoxin stimulated macrophages [215-217]. However, other studies have shown increased production of TNF-α by murine resident peritoneal macrophages after *ex vivo* stimulation with LPS [218, 219]. Fish oil supplementation has also been shown to decrease chemotaxis of neutrophils and monocytes towards chemoattractants like LTB_4, bacterial peptides and human serum. The mechanism behind the reduced chemotaxis is not clear but may relate to reduced expression of chemoattractant receptors [185].

Adhesion molecules help leukocytes to interact with the blood vessel wall and to leave the blood stream to enter the site of inflammation. Fish oil supplementation in animal feeding studies and studies with cultured cells exposed to n-3 PUFA have reported decreased expression of some adhesion molecules on the surface of monocytes [220], macrophages [221], lymphocytes [222] and endothelial cells [223] and some of the studies showed decreased adhesion between leukocytes and endothelial cells [185]. When studied under flow conditions, both EPA and DHA reduced the interaction between monocytes and endothelial monolayers [185]. A recent study also showed that n-3 PUFA reduced the number of neutrophils adhering to and transmigrating through the endothelium by incubating human umbilical vein endothelial cells (HUVEC) with EPA. This was shown to be dependent on PGD_2-mediated secondary signalling, which is necessary for full activation and transmigration of neutrophils. EPA is a precursor for PGD_3 that competes with AA-derived PGD_2 for binding to the PGD_2 receptor (PD1) and by inhibiting the PGD_2-mediated secondary signalling EPA inhibits the transmigration of neutrophils through the endothelium [224]. In addition, dietary fish oil reduced the number of peritoneal neutrophils in mice 12 h after i.p. injection with LPS but increased their numbers at 48 h indicating a dampening of the inflammation early in the inflammatory reaction but an increased effect later in the inflammation [225].

Overall, n-3 PUFA seem to have anti-inflammatory effects in the induction phase of inflammation seen in the reduction of pro-inflammatory mediators and decreased expression of some adhesion molecules.

1.5.4 The effects of n-3 PUFA on the resolution phase of inflammation

As of today, there are no reports of research focusing on how dietary n-3 PUFA affect the resolution phase of inflammation. However, the n-3 PUFA-derived pro-resolving mediators Rvs and PDs have been studied extensively *in vitro* and *in vivo* and they have been shown to enhance resolution of inflammation in a number of ways. Rvs were detected in healthy humans after 3 weeks supplementation of DHA (1 g/d) and EPA (1.4 g/d) in concentrations that have been shown to have anti-inflammatory and pro-resolving properties *in vivo* and *in vitro* [226]. As well as being able to inhibit infiltration of neutrophils into sites of inflammation, RvD1 has been shown to inhibit IL-1β production and PD1 to inhibit TNF-α and IL-1β production [23, 160, 161, 227]. Furthermore, administration of RvE1 and PD1 in zymosan-induced peritonitis in mice has been shown to reduce the number of peritoneal neutrophils and shorten the resolution interval [228].

In a colitis model, increased levels of n-3 PUFA in fat-1 mice (transgenic mice that can produce n-3 from n-6 leading to abundant n-3 PUFA) led to increased Rv production and reduced inflammation and tissue injury [229]. Rvs have also been shown to reduce inflammation in animal models of inflammatory diseases, including arthritis, colitis and asthma [169, 230-232].

1.5.5 The effects of n-3 PUFA on the adaptive response

How n-3 PUFA affect the adaptive immune response is largely unknown, although their effects on both B and T cell responses have been studied in some detail, especially their effects on T cells *in vitro*. Both EPA and DHA inhibit T cell production of IL-2 *in vitro* and reduce T cell proliferation *in vivo* as well as *in vitro* [185].

When the effects of n-3 PUFA on B cell responses have been studied, n-3 PUFA have been demonstrated to reduce proliferation, inflammatory gene expression and secretion of IL-10, TNF-α and IFN-γ by B cells and B cell lines *in vitro* [233-235]. However, *ex vivo and in vivo* studies indicate that n-3 PUFA enhance B cell activation and secretion of pro-inflammatory cytokines [200, 236, 237]. The *ex vivo* studies demonstrated that dietary n-3 PUFA enhanced activation of LPS-stimulated B cells, which secreted more IL-6 and IFN-γ and expressed more CD69 than B cells from mice fed the control diet, but suppressed B cell stimulation of naive $CD4^+$ T cells [200, 236]. The *in vivo* study showed that mice fed dietary fish oil had enhanced B cell activation upon antigen stimulation, with an increase in surface and/or circulating IgM antibody production when compared with mice fed a control diet [237]. In addition, *in vitro* treatment of human B cells with the pro-resolving lipid mediators 17-hydroxydosahexaenoic acid (17-HDHA), RvD1 and PD1 enhanced both IgM and IgG production [238], indicating enhancement of both the innate and adaptive B cell responses.

Overall, the studies discussed above indicate that dietary fish oil may influence the adaptive immune response by suppressing the T cell response but in contrast enhancing the B cell response.

2 Aims

Chronic low-grade inflammation has emerged as a central component of many prevalent diseases in Western societies (i.e. arthritis, periodontal disease, cardiovascular diseases, cancer, and Alzheimer's disease). To date, research on the inflammatory process has primarily focused on the inductive phase of inflammation. However, evidence now suggests that the resolution phase of inflammation is an active endogenous process that may be of major importance in recovery and that inadequate resolution may contribute to the unremitting inflammation that characterizes many chronic diseases. N-3 PUFA have been shown to influence the inductive phase but their effects on the resolution phase of inflammation have not been studied. As the n-3 PUFA, EPA and DHA, are substrates for the recently discovered pro-resolving lipid mediators they may, in addition to affecting the inductive phase of inflammation, also influence the resolution phase. Furthermore, little is known about the effects of n-3 PUFA on the adaptive response in antigen-induced inflammation.

The primary goal of this project was to determine how dietary fish oil affects induction, resolution and the adaptive immune responses in antigen-induced inflammation.

Specific aims:

1. To determine the cellular and molecular events in the induction, resolution and adaptive phases of antigen-induced peritonitis.
2. To determine the effects of dietary fish oil on the inductive phase and regression of the inductive phase in antigen-induced peritonitis.
3. To determine the effects of dietary fish oil on the resolution phase of inflammation in antigen-induced peritonitis.
4. To determine the effects of dietary fish oil on the adaptive immune response in antigen-induced peritonitis.

3 Materials and methods

3.1 Animals and diets

All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland (license number: 0507-1503) and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Female C57BL/6 mice weighing 18-20 g were obtained from Taconic Europe (Ejby, Denmark). They were housed 8-12 per cage with a 12 h light/dark cycle at 23-25°C and 45-55% humidity. Mice were acclimatized for one week prior to the initiation of the experiment. They were randomly assigned to receive either a control (C) diet (D07121302; Research Diets Inc., New Brunswick, NJ) or fish oil (FO) diet (D07121303; Research Diets Inc.) for 4-6 weeks. The composition of the diets was based on a typical Western diet, i.e. the “US17” diet formulated by Monsanto (St. Louis, MO) and Research Diets Inc. with minor modification (see Table 1).

The fish oil diet was made by adding 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA) to the basal diet at the expense of safflower oil (Welch, Holme & Clark CO Inc., Newark, NJ). AA ethyl ester (Nu-Check-Prep, Elysian, MN) (0.5 g/kg) was added to the C diet to adjust for the AA content in the FO diet. Energy distribution of the diets was as follows: carbohydrate, 44%; fat, 35%; and protein, 21%. Relative amounts of selected FAs in the diets are shown in Table 2. The diets were divided into daily portions and stored at -70°C to prevent oxidation.

The mice were given fresh food daily and had free access to food and water. The weight of the mice was monitored throughout the experiment and did not differ between the two dietary groups.

Table 1. Composition of the control and fish oil diets

	Control diet	***Fish oil diet***
Ingredients	***g/kg***	
Casein	229	229
L-Cystine	3	3
Cornstarch	274	274
Maltodextrin 10	86	86
Sucrose	114	114
Cellulose	57	57
Cocoa Butter, Deodorized	43	43
Linseed Oil, RBD[a]	5	5
Palm Oil, Bleached, Deodorized	60	60
Safflower Oil, USP[b]	32.5	4.5
Sunflower Oil, Trisun Extra	31	31
Menhaden Fish Oil	0	28
20:4 n-6 ethyl ester	0.5	0
Mineral Mix S10026[c]	11	11
Di Calcium Phosphate	15	15
Calcium Carbonate	6	6
Potassium Citrate, 1 H_2O	19	19
Vitamin Mix V13401[d]	11	11
Choline Bitartrate	2	2
alpha Vitamin E Acetate[e]	0.15	0.15
t-BHQ	0.03	0.03

Information as provided by the manufacturer

[a] Refined, Bleached, Deodorize
[b] United States Pharmacopeia
[c] Containing the following (g/kg mineral mix): sodium chloride, 259; magnesium oxid, 41.9; magnesium sulfate $7H_2O$, 257.6, chromium KSO_4 $12H_2O$, 1.925; cubric carbonate, 1.05; sodium fluoride, 0.2; potassium iodate, 0.035; ferric citrate, 21; manganous carbonate, 12.25; ammonium molybdate $4H_2O$, 0.3; sodum selenite, 0.035; zink carbonate, 5.6; sucrose 399.105.
[d] Containing the following (g/kg vitamin mix). Retinyl pamitate, 0.8; cholecalciferol, 1.0; menadione sodium bisulfate, 0.08; biotin (1.0%), 2.0; cyancocobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin-HCl, 0.6; sucrose, 988.42
[e] 500IU/g
Changes in the commercial diet are grey shaded.

Table 2. Fatty acid composition of the control and fish oil diets

	Control diet	***Fish oil diet***
Fatty acid	***g/kg***	
10:0	ND[b]	ND
12:0	0.2	0.2
14:0	0.5	2.4
15:0	ND	0.1
16:0	36.8	39.2
16:1	0.1	2.9
18:0	19.5	19.5
18:1	70.1	69.5
18:2 (n-6)	36.2	14.9
18:3 (n-3)	3.4	3.8
20:0	0.7	0.8
20:1	ND	0.4
20:2	ND	0.1
20:3	ND	0.1
20:4 (n-6)	0.6	0.6
20:5 (n-3)	ND	4.0
22:1	ND	0.1
22:4	ND	0.1
22:6 (n-3)	ND	2.5
24:0	ND	0.1
Total saturated FA	57.7	62.5
Total MUFA[c]	70.2	72.9
Total PUFA[d]	40.2	26.5
Total (n-6)	36.8	15.6
Total (n-3)	3.4	10.6
P:S ratio[e]	0.7	0.4
(n-6):(n-3) ratio	10.7	1.5
(n-3):(n-6) ratio	0.09	0.68

Information as provided by the manufacturer[a]

[a] Values are expressed as g per 1 kg diet
[b] ND, not detected
[c] MUFA: monosaturated fatty acid
[d] PUFA: polyunsaturated fatty acid
[e] P:S ratio: polyunsaturated vs. saturated fatty acids ratio

3.2 Induction of peritonitis

Mice were immunized subcutaneously (s.c.) at the base of the tail with 100 µg of mBSA (Sigma Aldrich, St. Louis, MO) emulsified in an equal volume of complete Freund's adjuvant (Sigma Aldrich) in total volume of 50 µl. Two weeks later mice were given a booster injection of 100 µg of mBSA in incomplete Freund's adjuvant (IFA) (Sigma Aldrich). Three weeks after the initial injection mice were challenged i.p. with 100 µg of mBSA in saline (total volume 50 µl) and peritonitis induced. Before and at several time points after peritonitis induction (3 h, 6 h, 12 h, 24 h, 48 h, 5 d and 10 d) mice were anesthetized with a mixture of hypnorm (VetaPharma Ltd, Leeds, UK), dormicum (Roche, Basel, Switzerland) and sterile water (1:1:2). Blood was collected via axillary bleeding under anesthesia. After blood collection mice were killed by cervical dislocation.

3.2.1 Serum preparation

Blood was collected into Eppendorf tubes, allowed to clot for 30 min at room temperature and then centrifuged at 3000 rpm for 10 min and the serum collected. Serum was stored at -70°C until used for analysis.

3.2.2 Collection of peritoneal lavage

Peritoneal exudate (fluid and cells) was obtained by injecting 1.5 ml of cold phosphate buffered saline (PBS) without calcium or magnesium into the peritoneal cavity, massaging gently and then collecting the fluid. Cells and supernatant were separated by centrifugation. The supernatant was collected and stored at -70°C. Peritoneal cells were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% BSA, 0.01% NaN_3) and counted by Countess automated cell counter (Invitrogen, Paisley, UK).

3.2.3 Collection of spleens and splenocytes

Spleens were removed from mice postmortem and placed in cold sterile saline. Part of the spleen was removed for immunohistochemistry and snap frozen in OCT compound (Sakura Finetek, Europe B.V.) and stored at -70°C until cryosectioned. The rest of the spleen was passed through a cell strainer (70 µm) (BD Bioscience, San Jose, CA) to obtain a single cell suspension. Spleen cells were washed by centrifugation and red blood cells lysed with ACK lysing buffer (0.15 M NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA in PBS) After washing and debris removal, cells were resuspended in 5 ml FACS buffer and counted using Countess automated cell counter.

3.3 Characterization of cells by flow cytometry

Peritoneal or spleen cells (0.3 x 10^6) were incubated with 2% normal rat: normal mouse serum (1:1) (AbD Serotec, Kidlington, UK) for 20 min. Cells were stained with fluorochrome-labelled monoclonal antibodies (mabs) against CD11b, CD115, F4/80, c-kit, Gr-1, CD5, IgD, B220, CD90.2, CD4, CD8, NK1.1 (eBioscience, San Diego, CA), Ly6G, CXCR2, CD11b, F4/80, CD115, Gr-1 (clone 1A8), CD61, CCR7, CD138 (BD Biosciences), CCR3, D6, CXCR2 (R&D Systems, Abington, UK), and CD11c (MBL-Nordic Biosite, Täby, Sweden). Appropriate isotypic controls were used to set the quadrants and evaluate background staining. After staining, samples were washed twice with FACS buffer and were finally suspended in 200 µl FACS buffer. Ten thousand cells were collected on FACScalibur (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Inc, Ashland, OR).

3.4 Cytokine and chemokine analysis

Cytokines (IL-1β, IL-4, IL-6, IL-10, IL-12, G-CSF and TGF-β), soluble cytokine receptors (IL-1ra, sIL-6R and sTNFRI) and chemokines (CCL2, CCL3, CCL11, CXCL1 and CXCL2) were measured in peritoneal fluid using Duoset ELISA kits (R&D Systems). Detection limit for IL1-ra was 156.3 pg/ml, 46.9 pg/ml for sIL-6R, 31.2 pg/ml for G-CSF, IL-10 and IL-12, 15.6 pg/ml for IL-1β, IL-4 IL-6, CXCL1, CXCL2 and TGF-β, 7.8 pg/ml for CCL3, CCL11 and sTNFRI and 3.9 pg/ml for CCL2. The results were expressed as pg/ml.

3.5 Measurement of BSA-specific IgM, IgG, IgA and IgE antibodies

BSA-specific IgG, IgM, IgA and IgE antibodies in serum were measured by indirect ELISA. Maxisorp plates (Nunc, Invitrogen) were coated with 50 µg/ml BSA in bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were then washed with PBS containing 0.05% tween-20. The samples and standard (pool of serum from mBSA immunized mice) were diluted from 1/10,000 for measuring IgG anti-BSA antibodies and from 1/100 for measuring IgM, IgA and IgE anti-BSA antibodies. Diluted samples and standard were added to the plates and the plates incubated for 2 h at room temperature. Samples were then further incubated for 2 h with horseradish peroxidase (HRP)-labelled goat anti-mouse IgG, IgM, IgA or IgE (Southern Biotech, Birmingham, AL). Substrate buffer containing 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to wells to produce colour and the colour reaction stopped with 0.18 M H_2SO_4. The results were expressed as arbitrary units (AU) per ml that were calculated from the standard curves made from serial dilutions of the standard.

3.6 Immunohistochemical staining

Seven µm thick sections were cut from the spleen and the sections stored at -70°C until stained. Spleen sections were stained with biotin-labelled peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA) followed by HRP-labelled Streptavidin (AbD Serotec) to detect GC B cells and with HRP labelled antibodies against IgM and IgG (Southern Biotech) to detect isotype switching of B cells. Chromogen 3,3' diaminobenzidine (Dako, Glostrup, Denmark) was used for visualization. Stained spleen sections were evaluated blindly by two individuals using a light microscope. As the spleen sections varied in size, the results for the GCs are given as number of GCs per μm^2 and as average size of GCs in each sample. The level of IgM^+ and IgG^+ cells was determined according to predetermined scale, taking into account the distribution and intensity of the staining inside the follicles and in the red pulp.

3.7 Statistical analysis

All data are expressed as mean values ± standard error of the mean (SEM). Kolmogorov Smirnov test was used to test if the data were normally distributed.

One-way ANOVA followed by Tukey's post-hoc test was used to determine whether the effects of mBSA administration were statistically significant. Where data were not normally distributed, Kruskal-Wallis non-parametric ANOVA followed by Dunn's post-hoc test was used to determine whether group medians differed, but means ± SEM shown for clarity.

One-way ANOVA followed by Tukey's post-hoc test or, if the data were not normally distributed, Kruskal-Wallis non-parametric ANOVA followed by Dunn's post-hoc test was used to calculate the overall P values in the dietary studies. Unpaired Student´s test, or non-parametric Mann-Whitney Rank Sum test if the data were not normally distributed, was used to determine if the differences between the two dietary groups at a single time point were statistically significant.

Differences between means or medians were considered significant if $P < 0.05$. Statistical analysis was performed using SigmaStat software, version 3.2 (Systat software Inc., Chicago, IL). All experiments were performed at least twice.

4 Results

4.1 Section I – Cells and mediators in antigen-induced inflammation in mice (Paper I, II, III and unpublished data)

Characterization of the mBSA-induced peritonitis model used in this study was necessary for further assessment of the effects of the FO diet on the model. After two vaccinations with mBSA with complete and incomplete Freund's adjuvant, peritonitis was induced by injecting mBSA into the peritoneal cavity. After mBSA administration, the number of peritoneal cells increased, with the total number of cells peaking at day 2 and declining thereafter (Fig. 5).

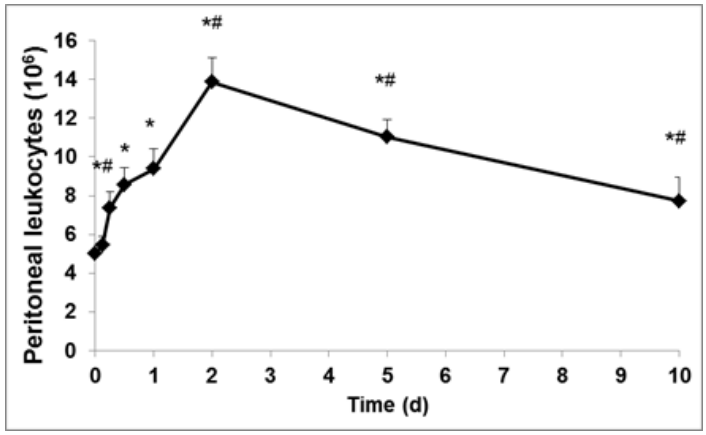

Figure 5. Effects of mBSA administration on the number of peritoneal cells.

Mice were immunized twice s.c. with BSA with two weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and analyzed by flow cytometry. Values are means ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$.

4.1.1 Peritoneal cell populations prior to and following intraperitoneal administration of mBSA (paper I)

Different cell populations were monitored prior to and at different time-points after mBSA administration to assess the changes occurring in the cell populations during the inflammatory response. Macrophages and lymphocytes constituted the majority of the peritoneal leukocytes in the peritoneal cavity prior to peritoneal administration of mBSA (Figs. 1A and B, paper I). Neutrophils appeared in the peritoneal cavity 3 h after mBSA administration, peaking in number at 6 h and declining rapidly thereafter (Figs. 1A and B, paper I and Table

3). Macrophages almost disappeared from the peritoneal cavity following administration of mBSA but monocytes appeared in the peritoneal cavity at 3 h and macrophages, that most likely developed from the monocytes, reappeared in the peritoneal cavity at 24 h and peaked at 48 h (Figs. 1A and B, paper I and Table 3). Eosinophils appeared in the peritoneal cavity 3 h after administration of mBSA and peaked at 48 h (Figs. 1A and B, paper I and Table 3). Lymphocyte numbers also increased following mBSA administration, peaking at day 5 (Figs. 1A and B, paper I). At that time point (day 5) lymphocytes were the dominating cell type in the peritoneal cavity. The number of different cell populations at different time points is summarised in Table 3.

Table 3. The number of cells belonging to different cell populations prior to and at different time-points following mBSA administration

Cell population	0 h	3 h	6 h	12 h	24 h	48 h	5 d	10 d
Neutrophils	N/D	2.05 ± 0.30 $_{a}$	3.29 ± 0.73 $_{a}$	1.30 ± 0.31 $_{a.b}$	0.96 ± 0.24	N/D	N/D	N/D
Monocytes	N/D	0.15 ± 0.02	0.97 ± 0.36	2.36 ± 0.26 $_{a}$	2.82 ± 0.42	1.80 ± 0.49 $_{a.b}$	N/D	N/D
Macrophages	2.01 ± 0.23	0.40 ± 0.07 $_{a}$	0.20 ± 0.02	1.63 ± 0.24 $_{b}$	3.31 ± 0.44	6.07 ± 0.42 $_{a.b}$	2.87 ± 0.10 $_{b}$	2.49 ± 0.10
Eosinophils	0.024 ± 0.004	0.23 ± 0.04	0.627 ± 0.31	1.26 ± 0.30	1.76 ± 0.34	2.87 ± 0.65 $_{a}$	1.53 ± 0.379	0.45 ± 0.22 $_{a.b}$
Mast cells	0.041 ± 0.005	0.0021 ± 0.0005 $_{a.b}$	0.0012 ± 0.0005 $_{a}$	0.0033 ± 0.0005 $_{a}$	0.0033 ± 0.0006 $_{a}$	0.0028 ± 0.0005 $_{a}$	0.0054 ± 0.001 $_{a}$	0.0045 ± 0.0003 $_{a}$
NK cells	0.024 ± 0.006	0.031 ± 0.007	0.063 ± 0.009	0.079 ± 0.005 $_{a}$	0.19 ± 0.02 $_{a.b}$	0.33 ± 0.03 $_{a}$	0.12 ± 0.02 $_{a}$	0.064 ± 0.01 $_{b}$
T cells	0.11 ± 0.01	-	-	0.20 ± 0.02	0.38 ± 0.05	0.94 ± 0.18 $_{a.b}$	0.63 ± 0.05 $_{a}$	0.40 ± 0.07
B cells	1.83 ± 0.30	-	-	1.81 ± 0.26	1.68 ± 0.18	3.82 ± 0.55 $_{a.b}$	4.84 ± 0.39 $_{a}$	3.74 ± 0.82 $_{a}$

Values are means (x10^{6}) ± SEM, n=4-7 per time-point. a different from 0 h, b different from previous time-point, $P < 0.05$

4.1.2 Peritoneal neutrophils following intraperitoneal administration of mBSA (paper I and II)

As the infiltration of neutrophils is known to play a role in the acute phase of several chronic inflammatory conditions and since the kinetics of the resolution process is measured by their disappearance, neutrophils were monitored prior to and after mBSA administration. Neutrophils were identified by their expression of the granulocyte marker Ly6G and the chemokine receptor CXCR2 (data not shown). Neutrophils were not present in the peritoneal cavity prior to mBSA administration, but appeared there at 3 h and peaked at 6 h (Figs. 1A and B, paper I and Table 3), when they constituted around 40% of total peritoneal cells. After peaking at 6 h their number declined and at 48 h after administration of mBSA, there were almost no neutrophils present in the peritoneal cavity (Figs. 1A and B, paper I). The R_i, i.e. the time it took for the peak number of neutrophils ($\Psi_{max}=3.3x10^6$) to be reduced by 50%, was around 5 h (Fig. 1A, paper II, dashed line). The neutrophils could be divided into two populations, N1 and N2, according to their size and granularity (Fig. 2A, insert, paper I). Neutrophils in the N1 population were smaller (FSC: 376.0±2.6) and less granular (SSC: 389.0±5.2) than the neutrophils in the N2 population (FSC: 620.7±1.5, $P<0.001$; SSC: 621.3±6.8, $P<0.001$). Both populations were present 3 h after administration of mBSA, with neutrophils in the N1 population constituting around 85% of total neutrophils and neutrophils in the N2 population around 15% (data not shown). At 6 h following administration of mBSA the N2 neutrophils were around 25% of total neutrophils, but they had almost disappeared at 12 h (Fig. 2A, paper I). Both N1 and N2 neutrophil populations expressed Ly6G, CXCR2 and the integrin CD11b, but the N2 neutrophils had higher expression of these surface molecules at all time points studied (Fig. 2B, paper I).

In summary these results show that the number of neutrophils peaked 6 h after mBSA administration and the R_i in this model was around 5 h. The neutrophils formed two populations, N1 and N2 that differed in size granularity and expression of Ly6G, CXCR2 and CD11b. N1 constituted the majority of the neutrophils at all time points studied.

4.1.3 Peritoneal macrophages prior to and following intraperitoneal administration of mBSA (paper I)

Since efficient clearance of dead cells (efferocytosis) by macrophages is necessary for proper resolution to take place, macrophages were monitored prior to and after mBSA administration. Macrophages were identified by their expression of the trans-membrane glycoproteins F4/80, CD11b (Fig. 3A, paper I)

and the colony stimulating factor 1 receptor (CSF1R or CD115) (data not shown). The resident peritoneal macrophages formed two populations, one with low expression of F40/80 (F4/80^{low} macrophages) and the other with high expression of F4/80 (F4/80^{high} macrophages). The former population constituted around 10% and the latter around 90% of total macrophages in the peritoneal cavity prior to administration of mBSA (data not shown). Following mBSA administration, the resident macrophages almost disappeared from the peritoneal cavity and CD115^{+} monocytes with medium expression of CD11b and no expression of F4/80 appeared in the peritoneal cavity (Fig. 3A, 6 h, paper I and Table 3). F4/80^{high} macrophages were observed again 24 h following mBSA administration (Fig. 3A, paper I). These macrophages were larger and more granular than the infiltrating monocytes but smaller and less granular than the resident macrophages (Fig. 1A, paper I). The F4/80^{high} macrophages at 24 and 48 h differed from the resident F4/80^{high} macrophages in having lower expression of CD11b and much higher expression of the atypical chemokine receptor D6 and the chemokine receptor CCR7 (Figs. 3A and C, 0, 24 and 48 h, paper I). The expression levels of both D6 and CCR7, molecules that are thought to be of importance in resolution of inflammation [97, 239, 240], peaked 2 d following mBSA administration and declined thereafter (Fig. 3C, paper I). F4/80^{low} macrophages were present again in the peritoneal cavity 48 h after mBSA administration (Fig. 3A, paper I). These macrophages expressed the DC marker CD11c and the integral membrane protein CD138 (syndecan-1), which is thought to modulate the activity of chemokines, cytokines, integrins and other adhesion molecules that play important roles in regulation of inflammation [241]. However, they did not express D6 nor CCR7 (Fig. 3B, paper I). The expression levels of CD138 on F4/80^{low} macrophages 5 and 10 d after mBSA administration were higher than prior to mBSA administration (Fig. 3D, paper I).

These results demonstrate that resident F4/80^{low} and F4/80^{high} macrophages disappeared from the peritoneal cavity early after mBSA administration. Their disappearance was followed by influx of monocytes and subsequently by the appearance of F4/80^{high} macrophages expressing D6 and CCR7 and CD11b although their CD11b expression was lower than that by the resident macrophages. The F4/80^{high} macrophages peaked in number at d 2. F4/80^{low} macrophages expressing CD138 and CD11c reappeared in the peritoneal cavity 2 d following mBSA administration.

4.1.4 Peritoneal eosinophils prior to and following intraperitoneal administration of mBSA (paper I)

As eosinophils have been shown to participate in resolution of zymosan-induced peritonitis [38] it was of interest to monitor them in the mBSA-induced inflammation. Eosinophils were identified by their expression of the chemokine receptor CCR3 and lack of expression of the lymphocyte markers B220, CD90.2 and NK1.1 (data not shown). There were few eosinophils present in the peritoneal cavity prior to mBSA administration (Fig. 1A, paper I and Table 3). At 3 h following mBSA administration, eosinophils appeared in the peritoneal cavity, peaking in number at 48 h and declining thereafter (Figs. 1A and B, paper I and Table 3). The eosinophils were more granular than the neutrophils and they had a wide range of granularity (Fig. 1A, paper I). The average granulation of the eosinophils did not change throughout the experiment (data not shown). Eosinophilic expression of CD11b dropped 6 h following administration of mBSA, but increased again before declining and remained low for the remainder of the study (Fig. 4, paper I). Expression levels of CCR3 increased after mBSA administration with peak expression levels at 2 d (Fig. 4, paper I).

In summary, eosinophils appeared in the peritoneal cavity 3 h after mBSA administration and peaked in numbers at d 2. Their granularity remained stable but their expression of CD11b decreased soon after they appeared in the peritoneal cavity and remained low throughout the study.

4.1.5 Peritoneal mast cells prior to and following intraperitoneal administration of mBSA (unpublished data)

As mast cells play an important role in the initiation of inflammation, mast cells were monitored prior to and after mBSA administration. Mast cells were identified by their expression of the mast/stem cell growth factor receptor c-kit (CD117). Mast cells were present in the peritoneal cavity prior to administration of mBSA, but almost disappeared following administration of mBSA (Fig. 6). Their numbers remained low throughout the study (Table 3).

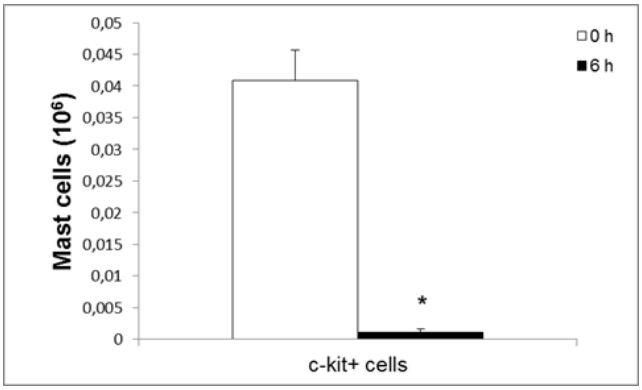

Figure 6. Effects of mBSA administration on the number of peritoneal mast cells.

Mice were immunized twice s.c. with BSA with two weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter, stained with fluorochrome-labelled monoclonal antibodies against c-kit and analyzed by flow cytometry. Values are means ± SEM, n=4-7 per time-point. * Different from 0 h, $P < 0.05$.

4.1.6 Peritoneal lymphocytes prior to and following intraperitoneal administration of mBSA (paper I and III)

Lymphocytes are not thought to participate in resolution of zymosan-induced peritonitis [117]. However, as lymphocytes, especially B cells, constitute a large part of the cells in the mouse peritoneum and since the current study used an antigen to induce the inflammation, lymphocyte numbers were monitored in the peritoneal cavity prior to and following induction of inflammation. NK cells were identified by their expression of the NK cell marker, NK1.1 (data not shown). There were few NK cells in the peritoneal cavity prior to mBSA administration. Twelve h after administration of mBSA the number of NK cells in the peritoneal cavity increased fourfold and peaked at d 2 when they constituted around 2% of the peritoneal cells (Fig. 5A, paper I and Table 3). The number of NK cells declined thereafter.

T cells were identified by their expression of the T cell marker CD90.2. Prior to administration of mBSA, T cells constituted around 2% of the cells in the peritoneal cavity (data not shown). After administration of mBSA the number of T cells in the peritoneal cavity increased and peaked at day 2 (Fig. 5B, paper I and Table 3), constituting around 20% of the peritoneal lymphocytes. Their number declined thereafter (Fig. 5B, paper I). Prior to mBSA administration $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells constituted around 60, 25 and 15% of total T cells, respectively and when they peaked in number 2 d following mBSA administration they constituted around 45, 20 and 35%, respectively (data not shown).

B cells were identified by their expression of the B cell marker B220. Prior to administration of mBSA, total B cells constituted around 40% of the cells in the peritoneal cavity (data not shown). B1 cells were identified as cells with low expression of B220 and IgD and intermediate expression of CD5 ($B220^{low}IgD^{low}CD5^{+/-}$) (Suppl. Fig. 1, paper III). They constituted around 75% of total B cells in the peritoneal cavity prior to administration of mBSA (data not shown). The total number of B cells increased following mBSA administration, peaking at 12 h and 5 d (Fig. 5C, paper I and Table 3). Of total B1 cells, B1a ($CD5^{+}$) cells constituted around 65% and B1b ($CD5^{-}$) cells around 35% (data not shown). The number of B1 cells increased following administration of mBSA peaking in numbers at 12 h, then declining and peaking again at d 5 (Fig. 5C, paper I). B2 cells were identified as cells having high expression of B220 and IgD and no expression of CD5 ($B220^{high}IgD^{high}CD5^{-}$) (Suppl. Fig. 1, paper III). B2 cells constituted around 25% of total B cells prior to administration of mBSA (data not shown). There were few B2 cells in the peritoneal cavity in the first 24 h following mBSA administration, after which their numbers increased and peaked at d 5 (Fig. 5C, paper I).

These results show that B cells constituted around 40% of resident peritoneal cells and that they peaked in numbers at d 5 (Fig 5C, paper I). In the beginning of the immune reaction the majority of the B cells were B1 cells. They peaked in number at 12 h and again at d 5, while the number of B2 cells started to increase at 24 h and peaked 5 d following mBSA administration (Fig 5C, paper I). The number of NK cells and T cells, although not constituting a large part of total peritoneal cells, peaked at d 2 (Figs. 5A and B, paper I and Table 3).

4.1.7 Antigen-specific IgM, IgG, IgA and IgE antibodies in serum prior to and following intraperitoneal administration of mBSA (paper I and unpublished data)

Since mBSA-induced peritonitis is an antigen-induced inflammation and B cells are the major cell type in the peritoneal cavity, serum levels of BSA-specific antibodies were measured. The level of BSA-specific IgM antibodies gradually increased following mBSA administration and peaked at day 5 (Fig. 6, paper I). Serum levels of BSA-specific IgG antibodies decreased following mBSA administration, probably because of immune complex formation, but increased again at 24 h and reached similar levels as prior to mBSA administration at day 10 (Fig. 6, paper I). Because of the high number of peritoneal B1 cells and the fact that B1 cells are thought to produce antigen-specific IgA in the gut, serum levels of BSA-specific IgA antibodies were measured. BSA-specific IgA was not detected in serum. In addition, serum levels of BSA-specific IgE antibodies were measured because of the high number of peritoneal eosinophils, but their levels were at or below the limit of detection.

4.1.8 Cytokines, chemokines and soluble cytokine recpetors in the peritoneal fluid prior to and following administration of mBSA (paper I and unpublished data)

Chemokines and cytokines play an important role in inflammation and therefore the kinetics of several pro-inflammatory mediators was determined prior to and after mBSA administration Peritoneal concentrations of the pro-inflammatory cytokines and chemokines IL-1β, IL-6, IL-12, CCL2, CCL3 and CCL11 increased rapidly after mBSA administration, peaking at 3 h, except for CCL11 and IL-12, which peaked at 6 and 12 h, respectively (Figs. 7A and B, paper I). IL-5 concentration in the peritoneal cavity was at or below the detection limit of the ELISA but somewhat higher levels were observed in some mice at 3 and 6 h following mBSA administration (data not shown). Basal levels of the pro-inflammatory cytokines and chemokines were reached 12-24 h after administration of mBSA, except for basal levels of IL-12 that were not reached until at 48 h (Figs. 7A and B, paper I).

Peritoneal concentration of the receptor antagonist, IL-1ra peaked 6 h after administration of mBSA and was close to basal levels at 24 h (Fig. 7D, paper I). Peritoneal concentration of sTNF-R peaked at 24 h, whereas concentrations of sIL-6R and TGF-β1 did not peak until 2 d after peritoneal administration of mBSA (Figs. 7C and D, paper I). Basal levels of these anti-inflammatory mediators were reached 10 d after mBSA administration (Figs. 7C and D, paper I). Peritoneal concentration of IL-10 was below the detection limit of the ELISA (data not shown).

These results show that the peritoneal concentration of the pro-inflammatory mediators, IL-1β, IL-6, IL-12, CCL2, CCL3 and CCL11 peaked in the first hours after mBSA administration, while the concentrations of most of the anti-inflammatory mediators peaked later, with IL-1ra peaking at 6 h, sTNF-R at 24 h and sIL-6R and TGF-β at 48 h.

4.2 Section II – The effects of dietary fish oil on the acute inflammatory response and the resolution phase of antigen-induced peritonitis (paper II and unpublished data)

4.2.1 Mouse weight, food intake and peritoneal cell count (paper II and unpublished data)

There was no difference in the average daily food intake or body weights of mice receiving either the C or the FO diet. Mice fed the FO diet had more peritoneal cells than mice fed the C diet 5 d after administration of mBSA but there was no difference in total peritoneal cell number at other time points (Fig.7).

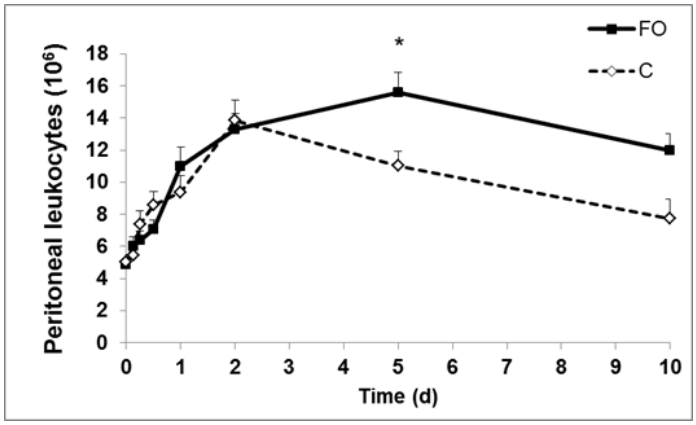

Figure 7. Effects of dietary fish oil on the number of peritoneal cells prior to and at different time points following administration of mBSA.

Mice received control diet (C, dashed line) or fish oil diet (FO, solid line) for 4-5 weeks. They were immunized twice s.c. with mBSA with two weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and analyzed by flow cytometry. Values are means ± SEM, n=4-7 per time-point. * Different from control diet, $P < 0.05$.

4.2.2 Effects of dietary fish oil on peritoneal neutrophils prior to and following administration of mBSA (paper II)

When the effects of dietary FO on peritoneal neutrophils were examined it became apparent that in mice fed the FO diet neutrophil numbers peaked at 3 h after mBSA administration while in mice fed the C diet neutrophil numbers continued to increase after 3 h and did not peak until 6 h after administration of mBSA (Fig. 1A, paper II). The R_i was shorter in mice fed the FO diet or 3 h compared to 5 h in mice fed the C diet (Fig. 1A, paper II). The expression levels

of CD11b on peritoneal neutrophils peaked 3 h after mBSA administration and at that time point there was no difference in expression levels of CD11b between the two dietary groups (Fig. 1B, paper II). At 6 h after mBSA administration CD11b expression on neutrophils from mice fed the C diet had decreased, whilst they had not changed on neutrophils from mice fed the FO diet (Fig. 1B, paper II). However, at 24 h after mBSA administration neutrophils from mice fed the FO diet did not express CD11b while neutrophils from mice fed the C diet had only slightly reduced expression levels of CD11b compared with that at 12 h (Fig. 1B, paper II). Expression levels of CD11b were higher on both N1 and N2 neutrophils from mice fed the FO diet than on these neutrophil populations from mice fed the C diet at 6 h following mBSA administration (Fig. 1B, insert, paper II).

These results show that mice fed the FO diet had lower peak number of neutrophils and shorter R_i than mice fed the C diet. In addition, the expression levels of CD11b decreased more slowly on neutrophils from mice fed the FO diet compared to that in mice fed the C diet.

4.2.3 Effects of dietary fish oil on peritoneal mast cells prior to administration of mBSA (paper II)

Mice fed the FO diet had a tendency towards having more mast cells in their peritoneal cavity prior to mBSA administration than mice fed the C diet (P=0.06) (Fig. 8). Mast cells almost disappeared from the peritoneal cavity of mice in both dietary groups after mBSA administration (data not shown).

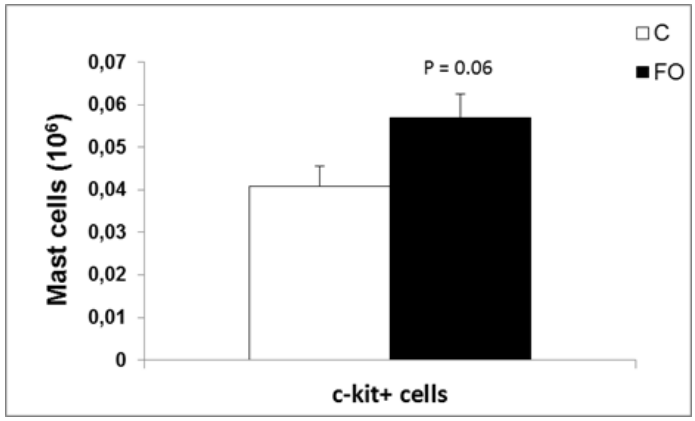

Figure 8. Effects of dietary fish oil on the number of peritoneal mast cells prior to administration of mBSA.

Mice received control diet (C) or fish oil diet (FO) for 4-5 weeks. They were immunized twice s.c. with mBSA with two weeks interval. Mice were sacrificed one week later and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with c-kit monoclonal antibodies and analyzed by flow cytometry. Values are means ± SEM, n=4-7 per time-point.

4.2.4 Effects of dietary fish oil on peritoneal NK cells prior to and following administration of mBSA (paper II)

Mice fed the FO diet had more peritoneal NK cells than mice fed the C diet 12 h after mBSA administration (Fig. 2, paper II). On the contrary, mice fed the FO diet had had fewer NK cells in their peritoneal cavity than mice fed the C diet at the time point when the NK cells peaked or 2 d after mBSA administration (Fig. 2, paper II). Furthermore, 10 d after mBSA administration, the number of peritoneal NK cells in mice fed the FO diet remained high, while the number of peritoneal NK cells in mice fed the C diet had declined and were similar to that prior to mBSA administration (Fig. 2, paper II).

4.2.5 Effects of dietary fish oil on peritoneal macrophages prior to and following administration of mBSA (paper II)

There was no difference between the two dietary groups in peak number of F4/80^{high} macrophages 2 d after mBSA administration (Fig. 3A, paper II). However, the number of peritoneal F4/80^{high} macrophages was higher in mice fed the FO diet than in mice fed the C diet 5 d following mBSA administration (Fig. 3A, paper II). Examination of the surface markers on F4/80^{high} macrophages revealed that expression of F4/80 peaked earlier on F4/80^{high} macrophages from mice fed the FO diet than on F4/80^{high} macrophages from mice fed the C diet (Fig. 4A, paper II). In addition, F4/80^{high} macrophages from mice fed the FO diet had higher peak expression levels of CCR7 and D6 than F4/80^{high} macrophages from mice fed the C diet (Figs. 4B and C, paper II).

The number of F4/80^{low} macrophages peaked 2 d following mBSA administration in mice fed the C diet while not until at d 5 in mice fed the FO diet (Fig. 3B, paper II). At that time point, the number of F4/80^{low} macrophages in mice fed the FO diet was more than double the number of those cells in mice fed the C diet (Fig. 3B, paper II). Expression levels of F4/80 and CD138 on F4/80^{low} macrophages did not differ between the two dietary groups at any of the time points studied (data not shown).

In summary, compared with mice fed the C diet, mice fed the FO diet had higher peak expression levels of CCR7 and D6 and earlier peak expression levels of F4/80 on F4/80^{high} macrophages. In addition, there was more gradual decline in peritoneal F4/80^{high} macrophages following mBSA administration and higher peak number of F4/80^{low} macrophages in the peritoneal cavity of mice fed FO diet than in mice fed the C diet.

4.2.6 Effects of dietary fish oil on peritoneal and splenic eosinophils prior to and following administration of mBSA (paper II)

The number of peritoneal eosinophils in mice fed the C diet peaked 2 d following mBSA administration while the number of eosinophils in mice fed the FO diet peaked at d 5 (Fig. 5A, paper II). At that time point the number of peritoneal eosinophils was more than twofold in mice fed the FO diet compared with that in mice fed the C diet (Figs. 5A and B, paper II) and the number of eosinophils remained higher in mice fed the FO diet than in mice fed the C diet at d 10 (Fig. 5A, paper II). There was no difference in expression levels of CD11b and CCR3 on eosinophils from mice fed the different diets at any of the time points studied (data not shown).

To examine whether the higher number of peritoneal eosinophils in mice fed the FO diet compared with that in mice fed the C diet was reflected in more eosinophils in the spleen, the proportion of eosinophils in spleen was examined. Mice fed the FO diet had almost twofold higher proportion of eosinophils in their spleen 5 d following mBSA administration than mice fed the C diet (Fig. 9).

In summary, these results show that mice fed the FO diet had a more prolonged increase in the number of eosinophils following mBSA administration than mice fed the C diet (Figs. 5A and B, paper II). In addition, mice fed the FO diet had a higher peak number of splenic eosinophils following mBSA administration when compared to mice fed the C diet.

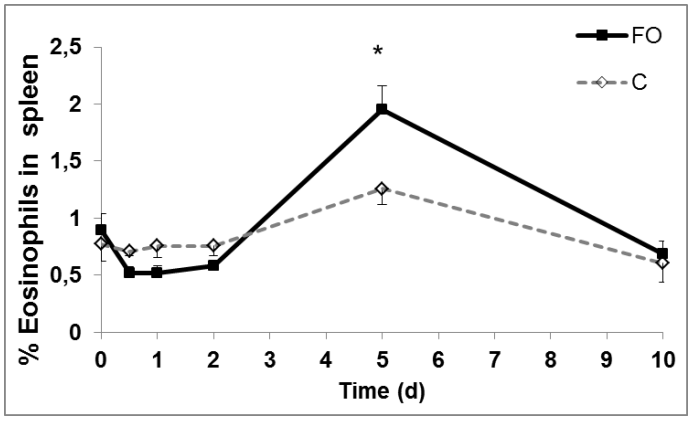

Figure 9. Effects of dietary fish oil on the proportion of splenic eosinophils prior to and at different time points following administration of mBSA.

Mice received control diet (C) or fish oil diet (FO) for 4-5 weeks. They were immunized twice s.c. with mBSA with two weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and spleens collected. Cells were counted using Countess automated cell counter and stained with CCR3 monoclonal antibodies and analyzed by flow cytometry. Values are means ± SEM, n=4-7 per time-point.

4.2.7 Effects of dietary fish oil on pro- and anti-inflammatory mediators in the peritoneal fluid prior to and following administration of mBSA (paper II and unpublished data)

Mice fed the FO diet had lower peritoneal concentrations of the neutrophil chemoattractant CXCL1 and the pro-inflammatory mediator IL-6 than mice fed C diet at the time when their levels peaked (3 h) (Figs. 6A and B, paper II). In addition, peritoneal concentration of the neutrophil growth factor and regulator ,G-CSF, peaked earlier in mice fed the FO diet (3 h) than in mice fed the C diet (6 h) (Fig. 6C, paper II). At the time point when G-CSF peaked in mice receiving the C diet the concentration of this mediator was much lower in mice receiving the FO diet (Fig. 6C, paper II). Mice fed the FO diet also had lower peak concentration of the eosinophil chemoattractant, CCL11, at 6 h following mBSA administration than mice fed the C diet (Fig. 6D, paper II). The FO diet did not affect peritoneal concentrations of the pro-inflammatory mediators CCL2, CCL3, IL-1β and IL-12 (data not shown).

Mice fed the FO diet had an earlier increase in peritoneal concentration of the anti-inflammatory mediator sIL-6R (6 h) than mice fed the C diet (12 h) (Fig. 6E, paper II). However, there was no difference in peritoneal concentrations of sIL-6R between the two dietary groups at the time when their levels peaked, i.e. at 2 d following mBSA administration (Fig. 6E, paper II). A similar trend was seen in the effects of the FO diet on peritoneal concentration of the anti-inflammatory cytokine TGF-β. Peritoneal concentration of TGF-β peaked 6 h after administration of mBSA in mice fed the FO diet but was at that time point similar to that prior to mBSA administration in mice fed the C diet (Fig. 6F, paper II). Peritoneal concentrations of TGF-β peaked again 2 days following mBSA administration in mice fed both diets with no difference between the two groups (Fig. 6F, paper II). There was a sharper peak in the concentration of the anti-inflammatory mediator IL-1ra in mice fed the FO diet than in mice fed the C diet but there was no difference in the peak levels of IL-1ra between the groups (Fig. 10A). Peritoneal concentration of sTNFR1 in mice fed the C diet peaked at 24 h but at 48 h in mice fed FO diet (Fig. 10B). However, there was no difference in the peak concentrations of sTNFR1 between the dietary groups (Fig. 10B).

In summary, these results show that mice fed the FO diet had lower peak concentrations of the pro-inflammatory mediators CXCL1, IL-6 and G-CSF and earlier peak concentration of G-CSF. In addition, in mice fed the FO diet there was an earlier increase in the concentrations of the anti-inflammatory mediators sIL-6R and TGF-β than in mice fed the C diet.

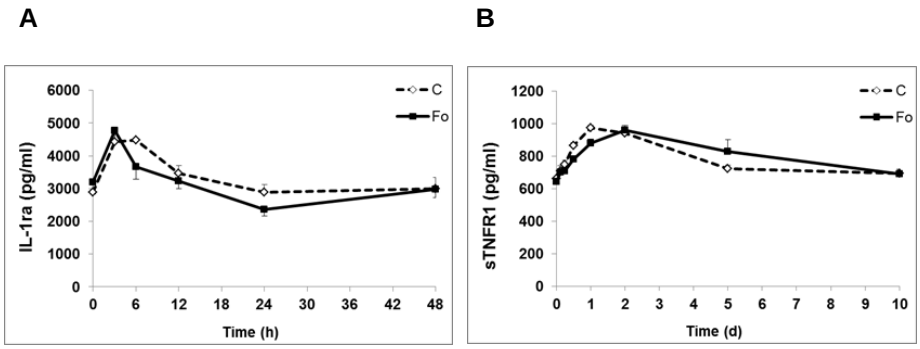

Figure 10. Effects of dietary fish oil on peritoneal concentrations of IL-1ra (A) and sTNFR1 (B) prior to and at different time points following administration of mBSA.

Mice received control diet (C, dashed line) or fish oil diet (FO, solid line) for 4-5 weeks. They were immunized twice s.c. with mBSA with two weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected and concentrations of IL-1ra and sTNFR1 measured with ELISA. Values are means ± SEM, n=4-7 per time-point.

4.3 Section III – The effects of dietary fish oil on the adaptive response in antigen-induced peritonitis (Paper III)

4.3.1 Effects of dietary fish oil on peritoneal and splenic B cells prior to and following administration of mBSA (paper III)

As the inflammation model used in the study was antigen-induced and, therefore, likely to involve the adaptive immune system, the effects of the FO diet on peritoneal lymphocytes were evaluated. The majority of the peritoneal lymphocyes were B cells. Prior to mBSA administration there was no difference in the number of total B cells between the two dietary groups (Fig. 1A, paper III). However, in mice fed the FO diet the number of total B cells in the peritoneal cavity was higher 24 h after administration of mBSA than it was prior to mBSA administration whereas at that time point there had been no increase in the number of total B cells in the peritoneal cavity of mice fed the C diet (Fig. 1A, paper III). The FO diet did not affect the number of peritoneal B2 cells at any of the time points studied (Fig. 1C, paper III). The number of B1 cells was higher in mice fed the FO diet at 24 h and 5 d following mBSA administration than in mice fed the C diet and there was a tendency towards a higher number also 10 d following mBSA administration (Fig. 1B, paper III). In addition, mice fed the FO diet had a higher number of B1a cells in their peritoneal cavity at 24 h and 5 d following mBSA administration than mice fed the C diet (Fig. 1D, paper III).

However, mice fed the FO diet only had a higher number of peritoneal B1b cells than mice fed the C diet 10 d following mBSA administration (Fig. 1E, paper III).

To examine whether the higher number of peritoneal B cells in mice fed the FO diet compared with that in mice fed the C diet was reflected in higher number of B cells in the spleen, the proportion of splenic B cells was examined. There was little effect of mBSA administration on the proportion of B cells in the spleen and no difference in the proportion of splenic B cells between the two dietary groups at any of the time-points studied (Suppl. Fig. 2, paper III).

In summary, compared with mice fed the C diet, mice fed the FO diet had a higher number of total B cells in the peritoneal cavity at 24 h following mBSA administration and the number of B1 cells was higher at 24 h and 5 d. There was no difference between the dietary groups in the number of peritoneal B2 cells or in the proportion of B cells in the spleen.

4.3.2 Effects of dietary fish oil on peritoneal and splenic T cells prior to and following administration of mBSA (paper III)

The effects of the FO diet on peritoneal and splenic T cells were also examined. Mice fed the FO diet had more total T cells in their peritoneal cavity than mice fed the C diet 5 d after mBSA administration (Suppl. Fig. 3A, paper III). Mice fed the FO diet had more $CD4^+$ and $CD8^+$ T cells at 12 h and 5 d and a tendency towards more $CD4^+$ and $CD8^+$ T cells at 10 d after mBSA administration when compared with mice fed the C diet (Suppl. Figs. 3B and C, paper III). The number of $CD4^+$ T cells did not decline in mice fed the FO diet until 10 d after mBSA administration whereas in mice fed the C diet the number of T cells had declined at d 5 and declined further at d 10 (Suppl. Fig. 3B, paper III). The FO diet did not affect the number of $CD4^-CD8^-$ T cells at any of the time-points studied (data not shown).

To examine whether the higher number of peritoneal T cells in mice fed the FO diet compared with that in mice fed the C diet was reflected in more T cells in the spleen, the proportion of splenic T cells was examined. There was no difference in the proportion of splenic T cells between the two dietary groups at any of the time points studied (Suppl. Fig. 3D, paper III). The proportion of splenic T cells did not change following mBSA administration.

In summary, compared with mice fed the C diet, mice fed the FO diet had a higher number of total T cells in the peritoneal cavity 5 d following mBSA administration and the number of $CD4^+$ and $CD8^+$ T cells was higher at 12 h and 5 d. There was no difference between the dietary groups in the proportion of T cells in the spleen.

4.3.3 Effects of dietary fish oil on serum levels of BSA-specific IgM and IgG antibodies prior to and following administration of mBSA (paper III)

Next it was examined whether the FO diet affected serum levels of BSA-specific IgM and IgG antibodies. At d 5 following mBSA administration serum levels of IgM specific antibodies were higher in mice fed the FO diet than in mice fed the C diet and there was also a tendency towards higher serum levels 10 d after mBSA administration in mice fed FO diet (Fig. 2A, paper III). There was no difference in serum levels of BSA-specific IgG antibodies between the two dietary groups (Fig. 2B, paper III).

4.3.4 Effects of dietary fish oil on IgM^+ and IgG^+ cells in spleen prior to and following administration of mBSA (paper III)

We next examined whether higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet than in mice fed the C diet were reflected in more IgM^+ cells in spleen. Mice fed the FO diet had higher levels of IgM^+ cells in the red pulp of the spleen than mice fed the C diet 5 and 10 d following mBSA administration (Figs. 3A and B). However, there was no difference between the two dietary groups in the level of IgM^+ cells within or around the follicles (data now shown). IgG staining was mainly observed within the follicles, not in the red pulp. There was no difference between the two dietary groups in the level of IgG staining in the spleen (data not shown).

4.3.5 Effects of dietary fish oil on plasma cells and germinal centers in spleen prior to and following administration of mBSA (paper III)

To examine whether higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet when compared to mice fed the C diet were reflected in more GC formation in the spleen, the average size and number of GCs in spleens were determined. The average number of GCs peaked at day 5 following mBSA adminstration in both dietary goups (Fig. 4A, paper III). There was no difference in the average number of GCs between the two dietary groups at any of the time-points studied (Fig. 4A, paper III). The average size of the GCs was larger at d 2 following mBSA adminstration in mice fed the FO diet than in mice fed the C diet (Fig. 4B and C, paper III).

To examine whether the increase in the number of GCs in mice following mBSA administration was reflected in more plasma cells in the spleen, splenic sections were stained for expression of CD138, a plasma cell marker. However, no $CD138^+$ cells were detected in the spleen at any time-point (data not shown).

5 Discussion and conclusion

This thesis describes the kinetics of the cellular and molecular events in the induction phase and resolution phase of inflammation and the adaptive immune response in mBSA-induced peritonitis in mice. In addition, the effects of dietary fish oil on the same aspects are presented. The results show that in the induction phase, dietary fish oil reduced the number of infiltrating neutrophils, shortened the resolution interval and decreased the levels of the pro-inflammatory mediators CXCL1, IL-6, G-CSF and CCL11. Dietary fish oil also induced an earlier peak in the immunosuppressive molecules sIL-6R and TGF-β that was not seen in mice fed the control diet. In the resolution phase dietary fish oil increased expression levels of the atypical chemokine receptor D6 and the chemokine receptor CCR7 on macrophages and later in the resolution phase increased the number of peritoneal eosinophils and macrophages. The effects of dietary fish oil on peritoneal neutrophils, macrophages and eosionphils in the mBSA model are summarized in Figure 11.

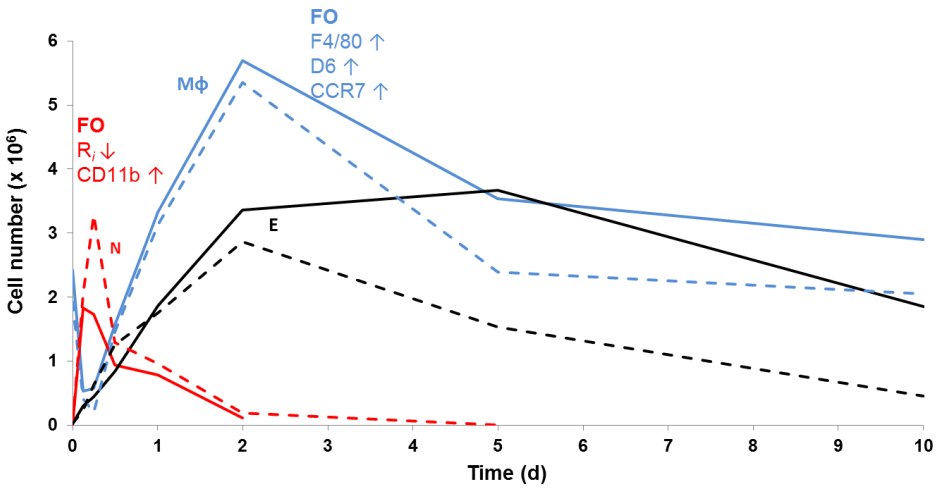

Figure 11. Effects of dietary fish oil on peritoneal neutrophils, macrophages and eosinophils prior to and at different time-points following administration of mBSA.

Number of peritoneal neutrophils (N), macrophages (Mφ) and eosinophils (E) in mice fed the control diet (C, dashed line) or the fish oil diet (FO, solid line) immunized with mBSA and peritonitis induced. The arrows indicate whether the FO diet induced an increase (↑) or a decrease (↓) in expression levels of certain surface molecules on the cells, as well as in the resolution interval (R_i).

In the present study, dietary fish oil also increased the number of peritoneal B1 cells, increased serum levels of BSA-specific IgM antibodies and more IgM^+

cells were found in spleens of mice receiving the fish oil diet compared with that in mice receiving the control diet.

The murine antigen-induced peritonitis model used in this study was adapted from a model established by Cook et al. [121]. This model is based on an antigen, rather than an irritant, like a wide range of other peritonitis models including the zymosan model. Therefore, it may be more relevant in investigating immunologic events in many inflammatory diseases. Resolution of inflammation has been investigated extensively in the zymosan model, where the short-lived sharp inflammation results from TLR activation, mimicking the early inflammatory response to bacteria and involves little or no activation of the adaptive immune response [117, 242-244]. In mBSA-induced peritonitis, both the innate and the adaptive immune responses are activated by immunizing (s.c.) with mBSA in CFA and mild peritonitis is induced later by peritoneal injection of mBSA. This model may, therefore, better reflect the immunologic events in many chronic inflammatory diseases where both the innate and the adaptive immune responses participate.

5.1 Neutrophils in mBSA-induced peritonitis and the effects of dietary fish oil

The results from the present study show that neutrophils infiltrated the peritoneal cavity in the induction phase of the inflammation, peaking in numbers 6 h after mBSA administration. These results are in agreement with results from a study by Vieira et al. [245] showing that neutrophils were present in the peritoneal cavity of the mice at 2 h and peaked at 6 h following induction of mBSA-induced peritonitis. However, these results do not agree with results from a study by Cook et al. [121] showing that the neutrophils peaked in numbers 2 d after mBSA administration. The mBSA immunization procedure in the study by Cook et al. [121] was different from the one in the present study and the study by Vieira et al. [245] in that both immunizations were in CFA in the study by Cook et al. whereas the second immunization was in IFA in the two latter studies. Therefore, it is possible that the different immunization procedure may have led to the later peak in neutrophil numbers in the study by Cook et al. [121]. However, it is also possible that what they report as neutrophils, peaking in numbers at 2 d, may have been monocytes identified as neutrophils, since in that study they used anti Gr1 antibody to identify the neutrophils, but anti Gr1 antibodies recognize Ly6C present on monocytes, in addition to Ly6G present on neutrophils [121]. In the present study an antibody detecting only Ly6G was used to identify the neutrophils.

When the induction phase of the mBSA-induced peritonitis in the present study was compared to the induction phase of the zymosan-induced peritonitis in the study by Bannenberg et al. [228], where 1 mg of zymosan was injected i.p., the cellular and molecular events were similar. However, the induction phase in the mBSA-induced peritonitis developed more rapidly, with T_{max} being 6 h compared with 12 h in the zymosan-induced peritonitis. In addition, the induction phase in the mBSA-induced peritonitis was less severe with fewer infiltrating neutrophils, their maximum number (ψ_{max}) being $3.3x10^6$ in the mBSA-induced peritonitis vs. $15x10^6$ in the zymosan-induced peritonitis. Furthermore, the inflammation in the mBSA-induced peritonitis resolved more rapidly with R_i=5 h vs. 8 h in the zymosan-induced inflammation. Shorter R_i in the mBSA model may be explained by the fact that it was a milder inflammation, with lower ψ_{max} and/or because the mice were immunized, and that the adaptive response that was activated in the beginning of the inflammatory response may have led to the reduced R_i. In the present study dietary fish oil reduced the peak number of neutrophils in the peritoneal cavity and accelerated the resolution as was evident by the shorter R_i. These results are similar to what has been seen in zymosan-induced peritonitis following administration of the n-3 PUFA-derived pro-resolving mediators RvE1 and PD1 [228]. Therefore, one could speculate that mice fed the FO diet have higher levels of RvE1 and/or PD1 than mice fed the C diet because of more n-3 PUFA substrate being available for their production and that these mediators drive the reduction in the resolution interval seen in mice fed the FO diet.

The lower ψ_{max} of neutrophils in mice fed the FO diet when compared with mice fed the C diet may be explained by less recruitment of peritoneal neutrophils, caused at least in part by less concentration of CXCL1 in mice fed the FO diet than in mice fed the C diet. The peak concentration of the growth factor G-CSF was also lower and peaked earlier in mice fed the FO diet compared with that in mice fed the C diet and that may have resulted in earlier and decreased release of neutrophils from the bone marrow in mice receiving the FO diet. Previous studies have shown that n-3 PUFA can reduce adhesion and migration of neutrophils *in vitro* [224, 246, 247] and indicate that n-3 PUFA can thereby suppress inflammatory responses [248]. In addition, a previous study from the laboratory demonstrated that dietary fish oil reduced the number of peritoneal neutrophils in mice 12 and 24 h after intraperitoneal administration of LPS but increased their numbers 48 h after LPS administration [225]. Furthermore, the n-3 PUFA-derived RvE1, RvD2 and PD1 have been shown to reduce neutrophil recruitment to the inflamed site *in vivo* [228, 249, 250]. These mediators may be produced in the mice fed the FO diet and may be responsible for the reduced neutrophil numbers in the peritoneal cavity of these mice.

The two populations of neutrophils that appeared in the peritoneal cavity in the induction phase in the present study, not only differed in size and granularity but also in their expression levels of CD11b, CXCR2 and Ly6G, indicating that they may have different roles in the inflammatory response. Neutrophil heterogeneity has been observed in several studies where neutrophils have different phenotypes and roles depending on the phase of inflammation [251]. In addition, a previous study from the laboratory demonstrated two neutrophil populations being present in the circulation in endotoxin-induced acute inflammation in mice, although the peritoneal neutrophils were a homogenous population in that study [252]. The higher CD11b expression levels seen on the N2 neutrophils than on the N1 neutrophils in the present study and on both N1 and N2 neutrophils from mice fed FO diet as compared with mice fed the C diet may indicate that these cells are in a more activated state, since increased CD11b expression has been linked to enhanced neutrophil activation [253-255]. CD11b expression has been shown to mediate adhesion of neutrophils to the endothelium and help their migration into inflamed tissues [256, 257] and a human neutrophil subset having higher mean expression levels of CD11b when compared with other subsets was released into the circulation during acute inflammation [258]. However, in the present study CD11b did not seem to enhance neutrophil recruitment into the peritoneal cavity as there were fewer N2 neutrophils there than N1 neutrophils and less neutrophils in mice fed the FO diet than in mice fed the C diet. Increased CD11b expression on neutrophils has also been linked to immunosuppressive properties [259] and, therefore, neutrophils from mice fed the FO diet in the present study may have more immunosuppressive properties than neutrophils from mice fed the C diet.

5.2 Macrophages in mBSA-induced peritonitis and the effects of dietary fish oil

In the present study there were two populations of resident peritoneal macrophages that differed in granularity and their expression levels of F4/80. This is similar to what has been seen by others [31, 260, 261]. However, in those studies the resident peritoneal macrophages also differed in size, which they did not in the present study. In the study by Dioszeghy et al. [260] there were two populations of CD11b^{high} cells, 95% of which were F4/80^{high}12/15-LOX^{+} while the rest were F4/80^{med}12/15-LOX^{-} and expressed the DC marker CD11c. In the study by Ghosn et al. [261] the F4/80^{high} macrophages were larger than the F4/80^{low} macrophages and were called large peritoneal macrophages (LPM), constituting around 90% of the total macrophages while the F4/80^{low} macrophages were called small peritoneal macrophages (SPM) and constituted around 10% of total macrophages. Both LPM and SPM had phagocytic activity

and produced nitric oxide (NO) in response to LPS stimulation *in vivo*. In addition, the SPM expressed MHCII and CD62L that indicates their role as antigen-presenting cells. These surface markers were not determined in the present study but the F4/80^{low} macrophages were a similar proportion of the total peritoneal macrophages as the SPM in the study by Ghosn et al. [261]. The disappearance of the resident peritoneal macrophages upon induction of inflammation in the present study is similar to what has been observed in other studies [260, 262]. Although Cassado et al. [262] only observed disappearance of the LPM and not the SPM, they may have missed the time point at which the SPM disappeared as they only looked at three time points, i.e. 0 h, 30 min and 48 h [262] not including the one that revealed the disappearance of the resident peritoneal macrophages in the present study, i.e. 6 h.

In the present study a population of F4/80^{high}D6^{high}CCR7^{+} macrophages appeared in the peritoneal cavity at 24 h and peaked in number at the time point when the resolution phase is probably peaking. Bystrom et al. [30] and Stables et al. [31] have identified a population of macrophages that appeared in the resolution phase of zymosan-induced peritonitis. These resolution phase macrophages expressed molecules necessary for antigen processing/presentation (MHCII, CD74, CD86), secreted T and B lymphocyte chemokines, as well as factors that enhance macrophage/DC development and promote DC/T cell synapse formation [30, 31]. As these surface markers and chemokines were not determined in the present study its results cannot be compared with those by Bystrom and Stables. However, the F4/80^{high}D6^{high}CCR7^{+} macrophages in the present study had lower expression of CD11b than the resident peritoneal macrophages and may, therefore, be similar to the pro-resolving CD11b^{low} macrophages that were recently reported to emerge *in vivo* during resolution of zymosan-induced peritonitis [263]. In that study, macrophages converted from CD11b^{high} to a CD11b^{low} phenotype upon interaction with apoptotic cells *ex vivo* [263]. Therefore, the F4/80^{high}D6^{high}CCR7^{+} macrophages in the present study may have decreased their expression of CD11b upon interaction with apoptotic cells and thereby become resolution phase or pro-resolving macrophages.

In the present study dietary fish oil did not influence peak numbers of the resolution phase macrophages or their expression of CD11b. However, dietary fish oil increased expression levels of F4/80, D6 and CCR7 and may, therefore, have affected macrophage function. Higher expression levels of F4/80 suggests increased macrophage maturation as F4/80 has been indicated to be a maturation marker on macrophages [264]. In addition, higher expression levels of CCR7 may indicate more effective emigration of these macrophages to lymph nodes, since CCR7 is involved in cell migration to lymph nodes [239]. Although

results from a recent study indicate that macrophage migration to lymph nodes is CCR7 independent and their removal is controlled by local death in the resolution phase of TM-induced peritonitis [35], other studies indicate that macrophages do indeed travel to the lymph nodes following ingestion of apoptotic neutrophils [24, 34, 38]. Higher expression levels of D6 on the resolution phase macrophages in the present study may also support effective emigration of macrophages to lymph nodes, since D6 expression on lymphatic endothelial cells has been shown to prevent association of inflammatory CC-chemokines with lymphatic surfaces and thereby facilitating cellular migration and fluid flow to lymph nodes [265]. Furthermore, many studies have demonstrated the importance of D6 for resolution of inflammation and shown that D6 deficient mice have exaggerated inflammatory response [93, 96-100, 266]. Higher expression levels of D6 on the resolution phase macrophages in mice fed the FO diet in the present study could, therefore, indicate enhanced resolution in mice fed the FO diet than in mice fed the C diet.

In the present study, the simultaneous peak in the concentration of the immunosuppressive molecule TGF-β and the number of $F4/80^{high}D6^{high}CCR7^{+}$ resolution phase macrophages indicate active resolution at 48 h or 2 d after mBSA administration. At this time point the macrophages may be recognizing and ingesting apoptotic cells but this recognition of apoptotic cells has been demonstrated to inhibit the release of pro-inflammatory mediators [267] and to induce TGF-β production, which helps in accelerating and regulating resolution of the inflammation [110, 268, 269]. In addition, a recent study demonstrated that D6 regulate macrophage clearance of apoptotic neutrophils in the resolution phase [242]. Furthermore, it may be speculated that the increased macrophage expression of D6 at this time point may be caused by the increased peritoneal concentration of TGF-β, as TGF-β has been shown to up-regulate D6 expression on THP-1 cells *in vitro* [270]. The FO diet did not affect the number of the resolution phase $F4/80^{high}$ macrophages at 2 days but the higher expression levels of F4/80, D6 and CCR7 on these macrophages in mice fed the FO diet may indicate that these macrophages are better equipped for resolution activity.

In the late or post resolution phase, i.e. 5 d following administration of mBSA, there were two populations of $F4/80^{high}$ macrophages that had different expression levels of CD11b. This could indicate that resident $F4/80^{high}CD11b^{high}$ macrophages were starting to repopulate the periteonal cavity at a time when the $F4/80^{high}CD11b^{low}$ resolution phase macrophages were facilitating homeostasis attainment. In the present study dietary fish oil increased the number of $F4/80^{high}$ macrophages at d 5 and that could indicate faster repopulation of resident peritoneal macrophages in mice fed the FO diet compared to that in mice fed the C diet.

The F4/80^{low} macrophages appeared in the peritoneal cavity again 48 h after mBSA administration. They expressed the DC marker CD11c but did not express CCR7, which indicates that they are not typical antigen-presenting DCs. They also expressed the heparan sulfate proteoglycan CD138 or syndecan-1 protein, an integral membrane protein that participates in cell proliferation, cell migration and cell-matrix interactions. In the present study the CD138 expression on F4/80^{low} macrophages indicates that they may have a regulatory role by reducing inflammation and/or supporting resolution of inflammation, since CD138 has been suggested to inhibit leukocyte adhesion, reduce expression of or inhibiting release of pro-inflammatory factors and to remove inflammatory chemokines [241]. In addition, CD138 has been suggested to help in attenuating lung inflammation [271] and to play a role in the resolution of neutrophilic inflammation in a mouse model of endotoxemia [272]. Furthermore, CD138 knockout mice have been shown to have impaired wound healing [273]. In the present study the expression levels of CD138 on the F4/80^{low} macrophages were extensively increased at d 5 and 10 following mBSA administration. These results indicate a regulatory role for these macrophages and that they may perhaps have a role in reaching homeostasis. Dietary fish oil increased the number of F4/80^{low} macrophages 5 d after mBSA administration indicating that mice fed the FO diet may have improved potential for reaching homeostasis than mice fed the C diet.

5.3 Eosinophils in mBSA-induced peritonitis and the effects of dietary fish oil

Eosinophils infiltrated the peritoneal cavity soon after mBSA administration in the present study. The presence of eosinophils is usually linked to allergic Th2-polarized environment with elevated levels of the cytokines IL-4, IL-5 and IL-13 and the generation of allergen-specific IgE antibodies. However, in the present study the peritoneal eosinophils were generated following immunization with CFA, a Th1-polarizing agent, and BSA-specific IgE antibodies were hardly detectable in the serum during the inflammatory response. In addition, eosinophils in allergic reactions, such as asthma, increase their expression levels of CD11b and high levels of CD11b expression have been linked to allergic diseases and oxidative burst [274, 275] but in the present study the mean expression level of CD11b on eosinophils was decreased at the time when their numbers peaked. Furthermore, during allergic reactions eosinophils degranulate and release an array of cytotoxic granule proteins but in the present study the mean granulation of the eosinophils, as measured by flow cytometry, did not change throughout the study. Therefore, it is unlikely that the eosinophils

in the mBSA model are linked to an allergic reaction or have a cytotoxic function, spilling out their cytotoxic granules and causing harm in the tissue.

Eosinophils peaked in number at the same time as the resolution phase $F4/80^{high}D6^{high}CCR7^{+}$ macrophages and the anti-inflammatory/immune-suppressive molecules TGF-β and sIL-6R, which could indicate that they are cells that participate in the resolution phase. As eosinophils have been shown to be a major source of TGF-β [276], the high peritoneal concentration of this immunosuppressive cytokine in the resolution phase may have come from peritoneal eosinophils. Yamada et al. [38] have recently described eosinophils as cells that promote resolution of zymosan-induced peritonitis and demonstrate in that study that eosinophil depleted mice have impaired resolution and sustained number of neutrophils in the inflamed tissue. In addition, these resolution phase eosinophils were identified as major producers of the pro-resolving n-3 PUFA-derived PD1 [38]. In the present study dietary fish oil prolonged the high number of peritoneal eosinophils, with mice fed the FO diet having a higher number of peritoneal eosinophils 5 and 10 d after mBSA administration than mice fed the C diet. It can also be speculated that eosinophils in mice fed the FO diet produced more PD1 than eosinophils in mice fed the C diet, since they have more n-3 PUFA available as substrate for its production and that could in part explain the lower number of peritoneal neutrophils seen in mice fed the FO diet compared with that in mice fed the C diet early in the resolution phase.

In the present study peak level of the eosinophil chemoattractant CCL11 was reached 6 h after mBSA administration and at that time point eosinophils were already present in the peritoneal cavity. However, when the number of peritoneal eosinophils peaked, CCL11 had reached basal levels indicating that other chemokines are responsible for the recruitment of eosinophils into the peritoneal cavity at later stages in the inflammatory response.

5.4 Mast cells in mBSA-induced peritonitis and the effects of dietary fish oil

Mast cells play an important role in the initiation of inflammation in both zymosan- [277] and mBSA-induced [245] peritonitis and mast cell-deficient mice have delayed inflammatory responses in TM-induced peritonitis [278]. Therefore, the tendency towards more mast cells being present in the peritoneal cavity of mice fed the FO diet than in mice fed the C diet prior to initiation of the inflammation would be expected to facilitate more inflammation in mice fed the FO diet than in mice fed the C diet. However, that was not the case as evident by lower number of neutrophils and lower concentrations of some pro-

inflammatory mediators in mice fed the FO diet when compared with mice fed the C diet after the administration of mBSA. Mast cells have also been shown to produce TGF-β [279] and, therefore, it is possible that the slightly higher concentration of TGF-β in mice fed the FO diet early in the inflammatory response may have been derived from the mast cells.

Mast cells are present in allergic reactions and, therefore, their disappearance from the peritoneal cavity following mBSA administration emphasizes that the presence of eosinophils in the peritoneal cavity is probably not allergy related.

5.5 NK cells in mBSA-induced peritonitis and the effects of dietary fish oil

The number of peritoneal NK cells increased greatly (4-10 fold at 12 and 24 h) following administration of mBSA and their numbers peaked at the same time as the resolution phase F4/80^{high}D6^{high}CCR7^{+} macrophages. Haworth et al. [49] recently demonstrated that NK cells are important in resolution of allergic airway inflammation and depleting NK cells or blocking them delayed resolution. NK cells have also been shown to be capable of inducing apoptosis of neutrophils [48]. Therefore, the early increase in NK cells in the present study in mice fed the FO diet that was not seen in mice fed the C diet may have led to the reduced neutrophil numbers in mice fed the FO diet compared with that in mice fed the C diet. As NK cells have been shown to produce TGF-β [280] the early rise in NK cell numbers may also have contributed to the early peak in TGF-β concentration seen in mice fed the FO diet but not in mice fed the C diet. The study by Haworth et al. [49] showed that NK cells express CMKLR1, a receptor for the pro-resolving n-3 PUFA-derived RvE1 and that depletion of NK cells decreased RvE1-mediated resolution of allergic inflammation. Therefore, in the present study the FO diet may have provided both more substrate for production of the pro-resolving RvE1 and more cells with a receptor for the RvE1, i.e. the NK cells, and both these mechanisms may have contributed to enhancing resolution of the inflammation.

5.6 Pro- and anti-inflammatory mediators in mBSA-induced peritonitis and the effects of dietary fish oil

The concentration of pro-inflammatory cytokines peaked in the first hours after mBSA administration and had reached basal levels when the number of resolution phase F4/80^{high}D6^{high}CCR7^{+} peritoneal macrophages and the concentration of the immune suppressive molecules sTNFR1, sIL-6R and TGF-β peaked. These molecular events in the mBSA model are similar to the ones

presented in the zymosan-induced peritonitis model [228]. In addition, the early rise in the concentration of the immunosuppressive molecule IL-1ra, peaking at 6 h, suggests that the resolution of inflammation is initiated very early in the inflammatory response. In the present study dietary fish oil decreased peak concentration of CXCL1, IL-6, G-CSF and CCL11 all of which considered to be pro-inflammatory and, therefore, the effect of the FO diet can be considered anti-inflammatory.

Dietary fish oil induced an earlier peak in the concentration of the immune suppressive molecules TGF-β that aids in regulating the initiation and resolution of inflammatory responses [110] and sIL-6R that has been associated with immune suppression and reduction of neutrophil accumulation in peritoneal inflammation [92]. In addition, dietary fish oil induced an earlier peak of the immunosuppressive molecule IL-1ra that helps in limiting the pro-inflammatory effects of IL-1 [281]. These results indicate that mice fed the FO diet may have an earlier anti-inflammatory response than mice fed the C diet.

5.7 Lymphocytes and the adaptive immune response in mBSA-induced peritonitis and the effects of dietary fish oil

Infiltration of both T and B cells into the peritoneal cavity of the mice in the mBSA-induced inflammation in the present study demonstrates the involvement of the adaptive immune response in this model and that the two mBSA immunizations very likely led to the production of memory lymphocytes. The number of total T cells peaked earlier than the number of total B cells in the peritoneal cavity following induction of inflammation. This early peak in T cell numbers may reflect the need of T cell-help for B cell activation. Also the $CD4^+$ T cells may be regulatory T_{REG} cells, however, that is only speculative, as the T cells were not studied in any detail in the present study. A recent study showed that T_{REG} cells, a subset of $CD4^+$, $CD25^+$ antigen dependent T cells that co-express the transcription factor Foxp3 and secrete the immune suppressive/pro-resolving cytokines IL-10 and TGF-β, participated in resolving LPS-induced acute lung injury model [282]. In that study, transfer of T_{REG} cells to RAG knockout mice increased apoptosis of alveolar neutrophils and macrophage efferocytosis, reduced the levels of pro-inflammatory alveolar cytokines and doubled the levels of alveolar TGF-β [282]. In this same study, isolated alveolar macrophages stopped producing TNF-α and increased TGF-β release when in contact with T_{REG} cells [282]. Another study showed that anti-inflammatory macrophages induced the differentiation of T_{REG} cells by their production of TGF-β [283]. These studies indicate a potential link between the innate and adaptive

immune systems in resolution of inflammation. In the present study T_{REG} cells, if involved, could have been influenced by the pro-resolving macrophages and TGF-β. That dietary fish oil increased the number of peritoneal T cells could perhaps indicate more T cell help being available for B cell activation in mice fed the FO diet compared with that in mice fed the C diet and/or higher number of $CD4^+$ T cells could indicate higher number of T_{REG} cells in mice fed the FO diet that could aid in the resolution of the antigen-induced inflammation.

Although previous studies have shown that lymphocytes repopulate the site of injury as inflammation resolves [118, 119, 284] it is not known whether they are necessary for resolution of inflammation to occur. Results from a study using RAG knockout mice indicate that lymphocytes are not necessary for resolution of zymosan-induced inflammation as the inflammation resolved similarly in RAG knockout and wild-type mice [117]. However, in that study lymphocytes were suggested to have a role in protecting against secondary infection, as wild-type mice were better protected against secondary infection than the RAG knockout mice [117].

In the mBSA-induced peritonitis in the present study, a high proportion of the peritoneal cells were B1 cells. This finding is in agreement with what has been described by Cook et al. [121] for mBSA-induced peritonitis and by Rajakariar et al. [117] for zymosan-induced peritonitis. B1 cells are innate type lymphocytes that are T cell independent and produce high levels of polyspecific natural IgM antibodies both during homeostasis and in infections [285]. Natural IgM antibodies are thought to be important in maintaining homeostasis in tissues where they participate in clearance of abnormal and apoptotic cells, inhibition of inflammation, removal of misfolded proteins and regulation of pathogenic autoantibody-producing B cells. B1a cells are known to spontaneously secrete protective polyspecific IgM antibodies [286] and these cells were increased earlier in mice fed the FO diet than in mice fed the C diet in the present study. The higher number of B1a cells in mice fed the FO diet may have led to more production of natural IgM antibodies than that in mice fed the C diet early in the resolution phase. However, that is only speculative, as serum levels of natural IgM antibodies could not be measured. Serum levels of BSA-specific IgM antibodies were measured and they were higher at 5 and 10 d following mBSA administration than prior to mBSA administration. BSA-specific IgM antibodies are most likely produced by B1b cells as B1b cells have been shown to secrete antigen-specific antibodies following stimulation via the B cell receptor [59] and they are required for long term protection against certain parasites and bacteria [287]. In the present study, dietary fish oil increased serum levels of BSA-specific IgM antibodies. These results are in agreement with results from a recent study by Teague et al. [237] showing that mice fed dietary fish oil had

enhanced B cell activation upon antigen stimulation with an increase in surface and/or circulating IgM antibody production. Overall the higher number of B1a and B1b cells and the higher serum levels of IgM in mice fed the FO diet compared with that in mice fed the C diet in the present study indicates that dietary fish oil may improve protection against secondary infections and enhance the potential for reaching homeostasis following antigenic challenge.

The newly discovered regulatory B10 cells are thought to belong to the B1 population [55] and, therefore, the higher number of B1 cells in the peritoneal cavity of mice fed the FO diet may also have included higher number of B10 cells than that in mice fed the C diet. However, that is only speculative, as B10 cells were not analysed in the present study.

The two immunizations with mBSA in the present study mounted an efficient B2 immune response, evident by the high levels of serum BSA-specific IgG antibodies. Following the peritoneal challenge with mBSA, the levels of IgG antibodies decreased temporarily, most likely because of immune complex formation between BSA-specific IgG antibodies and the mBSA. These immune complexes formed are likely to have induced the peritonitis. The increase in the number and size of GCs after mBSA administration indicates the formation of BSA-specific plasma and memory cells. In the present study dietary fish oil did not affect the B2 cell response, neither the number of peritoneal B2 cells nor the levels of BSA-specific IgG antibodies. These results are in agreement with results from a previous study showing that in mice with an enteric infection caused by respiratory enteric orphan virus (reovirus), reovirus-specific serum IgA and IgG2a antibody responses were similarly induced in mice fed control and DHA-rich diets [288]. However, the results from the present study are not in agreement with results from another study showing that dietary fish oil influenced serum levels of influenza-specific IgG antibodies by increasing its levels 5 d after infection and lowering its levels at d 7 [289]. In addition, the n-3 PUFA-derived 17-HDHA and RvD1 have been shown to increase IgG and IgM secretion by *in vitro* activated human B cells because of increased differentiation of the B cells into plasma cells [238].

The higher levels of IgM^+ cells in spleen from mice fed the FO diet compared with that in mice fed the C diet in the present study may be explained by the higher number of B1 cells in spleen as peritoneal B1 cells have been shown to migrate into the spleen and differentiate into antigenic and natural IgM-secreting cells [290-292]. The levels of IgG^+ cells in the spleen did not differ between the two dietary groups, which is in agreement with dietary fish oil not affecting peritoneal numbers of B2 cells.

As recent studies indicate a role for eosinophils in regulating antibody responses [293, 294], it is of interest to note that at the time when there were higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet compared with that in mice fed the C diet (5 d) the number of peritoneal eosinophils was also higher in mice fed the FO diet than in mice fed the C diet. Eosinophils have been shown to be critical for the maintenance of plasma cells in the bone marrow by secretion of the proliferation-inducing ligand APRIL and IL-6 [293] and eosinophil-deficient mice had impaired early antigen-specific IgM antibody responses to alum that could be restored by adoptive transfer of eosinophils [294]. Eosinophils have also been shown to be important for priming B cells and their secretion of antigen-specific IgM antibodies [294]. Therefore, it can be speculated that higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet compared with that in mice fed the C diet in the present study may be related to more peritoneal eosinophils in these mice. Dietary fish oil also increased the proportion of splenic eosinophils and, as most of the production of IgM by murine B1 cells occurs in the spleen [295], the higher proportion of splenic eosinophils may therefore also explain the higher IgM production in mice fed the FO diet compared with that in mice fed the C diet.

5.8 Limitations of the study

Using animal models as a tool to investigate cells and molecules of the immune system has yielded great insight into the mechanisms of the human immune system. However, because of the difference between mice and humans [296] one should keep in mind that responses in mice and humans may not be exactly the same. Mice are more sensitive to dietary n-3 PUFA in that their immune cell membranes seem to accumulate more n-3 PUFA than immune cells of humans [141] and for that reason n-3 PUFA may have more influence on immune function in mice than in humans. In the present study the amount of n-3 PUFA in the FO diet was kept low to decrease the possibility of n-3 PUFA accumulation into cell membranes far exceeding what has been observed in immune cells in humans. The FO diet provided 1.4% EPA and 1.4% DHA, which equals to about 3 grams of these long chain PUFA in a 2000 kcal human diet and is believed to be safe for human consumption. As the menhaden fish oil used in the diet contained little amount of AA, the same amount of AA was added to the C diet. The FO diet did not contain vitamin A or vitamin D and, therefore, both diets in this study had equal amounts of vitamin A, D, and E, which is important since these vitamins can affect immune responses [297-299].

One limitation in this study was the number of molecules (four) that could be detected simultaneously on each cell by the flow cytometer available. Because

of this limitation the cell populations investigated could not be studied in as much detail as they could have if a newer version of the flow cytometer had been available at the time of the study. This hampered for example a detailed study of the B1 cells, since the recognition of four molecules was necessary for their identification.

In this study there were no functional studies performed on the cells so the effects of the FO diet on cell function were only speculative based on its effects on the expression levels of different surface molecules and their time of appearance in the immune reaction.

It would also have been informative to have results on the concentrations of the resolution phase molecules LXA_4, Rvs and PDs. An attempt was made to measure LXA_4 and PGD_2 by ELISA, but the results were inconsistent and not reliable. Samples were also collected for measurement of Rvs and PDs concentrations in peritoneal fluid. However, there were problems with the technique that was to be set up by a collaborator in Iceland, and by the time that was evident, the samples had been stored for too long to be of value.

5.9 Summary and conclusion

The results in this thesis demonstrate that the mBSA-induced peritonitis model may be a good model to study initiation and resolution of inflammation as well as the adaptive immune response in antigen-induced inflammation. The initiation phase of the inflammation in this model was characterized by the infiltration of two populations of peritoneal neutrophils and by an increase in the peritoneal concentrations of the pro-inflammatory mediators IL-1β, IL-6, CCL2, CCL3, G-CSF, CXCL1 and CCL11. The neutrophils may have produced the anti-inflammatory mediator IL-1ra, which was seen early in the inflammatory response and is thought to be important in the initiation of the resolution phase. The resolution phase was characterized by the disappearance of the neutrophils and the appearance of resolution phase macrophages that expressed D6 and CCR7, molecules linked with resolution. Concentrations of the anti-inflammatory mediators, TGF-β, sIL-6R and sTNFR1, were also increased in the resolution phase. Eosinophils peaked simultaneously with the resolution phase macrophages and the anti-inflammatory mediators and because of their role in resolution of zymosan-induced peritonitis [38], it is likely that they may also have a role in resolving mBSA-induced inflammation. The peritoneal lymphocytes in the mBSA-induced peritonitis peaked late in the resolution phase, like they have been shown to do in zymosan-induced inflammation, with the majority of the lypmhocytes being B1 cells. B1 cells may have a role in the post resolution

phase like has been suggested that they do in the zymosan-induced inflammation [117].

Dietary fish oil reduced the inflammatory response and enhanced the resolution phase of the inflammation in the mBSA-induced peritonitis model. Reduced inflammatory response was evident by the lower number of infiltrating peritoneal neutrophils and the lower peritoneal concentrations of the pro-inflammatory mediators CXCL1, IL-6, G-CSF and CCL11 in mice fed the FO diet compared with that in mice fed the C diet. Enhanced resolution was manifest by the shorter R_i in mice fed the FO diet compared with that in mice fed the C diet. Enhancement of the resolution phase was also indicated by higher expression of the resolution linked molecules, D6 and CCR7, on peritoneal macrophages in mice fed the FO diet compared with that in mice fed the C diet. In addition, mice fed the FO diet showed an earlier anti-inflammatory response when compared with that in mice fed the C diet, seen in an earlier increase in the number of NK cells, peritoneal concentrations of TGF-β and sIL-6R and expression of F4/80 on macrophages. Furthermore, dietary fish oil may have improved the late-resolution response since mice fed the FO diet had higher number of peritoneal eosinophils and $CD138^+$ macrophages than mice fed the C diet.

Dietary fish oil also affected the adaptive immune response as it increased the number of total peritoneal B and T lymphocytes following mBSA administration. Mice fed the FO diet had a higher number of both $CD4^+$ and $CD8^+$ T cells 2 d following mBSA administration when compared with mice fed the C diet. In addition, mice fed the FO diet had increased B1 cell response when compared with mice fed the C diet, seen in higher number of peritoneal B1 cells, higher levels of splenic IgM^+ cells in the red pulp of the spleen and higher serum levels of BSA-specific IgM. However, dietary fish oil did not affect the number of peritoneal B2 cells or serum levels of BSA-specific IgG antibodies.

The results from this thesis indicate that dietary fish oil may dampen the inflammatory response, accelerate the onset of the resolution phase and enhance the resolution phase of inflammation. In addition, the results indicate that dietary fish oil may enhance the B1 cell response and thereby possibly improve protection against secondary infections.

5.10 Future tasks

The work presented in this thesis raised many questions, both regarding the mBSA-induced peritonitis model and the effects of fish oil on the inflammatory response.

Although many surface markers were evaluated on different cell populations present in the peritoneal cavity of the mice in the present study, it would be of

interest to study in more detail surface marker expression on several cell populations. For example, a more detailed analysis of the different macrophage populations, present at different time points in the peritoneal cavity, could be performed by determination of molecules like MHCII and CD62L. A more complex evaluation of the NK cells could also be performed as the NK cells were only identified with one surface marker. Further evaluation of surface molecules on the NK cells could provide more information regarding their potential role in the inflammation and resolution of inflammation. Finally, since the FO diet increased the number of $CD4^+$ T cells in the peritoneal cavity it would be interesting to see if it increased the number of T_{REG} cells as well.

Functional studies of the different cell populations appearing and disappearing in the peritoneal cavity would also provide another dimension to the image we have tried to draw up of the course of events during the inflammatory response. Therefore, it would be of interest to collect cells at different time points and study their function *in vitro*. It has to be pointed out though, that because the cells are already stimulated *in vivo* it may not be possible to stimulate them *ex vivo* and hence there are limits to what information can be gained by such studies.

Cell knockout studies or cell depletion studies can also be performed to gain more insight into the role that specific cell populations, like macrophages, eosinophils, NK cells and B1 cells, play at different time points during the inflammatory response.

In addition to the pro- and anti-inflammatory mediators measured in the present study it would have been of interest to determine the effects of the FO diet on the concentration of the lipid mediators PGD_2 and LXA_4, as PGD_2 is important in regulating inflammation and LXA_4 is an AA-derived pro-resolving mediator. Furthermore, although the FO diet is expected to have increased the concentration of the Rvs and the PDs in the peritoneal cavity, because the FO diet contains the fatty acids that are substrates for these mediators, it would be of interest to verify that as well as to determine the time-course of the presence of these mediators in the peritoneal cavity.

An evaluation of what cell types produce pro-, anti- and resolution type mediators during the inflammation would also be of interest. For example, a determination of whether the eosinophils produce the resolution enhancing mediator, PD1, would be of interest and whether the FO diet enhances the production of this mediator could be of great importance to the course of the resolution of the inflammation.

As dietary fish oil markedly increased the number of B1 cells in the present study it would be of great interest to further evaluate their role in the

inflammation, both within the peritoneal cavity as well as in the spleen. Since the recently discovered B10 cells are thought to belong to the B1 population it would be interesting to investigate whether B10 cells can be found in both the peritoneal cavity and the spleen in the mBSA-induced peritonitis and to see how dietary fish oil affects these cells. Furthermore, as eosinophils have been shown to be important for priming B cells and for their secretion of antigen-specific IgM antibodies it would be interesting to investigate whether eosinophil depletion in the mBSA model would result in lower serum levels of BSA-specific IgM.

At last, since repopulation of the peritoneal cavity with lymphocytes in the post-resolution phase has been shown to be important for protection against secondary infection it is of interest to examine if mice fed the FO diet, which in the present study increased the number of peritoneal T and B1 cells, would be better protected against secondary infections.

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Paper I

Murine antigen-induced inflammation - a model for studying induction, resolution and the adaptive phase of inflammation

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Abstract

Murine zymosan-induced peritonitis is the model most frequently used to study resolution of inflammation. However, the antigen-induced peritonitis model may be better suited for studying resolution of inflammation and the adaptive phase that follows. The objective of this study was to provide an evaluation of the kinetics of cells and mediators during induction, resolution and the adaptive immune phases of a murine antigen-induced inflammation.

Female C57BL/6 mice were immunized twice subcutaneously with mBSA and three weeks after the initial immunization they were injected intraperitoneally (i.p.) with mBSA, which induced peritonitis. Peritoneal cells were counted and expression of surface molecules and chemokine receptors analyzed with flow cytometry. Chemokine and cytokine concentrations in peritoneal fluid were determined by ELISA.

Two neutrophil populations, differing in size and granularity and slightly in expression of surface molecules, were observed in the peritoneal cavity after induction of inflammation. Macrophages disappeared from the peritoneal cavity following i.p. administration of mBSA but appeared again as they differentiated from recruited monocytes and peaked in numbers at 48 h. At that time-point two distinct populations of macrophages were present in the peritoneal cavity; one with high expression of F4/80, also expressing the atypical chemokine receptor D6 as well as CCR7; the other expressing low levels of F4/80 and also expressing CD11c and CD138. Eosinophils appeared in the peritoneum 3 h following i.p. administration of mBSA and peaked at 48 h. At that time-point they had upregulated their expression of CCR3 but decreased their expression of CD11b. Peritoneal levels of CCL11 peaked at 6 h and may have led to recruitment of the eosinophils. NK cells and T cells peaked at 48 h, whereas B cells peaked at 5 days, with the majority being B1 cells. Peritoneal concentrations of pro-inflammatory cytokines (IL-β and IL-6,) and chemokines (CCL2 and CCL3) peaked at 3 h, whereas IL-1ra peaked at 6 h, sTNF-R at 24 h and sIL-6R and TGF-β at 48 h.

The results show kinetic alterations in cell populations and mediators in a murine model that may be an excellent model to study initiation and resolution of inflammation and the following adaptive phase.

Keywords Antigen-induced inflammation, Murine model, Induction of inflammation, Resolution of inflammation

1. Introduction

The inflammatory response consists of an initial induction phase that evolves into a resolution phase. The fine tuning of these responses is essential for maximizing the benefits and minimizing the harm ensuing from activation of the inflammatory cascade. Although the inflammatory response is customarily initiated by activation of the innate immune system, adaptive immunity can serve a valuable role in diminishing inflammatory responses and accelerate recovery following re-exposure to an antigen.

Induction and resolution of inflammation has been thoroughly studied in the zymosan-induced peritonitis model (reviewed in (Serhan, 2007)), as well as in allergic airway inflammation (Haworth et al., 2011; Rogerio et al., 2012). Zymosan triggers the inflammatory response by binding to toll-like receptor (TLR)-2 on tissue monocytes/macrophages, thereby inducing cytokine and chemokine secretion. Marked but transient influx of neutrophils into the peritoneum is followed by infiltration and activation of monocytes/macrophages. Initially, the macrophages are pro-inflammatory (M1 macrophages), secreting tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 and vigorously phagocytosing foreign antigens and apoptotic neutrophils, but gradually their phenotype changes to an anti-inflammatory or pro-resolution type (M2 or rM macrophages, respectively) (Gilroy et al., 2004; Schif-Zuck et al., 2011), characterized by predominant production of pro-resolution lipid mediators (i.e. lipoxins, resolvins, protectins and maresins) and anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor (TGF)-ß) (reviewed in (Ariel and Timor, 2013)). This resolution process may be further strengthened by the emergence of a recently described resolution-promoting eosinophils (Yamada et al., 2011). Eventually, the inflammatory lesion recedes and post inflammation homeostasis is restored.

Induction of inflammation within the sterile peritoneal cavity represents an ideal location to study the evolution of the inflammatory response due to the easy access to the

peritoneal exudate for analysis of cells and soluble mediators. Therefore, zymosan-induced peritonitis is an excellent model to study an intense self-limited acute inflammation resulting from TLR activation. However, the zymosan-induced peritonitis model entails little or no activation of the adaptive immune response (Kolaczkowska et al., 2008) and may, therefore, not accurately reflect the kinetics of the induction and resolution phases during naturally occurring infections or during immune reactions that have the potential to evolve into chronic inflammation, including autoimmune processes. In the methylated BSA (mBSA)-induced peritonitis model, both the innate and adaptive elements of the immune response are initially activated by immunizing subcutaneously (s.c.) with mBSA and complete Freund´s adjuvant. Peritonitis is subsequently induced by intraperitoneal (i.p.) injection of mBSA without an adjuvant, resulting in reactivation of the adaptive immune response. In previous studies, Cook et al. described temporal changes in the number of neutrophils and macrophages in mBSA-induced peritonitis (Cook et al., 2003; Cook et al., 2004) but only provided analysis of surface markers and soluble mediators within the peritoneal cavity on day 4 following induction of inflammation. In the present study we provide a detailed analysis of cells and soluble mediators during the induction and resolution phases as well as the subsequent adaptive phase of inflammation in murine mBSA-induced peritonitis.

2. Materials and Methods

2.1. Mice

All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland (#0507 – 1503) and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Female C57BL/6 mice, weighing 18-20 g, were obtained from

Taconic Europe (Ejby, Denmark). They were housed 8 per cage with a 12 h light/dark cycle at 23-25°C and 45-55% humidity. Mice were acclimated for one week prior to initiation of the experiments. Mice had free access to food and water.

2.2. Induction of peritonitis

Mice were immunized s.c. at the base of the tail with 100 µg of mBSA (Sigma Aldrich, St. Louis, MO) emulsified in an equal volume of CFA (Sigma Aldrich), the total injection volume being 50 µl. Two weeks later the mice were given a booster injection of 100 µg of mBSA in incomplete Freund's adjuvant (IFA) (Sigma Aldrich). Three weeks after the initial immunization mice were challenged i.p. with 100 µg of mBSA in 50 µl of saline. Before and at several time-points after peritonitis induction (3 h, 6 h, 12 h, 24 h, 48 h, 5 days and 10 days) mice were anesthetized with a mixture of hypnorm (VetaPharma Ltd, Leeds, UK), dormicum (Roche, Basel, Switzerland) and sterile water (1:1:2) and killed by cervical dislocation.

2.3. Collection of serum and peritoneal lavage

Serum was collected and stored at -70°C. Peritoneal exudate was obtained by injecting 1.5 ml of cold phosphate buffered saline (PBS), without calcium or magnesium, into the peritoneal cavity and collecting the fluid. Cells and supernatant were separated by centrifugation. The supernatant was collected and stored at -70°C. Peritoneal cells were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% BSA, 0.2 mM EDTA and 0.1% NaN_3) and counted by Countess automated cell counter (Invitrogen, Paisley, UK).

2.4. Characterization of cells by flow cytometry

Peritoneal cells (0.3×10^6) were incubated with 2% normal rat: normal mouse serum (1:1) (AbD Serotec, Kidlington, UK) for 20 min. Cells were stained with fluorochrome-labeled

monoclonal antibodies (mabs) against Gr1, F4/80, CD115, CD90.2, NK1.1, CD4, CD8, B220, IgD, CD5 (eBioscience, San Diego, CA), Ly6G (clone 1A8), CD11b, F4/80 (BD Bioscience, San Jose, CA), CCR3, CXCR2, D6, CCR7 (R&D Systems, Abington, UK) and CD11c (MBL-Nordic Biosite, Taby, Sweden). Appropriate isotype controls were used to set the quadrants and evaluate background staining. Cells were washed twice, resuspended in FACS buffer and 10,000 cells collected on FACScalibur (BD Biosciences). Data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR). The macrophages were identified as cells expressing F4/80 and CD11b, the monocytes as cells expressing CD115 and not F4/80, the eosinophils as cells expressing CCR3, granulocytes as cells expressing Ly6G, CD11b and CXCR2 and lymphocytes as cells expressing B220, CD90.2 and NK1.1.

2.5. Measurement of BSA-specific IgM and IgG antibodies in serum

BSA-specific IgM and IgG antibodies were measured in serum using indirect ELISA. Maxisorp plates (Nunc, Invitrogen) were coated overnight at 4°C with 50 μg/ml BSA (Sigma-Aldrich) in bicarbonate buffer (pH 9.6). Standard (pooled serum from mBSA immunized mice) and sera, diluted from 1/10,000 for measuring IgG anti-BSA antibodies and from 1/100 for IgM antibodies, were incubated for 2 h at room temperature and further incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgM and IgG (Southern Biotech, Birmingham, AL) for 2 h. The substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and the color reaction stopped with 0.18 M H_2SO_4. The results are expressed as arbitrary units calculated from standard curves made from serial dilution of the standard.

2.6. Cytokine and chemokine analysis

Concentrations of cytokines (IL-1β, IL-6, IL-10, IL-12 and TGF-β), chemokines (CCL2, CCL3 and CCL11) and soluble cytokine receptors (IL-1ra, sTNF-R and sIL-6R) were measured in peritoneal fluid using Duoset ELISA kits (R&D Systems).

2.7. Statistical analysis

Results are expressed as mean values ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's post-hoc test was used to determine whether the effects of mBSA administration were statistically significant. Where data were not normally distributed, one-way ANOVA of ranks followed by Dunn's post-hoc test was used to determine whether group medians differed, but means ± SEM shown for clarity. Differences between means or medians were considered significant if $P < 0.05$. Statistical analysis was performed using SigmaStat software, version 3.2 (Systat software Inc, Chicago, IL).

3. Results

3.1. Cell populations

Macrophages and lymphocytes were the main cell types in the peritoneal cavity prior to i.p. injection of mBSA (Fig. 1A). Three h after i.p. injection of mBSA, the macrophages had disappeared but monocytes, neutrophils and eosinophils had emerged (Fig. 1A). Subsequently, at 24 h after i.p. injection of mBSA, the number of neutrophils had decreased by 70%, but macrophages were present again, probably having developed from the monocytes (Figs. 1A and B). Macrophages and eosinophils peaked in numbers at 2 days following i.p. injection of mBSA and lymphocytes peaked at 5 days, at which time they were the dominating cell type (Figs. 1A and B).

3.2. Neutrophils

The number of neutrophils peaked in the peritoneal cavity 6 h after i.p. injection of mBSA but decreased by more than 50% within 12 h (Fig. 1B). The neutrophils formed two distinct populations with the majority (~80%) belonging to a population of cells that were smaller and less granular (N1) than neutrophils in the other subpopulation (N2) (Fig. 2A). Both populations peaked at 6 h after administration of mBSA, but the N2 population had disappeared from the peritoneal cavity by 12 h (Fig. 2A). Neutrophils in the N2 population expressed more CD11b, Ly6G and slightly more CXCR2 than neutrophils in the N1 population (Fig. 2B).

3.3. Macrophages

Resident peritoneal macrophages had either low or high expression of F4/80, with both populations expressing CD11b (Fig. 3A) and CD115 (data not shown). The F4/80^{high} macrophages constituted around 90% of total macrophages in the peritoneum prior to administration of mBSA. Following mBSA administration, the resident macrophages almost disappeared from the peritoneal cavity but monocytes, which did not express F4/80 emerged. At 24 h following i.p. injection of mBSA F4/80^{high} macrophages reappeared in the peritoneal cavity and peaked at 48 h (Fig. 3A). They were smaller, less granular and expressed less CD11b than the resident macrophages (Figs. 1A and 3A). These macrophages had much higher expression of the atypical chemokine receptor D6 and the chemokine receptor CCR7 than resident peritoneal macrophages (Fig. 3B). Expression levels of D6 and CCR7, which are thought to be of importance in resolution of inflammation (Forster et al., 1999; McKimmie and Graham, 2006; Di Liberto et al., 2008), peaked at 2 days (Fig. 3C). F4/80^{low} macrophages reappeared in the peritoneum 48 h after administration of mBSA (Fig. 3A). They did not express D6 or CCR7, but expressed the dendritic cell (DC) marker CD11c and the integral

membrane protein CD138 (syndecan-1) (Fig. 3B). Expression levels of CD138 were highest at 10 days following mBSA administration (Fig. D).

3.3. Eosinophils

Eosinophils appeared in the peritoneal cavity 3 h after administration of mBSA, peaked at 48 h and declined thereafter (Figs. 1A and B). When the number of eosinophils had reached half of their maximum number, the number of neutrophils had decreased by half and by the time the number of eosinophils peaked the neutrophils had almost disappeared (Fig. 1B, insert). The eosinophils had a wide range of granularity (Fig. 1A) but their average granularity did not change throughout the experiment (data not shown). Expression levels of the chemokine receptor CCR3 peaked at 48 h after administration of mBSA, whereas mean expression levels of CD11b peaked at 6 h and declined rapidly thereafter (Fig. 4).

3.4. NK, T and B cells

NK ($NK1.1^{+}$) cells appeared in the peritoneal cavity of mice 3 h after i.p. injection of mBSA (Fig. 5A). They were scant at 3 and 6 h, but subsequently their numbers increased dramatically and at 48 h they constituted around 2% of total peritoneal cells (Fig. 5A). Lymphocytes constituted 42% of total peritoneal cells prior to induction of peritonitis, but around 50% when their numbers peaked at 5 days (Fig. 1B). The number of T cells in the peritoneum increased after i.p. injection of mBSA, peaking at 2 days (Fig. 5B) when they constituted around 20% of lymphocytes, with $CD4^{+}$, $CD8^{+}$ and $CD4^{-}CD8^{-}$ cells comprising 43, 23 and 34% of total T cells, respectively (Fig. 5B). B cells constituted around 40% of peritoneal lymphocytes prior to mBSA administration with B1 cells ($B220^{low}IgD^{-}$) comprising 75% and B2 cells ($B220^{low}IgD^{high}CD5^{-}$) 25% of the total B cells (Fig. 5C). The number of B cells increased following mBSA administration and after a slight drop in their numbers at 24

h, increased again peaking at 5 days (Fig. 5C). There were few B2 cells in the peritoneal cavity prior to and early after mBSA administration, but their numbers had increased at 2 and 5 days. B1a ($CD5^+$) cells constituted 65% and B1b ($CD5^-$) cells 35% of total B1 cells prior to mBSA administration (data not shown). Both B1a and B1b cells upregulated CD11b on their surface after mBSA administration (data not shown).

3.5. IgM and IgG levels.

Serum levels of mBSA-specific IgM antibodies increased gradually and peaked 5 days after i.p. injection of mBSA (Fig. 6). Serum levels of mBSA-specific IgG antibodies transiently decreased following administration of mBSA, most likely because of formation of IgG-mBSA immune complexes, but increased again and were similar to that prior to mBSA administration at 10 days (Fig. 6).

3.6. Cytokines, chemokines and soluble cytokine receptors

I.p. administration of mBSA resulted in a rapid increase in peritoneal concentrations of the pro-inflammatory cytokines and chemokines IL-1β, IL-6, IL-12, CCL2, CCL3 and CCL11, with peak levels occurring at 3 h, except for CCL11, which peaked at 6 h, and IL-12, which peaked at 12 h (Figs. 7A and B). Peritoneal concentration of IL-1ra peaked 6 h after administration of mBSA, sTNF-R at 24 h, whereas concentrations of sIL-6R and TGF-β1 did not peak until 2 days after peritoneal administration of mBSA (Figs. 7C and D).

4. Discussion

The resolution phase of acute self-limited inflammation has been thoroughly described in murine zymosan-induced peritonitis (Bannenberg et al., 2005; Stables and Gilroy, 2011). In that model, the induction of acute inflammation is characterized by a rapid increase in

neutrophils with subsequent influx of macrophages and this is followed by resolution, characterized by a marked decline in neutrophils and a change from inflammatory to resolution-type macrophages. This process is also reflected in changes in immune mediators, which evolve from inflammatory cytokines to anti-inflammatory cytokines and pro-resolution type mediators (Bannenberg et al., 2005; Stables and Gilroy, 2011). Since zymosan-induced peritonitis does not (at all) or minimally activate the adaptive immune response (Rajakariar et al., 2008) it may be important to also study resolution of inflammation in a murine model that involves the adaptive immune response. In the current study, we have examined the kinetics of cellular and immune mediator responses during the resolution phase of inflammation in murine mBSA-induced acute peritonitis.

The induction phase of the antigen-induced inflammation in the present study was more rapid (T_{max}=6 h) but less severe (Ψ_{max}=3.3x10^6) and resolved more rapidly (R_i=5 h) than the zymosan-induced peritonitis (T_{max}=12 h; Ψ_{max}=15x10^6; R_i=8 h) (Bannenberg et al., 2005). Two populations of neutrophils were observed in the present study, one population (N2), which included neutrophils that were larger and more granulated than the majority of the neutrophils in the other population (N1), and may have been in a more activated state, as they expressed more CD11b. We have previously described two circulating neutrophil populations in endotoxin-induced acute inflammation, but in that model peritoneal neutrophils were a homogenous population (Arnardottir et al., 2012). Several other studies have shown heterogeneous neutrophil populations with different phenotypes and roles, changing during the different phases of the inflammatory process (reviewed in (Beyrau et al., 2012)).

The resolution phase of inflammation is not only characterized by the disappearance of neutrophils from the inflammatory exudate but also by the emergence of resolution phase macrophages (Bystrom et al., 2008; Stables et al., 2011). In the present study the two populations of macrophages emerging following i.p. injection of mBSA, i.e. the F4/80^{high}

macrophages, appearing at 24 h and peaking at 48 h, and the F4/80^{low} macrophages, appearing at 48 h, both expressed high levels of molecules linked with resolution. The F4/80^{high} macrophages expressed high levels of the chemokine receptor CCR7 and the atypical chemokine receptor D6. CCR7 facilitates leukocyte migration to regional lymph nodes (Forster et al., 1999), hence the high expression of this chemokine receptor on macrophages present in the peritoneal cavity at 48 h in the present study may indicate a high potential of these macrophages to migrate to regional lymph nodes. D6 is a decoy receptor that binds most inflammatory CC chemokines and has been suggested to play a role in resolution of CC-chemokine-driven inflammatory responses (Jamieson et al., 2005). Support for a role for D6 in resolution comes from studies showing that D6 deficient mice have exaggerated inflammatory response (Jamieson et al., 2005; Di Liberto et al., 2008; Graham, 2009; Vetrano et al., 2010).

The F4/80^{high} macrophages present in the peritoneal cavity 48 h after induction of inflammation in the present study, had lower expression of CD11b than the resident peritoneal macrophages and may, therefore, be similar to the pro-resolving CD11b^{low} macrophages that were recently reported to emerge *in vivo* during resolution of zymosan-induced peritonitis (Schif-Zuck et al., 2011). In that study, macrophages converted from a CD11b^{high} to a CD11b^{low} phenotype upon interaction with apoptotic cells *ex vivo* (Schif-Zuck et al., 2011). Recognition of apoptotic cells by macrophages has also been shown to evoke signaling events that block the release of pro-inflammatory mediators from macrophages (Patel et al., 2006) and induce the production of TGF-β, resulting in accelerated resolution of inflammation (Huynh et al., 2002; Mitchell et al., 2002). In the present study TGF-β concentration peaked simultaneously with the number of F4/80^{high} macrophages, indicating active resolution of inflammation at 48 h after induction of inflammation in the antigen-induced peritonitis.

Although the F4/80^{low} macrophages expressed the DC marker CD11c, their expression of F4/80 and lack of CCR7 expression indicates that they are not typical antigen-presenting DCs (Dieu et al., 1998). These cells also expressed CD138, a surface heparin sulfate proteoglycan that has been shown to bind to and modulate the activity of chemokines, selectins, integrins and other adhesion molecules (Gotte, 2003; Gotte and Echtermeyer, 2003; Bartlett et al., 2007). CD138 has been proposed to play a role in resolution of inflammation by inhibiting leukocyte adhesion, reducing or inhibiting expression of pro-inflammatory factors or by removing inflammatory chemokines (Teng et al., 2012). Expression of CD138 on the F4/80^{med} macrophages was highest 5 and 10 days after induction of inflammation indicating that these macrophages are regulatory and may play a role in return to homeostasis.

In the present study, a considerable influx of eosinophils into the peritoneum was observed, already detectable 3 h after the initiation of inflammation and peaking at 48 h. The presence of eosinophils in an inflammatory exudate is conventionally believed to signify unwanted allergic type hypersensitivity reaction. This is unlikely to be the case in the mBSA-induced inflammation as the eosinophils emerge in a CFA-induced, Th1-polarized environment and BSA-specific IgE antibodies were hardly detectable in the serum of these mice at any time-point (data not shown). In allergic eosinophilic reactions, such as in asthma, there is commonly increased CD11b expression on eosinophils (Walker et al., 1993; Chiba et al., 2005), but the mean expression levels of CD11b on peritoneal eosinophils in the present study was decreased at the time when their numbers peaked. Neither is it likely that the eosinophils have a cytotoxic function or release cytotoxic granules in these mice, as their mean granularity did not change throughout the study.

Several observations in the present study support the view that the influx of eosinophils may have an important role in the resolution process. First, at the same time as the number of eosinophils increased in the peritoneum there was a reciprocal decrease in the number of

neutrophils, similar to that observed by (Yamada et al., 2011) using the zymosan-induced peritonitis model and demonstrating a resolution promoting role for the eosinophils; second, the eosinophils peaked in numbers at the same tima as the resolution type F4/80^{high} macrophages expressing D6 and CCR7; and third, the levels of the anti-inflammatory/immunosuppressive molecules TGF-β and sIL-6R reached maximum as the eosinophils peaked in numbers. The TGF-β may have been produced by the peritoneal eosinophils as eosinophils have been shown to be a major source of this immunosuppressive cytokine (Kadin et al., 1993).

A role for NK cells in resolution of inflammation has recently been suggested as they induce neutrophil apoptosis *in vitro* (Thoren et al., 2012) and are essential for effective resolution of allergic lung inflammation *in vivo* (Haworth et al., 2011). The kinetics of the NK response in the current study differed from that in the allergic lung inflammation study as the number of NK cells peaked earlier (24 h vs 48 h) and the increase in NK cell number was much greater in the current study (more than thirteen fold) (Haworth et al., 2011). In the present study, the number of NK cells peaked at the same time as the eosinophils and the resolving macrophages, suggesting that they may play a role in the resolution of inflammation.

The early infiltration of both T and B cells into the peritoneal cavity of the mice following induction of inflammation by i.p. injection of mBSA indicates involvement of the adaptive immune system in the antigen-induced response and the presence of memory lymphocytes. The number of T cells peaked earlier (48 h) than the number of B cells (5 days), perhaps reflecting the need of T cell-help for B cell activation. Similar to what has been reported for the mBSA-induced and zymosan-induced peritonitis (Cook et al., 2003), the majority of the peritoneal B cells, prior to and at all time-points during the inflammation, were B1 cells. Although B1 cells are known to produce high levels of polyspecific IgM antibodies,

both during homeostasis and infections, B1b cells are able to secrete antigen-specific antibodies following stimulation via the B cell receptor (Foote and Kearney, 2009). Therefore, the B1b cells in the present study are most likely the producers of the BSA-specific IgM antibodies observed in the serum.

The immunization with mBSA resulted in high levels of BSA-specific IgG antibodies in serum of the mice prior to induction of peritonitis. Upon induction of peritonitis the IgG levels dropped temporarily, undoubtedly because of immune complex formation between the IgG antibodies and the mBSA. These immune complexes are most likely the inducers of the peritonitis in the mice.

It has been demonstrated that resolution of inflammation is initiated within 6 hours in models of acute self-limited inflammatory response to carrageenan and zymosan (Bannenberg et al., 2005). In the current study the early rise in the concentration of the anti-inflammatory mediator IL-1ra, peaking at 6 h, indicates early induction of resolution of inflammation in the antigen-induced peritonitis model. Other immunosuppressive molecules that were analyzed, sTNF-R, sIL-6R, and TGF-β, peaked at 24-48 h, the time at which neutrophils were disappearing from the peritoneal cavity, and may have been involved in reducing their numbers as they have previously been associated with reducing neutrophil accumulation in peritoneal inflammation (Hurst et al., 2001).

In summary, we have delineated cellular and molecular responses in the induction, resolution and adaptive immune phases of the mBSA-induced peritonitis model. Our study suggests that numerous cell types play an active role in the resolution and adaptive phases of the immune response, including neutrophils, resolution-type macrophages, eosinophils, NK cells and B and T lymphocytes. Our results indicate that the mBSA-induced peritonitis model is an excellent model to study resolution of inflammation and the role of the adaptive immune responses in later stages of inflammation. We have previously used this model to demonstrate

the ability of dietary omega-3 polyunsaturated fatty acids to affect the induction, resolution and the adaptive immune phases of the inflammation (Tomasdottir et al., 2014; Tomasdottir et al., 2013).

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Figure legends

Fig. 1 Effects of mBSA administration on peritoneal cell populations. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points. Cells were stained with fluorochrome-labeled monoclonal antibodies against surface molecules as listed in Materials and methods and analyzed by flow cytometry. Cell numbers were determined using Countess automated cell counter. A Dot plots with forward and side scatter analysis of peritoneal leukocytes prior to and at different time-points following mBSA administration. Results are representative of 4 independent experiments. B Number of neutrophils, monocytes, macrophages, eosinophils and lymphocytes prior to and at different time-points following mBSA administration. Data are expressed as mean ± SEM, n= 4-7 per time-point. Insert shows the number of the peritoneal cells during the first 24 h following induction of inflammation. * Different from 0 h, # different from previous time-point, $P < 0.05$. L, Lymphocytes (identified by expression of B220, CD90.2 or NK1.1); Mφ, Macrophages (detected by expression of F4/80 and CD11b); N, Neutrophils (detected by expression of CD11b, Ly6G and CXCR2); E, Eosinophils (detected by expression of CCR3); M, Monocytes (detected by expression of CD115 and lack of expression of F4/80).

Fig. 2 Effects of mBSA administration on N1 and N2 neutrophils. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points. Cells were stained with fluorochrome-labeled monoclonal antibodies against CXCR2, Ly6G and CD11b and analyzed by flow cytometry. Cell numbers were determined using Countess automated cell counter. A Number of N1 and N2 neutrophils prior to and at different time-points following mBSA administration. Insert shows a dot plot with forward and side scatter analysis of neutrophils 6 h following mBSA

administration. B Mean fluorescence of CD11b, Ly6G and CXCR2 on N1 (thick black line) and N2 (thin black line) neutrophils 6 h following administration of mBSA. Data are expressed as mean ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$.

Fig. 3 Effects of mBSA administration on peritoneal macrophages. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points. Cells were stained with fluorochrome-labeled monoclonal antibodies against surface molecules as listed in Materials and methods and analyzed by flow cytometry. Circular gates are drawn around the different macrophage populations, expressing high or medium levels of F4/80 and at 5 days the F4/80^{high} macrophages are also divided into macrophages expressing medium and high levels of CD11b. A. FACS analysis of peritoneal leukocytes at different time-points prior to and following peritoneal mBSA administration. B. Mean fluorescence of D6, CCR7, CD11c and CD138 on F4/80^{high} (thick black line) and F4/80^{med} (thin black line) macrophages 48 h following administration of mBSA, with isotypic control being depicted in light grey. C. Mean expression levels of D6 and CCR7 on F4/80^{high} macrophages at different time-points following mBSA administration. D. Mean expression levels of CD138 on F4/80^{med} macrophages at different time-points, prior to and following mBSA administration. Data are expressed as mean ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$. MFI, Mean expression levels.

Fig. 4 Effects of mBSA administration on CCR3 and CD11b expression on peritoneal eosinophils. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points. Cells were stained with fluorochrome-labeled monoclonal antibodies against CCR3 and CD11b and analyzed by flow cytometry. Data are expressed as mean ± SEM, n= 4-7 per time-point. *

Different from 0 h, # different from previous time-point, $P < 0.05$. MFI, mean expression levels.

Fig. 5 Effects of mBSA administration on peritoneal lymphocytes. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points. Cells were stained with fluorochrome-labeled monoclonal antibodies against surface molecules as listed in Materials and methods and analyzed by flow cytometry. A. Number of NK cells prior to and at different time-points following mBSA administration. B. Number of T cells prior to and at different time-points following mBSA administration. C. Number of B cells prior to and at different time-points following mBSA administration. Data are expressed as mean ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$.

Fig. 6 Effects of mBSA administration on serum levels of mBSA specific IgM and IgG. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Serum was collected at indicated time-points and concentrations of BSA-specific IgM and IgG antibodies measured by ELISA. Values are in arbitraty units (AU). Data are expressed as mean ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$.

Fig. 7 Effects of mBSA administration on peritoneal concentrations of cytokines, chemokines and soluble cytokine receptors. Mice were immunized twice with mBSA and injected one week later intraperitoneally with mBSA. Peritoneal fluid was collected at indicated time-points and concentrations of cytokines, chemokines and soluble cytokine receptors measured by ELISA. A. IL-1β, IL-6 and IL-12. B. CCL2, CCL3 and CCL11 (eotaxin1). C. sIL-6R and sTNF-R1. D. IL-1ra and TGF-β. Data are expressed as mean ± SEM, n= 4-7 per time-point. * Different from 0 h, # different from previous time-point, $P < 0.05$.

Figure 1

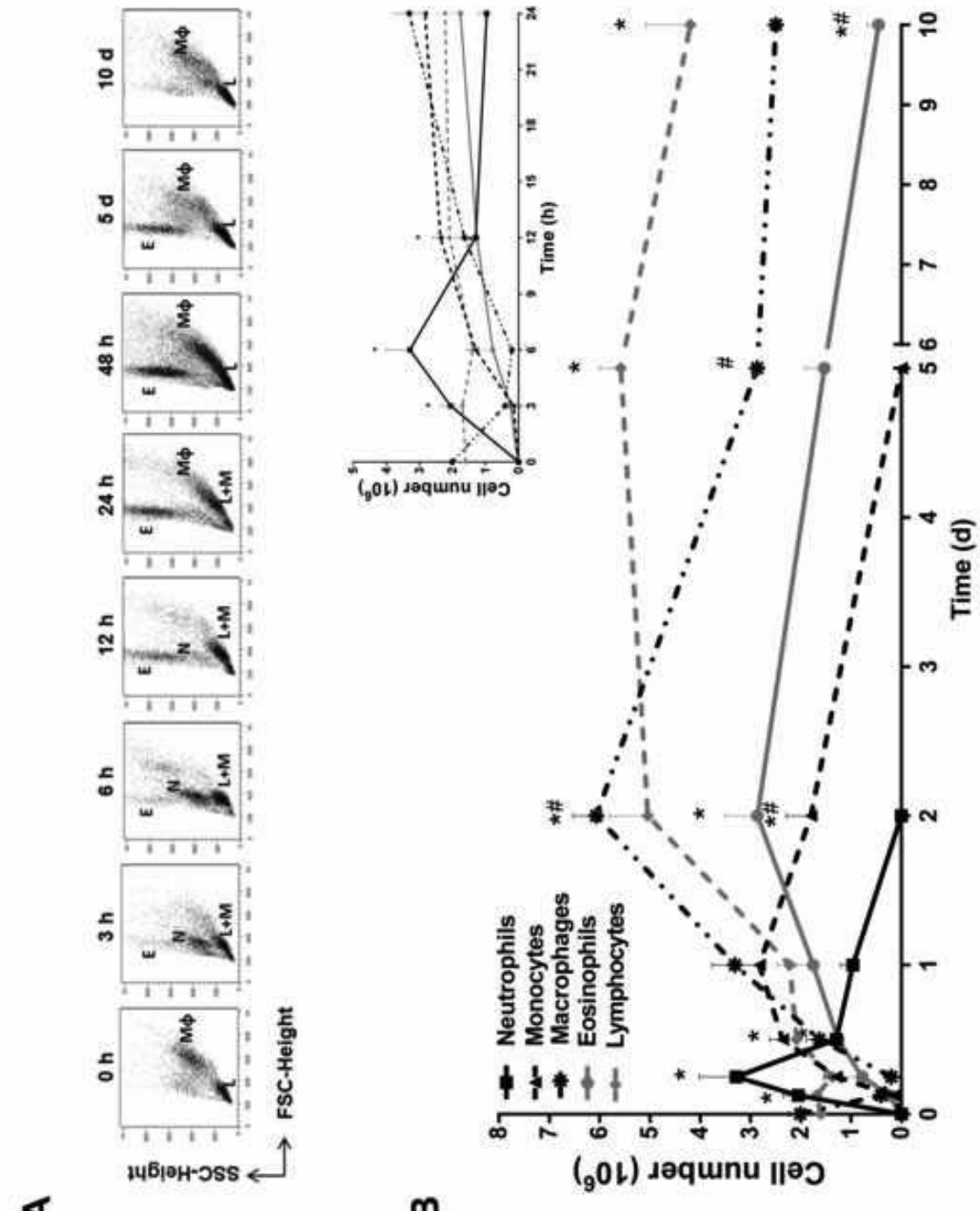

A
0 h
3 h
6 h
12 h
24 h
48 h
5 d
10 d
SSC-Height
FSC-Height
B
Neutrophils
Monocytes
Macrophages
Eosinophils
Lymphocytes
Cell number (10⁶)
Time (d)
Time (h)

Figure 2

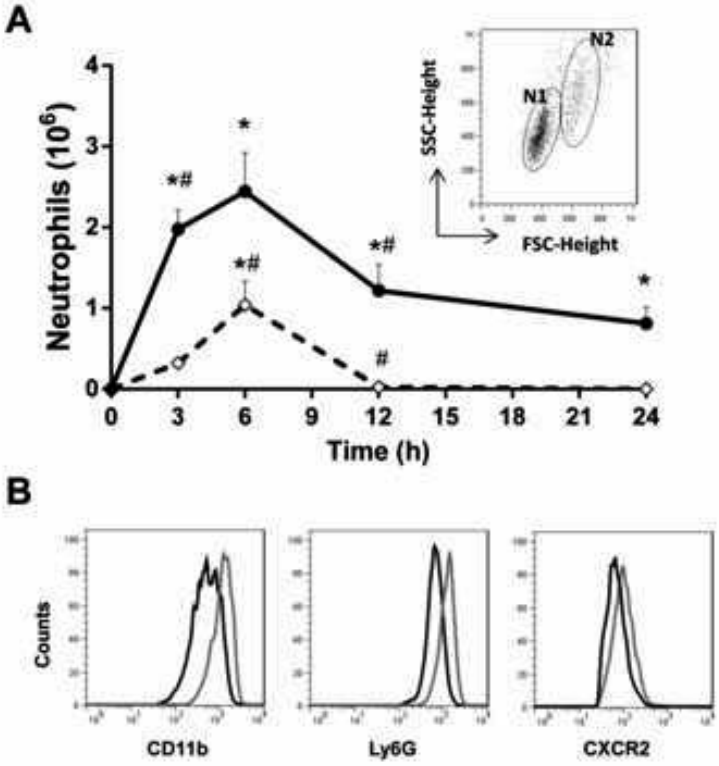

A
Neutrophils (10^6)
Time (h)
SSC-Height
FSC-Height
N1
N2
B
Counts
CD11b
Ly6G
CXCR2

Figure 3

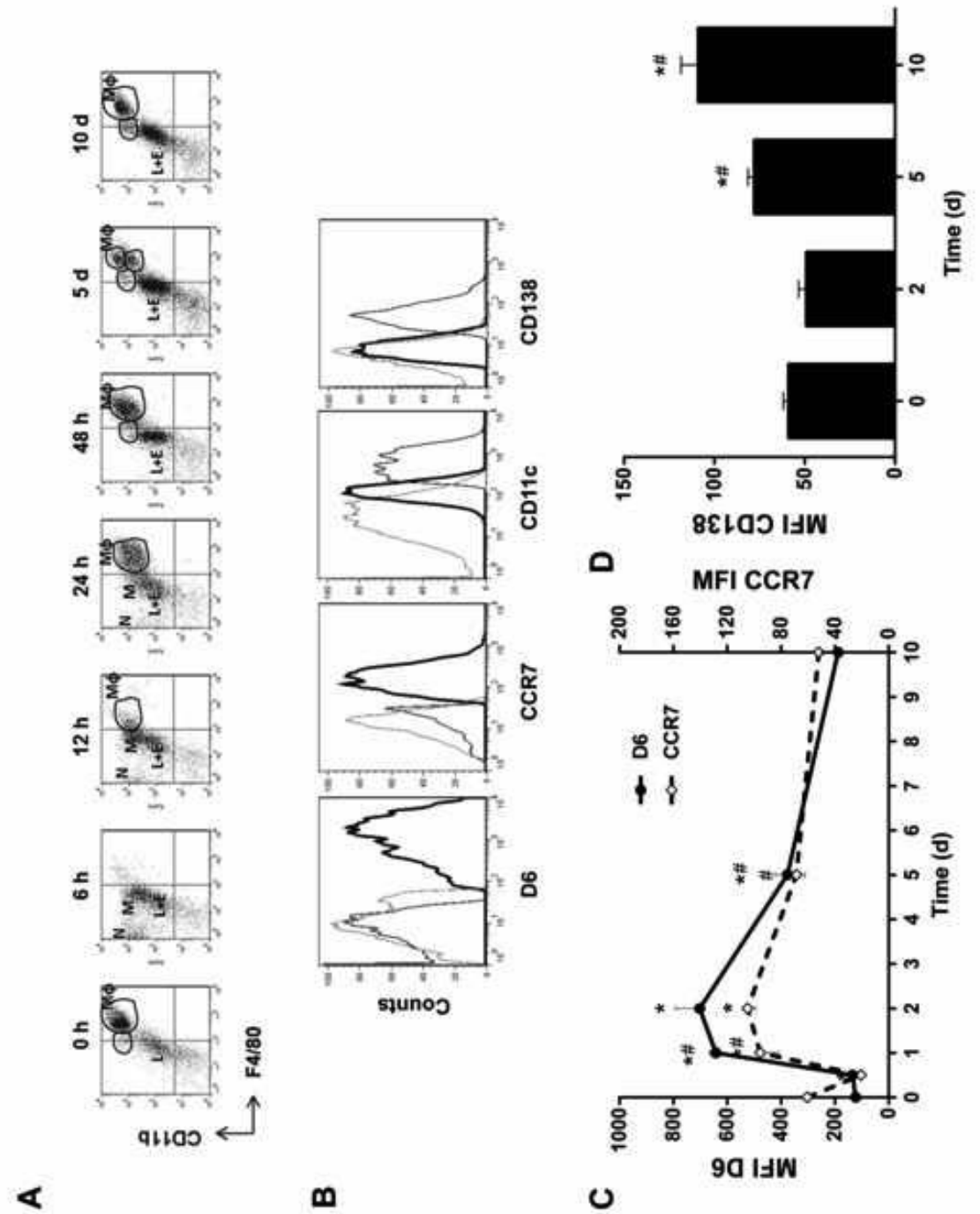

A
0 h
6 h
12 h
24 h
48 h
5 d
10 d
CD11b
F4/80
B
Counts
D6
CCR7
CD11c
CD138
C
MFI D6
D6
CCR7
Time (d)
D
MFI CD138
Time (d)

Figure 4

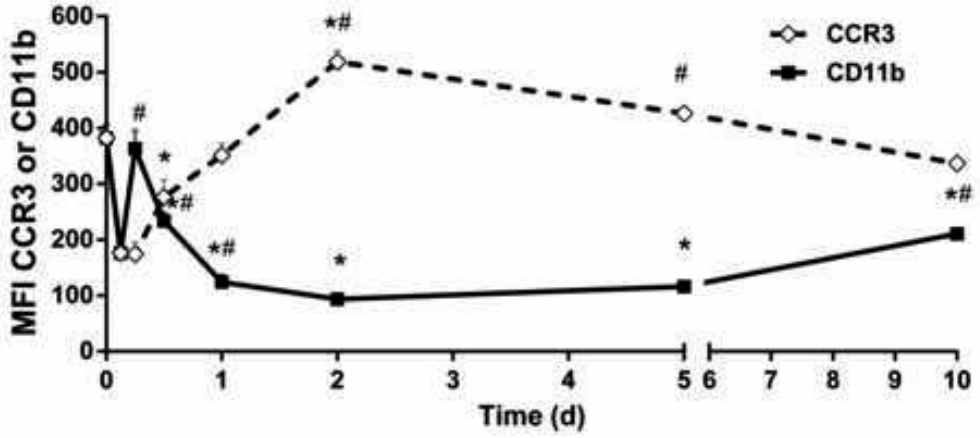

MFI CCR3 or CD11b
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
Time (d)
CCR3
CD11b
*#
#
*
*#
*#
*
#
*
*#

Figure 5

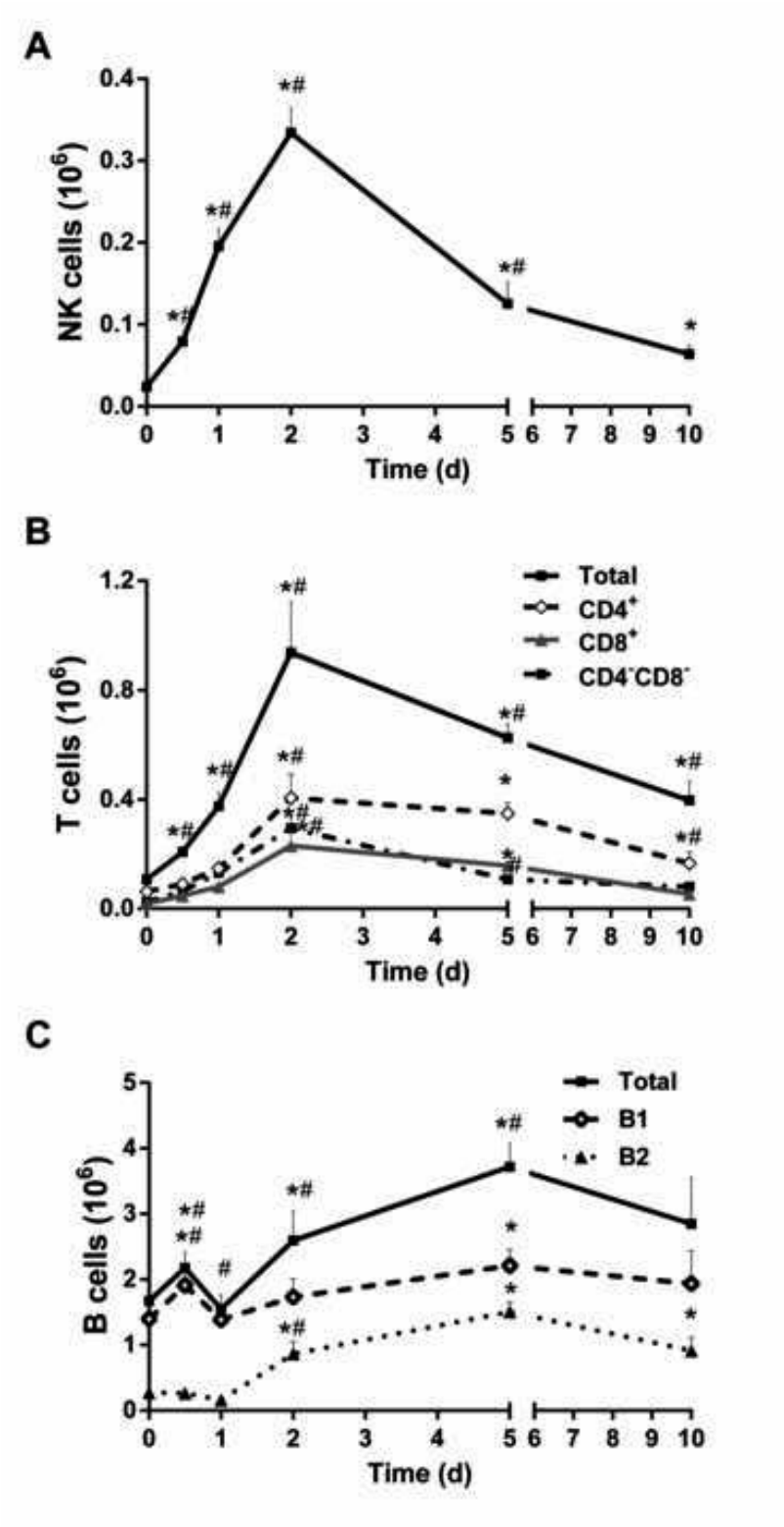

A
NK cells (10^6)
0.0
0.1
0.2
0.3
0.4
0 1 2 3 4 5 6 7 8 9 10
Time (d)
B
T cells (10^6)
0.0
0.4
0.8
1.2
Total
$CD4^+$
$CD8^+$
$CD4^-CD8^-$
0 1 2 3 4 5 6 7 8 9 10
Time (d)
C
B cells (10^6)
0
1
2
3
4
5
Total
B1
B2
0 1 2 3 4 5 6 7 8 9 10
Time (d)

Figure 6

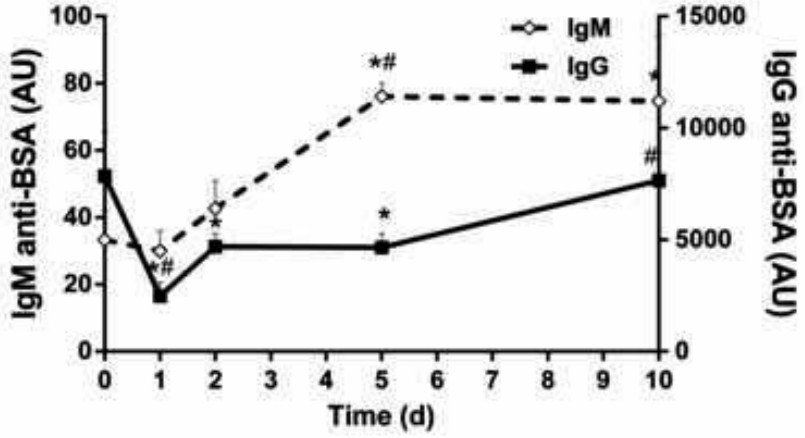
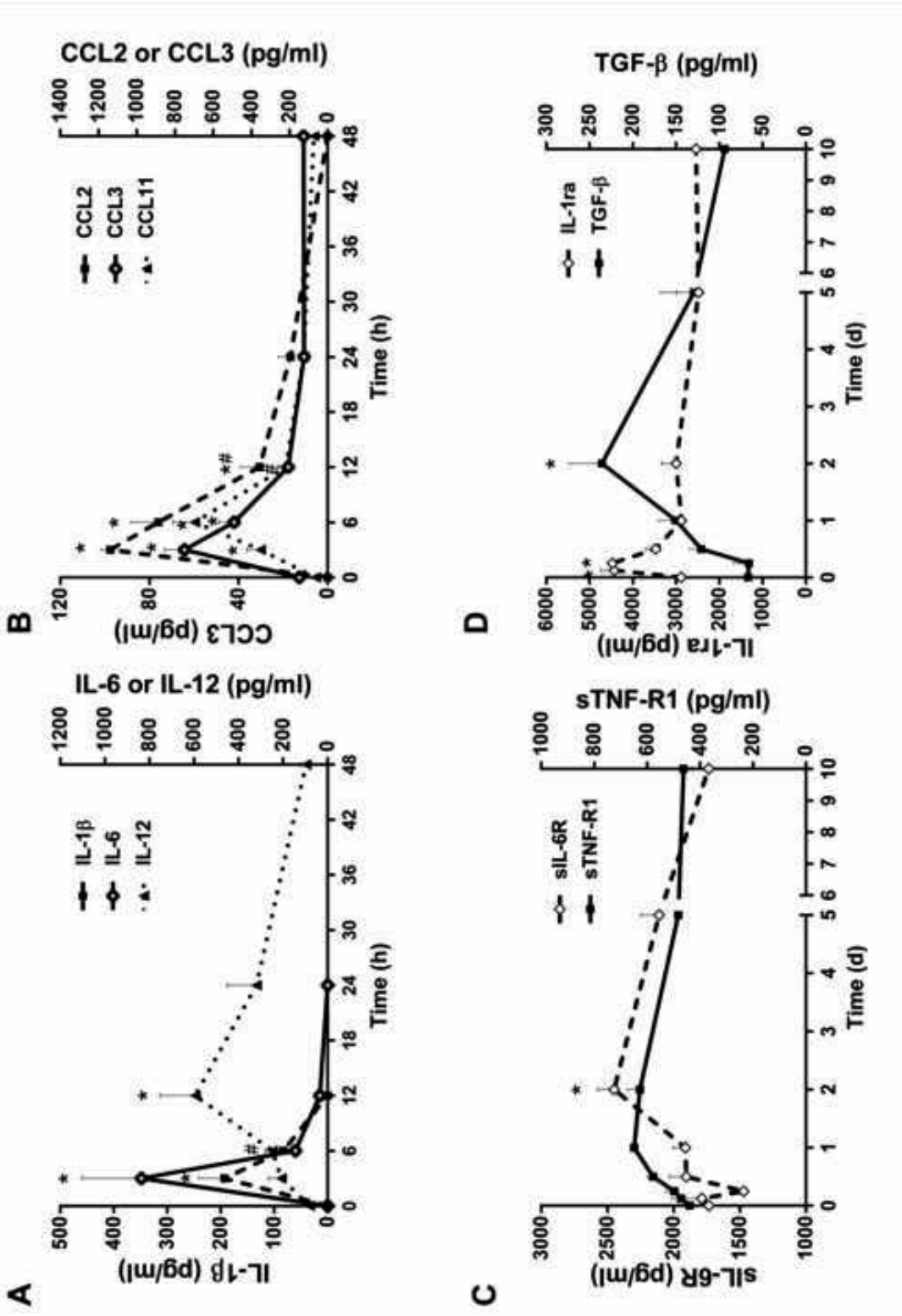

IgM
IgG
IgM anti-BSA (AU)
IgG anti-BSA (AU)
Time (d)
100
80
60
40
20
0
15000
10000
5000
0
0 1 2 3 4 5 6 7 8 9 10
*#
*
#

Figure 7


A
IL-1β (pg/ml)
IL-6 or IL-12 (pg/ml)
IL-1β
IL-6
IL-12
Time (h)
B
CCL3 (pg/ml)
CCL2 or CCL3 (pg/ml)
CCL2
CCL3
CCL11
Time (h)
C
sIL-6R (pg/ml)
sTNF-R1 (pg/ml)
sIL-6R
sTNF-R1
Time (d)
D
IL-1ra (pg/ml)
TGF-β (pg/ml)
IL-1ra
TGF-β
Time (d)

Paper II

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Dietary fish oil reduces the acute inflammatory response and enhances resolution of antigen-induced peritonitis☆

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Abstract

Dietary n-3 polyunsaturated fatty acids (PUFA) influence the inductive phase of inflammation but less is known about their effects on the resolution phase. This study examined the effects of dietary fish oil on induction and resolution of antigen-induced inflammation in mice. Mice were fed a control diet with or without 2.8% fish oil, immunized twice with methylated BSA (mBSA) and inflammation induced by intraperitoneal injection of mBSA. Prior to and at different time points after mBSA administration, peritoneal cells were analyzed and expression of surface molecules determined by flow cytometry. Concentration of chemokines, cytokines and soluble cytokine receptors was determined by ELISA. Mice fed the fish oil diet had fewer peritoneal neutrophils, shorter resolution interval and lower levels of pro-inflammatory cytokines and chemokines than mice fed the control diet. In mice fed the fish oil diet there was an early peak in peritoneal levels of the immunosuppressive molecules sIL-6R and TGF-β, that was not seen in mice fed the control diet. In the resolution phase, peritoneal macrophages from mice fed the fish oil diet expressed more of the atypical chemokine receptor D6 and peritoneal TGF-β levels were higher than that in mice fed the control diet. Furthermore, in the late-resolution phase there were more peritoneal eosinophils and macrophages in mice fed the fish oil diet than in mice fed the control diet. These results demonstrate a suppressive effect of n-3 PUFA on the inductive phase of inflammation and indicate an enhancing effect of n-3 PUFA on resolution of inflammation.

Keywords: Fish oil; mBSA; Antigen-induced inflammation; Macrophages; Eosinophils

1. Introduction

Dietary n-3 polyunsaturated fatty acids (PUFA) modulate inflammatory responses and are generally thought to be anti-inflammatory. Inflammation underlies many common conditions and diseases, such as rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, cancer and Alzheimer's disease. Recent studies indicate that some of these diseases can be ameliorated by increased consumption of n-3 PUFA [1–7].

Inflammation is characterized by a complex sequence of events involving an inductive phase followed by a resolution phase that is important for return to homeostasis. During the inductive phase pro-inflammatory eicosanoids and cytokines are generated and neutrophils are recruited to the inflamed site. Dietary n-3 PUFA have been shown to beneficially affect generation of pro-inflammatory eicosanoids and cytokines [8]. N-3 PUFA have also been shown to hinder neutrophil adhesion and migration in vitro [9–11] and we recently demonstrated fewer neutrophils at the inflamed site during the inductive phase of peritonitis in mice [12].

Resolution of inflammation is an active process and a necessary step in controlling inflammation [13]. Efficient resolution of inflammation depends on inhibition of neutrophil influx, promotion of monocyte recruitment and their development into macrophages, rapid clearance of apoptotic neutrophils and regeneration of disrupted tissue structures [14,15]. Specialized pro-resolving mediators, resolvins (Rvs), protectins (PDs) and maresins aid in the resolution of inflammation [16], and these are generated from the long chain n-3 PUFA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Therefore, it has been assumed that dietary n-3 PUFA are important for resolution of inflammation, but studies examining their effect as dietary supplement on the resolution phase of inflammation are lacking. Resolution of inflammation is necessary

Abbreviations: DHA, docosahexaenoic acid; C, control; CRP, C-reactive protein; EPA, eicosapentaenoic acid; FO, fish oil; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; i.p, intraperitoneal; mBSA, methylated BSA; mean fluorescence intensity, MFI; NK cells, natural killer cells; PBS, phosphate buffered saline; PDs, protectins; PUFA, polyunsaturated fatty acids; Rvs, resolvins; R_i, resolution interval; SEM, standard error of the mean.

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to minimize inflammation-related collateral damage and to promote tissue healing. Absent or insufficient resolution of inflammation may result in chronic inflammation, which can have serious consequences as chronic inflammation is now being recognized as an important component in the pathogenesis of many of the most prevalent chronic conditions, including atherosclerosis, cancer and Alzheimer's disease, as well as immune-mediated diseases, s.a. rheumatoid arthritis, asthma and inflammatory bowel disease.

The aim of this study was to determine the effects of dietary fish oil on the induction and resolution of inflammation in a methylated BSA (mBSA)-induced peritonitis, a model for subacute/chronic inflammation [17].

2. Methods and materials

2.1. Animals and diets

Female C57BL/6 mice, weighing 18–20 g, were purchased from Taconic Europe (Ejby, Denmark). All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with National Research Council's Guide for the Care and Use of Laboratory Animals [18]. Mice were housed 8 per cage with a 12 h light/dark cycle at 23–25°C and 45–55% humidity. Mice were acclimated for 1 week prior to initiation of the experiments, whereafter they were divided into two groups that were fed either a control (C) diet or a fish oil (FO) diet, starting 1 week before the immunization protocol and continuing throughout the experiment (4–5 weeks). The composition of the control diet was based on a typical American diet, i.e., the "US17" diet formulated by Monsanto (St. Louis, MO, USA) and Research Diets Inc (D07121302; Research Diets, New Brunswick, NJ, USA) with minor modification by the authors, as previously described [19]. Energy distribution of the diet was as follows: carbohydrate, 44%; fat, 35%; protein, 21%. The FO diet contained 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA, USA), which was added at the expense of safflower oil (Welch, Holme & Clark, Newark, NJ, USA). Arachidonic acid ethyl ester (Nu-Check-Prep, Elysian, MN, USA) (0.5 g/kg) was added to the control diet to adjust for the arachidonic acid content in the FO diet. In brief, the FO diet contained 10.6 g/kg n-3 PUFA (4.0 g/kg EPA and 2.5 g/kg DHA) and the C diet 3.4 g/kg n-3 PUFA (undetectable levels of EPA and DHA). All mice were provided fresh food daily and had free access to food and water. We have previously shown that healthy mice receiving the FO diet had a higher proportion of n-3 fatty acids and a higher ratio of n-3:n-6 PUFA in hepatic phospholipids than healthy mice receiving the C diet [19].

2.2. Antigen-induced peritonitis

Mice were immunized subcutaneously at the base of the tail with an emulsion containing 100 μg of mBSA (Sigma Aldrich, St. Louis, MO, USA) in an equal volume of complete Freund's adjuvant (Sigma Aldrich). Booster injection of 100 μg of mBSA in incomplete Freund's adjuvant (Sigma Aldrich) was given 2 weeks later. Three weeks after the initial immunization, peritonitis was induced by intraperitoneal (i.p.) injection of 100 μg of mBSA in saline. Prior to and at several time points after mBSA administration (3 h, 6 h, 12 h, 24 h, 48 h, 5 days and 10 days), mice were anesthetized with a (1:1) mixture of hypnorm (VetaPharma Ltd, Leeds, UK) and dormicum (Roche, Basel, Switzerland) and killed by cervical dislocation.

2.3. Collection of peritoneal lavage and spleen

Peritoneal fluid and cells were collected in 1.5 ml of cold phosphate buffered saline (PBS) without calcium or magnesium. Cells and fluid were separated by centrifugation and the peritoneal fluid stored at −70°C until analysis. The cells were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% BSA, 0.01% NaN_3). Peritoneal cells were counted by Countess automated cell counter (Invitrogen, Paisley, UK).

Spleens were removed postmortem and passed through a cell strainer (BD Bioscience, San Jose, CA, USA) to obtain a single cell suspension. Red blood cells were lysed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) and the cells were washed and resuspended in 5 ml Dulbecco's modified Eagle medium (Invitrogen). Spleen cells were counted by Countess automated cell counter.

2.4. Phenotypic characterization of leukocytes by flow cytometry

Peritoneal cells (3×10^5) and spleen cells (2.5×10^5) were incubated with a 1:1 mixture of 2% normal rat: normal mouse serum (AbD Serotec, Kidlington, UK) for 20 min. Peritoneal cells were stained with fluorochrome-labeled monoclonal antibodies against CD11b, F4/80, B220, CD90.2, NK1.1, CD117 (eBioscience, San Diego, CA, USA), Ly6G (clone 1A8), CXCR2, CD11b, F4/80, CCR7 (BD Biosciences), and CCR3, D6, CXCR2 (R&D Systems, Abington, UK). Antibodies against B220 (B cells), CD90.2 (T cells), NK1.1 (NK cells) were used for lymphocyte exclusion. NK cells were identified as $NK1.1^+$ cells, neutrophils as $Ly6G^+CXCR2^+$ cells, eosinophils as $CCR3^+$ cells, macrophages as $F4/80^{high}CD11b^+$ or $F4/80^{low}CD11b^+$ cells, and mast cells as $CD117^+$ cells. Eosinophils in spleens were stained with CCR3 and lymphocytes excluded as before. All samples were washed and resuspended in FACS buffer. Appropriate isotypic controls were used to set the quadrants and evaluate background staining. Samples were collected on FACScalibur (BD Biosciences) and data analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA). The results were expressed as percentage positive cells, mean fluorescence intensity (MFI) or total number of positive cells. The resolution interval (R_i) was determined as the time interval within which peak number of inflammatory cells in the peritoneum were reduced by 50% [20].

2.5. Chemokine and cytokine analysis

Cytokines/growth factors (IL-1β, IL-6, G-CSF and TGF-β), soluble cytokine receptors/agonists (IL-1ra, sIL-6R and sTNFR) and chemokines [CCL2, CCL3, CCL11 (eotaxin-1), CXCL1 (KC), and CXCL2] were measured in peritoneal fluid using Duoset ELISA kits (R&D Systems).

2.6. Data analysis

Data are expressed as mean values±standard error of the mean (S.E.M.). As the data were not distributed normally, Kruskal-Wallis nonparametric ANOVA was used to calculate overall *P* values. The nonparametric Mann–Whitney Rank Sum test was used to determine whether differences between the two dietary groups were statistically significant at a single time point. Statistical analysis was performed using SigmaStat software, version 3.2 (Systat software Inc., Chicago, IL, USA). $P<.05$ was considered significant. Values are means±S.E.M., $n=4$ for the 6 h and the 10 day time points and 7 for all other time points, making the total number of mice 100. Two other experiments with selected time points gave similar results.

3. Results

3.1. Mouse growth, dietary intake and peritoneal cell count

There was no difference in body weights of the mice or in daily food intake between the two dietary groups. There were more peritoneal cells in mice fed the FO diet ($15.6\pm1.3\times10^6$) than in mice fed the C diet ($11.0\pm0.9\times10^6$, $P<.05$) 5 days after administration of mBSA but no difference in total peritoneal cell number at other time points (data not shown).

3.2. Effects of dietary fish oil on peritoneal mast cells

Mast cells were present in the peritoneum of mice prior to induction of inflammation. There was a tendency towards more mast cells in the peritoneum of mice fed the FO diet than in peritoneum of mice fed the C diet ($5.7\pm0.6\times10^4$ vs. $4.1\pm0.5\times10^4$, $P=.06$).

3.3. Effects of dietary fish oil on peritoneal neutrophils

Administration of mBSA resulted in an influx of neutrophils into the peritoneum. Mice fed the FO diet had fewer neutrophils in their peritoneum than mice fed the C diet, at the time when the number of neutrophils in the peritoneum peaked (3 h for mice fed the FO diet, 6 h for mice fed the C diet) (Fig. 1A). The R_i was also shorter for mice receiving the FO diet (3 h) than for mice receiving the C diet (5 h) (Fig. 1A). Neutrophils from mice fed the FO diet had higher mean expression levels of the integrin CD11b 6 h after administration of mBSA than neutrophils from mice fed the C diet, although there were similar CD11b expression levels on neutrophils from mice in both dietary groups at 3 h, the time point at which CD11b expression peaked (Fig. 1B). At 24 h neutrophils from mice fed the fish oil diet did not express CD11b, whereas neutrophils from mice fed the control diet had similar expression of CD11b at 24 h as they had at 12 h after administration of mBSA.

The peritoneal neutrophils formed two distinct populations that differed in size and granularity. The majority (~90%) of the neutrophils belonged to a population (N1) that was smaller (FSC: 376±3) and less granular (SSC: 389±5) than the other population (N2) (FSC: 621±2, $P<.001$; SSC: 621±7, $P<.001$). The N2 neutrophils expressed more of the surface molecules CXCR2 (MFI: 102±5 vs. 66±

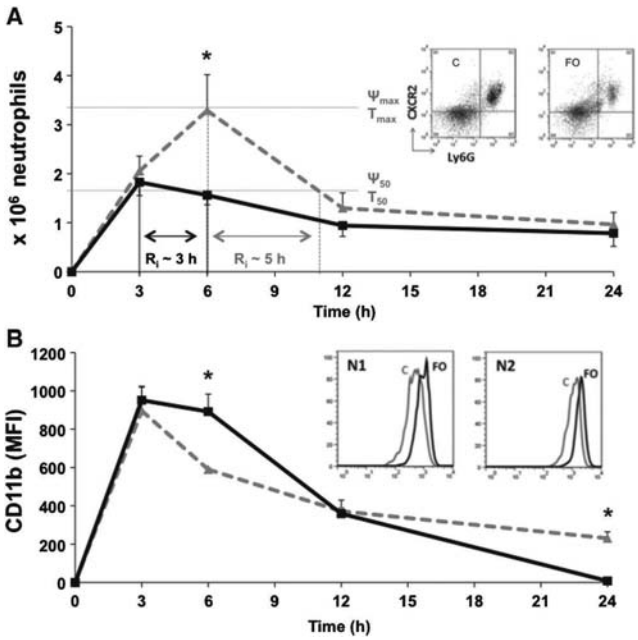

Fig. 1. Effects of dietary fish oil on the number of peritoneal neutrophils (A) and mean expression (MFI) of CD11b on neutrophils (B) at different time points after administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. Inserts are representative dot plots of peritoneal neutrophils ($CXCR2^+Ly6G^+$) from mice fed the C and the FO diets 6 h after administration of mBSA (A) and mean fluorescence of CD11b on N1 and N2 neutrophils from mice fed the C (grey line) and the FO (black line) diets 6 h after administration of mBSA (B). R_i, resolution interval; T_{max}, the time point when the neutrophil number reach maximum; T_{50}, the time point when the neutrophil number is reduced by 50%; Ψ_{max}, the maximum number of neutrophils, Ψ_{50}, half maximum number of neutrophils. *Different from control, $P<.05$.

4, $P=.004$), CD11b (MFI: 1067±25 vs. 474±12, $P<.001$) and Ly6G (MFI: 1097±66 vs. 686±75, $P=.015$). In mice fed the FO diet both N1 and N2 neutrophils had higher expression levels of CD11b than N1 and N2 from mice fed the C diet (Fig. 1B, insert).

3.4. Effects of dietary fish oil on peritoneal NK cells

There were few NK cells ($NK1.1^+$) in the peritoneum of mice prior to administration of mBSA but their number increased rapidly after induction of inflammation (Fig. 2). At 12 h after administration of mBSA there were more NK cells present in the peritoneum of mice fed the FO diet than in mice fed the C diet. However, there were fewer NK cells in peritoneum of mice fed the FO diet than in mice fed the C diet when the NK cells peaked, i.e., at 2 days after administration of mBSA (Fig. 2). At 10 days the number of NK cells in mice fed the C diet had nearly reached the number observed prior to administration of mBSA, whereas the number of NK cells in the peritoneum of mice fed the FO diet still remained high (Fig. 2).

3.5. Effects of dietary fish oil on peritoneal macrophages

The majority (90%) of the macrophages present in the peritoneum prior to induction of inflammation were $F4/80^{high}CD11b^{high}CD138^-$, with the rest being $F4/80^{low}CD11b^{high}CD138^+$. There was no difference in the number (Fig. 3A) or the proportion (data not shown) of the two macrophage populations in the peritoneum of healthy mice fed the different diets. After administration of mBSA, the $F4/80^{high}$ macrophages almost disappeared from the peritoneal cavity but new macrophages, developing from recruited monocytes, peaked at day 2 (Fig. 3A). At that time point there was no difference in the number of $F4/80^{high}$ macrophages in the peritoneum of mice fed the

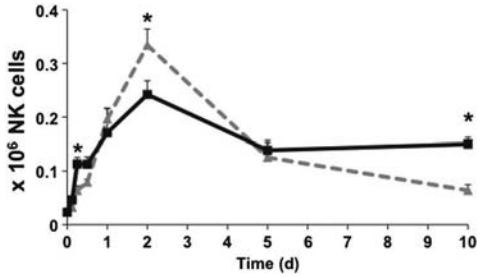

Fig. 2. Effects of dietary fish oil on the number of peritoneal NK cells prior to and at different time points after administration of mBSA. Mice received control diet (C, grey dashed line) or fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. *Different from control, $P<.05$.

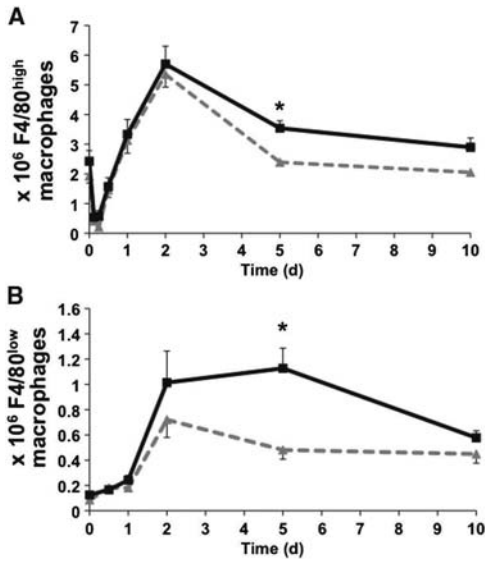

Fig. 3. Effects of dietary fish oil on the number of F4/80^{high} (A) and F4/80^{low} (B) peritoneal macrophages prior to and at different time points after administration of mBSA. Mice received control diet (C, grey dashed line) or fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. with mBSA 1 week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. *Different from control, $P<.05$.

two diets (Fig. 3A). However, at day 5 when the number of F4/80^{high} macrophages had started to decline, there were more macrophages in the peritoneum of mice fed the FO diet than in mice fed the C diet and at day 10 a tendency ($P=.064$) towards more macrophages being present in the peritoneum of mice fed the FO diet than in mice fed the C diet (Fig. 3A). The number of F4/80^{low} macrophages peaked 2 days after mBSA administration in mice fed the C diet but 5 days after mBSA administration in mice fed the FO diet, with the number of F4/80^{low} macrophages being more than twofold in mice fed the FO diet compared with that in mice fed the C diet (Fig. 3B).

Peak expression levels of F4/80 on F4/80^{high} macrophages from mice fed the two diets were similar, but were reached earlier (2 days after administration of mBSA) on F4/80^{high} macrophages from mice fed the FO diet than in mice fed the C diet (5 days after administration of mBSA) (Fig. 4A). Expression levels of the chemokine receptor CCR7 and the atypical chemokine receptor D6 peaked 2 days after administration of mBSA on F4/80^{high} macrophages from mice from both dietary groups, but F4/80^{high} macrophages from mice fed the FO diet had higher mean expression of both receptors at that time point (Figs. 4B and C). There was no difference in expression levels of F4/80 and CD138 on F4/80^{low} macrophages from mice fed the FO and the C diets (data not shown).

3.6. Effects of dietary fish oil on peritoneal eosinophils

Eosinophils appeared in the peritoneum 3 h after administration of mBSA (Fig. 5A). Their number peaked 2 days after administration of mBSA in mice receiving the C diet but 5 days after administration of mBSA in mice fed the FO diet, with their number at that time being more than double that in mice fed the C diet (Figs. 5A and B). The number of eosinophils was still higher at 10 days in mice fed the FO diet than in mice fed the C diet. Eosinophil expression of CD11b peaked 6 h after administration of mBSA, whereas CCR3 expression peaked at day 2 (data not shown). There was no difference in CD11b or CCR3 expression of peritoneal eosinophils from mice fed the different diets at any time point studied (data not shown).

3.7. Effects of dietary fish oil on pro- and anti-inflammatory mediators in the peritoneal fluid

Peritoneal concentration of the neutrophil chemoattractant CXCL1 and the pro-inflammatory mediator IL-6 peaked at 3 h and were at that time point lower in mice fed the FO diet than in mice fed the C diet (Figs. 6A and B). Peritoneal concentration of the neutrophil growth factor and regulator G-CSF peaked at 3 h in mice fed the FO diet but at 6 h in mice fed the C diet (Fig. 6C). At that time point the concentration of G-CSF was much less in peritoneum from mice fed

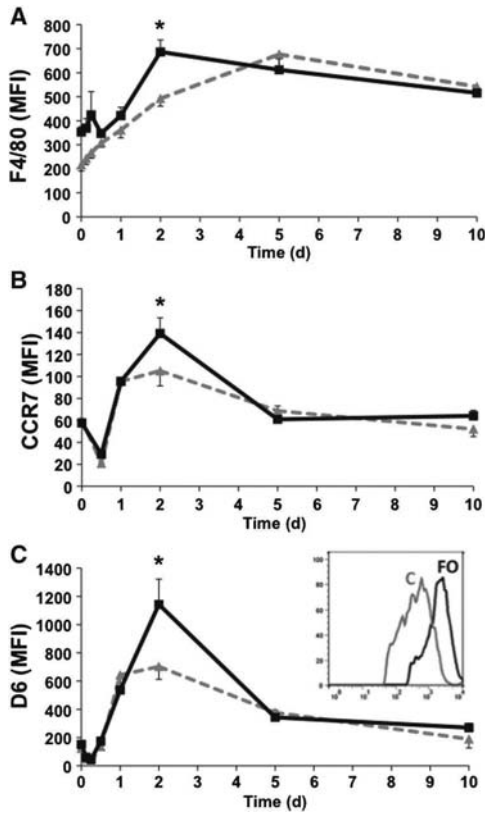

Fig. 4. Effects of dietary fish oil on mean expression levels (MFI) of F4/80 (A), CCR7 (B) and D6 (C) on F4/80^{high} peritoneal macrophages prior to and at different time points after administration of mBSA. Mice received control diet (C, grey dashed line) or fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. with mBSA 1 week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were stained with monoclonal antibodies and analyzed by flow cytometry. Insert shows MFI of D6 on F4/80^{high} macrophages from mice fed the C (grey line) and the FO (black line) diets 2 days after induction of inflammation. *Different from control, $P<.05$.

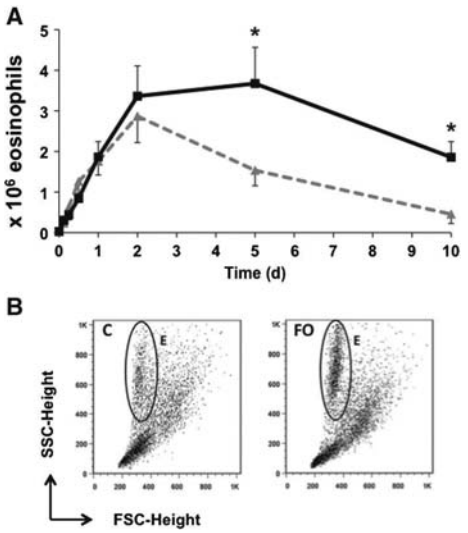

Fig. 5. Effects of dietary fish oil on the number of peritoneal eosinophils prior to and at different time points after administration of mBSA (A). Mice received control diet (C, grey dashed line) or fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. Representative forward and side scatter dot plots of peritoneal eosinophils ($CCR3^+B220^-CD90.2^-NK1.1^-$) from mice fed the C and the FO diets 6 h after mBSA administration (B). *Different from control, $P<.05$.

the FO diet than that in mice fed the C diet. Peritoneal concentration of the eosinophil chemoattractant CCL11 was much lower in mice fed the FO diet than that in mice fed the C diet at the time when its concentration peaked in mice fed the C diet (6 h) (Fig. 6D). Dietary fish oil did not affect peritoneal concentrations of CCL2, CCL3, IL-1β and IL-12 (data not shown).

In mice fed the FO diet peritoneal concentration of sIL-6R increased 6 h after mBSA administration, whereas at that time point there was a decrease in peritoneal concentration of sIL-6R in mice fed the C diet (Fig. 6E). Peritoneal concentration of sIL-6R peaked again at 2 days in mice fed the FO diet and at that time point it also peaked in mice fed the C diet, with the levels of sIL-6R being similar at that time point in peritoneum from mice in both dietary groups (Fig. 6E). Peritoneal levels of the anti-inflammatory cytokine TGF-β peaked 6 h after administration of mBSA in mice fed the FO diet, but had not changed at that time point from the level prior to mBSA administration in mice fed the C diet (Fig. 6F). TGF-β peaked again 2 days after administration of mBSA in mice fed the FO diet and also peaked at that time point in mice fed the C diet (Fig. 6F). Peritoneal concentration of IL-1ra peaked at 3 h in mice fed the FO diet but at 6 h in mice fed the C diet, but there was no difference in peak levels of IL-1ra between mice fed the different diets (data not shown). Peritoneal levels of sTNFR peaked at 48 h in mice fed the FO diet but at 24 h in mice fed the C diet, with no difference in peak concentrations of sTNFR between mice fed the different diets (data not shown).

4. Discussion

In the present study fewer neutrophils and less pro-inflammatory mediators were observed in the inductive phase of acute inflammation in mice fed the FO diet than in mice fed the C diet. In addition, an early peak in NK cell number and in the concentration of the anti-inflammatory/immunosuppressive molecules sIL-6R and TGF-β was observed in peritoneum of mice fed the FO diet but not in mice fed the C diet. Furthermore, in the resolution phase of acute inflammation, macrophages from mice fed the FO diet expressed more of the atypical chemokine receptor D6 and peritoneal concentration of sIL-6R and TGF-β were higher than that in mice fed the C diet. Finally, during the late resolution phase the number of macrophages and eosinophils were higher in mice fed the FO diet than in mice fed the C diet.

Although previous studies have indicated that n-3 PUFA can suppress the inflammatory response [21], it has not previously been shown that dietary n-3 PUFA can decrease neutrophil infiltration into an inflammatory site in vivo. Previous studies, however, have demonstrated that n-3 PUFA and mediators derived from them reduce neutrophil adhesion and migration in vitro [9–11] and that the n-3 PUFA derived pro-resolving mediators RvE1, RvD2 and PD1 reduce neutrophil recruitment to the inflamed site in vivo [20,22,23]. Furthermore, we have recently shown in a murine model of endotoxin-induced inflammation that the early influx of neutrophils into the peritoneal cavity was delayed in mice fed a diet rich in n-3 PUFA [12]. In the present study FO fed mice had both a lower peak of neutrophil infiltration into the peritoneum and accelerated resolution of the neutrophil infiltrate, as indicated by the shorter R_i. In fact, the lowering in peak neutrophil number and the shortening of the R_i is similar to what has been shown following administration of the n-3 derived pro-resolving mediators RvE1 and PD1 in zymosan-induced peritonitis [20]. Thus, the effects of the FO diet may be mediated by elevated RvE1 and/or PD1 concentration, resulting from more substrate being available for their production in mice fed the FO diet than in mice fed the C diet. These and other pro-resolving lipid mediators will be measured in follow-up studies.

In the present study, peak concentrations of the chemoattractant and growth factor for neutrophils, CXCL1 and G-CSF, respectively, were lower in peritoneum of mice fed the FO diet than in mice fed the C diet. Therefore, the lower peak number of neutrophils in the peritoneum of mice fed the FO diet may be the result of less release of neutrophils from the bone marrow and less recruitment of neutrophils into the peritoneum than in mice fed the C diet.

Neutrophils express the integrin CD11b, which may help in mediating their adhesion to the endothelium and migration into inflamed tissues [24,25] and high expression levels of CD11b have been linked to enhanced activation of neutrophils [26–28]. However, increased CD11b expression has also been seen on neutrophils with immunosuppressive properties [29]. In the present study, inflammation-induced up-regulation of CD11b in neutrophils reached similar levels in mice from both dietary groups but remained high in neutrophils from mice fed the FO diet at 6 h when it had declined in mice fed the C diet. The implication of more sustained levels of CD11b expression on neutrophils in mice fed the FO diet is unclear, but may indicate that neutrophils from mice fed the FO diet are more activated or that the neutrophils actually have more immunosuppressive properties than neutrophils from mice fed the C diet.

Resident immune cells, including NK cells and mast cells, have an important role in the early local inflammatory process [30,31], but may also, at the same time, participate in initiating the resolution phase. Resent data have shown that NK cells are important in resolution of inflammation in the respiratory system [32]. In the present study an early peak (6 h after induction of inflammation) in NK cell number in the peritoneum was observed in mice fed the FO diet but not in mice fed the C diet. As NK cells have been shown to be capable of inducing apoptosis of neutrophils [33], this early increase in NK cell number may have dampened the magnitude of the neutrophil response. Additionally, the early peak in the peritoneal concentration of the

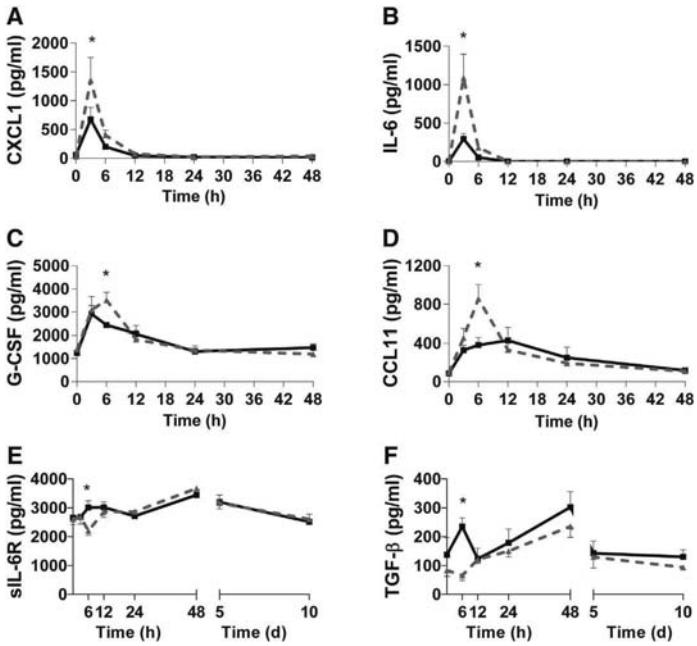

Fig. 6. Effects of dietary fish oil on peritoneal concentration of CXCL1 (A), IL-6 (B), G-CSF (C), CCL11 (D), sIL-6R (E) and TGF-β (F) prior to and at different time points after administration of mBSA. Mice received control diet (C, grey dashed line) or fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected i.p. one week later with mBSA. Mice were sacrificed at indicated time points and peritoneal fluid collected and concentration of cytokines, chemokines, growth factors and soluble cytokine receptors measured with ELISA. *Different from control, $P<.05$.

immunosuppressive mediators sIL-6R and TGF-β could theoretically be derived from NK cells as NK cells have been shown to produce TGF-β [34] and express IL-6R. This, however, is only speculative. Mast cells have also been shown to produce TGF-β [35] and, therefore, the increased peritoneal TGF-β production in mice fed FO diet may have been derived from mast cells, as there was a tendency towards more resident mast cells in the peritoneum of mice fed the FO diet than in mice fed the C diet. TGF-β aids in regulating the initiation and resolution of inflammatory responses [36] and sIL-6R has been associated with immune suppression and reduction of neutrophil accumulation in peritoneal inflammation [37]. Thus, the early peak in the concentration of these anti-inflammatory molecules in mice fed the FO diet indicates earlier initiation of the resolution phase in these mice. IL-1ra also peaked earlier in mice fed the FO diet (at 3 h in FO fed mice compared with 6 h in mice fed the C diet) and may thus have provided earlier anti-inflammatory effects in mice fed the FO diet.

FO diet did not affect the number of macrophages, neither the F4/80^{high} nor the F4/80^{low} macrophages, in the early phase of the inflammation (0–2 days). However, FO diet modulated the expression levels of several surface molecules and may, therefore, have affected macrophage function. The higher expression levels of F4/80 on F4/80^{high} macrophages from mice fed the FO diet suggests increased macrophage maturation as F4/80 has been indicated to be a maturation marker on macrophages [38]. The higher expression levels of CCR7 and D6 on F4/80^{high} macrophages from mice fed the FO diet may also indicate that they are better prepared for emigration into lymph nodes, since CCR7 has been shown to facilitate leukocyte migration to regional lymph nodes [39] and D6 expression on lymphatic endothelial cells has been shown to facilitate cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion [40]. D6 has also been shown to be important for resolution of inflammation [41–47], and therefore, the F4/80^{high} macrophages from mice fed the FO diet may have better resolution activity than F4/80^{high} macrophages from mice fed the C diet.

The F4/80^{low} macrophages were in much higher number in the peritoneum 5 days after induction of inflammation in mice fed the FO diet than in mice fed the C diet, and these macrophages expressed syndecan-1 (CD138). CD138 is a cell surface heparin sulfate proteoglycan that has been proposed to aid in attenuating lung inflammation [48] and facilitating resolution of neutrophil inflammation in a mouse model of endotoxemia [49]. Thus, expression of CD138 on the F4/80^{low} macrophages suggests that they may have a regulatory role by reducing inflammation and/or supporting resolution of inflammation. Furthermore, as impaired wound healing has been demonstrated in CD138 knockout mice [50] the higher number of F4/80^{low}CD138^{+} macrophages in peritoneum of mice fed the FO diet than in mice fed the C diet in the present study could indicate that mice fed the FO diet also had better potential for wound healing.

Although eosinophils have mostly been implicated in the pathogenesis of inflammatory processes, including parasitic helminth infections and allergic diseases [51], they have recently also been shown to be important for resolution of zymosan-induced inflammation, where they reduced neutrophil number and produced the n-3 PUFA derived pro-resolving mediator PD1 [52]. In the present study cellular kinetics in the peritoneal cavity at the initiation of inflammation (3–12 h) do not suggest that eosinophils play a role in neutrophil removal. However, it should be noted that eosinophils from mice fed the FO diet may have produced more of the n-3 PUFA derived mediator PD1

because of more substrate availability. In the study by Yamada et al. [52] PD1 was shown to restore neutrophil removal in eosinophil depleted mice. PD1 was not measured in the present study. It is not likely that the eosinophils in the peritoneum of the mice in the present study had a role related to allergic processes, as serum levels of IgE (BSA specific) were hardly detectable (data not shown) and the eosinophils, at the time when their number peaked, had down-regulated their expression of CD11b (data not shown) but high levels of CD11b expression on eosinophils has been linked to allergic diseases and oxidative burst [53,54]. The eosinophils peaked in number (in mice fed the C diet) at the same time as the levels of the immunosuppressive/pro-resolving mediators TGF-β and sIL-6R and therefore are more likely to play a role in the resolution of the inflammation, rather than processes related to allergy.

Interestingly, in mice fed the FO diet, the number of eosinophils peaked at 5 days after induction of inflammation and was more than double the number of eosinophils in peritoneum of mice fed the C diet. At this time point, B cells were the predominant cell type in the peritoneum (data not shown) and therefore it is possible that the eosinophils at this time point play a role in modulating the adaptive immune response as they have previously been shown to have the capacity to do [51]. This is supported by recent publications indicating a role for eosinophils in regulating antibody responses [55,56] by showing that eosinophils are critical for the maintenance of plasma cells in the bone marrow via production of plasma cell growth, maturation and survival factors [55] and that early antigen-specific IgM antibody responses to alum were impaired in eosinophil-deficient mice and could be restored with adoptive transfer of eosinophils [56].

The results from the present study show that mice fed the FO diet have reduced inflammatory response (fewer neutrophils and lower concentrations of CXCL1, IL-6, G-CSF and CCL11), more pronounced anti-inflammatory phase (early increase in number of NK cells, sIL-6R and TGF-β and expression of F4/80 on macrophages), enhanced resolution of inflammation (shorter R_i and more expression of CCR7 and D6 on macrophages) and increased late-resolution response (more eosinophils and CD138$^+$ macrophages). These results indicate that dietary fish oil dampens the inflammatory response, accelerates the onset of the resolution and may enhance the intensity and duration of the resolution phase and subsequent healing.

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Paper III

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Dietary omega-3 fatty acids enhance the B1 but not the B2 cell immune response in mice with antigen-induced peritonitis☆

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Abstract

The effects of omega-3 fatty acids on the adaptive immune response have mainly been analysed *in vitro* with varying results. How omega-3 fatty acids affect the adaptive immune response *in vivo* is largely unknown. This study examined the effects of dietary fish oil on the adaptive immune response in antigen-induced inflammation in mice, focusing on its effects on B cells and B cell subsets. Mice were fed a control diet with or without 2.8% fish oil, immunized twice with methylated BSA (mBSA) and peritonitis induced by intraperitoneal injection of mBSA. Serum, spleen and peritoneal exudate were collected prior to and at different time points after induction of peritonitis. Serum levels of mBSA-specific antibodies were determined by ELISA and the number of peritoneal and splenic lymphocytes by flow cytometry. The levels of germinal center B cells and IgM^+, IgG^+ and $CD138^+$ cells in spleen were evaluated by immunoenzyme staining. Mice fed the fish oil diet had more peritoneal B1 cells, more IgM^+ cells in spleen and higher levels of serum mBSA-specific IgM antibodies compared with that in mice fed the control diet. However, dietary fish oil did not affect the number of peritoneal B2 cells, splenic IgG^+ or $CD138^+$ cells or serum levels of mBSA-specific IgG antibodies in mice with mBSA-induced peritonitis. These results indicate that dietary fish oil can enhance the adaptive immune response, specifically the B1 cell response, which may lead to better protection against secondary infection as well as improvement in reaching homeostasis following antigenic challenge.

Keywords: Fish oil; Antigen-induced inflammation; B1 cells; IgM; Peritonitis

1. Introduction

How dietary omega-3 polyunsaturated fatty acids (PUFA) affect B cell responses *in vivo* is largely unknown. Several *in vitro* studies have demonstrated that omega-3 PUFA reduce proliferation, inflammatory gene expression and secretion of IL-10, TNF-α and IFN-γ by B cells and B cell lines [1–3]. In contrast, *in vitro* activation of human B cells in the presence of the pro-resolving lipid mediators 17-hydroxydosahexaenoic acid (17-HDHA) and resolvin D1, derived from omega-3 PUFA, resulted in increased IgG and IgM secretion [4]. In addition, *ex vivo* stimulated B cells from mice fed dietary omega-3 PUFA, secreted higher levels of IL-6 and IFN-γ and expressed higher levels of CD69 than B cells from mice fed a control diet [5]. Furthermore, dietary docosahexaenoic acid (DHA) increased CD40 expression and IL-6 and TNF-α secretion by *ex vivo* stimulated B cells from SMAD knockout mice, compared with that in SMAD knockout mice fed a control diet [6]. In the SMAD knockout mice, dietary DHA also led to a higher frequency of $B220^+$ cells (total B cells) being obtained from mesenteric lymph nodes and Peyer's patches, although not from the spleen, compared with that in mice fed the control diet [6]. A higher proportion of B cells were also obtained from the peritoneum of *Listeria monocytogenes*-infected mice fed omega-3 PUFA compared with that in mice fed a control diet [7]. In these studies, the total B cells were examined, and no attempt was made to investigate whether omega-3 PUFA had different effects on different B cell subclasses.

B cells are currently being divided into B1 and B2 cells, with B2 cells being the conventional B cells, also called follicular B cells. B1

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; GC, germinal center; HRP, horseradish peroxidase; Ig, immunoglobulin; ip, intraperitoneal; mBSA, methylated BSA; MFI, mean fluorescence intensity; MZ, marginal zone; NK, natural killer; PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acids; S.E.M., standard error of the mean.

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cells on the other hand are innate-like B cells with restricted B cell receptor repertoires, where antibody generation occurs without T-cell help, and they have limited somatic hypermutation, affinity maturation and class switching (reviewed in [8–10]). B1 cells reside in the peritoneal and pleural cavities and, to a lesser extent, in lymph nodes, bone marrow and spleen (reviewed in [11]). Peritoneal B1 cells play a key role in early protection against bacterial and viral infections via constitutive production of natural immunoglobulin (Ig)M antibodies and are the major natural antibody-producing B cell population in steady state, contributing to natural IgM antibodies in serum [12,13] and mucosal tissues of the respiratory tract [14]. Peritoneal B1 cells have also been shown to participate in protection of the gut barrier by producing secretory IgA against commensal bacteria [15,16]. In response to various stimuli, such as live bacteria, lipopolysaccharides, certain carbohydrates and cytokines, such as IL-5 and IL-10, peritoneal B1 cells migrate to the spleen or mucosa where they differentiate into IgM or IgA secreting cells, respectively (reviewed in [11]). The majority of IgM-secreting B1 cells reside in the bone marrow or spleen [17].

B1 cells have been subdivided into B1a and B1b cells, with B1a cells being the cells that spontaneously produce natural IgM antibodies (reviewed in [18]), whereas B1b cells can be induced to secrete antibodies following antigen specific cross-linking of the B cell receptor. The B1b cells can produce IgM or, following isotype switching, either IgG3 or IgA [19,20]. B1b cells are required for generating long-lasting protective responses against certain parasites and bacteria, although having both B1a and B1b cells has been shown to be important for generation of both natural and acquired bacterial antibody response and thus the greatest level of protection against infection [20]. The newly discovered regulatory B cells (B10 cells), defined by their ability to secrete IL-10, have been found amongst the B1a cells [10].

The aim of the present study was to determine the effects of dietary fish oil on the lymphocyte response in antigen-induced inflammation, focusing on the B cell response and distinguishing between B2 and B1 cells.

2. Methods

2.1. Animals and diets

Female C57BL/6 mice weighing 18–20 g were obtained from Taconic Europe (Ejby, Denmark). They were housed eight per cage with a 12-h light/dark cycle at 23–25°C and 45–55% humidity. All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with NRC's Guide for the Care and Use of Laboratory Animals. Mice were allowed to acclimatize for 1 week and were then divided into two groups, which were fed either the control diet (C) or the fish oil diet (FO) starting 1 week before the immunization protocol and continuing throughout the experiment. All mice were provided fresh food daily and had free access to food and water. The composition of the control diet was based on a typical American diet, that is, the "US17" diet formulated by Monsanto (St. Louis, MO, USA) and Research Diets Inc (D07121302; Research Diets Inc., New Brunswick, NJ) with minor modification by the authors, as previously described [21]. Energy distribution of the diet was as follows: carbohydrate, 44%; fat, 35%; and protein, 21%. The exact composition of the experimental diets has been reported previously [21]. The FO diet contained 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA, USA), which was added at the expense of safflower oil (Welch, Holme & Clark Co. Inc., Newark, NJ, USA). The amount of fish oil in the diet is moderate, corresponding to about 3 g of long chain omega-3 PUFA per 2000 kcal diet in humans. To adjust for the arachidonic acid (AA) content of the FO diet, AA ethyl ester (Nu-Check-Prep, Elysian, MN, USA) (0.5 g/kg) was added to the control diet. In brief, the FO diet contained 10.6 g/kg omega-3 PUFA (4.0 g/kg EPA and 2.5 g/kg DHA) and the control diet 3.4 g/kg omega-3 PUFA (undetectable levels of EPA and DHA). The fatty acid composition of the experimental diets and the ability of the FO diet to increase the proportion of omega-3 fatty acids and the ratio of omega-3:omega-6 PUFA in hepatic phospholipids have been reported previously [21].

2.2. Induction of mBSA-induced peritonitis

Mice were immunized subcutaneously at the base of the tail with 100 μg of mBSA (Sigma Aldrich, St. Louis, MO, USA) emulsified in an equal volume of complete Freund's adjuvant (Sigma Aldrich). Two weeks later, mice were given a booster injection of 100 μg of mBSA in incomplete Freund's adjuvant (Sigma Aldrich). Three weeks after the initial injection, peritonitis was induced by intraperitoneal (ip) injection of 100 μg of mBSA in saline. Before and at several time points (12 h, 24 h, 48 h, 5 days and 10 days) after peritonitis induction, mice were anesthetized with a mixture of hypnorm (VetaPharma Ltd, Leeds, UK), dormicum (Roche, Basel, Switzerland) and sterile water (1:1:2) and killed by cervical dislocation, followed by collection of blood, peritoneal lavage and spleen.

2.3. Collection of serum, peritoneal lavage and spleen

Serum was collected and stored at −70°C. Peritoneal cells and fluid were collected in 1.5 ml of cold phosphate buffered saline (PBS) without calcium or magnesium. The peritoneal cells were washed twice with PBS, resuspended in FACS buffer (PBS containing 1% BSA, 0.01% NaN_3) and counted by Countess automated cell counter (Invitrogen, Paisley, UK). Spleens were collected and fresh frozen in OCT compound (Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands) and stored at −70°C.

2.4. Characterization of splenic and peritoneal B and T cells by flow cytometry

Splenic or peritoneal cells (0.3×10^6) were incubated with 2% normal rat: normal mouse serum (1:1) (AbD Serotec, Kidlington, UK) for 20 min. Cells were stained with fluorochrome-labeled monoclonal antibodies (mabs) against IgD, B220, CD90.2, NK1.1, CD4, CD5 and CD8 (eBioscience, Vienna, Austria). All samples were washed twice with FACS buffer. Appropriate isotypic controls were used to set the quadrants and evaluate background staining. Samples were suspended in FACS buffer, and 10,000 cells collected on FACScalibur (BD Biosciences), and data were analyzed by FlowJo software (Tree Star, Inc, Ashland, OR, USA). B cells were identified as $B220^+CD90.2^-NK1.1^-$ cells, which were divided according to their expression of B220, IgD and CD5 into B1 ($B220^{low}IgD^{low}CD5^{+/-}$) and B2 ($B220^{high}IgD^{high}CD5^-$) cells. The B1 cell population was divided further into B1a ($CD5^+$) and B1b ($CD5^-$) subsets. The results are expressed as percentage positive cells, mean fluorescence intensity or total number of positive cells.

2.5. Measurement of BSA-specific IgM, IgG and IgA antibodies in serum

IgM, IgG and IgA-anti-BSA antibodies in serum was measured by indirect ELISA. Maxisorp plates (Nunc, Invitrogen) were coated with 50μg/ml BSA (Sigma-Aldrich) in bicarbonate buffer (pH 9.6) at 4°C overnight. Sera and standard (pool of serum from mBSA immunized mice), diluted from 1/10,000 for measuring IgG anti-BSA antibodies and from 1/100 for IgM and IgA anti-BSA antibodies, were incubated for 2 h at room temperature and further incubated with horseradish peroxidase (HRP)-labeled goat antimouse IgM, IgG or IgA (Southern Biotech, Birmingham, AL, USA) for 2 h. The substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added, and the color reaction stopped with 0.18 M H_2SO_4. The results are expressed as arbitrary units (AU) per ml calculated from standard curves made from serial dilution of the standard.

2.6. Immunohistochemical staining of spleen sections

Spleen sections (7 μm) were stained with biotin-labeled peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA, USA), followed by HRP-labeled Streptavidin (AbD Serotec) to detect germinal center (GC) B cells; monoclonal antibodies against CD138, followed by biotin-labeled goat-anti rat antibodies (both from BD Bioscience) and HRP-labeled Streptavidin to detect plasma cells; or HRP-labeled antibodies against IgM and IgG (Southern Biotech) to detect switched and unswitched B cells, respectively. The chromogen 3,3′ diaminobenzidine (Dako, Glostrup, DK) was used for visualization. The sections were viewed under a light microscope and evaluated blindly by two individuals. The number of GCs per μm^2 and the average size of the GCs were determined. The level of IgM^+ and IgG^+ cells was scored according to predetermined scoring system, taking into account the distribution and intensity of the staining inside the follicles and in the red pulp.

2.7. Data analysis and statistics

All data are expressed as mean values±standard error of the mean (SEM). All data were tested for normal distribution and equal variance using Kolmogorov–Smirnov test. If not normally distributed, Kruskal–Wallis nonparametric ANOVA was used to calculate overall *P* values, and the nonparametric Mann–Whitney rank sum test used to determine whether differences between the two dietary groups were statistically significant at a single time point. When the data were normally distributed, one-way ANOVA and Student's *t* test were used. Statistical analysis was performed using SigmaStat software, Version 3.2 (Systat software Inc., Chicago, IL, USA). $P<.05$ was considered significant.

3. Results

3.1. Effects of dietary fish oil on peritoneal and splenic B cells

B cells constituted around 40% of total peritoneal cells prior to ip challenge with mBSA (data not shown). The number of total B cells was the same in both dietary groups prior to administration of mBSA. At 24 h, the number of total B cells was higher in the peritoneum of mice fed the FO diet compared with that in mice fed the control diet (Fig. 1A). B1 cells ($B220^{low}IgD^{low}CD5^{+/-}$) constituted around 75% and B2 cells ($B220^{high}IgD^{high}CD5^{-}$) around 25% of the total B cells prior to mBSA administration (data not shown). Mice fed the FO diet had a higher number of B1 cells 24 h and 5 days after administration of mBSA and a tendency towards a higher number of B1 cells 10 days following mBSA administration than mice fed the control diet (Fig. 1B). There was no difference in the number of B2 cells in peritoneum from mice fed the different diets at any time point (Fig. 1C). B1 cells were divided into B1a cells ($CD5^{+}$) and B1b cells ($CD5^{-}$) (Suppl. Fig. 1). B1a cells comprised two thirds of the total B1 cells in the peritoneum and B1b cells 1/3. The mice fed the FO diet had higher number of B1a cells at 24 h and 5 days and higher number of B1b cells at 10 days in peritoneum after induction of inflammation than mice fed the control diet (Fig. 1D and E). The proportion of splenic B cells did not change following induction of inflammation, and there was no difference in the proportion of B cells in spleen from mice fed the two different diets (Suppl. Fig. 2).

3.2. Effects of dietary fish oil on peritoneal and splenic T cells

T cells were only around 10% of the peritoneal lymphocytes prior to peritonitis induction (Suppl. Fig. 3A). Their number increased sharply following induction of peritonitis with more total T cells (at 5 days) and $CD4^{+}$ and $CD8^{+}$ T cells (at 12 h and 5 days, and a tendency at 10 days) being present in the peritoneum of mice fed the FO diet compared with that of mice fed the control diet (Suppl. Figs. 3A–C). The proportion of total T cells in the spleen did not change following induction of peritonitis and was not affected by diet (Suppl. Fig. 3D), and the same was true for the $CD4^{+}$ and $CD8^{+}$ T cells (data not shown).

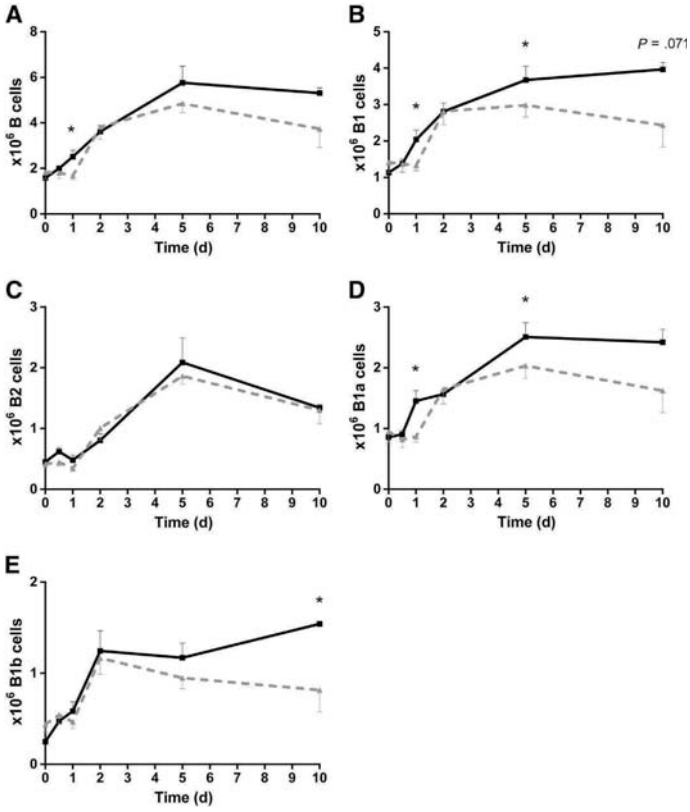

Fig. 1. Effects of dietary fish oil on the number of peritoneal total B cells (A), B1 cells (B), B2 cells (C), B1a cells (D) and B1b cells (E) prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were stained with monoclonal antibodies and analyzed by flow cytometry. Values are means±SEM, $n=3$ for the 10-day time point, $n=7$ for all other time points. *Different from control, $P<.05$.

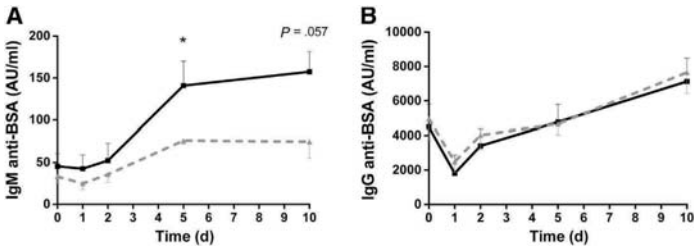

Fig. 2. Effects of dietary fish oil on serum levels of BSA-specific IgM (A) and IgG (B) antibodies prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points, serum collected and antibodies measured by ELISA. Values are in AU per ml and are means±SEM, $n=3$ for the 10-day time point, $n=7$ for other time points. *Different from control, $P<.05$.

3.3. Effects of dietary fish oil on BSA-specific IgM, IgG and IgA antibodies in serum

Serum levels of BSA-specific IgM antibodies did not change much following induction of inflammation in mice fed the control diet (Fig. 2A). However, in mice fed the FO diet, serum levels of BSA-specific IgM antibodies had increased by almost threefold 5 days after administration of mBSA and were higher than that in mice fed the control diet (Fig. 2A). There was also a tendency towards higher serum levels of IgM antibodies in mice fed the FO diet than in mice fed the control diet 10 days after mBSA administration (Fig. 2A). Serum levels of BSA-specific IgG antibodies decreased sharply following induction of inflammation, most likely because of formation of IgG-mBSA immune complexes and, then, increased again with no difference between the two dietary groups (Fig. 2B). There were no BSA-specific IgA antibodies detected in serum from mice fed either the control or the FO diet (data not shown).

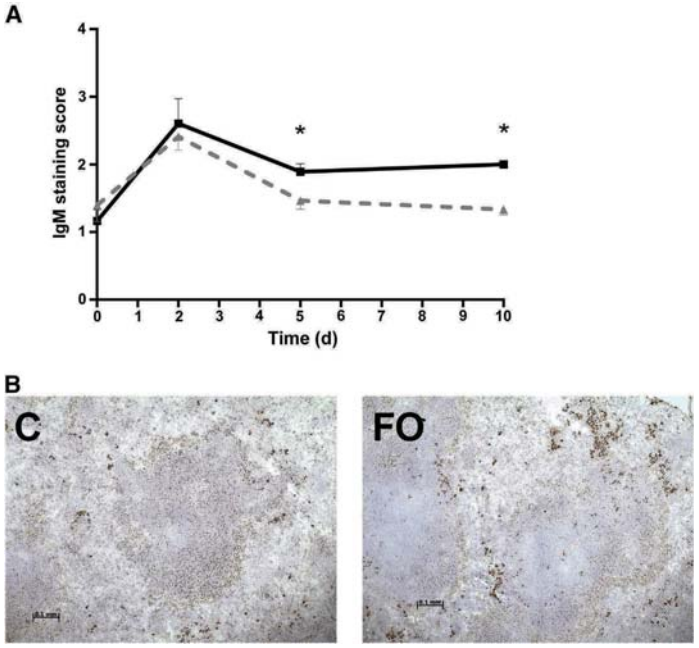

Fig. 3. Effects of dietary fish oil on the level of IgM^+ cells in red pulp of the spleen, prior to and at different time points following administration of mBSA (A). Representative histological sections from spleens collected at 5 days and stained with antibodies against IgM (B). Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points and spleen collected. Spleen sections were stained with immunohistochemistry and analyzed by light microscope blindly by two individuals. Scoring was performed according to a preformed scoring system, taking into account the number and intensity of the staining (see Suppl. Table 1). Values are shown as means±SEM, $n=3$ for the 10-day time point, $n=7$ for other time points. *Different from control, $P<.05$.

3.4. Effects of dietary fish oil on levels of IgM^+ and IgG^+ cells in spleen

Next, we determined whether higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet than in mice fed the control diet were reflected in higher levels of IgM^+ cells in spleen of mice fed the FO diet. Higher level of IgM^+ cells were detected in the red pulp of the spleen from mice fed the FO diet 5 and 10 days after mBSA administration than in the red pulp of the spleen from mice fed the control diet (Fig. 3A–B). There was no difference between the two dietary groups in levels of IgM^+ cells within the follicles. There was no difference in IgG staining levels between the two dietary groups with IgG staining mainly being observed within the follicles but not in the red pulp (Suppl. Fig. 4).

3.5. Effects of dietary fish oil on plasma cells and GCs in spleen

We then determined whether higher levels of BSA-specific IgM antibodies in serum of mice fed the FO diet compared with that of mice fed the control diet were reflected in an increase in GC formation or higher level of plasma cells in spleen. There was a slight increase in the average number and size of GCs in spleen following mBSA administration with the only difference between the two dietary groups being that the GCs were larger at 2 days in spleen from mice fed the FO diet compared with that in mice fed the control diet (Fig. 4A–C). There was no difference in the level of CD138 staining (plasma cells are $CD138^+$) at any time point following peritonitis induction in spleen from mice fed the different diets (data not shown).

4. Discussion

The results from the present study demonstrate that moderate amount of dietary fish oil affects the B cell response in antigen-induced peritonitis. The increase in the number of total B cells in peritoneum of mice fed the FO diet compared with that in mice fed the control diet, in the present study, is in agreement with results from two other studies. One showed a higher proportion of peritoneal B cells following infection with *L. monocytogenes* in mice fed omega-3 PUFA than in mice fed a control diet [7], and the other showed a higher frequency of total B cells in mesenteric lymph nodes and Peyer's patches in SMAD knockout mice, prone to colitis, that received a diet with a high content of DHA [6]. In the latter study, the DHA diet did not affect B cell numbers in spleen, which is in agreement with the results from the present study showing no effect of the FO diet on the proportion of splenic B cells. These results demonstrate that a diet with omega-3 PUFA can affect B cell numbers at the site of immune induction, although not systematically. In the present study, the higher number of total peritoneal B cells in mice fed the FO diet was due to a higher number of B1 cells, not follicular B2 cells, but neither of the previous studies determined the subclass of the B cells.

B1 cells formed the majority of the peritoneal B cells in the present study, which is in accordance with what has been shown previously [8,11,15,22]. The number of B1 cells was higher in mice fed the FO diet than in mice fed the control diet. B1 cells are important for production of natural IgM antibodies during steady-state, and these antibodies are important for maintaining tissue homeostasis via clearance of apoptotic and altered cells, inhibition of inflammation, removal of misfolded proteins and regulation of pathogenic autoantibody-producing B cells [23]. In addition, peritoneal B1 cells may play a vital role in protecting against secondary infection as they were the majority of the lymphocytes in the peritoneum of mice that had much better survival than RAG knockout mice following injection of live bacteria after inducing inflammation by zymosan [24]. The newly discovered regulatory B10 cells have been observed amongst the B1 population [10]. Since these were not analyzed in the present study, it cannot be concluded whether the increase in the number of peritoneal B1 cells observed in mice fed the FO diet was because of an increase in the regulatory B10 cell population. As the mice fed the FO diet in the present study had a higher number of peritoneal B1 cells and higher IgM antibody levels, they may be more protected against

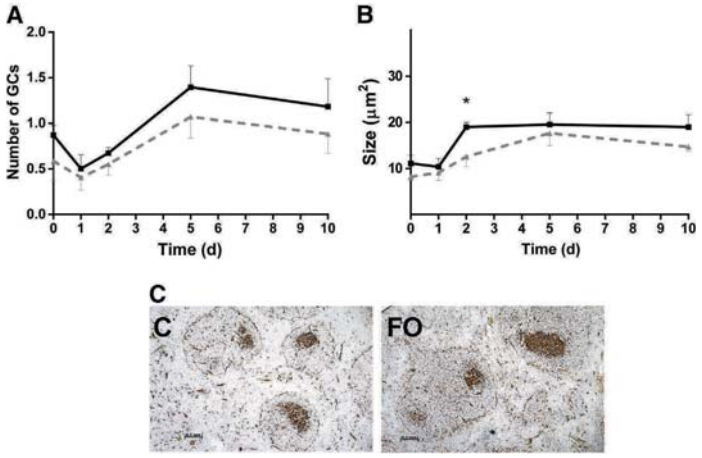

Fig. 4. Effects of dietary fish oil on the average number (A) and size (B) of GCs in spleen prior to and at different time points after administration of mBSA. Representative histological sections from spleens collected at 2 days and stained with PNA (C). Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points and spleen collected. Spleen sections were stained with immunohistochemistry and analyzed by light microscope blindly by two individuals. Values are means±SEM, $n=3$ for the 10-day time point, $n=7$ for other time points. *Different from control, $P<.05$.

secondary infections and more competent in reaching homeostasis following antigenic challenge.

Of the peritoneal B1 cells, the majority belonged to the B1a subclass with the number of both B1a and B1b cells being higher in mice fed the FO diet than in mice fed the control diet. Although little is known about the different roles of these two subpopulations of peritoneal B1 cells, recent studies show that B1a cells spontaneously secrete protective natural antibodies while some B1b cells are capable of producing antigen-specific IgM, IgG3 and IgA antibodies [19].

Although dietary fish oil increased the number of peritoneal B1 cells, it did not affect the B2 cell response, as demonstrated by it not affecting peritoneal B2 cell number, serum BSA-specific IgG levels, the level of IgG^+ cells in spleen or the number and size (except on Day 2) of the GCs in spleen. An efficient B2 immune response against mBSA following the two subcutaneous immunizations was demonstrated by high levels of BSA-specific IgG antibodies being present in serum prior to peritonitis induction, indicating the formation of BSA-specific plasma cells. BSA-specific memory cells were probably also formed as there was an increase in the number and size of the GCs and the number of peritoneal B2 cells increased following ip mBSA injection. There are few reports on the effects of dietary fish oil on antibody response following antigen challenge *in vivo*. The results from the present study are in agreement with results from a study in mice with an enteric infection by respiratory enteric orphan virus (reovirus), which showed that both reovirus-specific serum IgA and IgG2a antibody responses were similarly induced in mice fed control and DHA-rich diets [25]. However, the results from the present study are not in agreement with results from a study showing higher levels of serum influenza-specific IgG antibodies on Day 5 but lower levels on Day 7 in mice fed a fish oil diet compared with that in mice fed a beef tallow diet [26].

In the present study, as expected following two immunizations with mBSA and an adjuvant, the majority of the BSA-specific antibody-secreting cells were of the IgG class as indicated by serum levels of BSA-specific IgG antibodies being substantially higher than serum levels of BSA-specific IgM antibodies, indicating a class switch and hence few anti-BSA-specific IgM^+ memory B2 cells. Therefore, the increase in serum levels of BSA-specific IgM antibodies following induction of peritoneal inflammation in mice fed the FO diet is most likely dependent on peritoneal B1 cells. The peritoneal B1 cells may have been activated by BSA-IgG immune complexes formed following ip injection of mBSA. Although B1 cells mostly respond to T cell independent antigens, BSA-specific memory T cells produced during the immunization process may have provided the necessary help to induce a T cell-dependent response. As there were more T cells in the peritoneum of mice fed the FO diet than in mice fed the control diet, the B1 cells in peritoneum of mice fed the FO diet may have had more help from T cells than B1 cells from mice fed the control diet.

As peritoneal B1 cells have been shown to migrate into the spleen and differentiate into antigenic and natural IgM-secreting cells [27–29], the higher level of IgM^+ cells in spleen of mice fed the FO diet than in mice fed the control diet may be the result of the higher number of peritoneal B1 cells in mice fed the FO diet. That the IgM^+ cells are not B2 cells is supported by the finding that the level of IgG^+ cells in the spleen did not differ between the two dietary groups.

The higher serum levels of IgM antibodies in mice fed the FO diet in the present study may, in part, be linked to higher numbers of eosinophils in FO-fed mice than in mice fed a control diet, shown in our previous study [30], as eosinophils have been shown to be important for priming of B cells and their secretion of antigen-specific IgM antibodies [31].

B1 cells have been shown to induce an M2-like phenotype in peritoneal macrophages, both *in vitro* in co-culture experiments, as well as *in vivo* in transgenic mice overexpressing B1 cells [32]. Macrophages co-cultured with B1 cells expressed lower levels of IL-1β, TNF-α and CCL3 and higher levels of IL-10 than macrophages cultured without B1 cells or co-cultured with B2 cells, a phenotype that was also observed for peritoneal macrophages in mice over-expressing B1 cells [32]. In the present study, the higher number of peritoneal B1 cells in mice fed the FO diet may have led to an increase in M2-like peritoneal macrophages. Whether it did, we do not know, as in our previous study we did not determine the M1/M2 phenotypes of the peritoneal macrophages. However, we demonstrated a substantially higher number of $F4/80^{low}$ macrophages expressing CD138 (syndecan-1) in mice fed the FO diet than in mice fed the control diet [30], indicating an increase the number of macrophages linked with resolution of inflammation [33] similar to the M2 macrophage phenotype.

The results from the present study demonstrate that dietary fish oil can increase the number of peritoneal B1 cells, the level of splenic IgM^+ cells and the level of serum antigen-specific IgM antibodies in mice with antigen-induced peritonitis. These results indicate that dietary fish oil can enhance the adaptive immune response, specifically the B1 cell response, and lead to better protection against secondary infection as well as improvement in reaching homeostasis following antigenic challenge.

After submission of the present manuscript, Teague *et al.* [34] published a paper showing that dietary FO increased the number of B2 cells, as well as the innate-like marginal zone B cells, indicating that the effects of dietary FO on B cell response may be dependent on the antigen and the immunization procedures.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2013.09.010.

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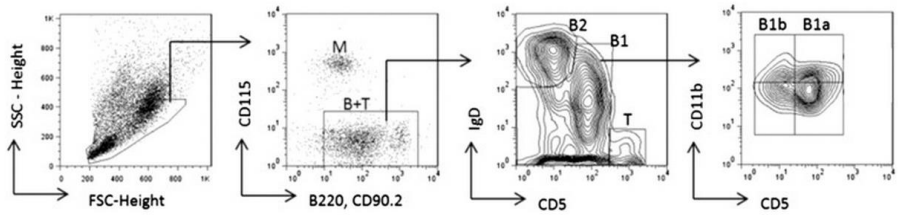
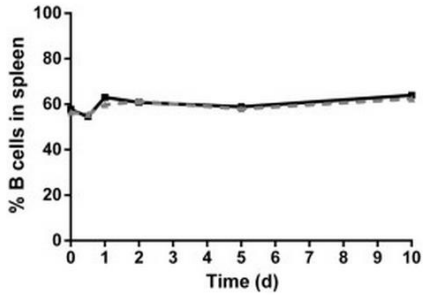
Paper III – Supplementary materials



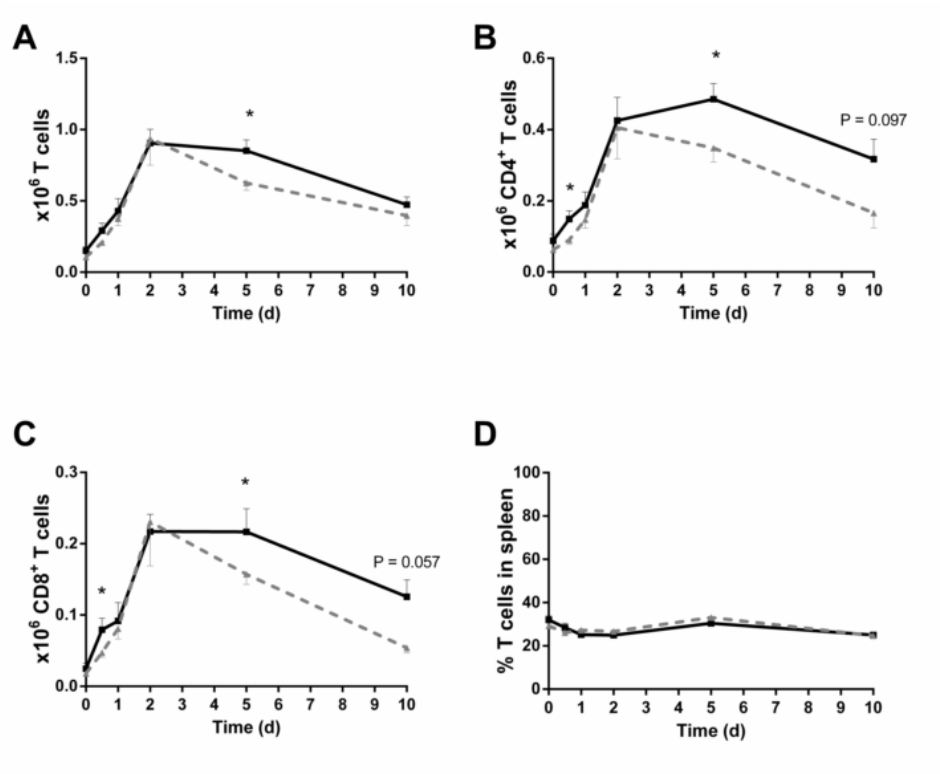
Suppl. Fig. 1.

Gating of B1a and B1b cells from the peritoneal cavity of mice fed a control diet. Mice were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at 2 days and peritoneal fluid collected and the peritoneal cells stained with monoclonal antibodies and analyzed by flow cytometry. M, macrophages; T, T cells; B, B cells; B2, B2 cells; B1, B1 cells; B1a, B1a cells; B1b, B1b cells.



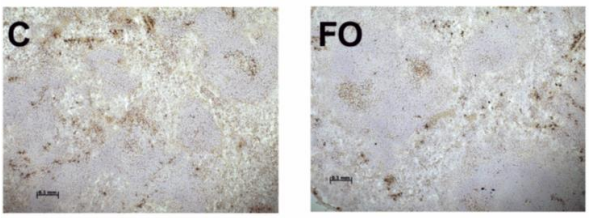
Suppl. Fig. 2.

Effects of dietary fish oil on the proportion of splenic B cells at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points, and spleen was collected. Splenocytes were stained with monoclonal antibodies and analyzed by flow cytometry. Values are means±SEM.



Suppl. Fig. 3.

Effects of dietary fish oil on the number of peritoneal total T cells (A), $CD4^+$ T cells (B) and $CD8^+$ T cells (C) and the proportion of splenic T cells (D) prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were stained with monoclonal antibodies and analyzed by flow cytometry. Values are means±SEM, n=3 for the 10-day time point, n=7 for all other time points. *Different from control, P<.05.



Suppl. Fig. 4.

The effects of dietary fish oil on the distribution of IgG staining in spleen 5 days following administration of mBSA. Mice were fed a control diet (C) or a fish oil diet (FO) for 4–5 weeks. They were immunized twice with mBSA with 2 weeks interval and injected ip with mBSA 1 week later. Mice were sacrificed at 5 days, and spleen was collected. Tissue sections were stained with antibodies against IgG and analyzed by light microscopy.

Supplementary Table 1. Staining score for spleen sections stained for IgM.

	Inside follicles		Marginal zone		Red pulp	
	Control	Fish oil	Control	Fish oil	Control	Fish oil
Day 0	1.6 ± 0.4	1.6 ± 0.3	2.2 ± 0.4	1.4 ± 0.4	1.4 ± 0.3	1.2 ± 0.2
Day 2	2.7 ± 0.2	2.6 ± 0.2	2.1 ± 0.2	2.1 ± 0.3	2.4 ± 0.2	2.6 ± 0.4
Day 5	1.7 ± 0.2	1.9 ± 0.3	1.2 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.9 ± 0.1*
Day 10	1.6 ± 0.2	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.3 ± 0.1	2.0 ± 0.0*

Mean ± standard error of the mean. * Significantly differs from the control group ($P<0.05$).