The effects of dietary fish oil on cell populations, cytokines, chemokines and chemokine receptors in healthy mice and mice with endotoxin-induced peritonitis

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Thesis for the degree of Philosophiae Doctor

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Áhrif fiskolíu í fæði á frumugerðir, frumuboðefni, flakkboða og flakkboðaviðtaka í heilbrigðum músum og músum sprautuðum með inneitri

Hildur Hrönn Arnardóttir

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Ágrip

Fiskolía, ríkt af n-3 fjölómettuðum fitusýrum (FÓFS), hefur jákvæð áhrif í sumum langvinnum bólgusjúkdómum, sýkingum og einnig í blóðeitrun. Sum þessara jákvæðu áhrifa fiskolíu á ónæmistengda sjúkdóma eru að hluta komin til vegna áhrifa hennar á myndun fituboðefna og frumuboða. Í ónæmistengdum sjúkdómum gegnir tog ónæmisfruma á bólgu og sýkingastaði mikilvægu hlutverki. Hins vegar hafa áhrif fiskolíu, eða n-3 FÓFS, á undirgerðir eða tog ónæmisfruma eða flakkboða og flakkboðviðtaka ekki verið mikið rannsökuð.

Markmið rannsóknarinnar var að ákvarða áhrif fiskolíu á a) frumugerðir í blóði, kviðarholi, milta og beinmerg músa; b) styrk flakk- og frumuboða í blóð- og kviðarholsvökva; c) tjáningu flakkboðaviðtaka á blóð-, kviðarhols-, miltis- og beinmergsfrumum í heilbrigðum músum og músum sprautuðum með inneitri; og d) frumu- og flakkboðamyndun staðbundinna kviðarholsfruma örvuðum með inneitri *ex vivo*.

Mýs fengu annaðhvort hefðbundið fóður eða fóður byggt á vestrænu fæði með eða án 2,8% fiskolíu í 6 vikur. Helmingur hvors fæðuhóps var sprautaður með 10 µg/20g af inneitri (lípópólísakkaríð, eða LPS) í kviðarhol. Músum sem fengu hefðbundið fóður, var fórnað fyrir (0 klst), eða 3, 8, 12, 24 eða 48 klst eftir sprautun með inneitri, en músum sem fengu fóður byggt á vestrænu fæði (með eða án 2,8% fiskolíu) var fórnað fyrir (0 klst) eða 3 eða 48 klst eftir sprautun með inneitri. Blóði, kviðarholsvökva, milta og beinmerg var safnað. Frumur voru taldar og greindar út frá stærð og kyrningu, og að auki tjáningu á flakkboðaviðtökum og öðrum yfirborðssameindum, með frumuflæðisjá. Styrkur frumu- og flakkboða í sermi, kviðarholsvökva og floti staðbundinna kviðarholsfruma örvuðum með inneitri *ex vivo*var ákvarðaður með ELISA aðferð.

Sprautun með inneitri olli skyndilegri fækkun hvítfruma í blóði, kviðarholi og beinmerg. Fjöldi hvítfruma hélst lágur fyrstu 24 klst í blóði og kviðarholi en jókst þar eftir. Hins vegar fækkaði hvítfrumum í beinmerg út tilraunartímann. Skammvinn aukning varð í heildarfjölda hvítfruma í milta í kjölfar sprautunar með inneitri, en síðan fækkaði þeim og 48 klst eftir sprautun með inneitri var fjöldi þeirra svipaður og fyrir sprautun.

Það dró hratt úr fjölda mónócýta/makrófaga í blóði, kviðarholi og beinmerg í kjölfar sprautunar með inneitri. Einnig fækkaði mónócýtum/makrófögum í milta í kjölfar skammvinnrar aukningar sem varð

í byrjun. Tveir undirflokkar mónócýta voru auðkenndir í blóði og milta, hefðbundir og óhefðbundnir, en einungis hefðbundnir mónócýtar voru til staðar í beinmerg. Makrófagar í kviðarholi skiptust einnig í tvo undirflokka.

Skammvinn minnkun varð í fjölda daufkyrninga í blóði í kjölfar sprautunar með inneitri, hins vegar fækkaði daufkyrningum í kviðarholi stöðugt út tilraunartímann. Fáir daufkyrningar voru til staðar í kviðarholi fyrir sprautun með inneitri en fjöldi þeirra jókst að 24 klst og í milta fjölgaði þeim þar til 12 klst eftir sprautun og hélst fjöldinn stöðugur út tilraunartímann (48 klst). Daufkyrningar í blóði músa sem ekki fengu inneitur voru mjög kyrndir en kyrningin minnkaði í kjölfar sprautunar með inneitri. Átta klst eftir sprautun með inneitri voru tvær gerðir af daufkyrningum til staðar í blóði og var önnur minni og meira kyrnd heldur en hin. Tjáning flakkboðaviðstakans CXCR2 minnkaði á daufkyrningum í kjölfar sprautunar með inneitri, en var til staðar á daufkyrningum 24 klst eftir sprautun með inneitri.

Styrkur frumu- og flakkboða í sermi og kviðarholsvökva jókst hratt í eftir sprautun með inneitri en lækkaði hratt aftur.

Heilbrigðar mýs sem fengu fiskolíufóður voru með lægra hlutfall af hefðbundnum mónócýtum í blóði miðað við mýs sem fengu viðmiðunafóður. Sprautun með inneitri jók hlutfall hefðbundinna mónócýta í blóði músa sem fengu fiskolíufóður en ekki músa sem fengu viðmiðunarfóður. Heilbrigðar mýs sem fengu fiskolíufóður höfðu lægri styrk af CCL2 í blóði en mýs sem fengu viðmiðunarfóður, en eftir sprautun með inneitri var styrkur CCL2 í blóði hærri í músum sem fengu fiskolíu en í músum sem fengu viðmiðunarfóður.

Heilbrigðar mýs sem fengu fiskolíufóður voru með færri frumur í kviðarholi og færri makrófaga í kviðarholi heldur en heilbrigðar mýs sem fengu viðmiðunarfóður. Fækkun kviðarholsfruma í kjölfar sprautunar með inneitri var minna áberandi í músum sem fengu fiskolíufóður heldur en í músum sem fengu viðmiðunarfóður, sem leiddi til þess að 3 klst eftir sprautun með inneitri voru fleiri frumur í kviðarholi músa sem fengu fiskolíufóður en í kviðarholi músa sem fengu viðmiðunarfóður.

Mýs sem fengu fiskolíufóður höfðu hærri styrk af CCL3 og CCL2 í kviðarholi 3 og 48 klst eftir sprautun með inneitri, í áðurnefndri röð. Styrkur IL-6 var hærri og það var tilhneiging til að vera hærri styrkur af TNF-α í kviðarholsvökva músa sem fengu fiskolíufóður 48 klst eftir sprautun með inneitri en í kviðarholsvökva músa sem fengu viðmiðunarfóður. Þegar staðbundnir makrófagar músa sem fengu fiskolíufóður voru örvaðir *ex vivo* mynduðu hlutfallslega færri makrófagar TNF-α og CCL3 þrátt fyrir að

meðalmyndun hverrar frumu væri meiri í músum sem fengu fiskolíufóður miðað við í músum sem fengu viðmiðunarfóður.

Heilbrigðar mýs sem fengu fiskolíufóður voru með lægra hlutfall hefðbundinna mónócýta í blóði en heilbrigðar mýs sem fengu viðmiðunarfóður. Hins vegar höfðu mýs sem fengu fiskolíufóður tilhneigingu til að vera með hærra hlutfall hefðbundinna mónócýta í blóði miðað við mýs sem fengu viðmiðunarfóður 48 klst eftir sprautun með inneitri. Einnig var hærra hlutfall hefðbundinna mónócýta 48 klst eftir sprautun með inneitri í miltum músa sem fengu fiskolíufóður miðað við í miltu músa sem fengu viðmiðunarfóður.

Heilbrigðar mýs sem fengu fiskolíufóður höfðu lægri styrk af CCL2 í blóðvökva en heilbrigðar mýs sem fengu viðmiðunarfóður. Þvert á móti, 3 og 48 klst eftir sprautun með inneitri var styrkur CCL2 í blóðvökva hærri í músum sem fengu fiskolíufóður en í músum sem fengu viðmiðunarfóður.

Þrátt fyrir að fiskolía í fæði hefði ekki áhrif á fjölda daufkyrninga í blóði heilbrigðra músa eða í blóði músa sprautuðun með inneitri (48 klst), þá voru heldur fleiri daufkyrningar í blóði músa sem fengu fiskolíu 12 og 24 klst eftir sprautun með inneitri en í blóði músa sem fengi viðmiðunarfóður. Mýs sem fengu fiskolíufóður voru með hærra hlutfall af minna kyrndum daufkyrningum í blóði heldur en mýs sem fengu viðmiðunarfóður. Einnig höfðu mýs sem fengu fiskolíufóður lægri styrk af CXCL2 og hærri styrk af CCL3 í sermi en mýs sem fengu viðmiðunarfóður 3 klst eftir sprautun með inneitri.

Mýs sem fengu fiskolíufóður voru með færri daufkyrninga í kviðarholi 12 klst eftir sprautun með inneitri og höfðu tilhneigingu til að vera með færri daufkyrninga í kviðarholi 24 klst eftir sprautun með inneitri miðað við mýs sem fengu viðmiðunarfóður. Þvert á móti, þá höfðu mýs sem fengu fiskolíufóður fleiri daufkyrninga í kviðarholi 48 klst eftir sprautun með inneitri miðað við mýs sem fengu viðmiðunarfóður.

Niðurstöður verkefnisins benda til þess að fiskolía í fæði hafi dempandi áhrif á bólguvirkni í jafnvægi en auki hins vegar bólguviðbragð eftir sýkingu og gæti mögulega unnið á móti minnkaðri virkni mónócýta og vanvirkni daufkyrninga sem hefur orðið vart við á seinni stigum blóðeitrunar.

Lykilorð:

Fiskolía, inneitur, daufkyrningar, mónócýtar, flakkboðar

Abstract

Fish oil, rich in n-3 polyunsaturated fatty acids (PUFAs), has immune-modulatory properties and may have beneficial effects in several immune disorders, including sepsis. Some of the beneficial effects of dietary fish oil on immune disorders have been attributed to their effects on production of eicosanoids and cytokines. In immune disorders the recruitment of specific populations of immune cells to the sites of infection or inflammation is very important. However, the effects of dietary fish oil, or n-3 PUFAs, on specific immune cell subpopulations, immune cell recruitment or on chemokines and chemokine receptors have not been studied in detail.

The objective of this study was to determine the effects of dietary fish oil on a) mouse cell populations in blood, peritoneum, spleen and bone marrow; b) cytokine and chemokine concentrations in serum and peritoneal fluid; c) chemokine receptor expression on blood, peritoneal, spleen and bone marrow cells in healthy mice and mice with severe endotoxin-induced peritonitis; and d) cytokine and chemokine production in lipopolysaccharide (LPS)-stimulated peritoneal cells *ex vivo*.

Mice were either fed a regular chow or a Western-type diet with or without 2.8% fish oil for six weeks. Half of the mice in each dietary group were injected intraperitoneally with 10 µg/20g body weight of LPS. Mice fed regular chow were sacrificed before (0 h) or 3, 8, 12, 24 or 48 h after LPS administration, but mice fed the experimental diets (Western-type diet with or without 2.8% fish oil) were sacrificed before (0 h) or 3 or 24 h following LPS administration. Blood, peritoneal fluid, spleen and bone marrow were collected. The cells were counted and analyzed for size and granulation as well as expression of chemokine receptors, other surface molecules and for intracellular cytokine production by flow cytometry. Cytokine and chemokine concentrations in serum, peritoneal fluid and supernatant from LPS-stimulated resident peritoneal macrophages were determined by ELISA.

Administration of LPS led to a rapid decrease in leukocyte numbers in blood, peritoneum and bone marrow, which remained low for 24 h in blood and peritoneum and then increased, but their numbers in bone marrow continued to decrease throughout the experiment. The number of total leukocytes in spleen increased transiently following LPS administration, after which their numbers decreased and at 48 h their numbers were similar to that before LPS administration.

The numbers of monocytes/macrophages in blood, peritoneum and bone marrow were rapidly reduced following LPS administration as well as in spleen following a transient increase in monocyte numbers. Two subpopulations of monocytes were identified in blood and spleen, classical and non-classical monocytes, whereas in the bone marrow only the classical monocytes were present. Macrophages in the peritoneum were also divided into two subpopulations.

There was a transient decrease in neutrophil numbers in blood following LPS administration, whereas in bone marrow the number of neutrophils did not increase after the initial decrease. In peritoneum few neutrophils were detected prior to LPS administration but their numbers kept increasing until 24 h after administration and in spleen their numbers increased until 12 h after administration of LPS, and stayed constant thereafter. The neutrophils in blood from mice that were not administered LPS were highly granular but their granularity decreased following administration of LPS. Eight hours following LPS administration the neutrophils had divided into two subpopulations, one consisting of smaller but more granular neutrophils than the other. Expression of the chemokine receptor CXCR2 on neutrophils was down-regulated following LPS administration but was present again on neutrophils 24 h following LPS administration.

Administration of LPS induced a rapid increase in serum and peritoneal concentrations of chemokines and cytokines, followed by a rapid decline in their levels.

Healthy mice fed the fish oil diet had a lower proportion of classical monocytes in blood than mice fed the control diet. Administration of LPS increased the proportion of classical monocytes in blood in mice fed the fish oil diet but not in mice fed the control diet. Healthy mice fed the fish oil diet had a lower serum concentration of CCL2 than mice fed the control diet, but following LPS administration CCL2 concentration was higher in mice fed the fish oil diet than in mice fed the control diet.

Healthy mice fed dietary fish oil had fewer peritoneal cells and fewer peritoneal macrophages than healthy mice fed the control diet. The decrease in peritoneal cell numbers following LPS administration was less pronounced in mice fed the fish oil diet than in mice fed the control diet, resulting in a higher number of peritoneal cells in mice fed the fish oil diet compared with that in mice fed the control diet 3 h following LPS administration.

Mice fed the fish oil diet had a higher concentration of CCL3 and CCL2 in the peritoneum 3 and 48 h following LPS administration, respectively. The concentration IL-6 was higher and there was a trend towards higher

concentration of TNF-α in peritoneal fluid from mice fed the fish oil diet 48 h following LPS administration than in peritoneal fluid from mice fed the control diet. When macrophages from mice fed the fish oil diet were stimulated with LPS *ex vivo*, the proportion of macrophages that secreted TNF-α and CCL3 was lower although the mean CCL3 production per cell was higher than among cells from mice fed the control diet.

Healthy mice fed the fish oil diet had a lower proportion of classical monocytes in blood than mice fed the control diet. But mice fed the fish oil diet had a tendency towards a higher proportion of classical monocytes in the circulation than mice fed the control diet 48 h following LPS administration. There was also a higher proportion of classical monocytes in spleen from mice (at 48 h) fed the fish oil diet compared with that in mice fed the control diet 48 h following LPS administration.

Healthy mice fed the fish oil diet had lower serum CCL2 levels than mice fed the control diet. On the other hand, mice fed the fish oil diet had higher serum concentrations of CCL2 than mice fed the control diet 3 h and 48 h following LPS administration.

Although dietary fish oil did not affect the number of total neutrophils in healthy mice nor in mice 48 h following LPS administration, 12 and 24 h following administration of LPS there was a trend towards a higher number of neutrophils in blood in mice fed the fish oil diet compared with that in mice fed the control diet. Forty-eight hours following LPS administration mice fed dietary fish oil had a larger proportion of the less granular neutrophils in blood compared with mice fed the control diet. Mice fed the fish oil diet had lower serum concentrations of CXCL2 and higher serum concentrations of CCL3 than mice fed the control diet 3 h following LPS administration.

Mice fed the fish oil diet had fewer neutrophils in peritoneum 12 h after administration of LPS and there was a tendency towards fewer neutrophils 24 h after administration of LPS than in peritoneum of mice fed the control diet. In contrast, 48 h after administration of LPS, mice fed the fish oil diet had a higher number of neutrophils in peritoneum than mice fed the control diet.

These results indicate that dietary fish oil may attenuate the immune activation state during homeostasis but intensify the immune response in infection and possibly counteract the monocyte deactivation and neutrophil impairment observed in the later stages of sepsis.

Keywords:

Endotoxin-induced peritonitis, fish oil, neutrophils, monocytes, chemokines

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Contents

List of abbreviations

AA	Arachidonic acid
AIN	American Institute of Nutrition
ALA	α-linolenic acid
ALI	Acute lung injury
ANOVA	Analysis of Variance
ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
t-BHQ	t-Butylhydroquinone
BHT	Butylated hydroxytoluene
CARS	Compensatory anti-inflammatory response syndrome
CAV	cardiac allograt vasculopathy
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
COX	Cyclooxygenase
DC	Dendritic cell
DGLA	dihomogammalinolenic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle medium
DON	Deoxinivalenol
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FLAP	5-lipoxygenase activating protein
GC	Gas chromatography
G-CSF	granulocyte colony-stimulating factor
GLA	γ-linolenic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor

HETE	hydroxyeicosatetraenoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
HLA	human leukocyte antigen
ICAM	Intracellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneally
i.t.	intratracheally
i.v.	Intravenously
HSF	High saturated fat
HUVEC	human umbilical vein endothelial cell
KC	Keratinocyte chemoattractant
LA	Linoleic acid
LBP	LPS binding protein
LC-PUFA	Long-chain polyunsaturated fatty acid
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxin
LFA	Lymphocyte function associated antigen
LT	Leukotriene
mab	Monoclonal antibody
MALP	Macrophage activating lipopeptide
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
MSR-A	Macrophage scavenger receptor A
MUFA	Monounsaturated fatty acid
NADP	Nicotinamide adenine dinucleotide phosphate

NF	Nuclear factor
NK cell	Natural killer cell
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PD	Protectin
PD-1	PGD_2-receptor
PG	Prostaglandin
PGI	Prostacyclin
PHA	Phytohaemagglutinin
PL	Phospholipid
PLA2	Phospholipase A2
PMN	Polymorphnuclear
PPG	Peptidoglycan
PRRs	Pattern recognition receptors
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid Arthritis
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
ROS	Reactive oxygen species
RPM	Revolutions per minute
Rv	Resolvin
SDF-1	Stromal derived factor-1
SEM	Standard error of the mean
SIRS	systemic inflammatory response syndrome
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TX	Thromboxane
VCAM	Vascular cell adhesion molecule

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Declaration of contribution

I participated in designing the hypothesis of this study, analyzed the data and have written 3 manuscripts with the assistance of my mentors Dr. Hardardottir and Dr. Freysdottir. I took part in maintaining the laboratory animals in collaboration with Valgerður Tómasdóttir and Guðný Ella Thorlacius. I changed the experimental diets daily for the duration of the dietary experiments (6 weeks). I injected the mice with LPS and/or euthanasia and obtained the cells with assistance from Valgerður and Guðný Ella, and performed all the assays used (ELISA, flow cytometry, etc).

1 INTRODUCTION

A well balanced diet is important for good health, and the role of nutrition in improving health is continuously being emphasized by ongoing research. Nutritional research is a complex field pertaining to all systems of the body and their susceptibility to dietary influences. The emerging evidence of the effects of dietary fats in health and disease has stimulated a growing interest in the field of fatty acids (FAs).

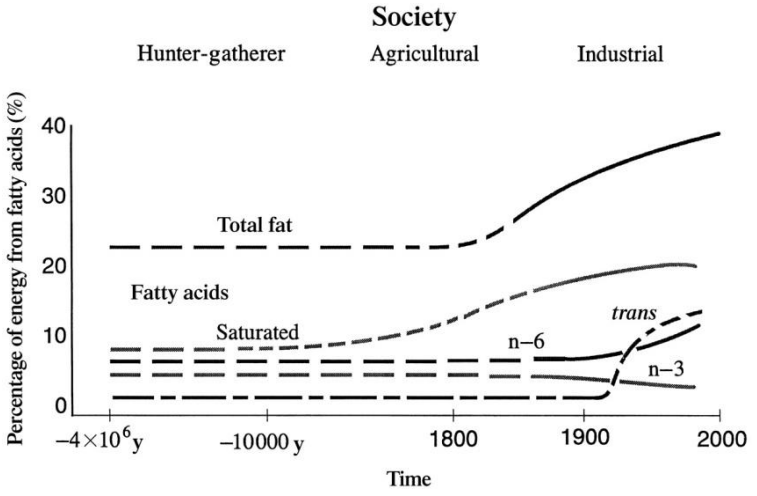
Lipids are important dietary components. They provide a concentrated source of energy and are a source of essential fatty acids (EFAs), i.e. FAs that humans and other animals cannot synthesize *de novo*. Among dietary lipids are FAs that provide structural components for cell membranes and some FAs serve as substrates for the synthesis of many signaling molecules. Polyunsaturated FAs (PUFAs) are a subclass of dietary FAs that are considered to be important nutritional components because of their modulatory effects on the immune system and their therapeutic potential.

The two major families of PUFAs are the omega-3 (n-3) and the omega-6 (n-6) families. FAs from these two families compete for incorporation into cell membranes and for being used as substrates for synthesis of lipid mediators, such as the eicosanoids. Eicosanoids and other lipid mediators have several important cellular functions and their activity is dependent on the precursor from which they are made. The biological effects of n-3 PUFAs are considered to be more anti-inflammatory than the effects of the n-6 PUFAs, as they are precursors for less potent inflammatory mediators. PUFAs may alter the immune response in several ways, e.g. by changing membrane composition and function and modifying production of several lipid mediators, cytokines and chemokines (Fritsche, 2006).

Over the past 100 years there have been dramatic changes in our dietary fat intake, predominantly in the type and amount of essential FAs (Fig. 1). During evolution the ratio of n-6 to n-3PUFAs in the diet has been estimated to be approximately 1:1. However, over the past 100-150 years there have been major changes in the dietary intake of n-6 and n-3PUFAs, favoring the n-6 PUFAs. Today in Western societies the consumption of n-6PUFAs has become approximately 20 times greater than the consumption of n-3PUFAs (reviewed in (Simopoulos, 2006, 2010)).

It has been postulated that a diet with a high ratio of n-6 to n-3 PUFAs promotes the pathogenesis of numerous diseases, such as cardiovascular

disease, arthritis, cancer and inflammation. In contrast, an increase in the intake of n-3 PUFAs (i.e. a lowern-6/n-3 ratio) may have suppressive effects on the development of these same diseases (reviewed in (Simopoulos, 2006)). The immunosuppressive effects of n-3 PUFAs has lead to attempts at therapeutic applications of n-3PUFAs in autoimmune and inflammatory disorders as well as in surgical and critically ill patients. There is evidence showing beneficial effects of dietary fish oil in autoimmune and chronic inflammatory diseases, however, there are some inconsistencies in existing data, where it has also been shown that dietary fish oil has no or even adverse effects on these diseases (reviewed in (Fritsche, 2006)). Even though diminished inflammatory response can be favorable in some diseases, it may interfere with the ability of the host to fight infections.



Simopoulos, Am J Clin Nutr (1999;70:570S-575S), American Society for Nutrition.

Figure 1. Hypothetical scheme of the changes in consumption of different fatty acid families.

Over the last decade there has been a rising interest in the potential use of n-3 PUFAs in critically ill patients, especially in septic patients, as sepsis is the leading cause of death in critically ill patients in the United States (reviewed in (Stapleton et al., 2010)). The poor outcome of patients with sepsis is believed to be a result of not only the massive activation of the immune system but also the subsequent immunosuppression. Beside caloric support, the immunomodulatory properties of lipids used for intravenous nutrition have been noted in the critically ill. Animal studies and

few recent clinical trials indicate beneficial effects of using n-3 PUFAs in critically ill patients However, there are some conflicting data (reviewed in (Stapleton et al., 2010)) and further research is necessary to determine the possible benefits of n-3 PUFAs in critically ill patients.

Studies on the effects of n-3 PUFAs on the immune system have provided inconsistent outcomes. The contrasting results may in part be because of the different experimental setup of the studies. These studies have for example used different species or different strains within species and they have used different types of cells or cells from different tissues. In addition, the studies have been *in vivo*, *ex vivo* or *in vitro* studies and the amount and time of n-3 PUFAs given has varied. Considering nutritional studies in critically ill patients there are a number of factors that make them hard to conduct and interpret; 1) time of enrollment of the patients, 2) heterogeneous patient population with a wide variety of medical illnesses, 3) small number of patients in each study, 4) mixed formulas with several nutritional ingredients, and 5) doses and the time of n-3PUFAs given. For successful use of dietary constituents such as lipids in therapy or as a support to conventional therapy it is important to understand the mechanism by which they exert their effects. The purpose of this study was to determine the effects of dietary fish oil or n-3 PUFAs on mouse leukocyte populations, chemokine production and chemokine receptor expression and explore the mechanism by which dietary fish oil affects cell populations and their migration in healthy mice and in mice with severe endotoxin-induced peritonitis. These results should contribute to a better understanding of the effects of n-3 PUFAs and the mechanism by which they exert their effects on the innate immune system.

1.1 Fish oil and polyunsaturated fatty acids

Fish oil contains high levels of n-3 PUFAs. The most abundant n-3 PUFAs in fish oil are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The n-3 PUFAs are believed to be the major contributors of the immune modulating effects of most fish oils.

FAs are hydrocarbon chains with a methyl group on one end (the omega end) and a carboxyl group on the other (the alpha end). FAs vary both in length and saturation (Fig. 2). Saturated FAs do not contain double bonds, whereas monounsaturated FAs (MUFAs) have one double bond and PUFAs have two or more double bonds. The position of the double bond from the omega end determines the subclass of PUFAs the FA

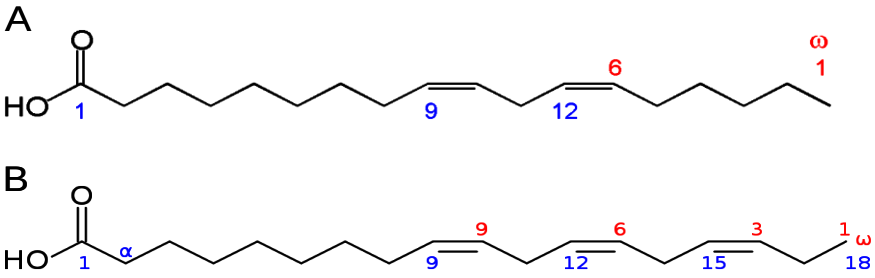


Figure 2. Structure of (A) linoleic acid (18:2 n-6) and (B) α-linolenic acid (18:3 n-3).

belongs to. In n-6 PUFAs the first double bond is on the sixth carbon from the methyl, or the omega end, whereas in n-3 PUFAs the first double bond is three carbons away from the methyl end.

Mammalian cells do not have the desaturase enzymes necessary to insert double bonds between the ninth carbon and the methyl end and thus they cannot synthesize *de novo* 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) (Fig. 2) (Cook & McMaster, 2002; Simopoulos, 2006). Since LA and ALA are the precursors for the n-6andn-3long-chain PUFAs (LC-PUFAs; C ≥ 20), respectively, they are essential in the diet, i.e. they are EFAs. LA is found in most vegetable oils, e.g. corn oil and safflower oil and represents ~90% of the total PUFAs in U.S. diets (*IOM*, 2002). ALA is found in some vegetable oils, such as rapeseed, flaxseed and soybean oils, as well as green vegetables and nuts, and provides ~90% of the n-3 PUFAs in Western diets (*IOM*, 2002). LA and ALA can be further elongated and unsaturated in mammalian cells, where LA is a metabolic precursor for arachidonic acid (AA, 20:4, n-6) and ALA for EPA (20:5, n-3) and subsequently DHA (22:6, n-3) (Fig. 3) (Wallis et al., 2002). Desaturation and elongation of LA to AA and ALA to EPA follows the same pathway, resulting in a competition between LA and ALA as substrates for the enzymes that desaturate and elongate them. Since the conversion of ALA to EPA and DHA in humans is limited (Brenna et al., 2009), EPA and DHA may have to be obtained directly from the diet, e.g. from fatty fish and fish oil. Following consumption of PUFAs, they are absorbed in the intestines and transported to virtually all cell types where they are incorporated into cell membranes (reviewed in (Jump, 2002)). Increased intake of EPA and DHA increases their availability in the plasma or extracellular milieu that leads to increased incorporation into cell membranes in a dose dependent manner (reviewed in (Fritsche, 2007)),

reaching a plateau after about 4 weeks (reviewed in (Calder, 2007a)). The incorporation of EPA and DHA into cell membranes is partly at the expense of AA, and therefore incorporation of EPA and DHA into cell membranes alters the membrane phospholipid pool (Calder, 2008; Healy et al., 2000). The AA content in rodent lymphocytes is more readily altered by dietary EPA and DHA than AA content in human lymphocytes and they can accumulate higher levels of EPA. The percentage of EPA in human lymphocytes never exceeds 4% of total FAs but in rodent lymphocytes it can reach up to 12% of total FAs (Fritsche, 2007). FA composition of the cell membrane influences immune cell function by various mechanisms, e.g. by modulating membrane integrity, affecting intracellular signaling and gene expression, biosynthesis of lipid mediators, like eicosanoids, and lipid raft structure (Stulnig, 2003).

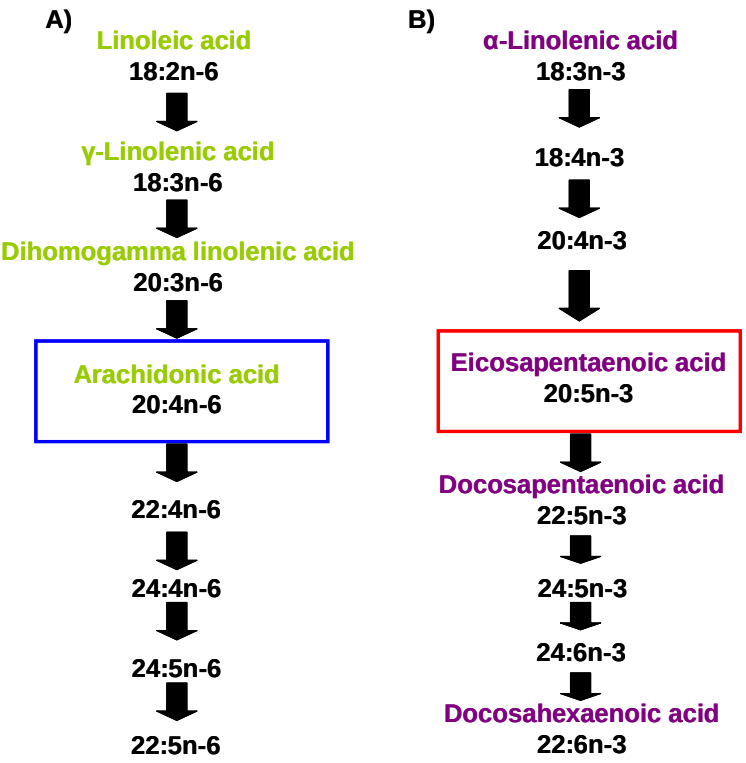


Figure 3. Members of the (A) n-6 and (B) n-3 families of polyunsaturated fatty acids.

Lipid rafts are subdomains located on plasma membranes that contain high concentrations of glycolipids, cholesterol and sphingolipids (reviewed in (Kobayashi et al., 2006)). These domains are small and detergent resistant but are thought to constitute a relatively large fraction of the phospholipid bilayer (Pike, 2003). However, the lipid rafts are difficult to examine and therefore their size and lifespan remain a subject of debate. Lipid rafts serve a role as "signaling platforms" containing high amounts of proteins important for signal transduction (reviewed in (Zeyda & Stulnig, 2006)). They are thought to be important in lipopolysaccharide (LPS)-induced signaling and signaling via the T cell receptor (Moran & Miceli, 1998). Incorporation of n-3 PUFAs into cell membranes can alter the characteristics of lipid rafts by affecting their size and stability as well as the composition of signaling proteins within the lipid rafts (Kim et al., 2008; Ruth et al., 2009).

1.1.1 Eicosanoids

Eicosanoids are bioactive lipid mediators derived from the 20-carbon PUFAs, dihomo-γ-linolenic acid (DGLA), AA and EPA. They are important mediators of immune responses, both in inflammation and homeostasis. Among the eicosanoids are leukotrienes (LTs) and prostanoids, which can be further divided into prostaglandins (PGs), prostacyclins (PGIs), and thromboxanes (TXs) (reviewed in (Kantarci & Van Dyke, 2003)). DGLA, AA and EPA are precursors for different families of eicosanoids and compete as substrates for the same metabolic enzymes for the production of eicosanoids (Smith, 2005). In addition EPA and DGLA partially inhibit AA metabolism by the cyclooxygenases (COX) and the lipooxygenases (LO or LOX) enzymes (Stulnig, 2003). Thus, the availability of DGLA, AA and EPA in cell membranes directly influences what family of eicosanoids is formed. DGLA is a precursor for the 1-series of prostanoids and 3-series LTs. AA is a precursor for the 2-series prostanoids and the 4-series LTs, whereas EPA is a precursor for the 3-series prostanoids and 5-series LTs (Stulnig, 2003). The 1- and 3-series prostanoids and the 3- and 5-series LTs are in general considered less potent or less inflammatory than the 2-series prostanoids and the 4-series LTs derived from AA (Dooper et al., 2002) and (reviewed in (Calder, 2010)). It is believed that increased proportion of EPA in cell membranes leads to an anti-inflammatory or less inflammatory immune response. The oxidation of PUFAs into prostanoids and LTs is driven by COX and LO, respectively. n-3 LC-PUFA affect the expression of

some of these enzymes and can therefore alter the production of lipid mediators (reviewed in (Stulnig, 2003)).

The cyclooxygenase pathway: There are three known isoforms of COX: COX-1, COX-2 and COX-1b (COX-3, COX-1v). COX-1 is expressed constitutively in most cells, whereas COX-2 is induced by various stimuli such as cytokines and mitogens (reviewed in (Chell et al., 2006; Fetterman & Zdanowicz, 2009)). Little is known about COX-1b but it is currently believed that it does not exert a cyclooxygenase activity and therefore does not play a role in production of fever and pain in rodents and humans (Chandrasekharan et al., 2002; Kis et al., 2006). Phospholipase (PL) A2 releases AA from cell membranes and the free FAs are readily metabolized to PGH_2, an unstable intermediate, by COX. This is the rate limiting step in the synthesis of prostanoids. PGH_2 is then converted into a series of bioactive prostaglandins PGF_2, PGI_2, PGE_2, PGD_2 or TXA_2, by cell specific prostanoid synthases (reviewed in (Chell et al., 2006; Goetzl et al., 1995)).

The lipoxygenase pathway: The three main human LOs, are 5-, 12- and 15-LO. 15-LO is not expressed in mice, but mice do express 5-LO, 8-LO and 12S-LO among others (Heidt et al., 2000; Schneider et al., 2004; Silverman et al., 2002). Different cell types express different LO, 5-LO is normally expressed in neutrophils, 12-LO in platelets and 15-LO in endothelial/epithelial cells (reviewed in (Kantarci & Van Dyke, 2003)), whereas monocytes, dendritic cells (DCs) and macrophages express both 5- and 15-LO (Christmas et al., 1999; Spanbroek et al., 2001). Induction of the 5-LO pathway starts with the release of free AA from phospholipids (reviewed in (Newcomer & Gilbert, 2010; Stulnig, 2003)). Free AA sequesters at the nuclear membrane where it is brought in contact with 5-LO by its "helper" protein, the nuclear envelope protein 5-lipoxygenase activating protein (FLAP) (reviewed in (Newcomer & Gilbert, 2010)). AA is catalyzed by 5-LO to 5- hydroperoxyeicosatetraenoic acid (HPETE) and subsequently to the intermediate LTA_4, which is further converted to LTB_4 or LTC_4 by LTA_4 hydrolase (LTA_4H) or LTC_4 synthase, respectively. LTC_4 can be further catalyzed by ectoenzymes to LTD_4 and LTE_4, which are biologically active metabolites (Keppler et al., 1987; Smith & Murphy, 2002).

AA is also converted to 12-HPETE and 15-HPETE with 12- and 15-LO, respectively. HPETE is unstable and rapidly dehydrated to hydroxyeicosatetraenoic acid (HETE)(Smith & Murphy, 2002). As said before, 12-LO is mainly expressed in platelets and its metabolite 12-HETE has been reported to augment metastasis and tumorigenesis (Natarajan & Nadler, 1998; Yoshimura et al., 2004). The metabolite of 15-LO, 15-HETE, has been shown to inhibit LTB_4 activity (Profita et al., 2000) and induce

angiogenesis (Zhang et al., 2005). The LO enzymes are also important initiators of the first steps in the synthesis of the pro-resolving lipid mediators, lipoxins (LX), resolvins (Rv), protectins (PD) and maresins (reviewed in (Ariel & Serhan, 2007; Serhan et al., 2009)).

1.1.2 Pro-resolving lipid mediators

A novel group of bioactive lipid mediators has been identified that in contrast to the classical pro-inflammatory eicosanoids are involved in the counter-regulation of inflammation. Resolution of acute inflammation was, in the past, thought to be a passive process but recent evidence has demonstrated that resolution is an active process, which is coordinated by a group of pro-resolving lipid mediators. These lipid mediators are metabolic derivatives of PUFAs and exert immunoregulatory actions at concentrations in the nano- and picomolar range (Serhan et al., 2002). LXs are the only pro-resolving mediators synthesized from the n-6PUFA AA, whereas Rvs, PDs and the newly identified maresins are synthesized from the n-3PUFAs EPA and DHA (reviewed in (Serhan, 2010)).

Lipoxins:

LXs were the first pro-resolving lipid mediators that were identified (Serhan, 2005). Biosynthesis of LXs is activated by cell-cell interaction at the onset of inflammation or infection (Levy et al., 2001). LXs can be generated from AA by several different transcellular biosynthetic pathways. The first described pathway of LX biosynthesis, involves the primary synthesis of 15-HPETE initiated by 15-LO in macrophages, basophils or epithelial cells. 15-HPETE is a 5-LO substrate and is taken up by leukocytes that convert it to LXA_4 or LXB_4 (reviewed in (Kantarci & Van Dyke, 2003; Serhan, 2007)). Another main route of LX synthesis is the 5-LO initiated pathway that occurs as the result of interaction of neutrophils with platelets. 5-LO, which is present in most leukocytes, converts AA to LTA_4, an unstable intermediate which can be taken up by platelets and converted to LXA_4 or LXB_4 by 12-LO (reviewed in (Kantarci & Van Dyke, 2003; Serhan, 2005)). Another class of LXs is the aspirin-triggered lipoxins (ATL), which are produced by COX-2(reviewed in (Serhan, 2007)). In the presence of aspirin, COX-2 becomes acetylated which changes its catalytic activity from prostanoid synthesis to production of 15R-HETE. 15R-HETE is then transformed by leukocytes to 15-epi-lipoxins, 15-epiLXA_4 and 15-epi-LXB_4, through the action of 5-LO (reviewed in (Kantarci & Van Dyke, 2003)).

The bioactivity of LXs is not fully understood but is under current investigation. Their main function seems to be in counter-regulation of inflammation with inhibitory actions on neutrophils, eosinophils and natural killer (NK) cells, but stimulating actions on monocytes and macrophages. LXs down-regulate neutrophil recruitment, adhesion and transmigration and promote monocyte recruitment without stimulating pro-inflammatory gene pathways and subsequent pro-inflammatory gene products and enhance macrophage engulfment of apoptotic neutrophils (reviewed in (Serhan, 2010)). These actions of LXs result in stimulation of the “clean-up process” following inflammatory responses.

Resolvins:

Rvs are bioactive lipid mediators that are synthesized from the n-3 PUFAs EPA and DHA. There are two classes of Rvs, each with chemically unique structural form. The E series resolvins (RvE) are synthesized from EPA, whereas the D series resolvins (RvD) are synthesized from DHA (reviewed in (Schwab & Serhan, 2006; Serhan et al., 2004)). The mechanism of RvE biosynthesis has been partially elucidated and is initiated by the conversion of EPA to 18R- and 18S-HEPE by aspirin-modified COX-2 and mammalian or microbial CYP450(Serhan, 2007). Both 18-HEPE isomers are substrates for 5-LO and are either reduced into RvE2 and 18S-RvE2 or hydrolyzed further by LTA_4H to RvE1 and 18S-RvE1 (Oh et al., 2011). Rvs, similar to LXs, are potent pro-resolving mediators and may contribute to the return of homeostasis of the inflamed tissue (Arita et al., 2006). RvE1 reduces neutrophil infiltration and prevents leukocyte mediated damage of tissues by reducing production of pro-inflammatory cytokines and chemokines (Arita et al., 2005).

The D series resolvins are synthesized from DHA either by12/15-LO (in humans, 12-LO in mice) producing the 17S-HPDHA which gives rise to RvD1-4, or by aspirin-modified COX-2 function forming the 17R-HPDHA intermediate that gives rise to the AT-RvD1-4 (Serhan, 2010). The E and D series Rvs exhibit similar biological functions.

Protectins:

PDs are another family of pro-resolving lipid mediators. They are synthesized from DHA in the presence of aspirin and have a conjugated triene double bond structure (Serhan et al., 2006). DHA is converted to 17S-HPDHA intermediate though a LO mechanism but is rapidly converted to a 16(17)-epoxide that is subsequently enzymatically opened to form 10,17S-docosatriene or PD1 (or neuroprotectin when produced by neural

tissues) (Hong et al., 2003). PDs are potent anti-inflammatory, pro-resolving lipid mediators similar to LXs and Rvs. They reduce neutrophil influx and stimulate macrophage efferocytosis and promote wound healing (reviewed in (Serhan, 2007) and (Serhan et al., 2008)). PD1 has also been shown to upregulate CC chemokine receptor (CCR) 5 on dying neutrophils and thus facilitate chemokine removal (Ariel et al., 2006). Along with its immunoregulatory effects, PDs are also believed to be neuroprotective as it promotes brain survival (Marcheselli et al., 2003).

Maresins:

Maresins are recently discovered macrophage-derived pro-resolving lipid mediators. Maresins are produced by human and mouse macrophages and are synthesized from DHA, like RvDs and PDs (Serhan et al., 2009). The biosynthetic pathway of maresins is yet to be elucidated but it is proposed that following the conversion of DHA to 14S-HPDHA by 12/15-LO, 14S-HPDHA serves as a substrate for subsequent enzymatic epoxidation and hydrolyzation yielding the product 7,14-diHDHA denoted maresin 1 (MaR1) (Serhan et al., 2009). After the initial 12/15-LO step the14S-HPDHAintermediate can be converted by 5-LO to7S,14S-diHDHA,an active but less potent isomer of MaR1 (Serhan et al., 2009). Maresins are potent in inhibition of neutrophil infiltration and stimulation of macrophage efferocytosis (Serhan et al., 2009).

1.2 The innate immune system

Our survival is greatly dependent on a properly functioning immune system; a complex network of proteins, cells, tissues and organs that work together in a synchronized manner to protect us from foreign pathogens and noxious insult. The immune system is tightly regulated where it can discriminate between self and non-self molecules, as well as between harmful and non-harmful non-self molecules assuring tolerance and elimination of harmful non-self antigens. The cells of the innate immune system provide the first line of defense, they are the first cells to arrive at the site of insult, where they recognize and eliminate threatening pathogens and secrete inflammatory mediators such as cytokines and chemokines. If the innate immune system is not successful in eliminating the infectious agents the adaptive immune system is activated, providing immunological memory and preventing repeated infections. Thus the innate immune response plays an important role in controlling the initial infection

until the adaptive immune system becomes activated and together they eliminate the threat.

The cells of the immune system all derive from the hematopoietic stem cells (HSCs) in the bone marrow. The HSCs give rise to stem cells that have more limited potentials and are immediate progenitors of the different types of immune cells (reviewed in (Ratajczak, 2008)). Most of the cells of the innate immune system are derived from the common myeloid progenitor (CMP), including mast cells, granulocytes, monocytes/ macrophages and DCs (reviewed in (Weissman & Shizuru, 2008)). There is one exception, the NK cells, which are derived from the common lymphoid progenitor. NK cells are different from other cells derived from common lymphoid progenitor as they do not express traditional antigen receptors. They are activated during the early immune response, and are thus part of the innate immune system but not the adaptive immune system as the other common lymphoid progenitorderived cells (reviewed in (Paust et al., 2010)). The primary role of NK cells is killing abnormal cells, mainly tumor cells and those infected with viruses.

Granulocytes are cells that are densely packed with granules in their cytoplasm. They are also termed polymorphonuclear (PMN) leukocytes because of their oddly shaped nuclei. Granulocytes consist of three different cell types, the eosinophils, the basophils and the neutrophils. Along with mast cells, basophils and eosinophils mainly act as secretory cells releasing their granular contents (enzymes and toxic proteins) upon activation (reviewed in (Stone et al., 2010)). The main function of eosinophils is thought to be protection of the host against parasites, whereas basophils and mast cells play a role in allergic inflammation. These cells will not be discussed in further detail in this thesis.

1.2.1 Neutrophils

Neutrophils are the most abundant white blood cells in mammals and their primary function is to protect the host from microbial invaders (reviewed in (Borregaard, 2010)). Neutrophils are short lived cells, and reside in a resting state within the circulation only for several hours in the absence of inflammatory signals, but in response to infection or inflammation they are recruited out of the bloodstream. The neutrophils are fast acting and provide an essential part of the innate system. They are the first cells to arrive at the site of infection where their role is to eliminate the invading pathogen through three different mechanisms: microbial uptake, the

secretion of antimicrobial proteins and peptides, and as has been recently described, the release of neutrophil extracellular traps (NETs) (reviewed in (Papayannopoulos & Zychlinsky, 2009)). However, despite their essential role in host defense, they are a double edged sword as they can also promote persistent inflammatory responses and tissue injury which can be detrimental to the host. Thus, the balance between neutrophil production, neutrophil release from the bone marrow and their clearance from the circulation needs to be tightly regulated. The number of neutrophils in blood needs to provide the host with an adequate pool of neutrophils that can be readily deployed to sites of infection when needed. Too few neutrophils, or neutropenia, can be harmful to the host during bacterial infections as it can lead to bacterial overgrowth. On the other hand, excessive neutrophil recruitment and activation can contribute to tissue damage in some inflammatory disorders, such as arthritis (reviewed in (Eash et al., 2009)).

Neutrophils develop in the bone marrow, where they originate from self-renewing myeloid stem cells. Upon maturation they emigrate to the vasculature. Neutrophil maturation and differentiation within the bone marrow is mediated by variety of hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte/ macrophage colony-stimulating factor (GM-CSF). Neutrophil production, differentiation and maturation is dependent on the interaction of the growth factors with their respective receptors expressed by the neutrophil myeloid precursors and impairment in their function or expression can lead to myeloid maturation arrest and neutropenia (reviewed in (Seely et al., 2003)). Neutrophils are short lived, with a half life of 8-16 h under normal conditions (reviewed in (Eash et al., 2009)) and need to be efficiently cleared from the circulation. Neutrophils are mainly cleared in the liver, spleen or bone marrow where they are phagocytosed by macrophages, but the mechanism of their destruction is poorly defined (Furze & Rankin, 2008).

Neutrophil release from the bone marrow is negatively regulated by the interaction of the CXC chemokine receptor (CXCR) 4, expressed by neutrophils and most hematopoietic cells, and the CXC chemokine ligand (CXCL) 12 (stromal derived factor-1, SDF-1) which is constitutively expressed by cells in bone marrow stroma under normal conditions (reviewed in (Borregaard, 2010)). During infection there is a significant increase in G-CSF production that in turn causes a decrease in CXCL12 expression in the bone marrow along with down-regulation of CXCR4 on neutrophils which may in part

mediate the release of neutrophils from the bone marrow (Kim et al., 2006; Levesque et al., 2003; Semerad et al., 2005).

When neutrophils are released from the bone marrow under normal conditions they circulate within the vasculature in a resting state or move to marginating pools in the capillaries of certain tissues, such as the lungs (Downey et al., 1993; Seely et al., 2003). The marginating pools allow for a rapid influx of neutrophils into tissues in response to an inflammatory insult. Neutrophils express selectins (e.g. L-selectin) and integrins (e.g. CD11b) that mediate their adherence and firm adhesion to the endothelium and their consequent transmigration into the inflamed tissue, which is directed by chemokines (reviewed in ((Granger & Kubes, 1994; Seely et al., 2003). Neutrophil transmigration is a tightly regulated multistep process involving several sequential events, with each step being important for the progression to the next. The process of neutrophil recruitment will be discussed in more detail in section 1.2.6.1.

Neutrophils are well suited for the elimination of pathogens as they possess several mechanisms to kill microbes, such as phagocytosis, release of soluble antimicrobial products and generation of NETs as mentioned earlier (reviewed in (Borregaard, 2010; Hickey & Kubes, 2009)). The NETs are made from chromatin and they, along with serine proteases, aid with the trapping and killing of bacteria extracellularly (reviewed in (Borregaard et al., 2007)).

One of the main characteristics of neutrophils is their numerous granules, in which they store preformed proteins (Soehnlein et al., 2009) that are secreted upon activation. The different granules have specialized functions (reviewed in (Borregaard et al., 2007)). Secretory vesicles are unique granules that are important for providing membrane associated receptors, such as CD11b, that ensure firm adhesion to the activated endothelium and therefore facilitate transmigration (reviewed in (Borregaard et al., 2007)). Gelatinase granules, or tertiary granules, contain metalloproteases, such as matrix metallopeptidase (MMP)-9 that are secreted during transmigration of the neutrophils and degrade the basal membrane to allow for more efficient neutrophil migration (Soehnlein et al., 2009). Azurophilic (primary) and specific (secondary) granules contain cytotoxic and matrix-degrading enzymes, such as collagenase, serine proteases and lysozyme that are important in bacterial clearance. The content of the azurophilic granules is mainly released within the phagosomes, whereas the content of the specific granules is secreted both

into the phagolysosomal vacuoles and to the surroundings (Hager et al., 2010).

Upon phagocytosis, neutrophils activate both oxygen-dependent and oxygen-independent antimicrobial systems with formation and secretion of reactive oxygen species (ROS) and hydrolytic enzymes that assist with the degradation of the internalized pathogens (Murphy et al., 2008). The membrane of the phagolysosome contains NADPH oxidase that, along with other enzymes such as myeloperoxidase, drive the production of other potent oxidants that can destroy the pathogen (Hager et al., 2010). Despite the vital role of these enzymes in microbial killing, they can prove to be harmful to the host if they are released inappropriately (reviewed in (Borregaard et al., 2007)).

In addition to their capacity to eliminated pathogens, neutrophils secrete a vast number of mediators that activate or attract other cells also involved in immune defense.

1.2.2 Monocytes

Circulating monocytes are among the key effector cells of the innate immune system (reviewed in (Serbina et al., 2008; Wang & Deng, 2008)). Monocytes are a heterogeneous population of cells that are in a transitional state in the circulation. When they arrive in tissues they become macrophages or in some cases DCs. Thus, monocytes are “plastic” cells that give rise to distinct cell populations with distinct functions, where their local microenvironment and maturation status determine their development and function. Migration of monocytes to healthy or infected tissues is mediated by chemokine receptors and adhesion molecules and subsets of monocytes are defined based on their expression of these molecules.

Monocytes develop in the bone marrow and then emigrate to the blood (Varol et al., 2009). They can circulate between blood and spleen before they finally reside in various tissues where they become long-lived macrophages. There are two major subsets of monocytes that have been described in human and mice (reviewed in (Geissmann et al., 2003; Gordon & Taylor, 2005; Sunderkotter et al., 2004)) termed classical and non-classical monocytes. These subtypes express different chemokine receptors and adhesion molecules and differ in their level of maturation, differentiation potential and migration pattern (Table 1). $CD14^{low}CD16^{hi}$ monocytes in humans behave similar to $CX_3CR1^{hi}Ly6C^{low}$ subtype of monocytes in mice and in both species these monocytes express low levels

of the chemokine receptor CCR2 (reviewed in (Geissmann et al., 2003; Tacke & Randolph, 2006)). These monocytes are called non-classical monocytes. The classical $CD14^{hi}CD16^{low}CCR2^{hi}$ monocytes in humans resemble the murine $CX_3CR1^{low}Ly6C^{hi}CCR2^{hi}$ monocytes (reviewed in (Geissmann et al., 2003; Sunderkotter et al., 2004; Tacke & Randolph, 2006)). Classical monocytes express high levels of the chemokine receptor CCR2(reviewed in (Geissmann et al., 2003; Serbina et al., 2008; Strauss-Ayali et al., 2007; Sunderkotter et al., 2004; Tacke & Randolph, 2006; Yona & Jung, 2010)). In humans classical monocytes constitute 90-95% of total monocytes, but in mice they only constitute around 50% of total monocytes (Tacke & Randolph, 2006).

Table 1. Phenotype of mouse monocyte subsets[1]

	Classical	Non-classical
Monocyte markers		
CD115	+	+
CD11b	+	+
F4/80	+	+
GR-1 (Ly6C/Ly6G)	+	−
Chemokine receptors		
CX_3CR1	Lo	hi
CCR2	+	−
Adhesion molecules		
CD31	++	+
LFA1	+	++
VLA2	+	−
VLA4	+	+
L-selectin (CD62L)	+	−
T, B, NK, IPC, DC markers		
CD90.2	−	−
B220 (CD45R)	−	−
NK1.1	−	−
CD11c	−	−
TCRβ	−	−

[1]Based on (Geissmann et al., 2003)

The surface markers Gr-1 and Ly6C, both recognizing Ly6C, have both been used for identification of monocyte subpopulations. However, the antibody against Gr-1 not only recognizes Ly6C but also the neutrophil surface marker Ly6G (Daley et al., 2008; Fleming et al., 1993)). Ly6C is expressed by both monocytes and neutrophils (and other hematopoeitic cells), whereas Ly6G is only expressed by neutrophils (reviewed in (Geissmann et al., 2003; Lagasse & Weissman, 1996; Sunderkotter et al., 2004)). The surface receptor CD115 (the receptor for macrophage colony stimulating factor (M-CSF)) has been established as a useful monocyte marker (Breslin et al., 2011; Sunderkotter et al., 2004), whereas the use of CD11b can be problematic because of its expression by other cell types, e.g. NK cells, activated T cells, and B cells (reviewed in (Breslin et al., 2011)).

When monocytes are released from the bone marrow they exhibit a phenotype of classical monocytes, but in the circulation they can serve as precursors for the more mature non-classical monocytes (reviewed in (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008; Strauss-Ayali et al., 2007; Sunderkotter et al., 2004; Tacke & Randolph, 2006; Yona & Jung, 2010)). Table 1 shows the phenotypes of classical and non-classical subpopulations of mouse monocytes (Geissmann et al., 2003). In response to infection or inflammation, classical monocytes emigrate massively from the bone marrow to the blood in response to high concentrations of the CC chemokine ligand (CCL) 2, the ligand for the chemokine receptor CCR2. They are also selectively recruited to inflamed tissue and lymph nodes in response to CCL2. Classical monocytes produce high levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1 during infection and inflammation (reviewed in (Geissmann et al., 2003; Serbina et al., 2008; Serbina & Pamer, 2006; Sunderkotter et al., 2004)) and they contribute to the clearance of bacteria during infections (Robben et al., 2005)).

Because of the apparent role of the classical monocytes in inflammatory responses, earlier reports use the term inflammatory monocytes for these murine Ly6C^{hi} monocytes. The Ly6C^{low} non-classical monocytes are generally thought not to be recruited into inflamed or infectious sites but rather to migrate to tissues under homeostatic conditions to replace tissue macrophage or DCs ((Drevets et al., 2004) and reviewed in (Geissmann et al., 2003)). The non-classical monocytes patrol the blood vessels and use lymphocyte-function associated antigen (LFA)-1 dependent crawling on the endothelium for that purpose. In the blood vessels they play an important role in scavenging oxidized lipid, dead cells

and potential pathogens (reviewed in (Auffray et al., 2009)). Due to their close proximity with the endothelial vessel walls, the non-classical monocytes rapidly invade tissues in case of inflammation or damage. Their infiltration into tissues even precedes that of neutrophils and classical-monocytes and occurs as early as within 1 h from initial insult or inflammation (reviewed in (Auffray et al., 2009)). Non-classical monocytes may also have a role later in inflammation as they have been shown to be recruited at a late phase to the ischemic myocardium, where they express vascular endothelial growth factor (VEGF) and are proposed to promote healing via myofibroblast accumulation, angiogenesis and deposition of collagen (reviewed in (Geissmann et al., 2003)).

Recently, a reservoir of undifferentiated monocytes was identified in the spleen that can be readily recruited in response to injury. The reservoir contains a large number of monocytes, both the classical and non-classical subpopulations, and the ratio between the monocyte subtypes is similar to that in the blood. These splenic monocytes resemble their blood counterparts morphologically and exceed them in number (Swirski et al., 2009).

1.2.3 Macrophages

Macrophages are widely distributed throughout the body. The majority of tissue macrophages are derived from circulating monocytes, although some populations of macrophages are replenished by self-renewal under normal conditions (reviewed in (Gordon & Taylor, 2005)). Macrophages comprise a very heterogeneous population of cells. The development of macrophage phenotypes depends upon the type of stimuli encountered and tissue localization. They develop distinct functions as well as expression of different surface molecules (reviewed in (Gordon & Taylor, 2005)). Osteoclasts in bones and Kupffer cells in liver are examples of macrophages in different tissues that have developed different phenotypes and functions. In addition, within different compartments of the spleen there are several phenotypes of macrophages, such as splenic white pulp macrophages, marginal zone metallophilic macrophages and red pulp macrophages (reviewed in (Gordon & Taylor, 2005)). Macrophages have diverse functions both in steady state as well as during innate and adaptive immune responses. Under normal conditions they are involved in the maintenance of tissue homeostasis through clearance of senescent and tumor cells. In inflammation the main functions of macrophages are phagocytosis, eradication of apoptotic cells, clearance of pathogens,

biosynthesis of inflammatory mediators and wound healing (reviewed in (Gordon, 2007)). Macrophages, along with DCs, can also act as antigen presenting cells as they can present antigens to T cells and activate them.

Macrophages express a variety of surface markers and receptors that are necessary for their function, such as adhesion molecules that aid their adherence to extracellular matrix and other cells. Macrophages express several types of recognition receptors that can be divided into phagocytic/endocytic receptors and pathogen recognition receptors that do not mediate phagocytosis but are instead important for sensing pathogens and alerting the immune system. Fc receptors and complement receptors belong to the former, as they recognize opsonized particles, and enable their phagocytosis (reviewed in (Gordon, 2007; Taylor et al., 2005)). Scavenger receptors, as well as being pattern recognition receptors (PRRs), also act as phagocytic receptors, but unlike Fc and complement receptors they are not opsonizing receptors but recognize bacteria, apoptotic cells and low density lipoproteins (LDL) (reviewed in (Taylor et al., 2005)). Most PRRs, such as toll-like receptors (TLRs), belong to the non-phagocytic PRRs, and facilitate the recognition of pathogen-associated molecular patterns (PAMPs) expressed on pathogens and initiate effector function upon activation without mediating phagocytosis (reviewed in (Gordon, 2007; Taylor et al., 2005)). TLRs are transmembrane glycoprotein receptors expressed either on the cell surface (TLR1, -2, -4, -5, and -6) or intracellularly in endozomes/lysosomes (TLR3, -7, -8 and -9) (Kawai & Akira, 2007). TLR4 is one of three components of the mammalian LPS receptor; the other two being CD14 and MD-2. LPS is the major constituent of the outer wall of Gram-negative bacteria. TLR2 signaling is also enhanced by CD14, but unlike TLR4, TLR2 recognizes a broad range of microbial products such as peptidoglycan (PPG), macrophage activating lipopeptide (MALP)-2, lipoproteins, lipopeptides and fungal cell wall molecules (e.g. zymosan) (reviewed in (Dziarski, 2003)). TLR2 can form different heterodimers with TLR1 and TLR6, which contributes to its diverse recognition range (reviewed in (Schenk et al., 2009)).

1.2.4 Cytokines

Cytokines are small (10-25 kDa) soluble proteins that mediate communication between cells (Murphy et al., 2008). They constitute a diverse group of proteins with miscellaneous functions. Cytokines have been found to participate in virtually all physiological processes, e.g. from regulating embryogenesis to mediating inflammation. They are important

mediators of immune responses as they direct the development, maturation, localization, interactions, activation and life span of immune cells. Cytokines are frequently produced in response to activating stimuli and serve as signaling molecules orchestrating inflammatory responses by binding to specific receptors (Murphy et al., 2008). As cytokines play an important role in orchestrating the immune responses, their expression is tightly regulated, whereas unregulated production can result in serious pathological conditions.

The functions of cytokines are normally pleiotropic and redundant and one cytokine can influence the synthesis of other cytokines, enhancing or suppressing their production. In addition, cytokines can often influence the action of other cytokines, providing antagonistic, additive or even synergistic effects. Cytokines can be clustered into several families. These families include colony stimulating factors (CSF), ILs, TNF family, interferons (IFN), transforming growth factors (TGF) and chemokines (that will be discussed in detail below). The cytokines are sometimes further categorized according to their pro-inflammatory (e.g. TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-17, IL-18, IL-21, IL-22, IL-23, IL-27, IL-33 and IFN-γ) or anti-inflammatory (e.g. IL-4, IL-10, IL-13, IL-35, IL-37, TGF-β) actions (D'Acquisto et al., 2010).

TNF-α: TNF-α is a pro-inflammatory cytokines and is the defining member of the TNF family of cytokines. It is known as the principle cytokine that mediates acute inflammation and in excessive amounts it can cause systemic complications, such as the ones seen in septic shock.

TNF-α is produced by activated leukocytes, primarily monocytes and macrophages, but also by T cells, B cells and NK cells (Elenkov & Chrousos, 2002; Kruglov et al., 2008). TNF-α production is induced by cytokines, such as IFN-γ, IL-1 and IL-2, or by TLR activation by bacterial constituents such as LPS (Mannel & Falk, 1989). TNF-α can both function as a cell-associated or secreted protein that interacts with receptors called tumor necrosis factor receptors (TNFRs). The pre-formed membrane bound protein is cleaved off by a proteolytic enzyme, the TNF-α converting enzyme, rendering the protein soluble (Feldmann & Maini, 2001). Its production can in turn be inhibited by several factors like IL-10, PGE_2 and glucocorticoids (Elenkov & Chrousos, 2002).

TNF-α exerts diverse biological activities. It plays an important role in the induction of the immune response to infection. The primary role of TNF-α is immune cell regulation. It alters the cell-cell junctions in the vascular endothelium, increasing its permeability, making it easier for the cells, and

other factors, to pass through the endothelium from the circulation (Murphy et al., 2008). Secondly, TNF-α induces the production of multiple cytokines, chemokines and adhesion molecules that participate in the recruitment of phagocytes and other immune cells to the site of the release of these proteins. TNF-α is also important in the regulation of the production of other cytokines and chemokines and enhances the production of cytokines such as IL-1 and IL-6 (Feldmann & Maini, 2001) and chemokines like CXCL2 (Tessier et al., 1997). TNF-α activates neutrophils and macrophages and stimulates phagocytosis and enhanced killing of bacteria during infection. Another important function of TNF-α is its ability to facilitate blood clotting, which inhibits bacteria from spreading (Murphy et al., 2008). Production of TNF-α is not always beneficial as overexpression of the cytokine is linked to the pathogenesis of several inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (Kollias, 2005) and also in more severe conditions such as cachexia and sepsis (Tracey et al., 1987).

IL-6: IL-6 is a pleiotropic cytokine with a wide range of biological activities, but similar to TNF-α, IL-6 is well known for its pro-inflammatory actions. It is produced by a number of cells including T cells, B cells, macrophages, astrocytes and endothelial cells (Hirano, 2010) in response to cytokines or LPS (Beyaert et al., 1996; Medzhitov & Horng, 2009; Navarro et al., 1989). IL-6 acts on many cell types and induces an extensive range of responses. Its key function is to induce the production of acute phase proteins by hepatocytes, a function that it shares with TNF-α and IL-1 (Kopf et al., 1994). IL-6 has diverse effects on the immune system. It simulates B cell antibody production and T cell differentiation (Naka et al., 2002). It also induces IL-2 production and IL-2 receptor expression (Park & Pillinger, 2007). IL-6 also plays a role in leukocyte recruitment to infected or inflamed tissues, as it activates endothelial cells and induces production of chemokines and expression of adhesion molecules (Cronstein, 2007). As a part of its pro-inflammatory effects, IL-6 induces the expression of PLA2, which in turns promotes the generation of LTs, PGs and platelet-activating factor (PAF) (Tilg et al., 1997). IL-6 and PAF synergistically prime PMNs but priming of PMNs appears to be an important event in the pathogenesis of hyper-inflammatory states (Biffl et al., 1994). Although, IL-6 is more recognized for its pro-inflammatory effects, it can also have anti-inflammatory effects. *In vitro* studies have demonstrated that IL-6 inhibits production of TNF-α and the release of IL-1 inhibitor (Heinrich et al., 1990; Naka et al., 2002; Nijsten et al., 1991; Scheller & Rose-John, 2006; Tilg et al., 1997).

The production of IL-6 is implicated in the pathogenesis of a variety of diseases such as diabetes (Kristiansen & Mandrup-Poulsen, 2005) and RA

(Nishimoto, 2006). Increased serum concentrations of IL-6 have been found in surgical patients, with the concentration of IL-6 being proportional to the magnitude of the surgical stress (Kashiwabara et al., 2007; Ohzato et al., 1992; Yahara et al., 2002). In addition serum concentrations of IL-6 correlated with severity of sepsis in septic patients and increased concentrations of IL-6 have been correlated with adverse outcome in septic patients (Damas et al., 1992; Hack et al., 1989).

IL-1β: IL-1β is a member of the IL-1 cytokine family and is a pleiotropic cytokine. Under homeostatic conditions IL-1β regulates feeding, sleep and temperature (reviewed in (Dinarello, 1996)). IL-1β is also a known pro-inflammatory cytokine that plays an important role in chronic and acute inflammatory immune responses. It acts synergistically with TNF-α and mediates acute inflammatory responses. The functions carried out by IL-1β include promotion of inflammation, activation of the coagulation pathway and stimulation of acute phase protein production (Murphy et al., 2008). IL-1β activates macrophages and stimulates the production of other pro-inflammatory cytokines, like TNF-α and IL-6, and the expression of adhesion molecules that promote transmigration (Murphy et al., 2008). Furthermore, IL-1β also promotes a Th17 bias in the cellular adaptive responses (Chung et al., 2009). IL-1β is produced by many cell types, such as monocytes/macrophages, fibroblasts and endothelial cells (Shirakawa et al., 1993). It is produced as an inactive pro-IL-1β, which is cleaved by inflammasomes to generate the bioactive, pro-inflammatory form (reviewed in (Netea et al., 2010)). Overproduction of IL-1β is implicated in many inflammatory disorders, such as RA, vascular disease and multiple sclerosis (reviewed in (Braddock & Quinn, 2004; Dinarello, 1996)) and is well known as a contributor to the development of sepsis (reviewed in (Dinarello, 2000)).

IL-33: IL-33 is a recently identified cytokine that belongs to the IL-1 superfamily of cytokines. IL-33 is a nuclear protein that is also released into the extracellular space, and thus acts as a dual-function molecule. Like IL-1β, IL-33 is produced in a precursor form that is cleaved by inflammasomes to render the activated form (reviewed in (Schmitz et al., 2005)). It is mainly expressed by structural and lining cells like fibroblasts (Moussion et al., 2008; Sanada et al., 2007), epithelial cells (Moussion et al., 2008) and endothelial cells (Carriere et al., 2007; Miller et al., 2008; Moussion et al., 2008) and appears to be released as an alarmin, i.e. an endogenous danger signal provided by stressed cells.

IL-33 is a ligand for the receptor ST2 (or IL-1RL1) which is a member of the IL-1 receptor family. ST2 is mainly expressed by Th2 cells and IL-33 is thought to play a role in Th2 cell mobilization (Komai-Koma et al., 2007) and to enhance the secretion of Th2-type cytokines, like IL-4 (Bourgeois et al., 2009; Smithgall et al., 2008). However, IL-33 enhances production of Th1 type cytokines by NK and NKT cells (Bourgeois et al., 2009; Smithgall et al., 2008). IL-33 activates mast cells and stimulates their production of pro-inflammatory cytokines and chemokines, particularly IL-1β, IL-6, IL-13, TNF-α, CCL2 and CCL3 (reviewed in (Liew et al., 2010)). Granulocytes are also targets of IL-33 (Alves-Filho et al., 2010; Cherry et al., 2008; Pushparaj et al., 2009; Suzukawa et al., 2008a; Xu et al., 2008), in which it induces degranulation, adhesion and survival (Cherry et al., 2008; Smithgall et al., 2008; Suzukawa et al., 2008a; Suzukawa et al., 2008b). The activation of neutrophils by IL-33 has been indicated to prevent polymicrobial-mediated sepsis as it prevents TLR mediated down regulation of CXCR2 both in human and mouse neutrophils (Alves-Filho et al., 2010). The preservation of CXCR2 on neutrophils enhances their migration to the site of infection for bacterial clearance and prevents their sequestration into the lungs (Alves-Filho et al., 2010). It has been implied that IL-33 plays a role in the pathogenesis of several diseases, such as asthma, arthritis, atopic allergy and Alzheimer's disease, although it has been suggested to have a protective role in other disease such as arthrosclerosis and infections (reviewed in (Liew et al., 2010)).

IL-10: IL-10 is an important anti-inflammatory cytokine and it is the defining member of the IL-10 cytokine family. It is produced by a variety of cells with the major cellular source of *in vivo* production of IL-10 being from monocytes and macrophages, but some T cell subsets, some B cells, NK cell, DCs and mast cells can also produce IL-10 (Sabat et al., 2010). IL-10 production is induced in response to pro-inflammatory cytokines (e.g. IL-12 and IL-6). Increased IL-10 production in turn inhibits further formation of pro-inflammatory cytokines, providing a form of negative feedback inhibition that plays an important role in tissue protection (Ouyang et al., 2011). Originally, IL-10 was described as a cytokine synthesis inhibitory factor (Fiorentino et al., 1989). It inhibits the production of several pro-inflammatory mediators including TNF-α, IL-1β, IL-6, G-CSF, CXCL8 and CCL3 by activated monocytes/macrophages (Lim et al., 2002; Moore et al., 2001). IL-10 can inhibit cytokine production at several different levels, it can reduce cytokine gene transcription, promote cytokine mRNA degradation and deactivate accessory cell function (reviewed in (Moore et al., 2001)

and (Pretolani, 1999)). IL-10 can also inhibit free oxygen radical release and nitric oxide (NO)-dependent microbicidal activity of macrophages as well as reduce their capacity to produce PGs (reviewed in (Moore et al., 2001)). The IL-10 family members mediate their biological activity through two receptors, namely IL-10R1 and IL-10R2. These receptors are widely expressed on cells, especially by cells of the hematopoietic lineage (Ouyang et al., 2011; Saraiva & O'Garra, 2010).

G-CSF: G-CSF is a hematopoietic cytokine that is a major regulator of neutrophil production (Lieschke et al., 1994). G-CSF stimulates the survival, proliferation, differentiation and function of neutrophil precursors and mature neutrophils (Roberts, 2005). Non-hematopoietic cells such as endothelial cells, fibroblasts and bone-marrow stromal cells are the main producers of G-CSF, although it can also be produced by cells of the myeloid lineage (e.g. monocytes, macrophages and neutrophils) following activation (Roberts, 2005). G-CSF binds to the G-CSF receptor (G-CSFR or CD114). The G-CSFR is expressed by cells of the granulocyte lineage, such as progenitors and differentiating myeloid cells in the bone marrow, mature neutrophils in the circulation, as well as endothelial cells and some carcinoma cell lines (reviewed in (Cornish et al., 2009)). Binding of G-CSF to G-CSFR initiates the proliferation and differentiation of precursor bone marrow cells into mature granulocytes (reviewed in (Panopoulos & Watowich, 2008)).

Under homeostatic conditions serum levels of G-CSF are low but they are induced in stressful conditions in an attempt to sustain the output of circulating neutrophils in response to increased demand (reviewed in (Cornish et al., 2009)). G-CSF production is induced in response to several inflammatory stimuli such as bacterial endotoxin, TNF-α and IL-1β. Increased G-CSF production results in increased neutrophil production within the bone marrow and increased neutrophil mobilization to the vasculature (Demetri & Griffin, 1991). G-CSF is essential for the increased granulopoiesis that occurs in response to bacterial infection which has been termed “emergency” or “demand-driven” granulopoiesis (reviewed in (Panopoulos & Watowich, 2008)). Increase in the proliferation rate of bone marrow neutrophils as during infection, leads to an increased proportion of early neutrophil forms, such as myeloblasts and promyelocytes in bone marrow compared to basal conditions (reviewed in (Panopoulos & Watowich, 2008)).

1.2.5 Chemokines

Chemokines are chemotactic cytokines that are essential regulators of cell migration. Chemokines exert a variety of important functions as they are involved in many diverse biological processes such as maintenance of homeostasis, angiogenesis/angiostasis, cellular differentiation and activation, wound healing, tumor growth and metastasis, lymphocyte homing and development of lymphoid tissue (Coelho et al., 2005).

Over 50 different chemokines have been identified in humans as well as 20 chemokine receptors (Table 2) (O'Hayre et al., 2008). The chemokines are low molecular weight (8-10 kDa) heparin bound proteins. They are traditionally classified according to the position of the N-terminal cysteine residues, into C chemokines (XCL), CC chemokines (CCL), CXC chemokines (CXCL) and CX_3C (CX_3CL) chemokines. The C chemokines have only two cysteines, one N-terminal cysteine and one cysteine downstream. CC chemokines have two adjacent cysteines near their amino end, whereas the CXC chemokines have a single amino acid separating the two N-terminal cysteines and CX_3C chemokine has three amino acids separating the two N-terminal cysteines (reviewed in (Rollins, 1997)). The CXC chemokines can be further subdivided into two categories, one with and one without glutamic acid-leucine-arginine (or ELR) sequence (ELR-positive or ELR-negative, respectively). The ELR-positive CXC chemokines are thought to specifically promote neutrophil chemotaxis (reviewed in (Rollins, 1997)). There are only two known chemokines in the C chemokine family, XCL1 and XCL2 (lymphotactin-α and lymphotactin-β, respectively). These chemokines attract T cell precursors to the thymus (Murphy et al., 2008). Only one CX_3C chemokine has been identified, i.e. CX_3CL1 or fractalkine, a membrane bound protein which serves both as a chemoattractant and as an adhesion molecule (reviewed in (Rollins, 1997; Worthylake & Burridge, 2001)).

Functionally, chemokines can also be classified as “homeostatic” chemokines or “inflammatory” chemokines, where the former are constitutively expressed and direct leukocyte migration during immune surveillance, whereas the latter are induced and control the recruitment of inflammatory cells to the infected or inflamed site (Moser & Willimann, 2004; O'Hayre et al., 2008). The majority of chemokines belong to the “inflammatory” family.

Chemokines exert their effects by signaling through seven-transmembrane domain G-protein coupled receptors (GPCR), except for the chemokines CX_3CL1 and CXCL16. The chemokine receptors have been grouped into a C chemokine receptor (XCR), CC chemokine receptors (CCRs), CXC chemokine receptors (CXCRs), and a CX_3C chemokine receptor (CX_3CR) (Weber, 2003).

Each chemokine receptor can bind several chemokines and one single chemokine is generally able to interact with more than one receptor (Rot & von Andrian, 2004) as shown in Table 2. The chemokine-chemokine receptor interaction can direct several distinct sequential events during leukocyte recruitment starting with mobilization from the bone marrow into the blood, migration from the blood into lymph nodes and tissues and finally directional migration within the tissues.

Table 2. Chemokines and chemokine receptors mentioned in this thesis1

Chemokine	Other name	Chemokine receptor
***CC chemokines*:**		
CCL2	MCP-1, MCAF, murine JE	CCR2, CCR10
CCL3	MIP-1α, LD78α	CCR1, CCR5
CCL4	MIP-1β, Act-2, HC21	CCR5, CCR8
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR10
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin-1	CCR3, CCR2 antagonist
CCL12	murine MCP-5	CCR5
CCL13	MCP-4, CKβ10	CCR1, CCR2, CCR3
CCL15	HCC-2, MIP-1δ, MIP-5, LKN-1	CCR1, CCR3
CCL16	HCC-4	CCR1
CXC chemokines		
CXCL1	GROα, MGSAα, murine KC	CXCR2, CXCR1 (low affinity)
CXCL2	GROβ, MGSAβ, MIP-2α	CXCR2 CXCR2 (low affinity)
CXCL8	IL-8, NAP-1, MDNCF, murine MIP-2	CXCR1, CXCR2
CXCL12	SDF-1, PBSF	CXCR4
CX_3C chemokine		
CX_3CL1	Fractalkine, neurotaxin, ABCD-3	CX_3CR1
C chemokine		
XCL1	Lymphotactin, SCM-1α	XCR1

[1] Based on (Coelho et al., 2005)

Pro-inflammatory cytokines and TLR ligands induce production of chemokines during inflammation or infection and in turn promote the recruitment of immune cells to the site of inflammation (Luster, 2002). Furthermore, cytokines and TLR ligands can also alter the repertoire of

chemokine receptors on the cell surface, e.g. by down regulating the expression of chemokine receptors and subsequently direct the migration of individual cell within the tissue (Khandaker et al., 1999).

1.2.5.1 CC chemokines

CCL2, also termed monocyte chemoattractant protein (MCP)-1, binds to the chemokine receptor CCR2, which is mainly expressed on a subpopulation of monocytes and activated T cells. CCL2 is produced in response to various stimuli by various immune and non-immune cells, such as fibroblasts, endothelial cells, vascular smooth muscle cells, T cells, monocytes and macrophages (Chabot et al., 1998; Chensue et al., 1996). CCL2 is mainly involved in the chemotaxis of monocytes, both during homeostasis and in inflammation (Conti & DiGioacchino, 2001; Lu et al., 1998; Weber, 2003) but has also been shown to be involved in the chemotaxis of T cells, mast cells and basophils (Leonard & Yoshimura, 1990). CCL2 is constitutively expressed during homeostasis and it plays an important role in maintaining sufficient levels of monocytes within the vasculature as it recruits monocytes from the bone marrow to the circulation. However, the production of CCL2 is dramatically increased during inflammation, which subsequently induces a more rapid release of monocytes from the bone marrow to the circulation and recruits classical monocytes to the site of infection or inflammation. CCL2 plays a role in the pathology of several inflammation-related diseases such as atherosclerosis (Shin et al., 2002), arthritis (Taylor et al., 2000) and cancer (O'Hayre et al., 2008), and excessive influx of macrophages has been suggested to play a part in the pathology of these diseases. Therefore, impairment of CCL2-CCR2 interaction which results in impaired recruitment of classical monocytes during infection can lead to overwhelming bacterial growth and cause harm to the host (Serbina et al., 2003a). In murine tissues, increased levels of CCL2 lead to PMN accumulation and organ damage by increasing the levels of CXCL1 (Frink et al., 2007). CCL2 enhances secretion of IL-4 by T cells and its production has been associated with the development of polarized Th2 responses (Fujita et al., 2006; Gu et al., 2000; Lukacs et al., 1997).

CCL3, or MIP-1α, binds to the chemokine receptors CCR1 and CCR5 with high affinity, but these receptors are expressed by several different immune cells like monocytes, T cells, NK cells and neutrophils (Ramos et al., 2005). CCL3 is mainly known for its role in the recruitment of monocytes/macrophages and neutrophils to inflamed or infected tissues. CCL3 is secreted by a variety of cells such as macrophages, lymphocytes, neutrophils, DCs, mast cells, basophils and epithelial cells (Maurer & von

Stebut, 2004). CCL3 production is induced by several factors such as LPS, TNF-α, IFN-γ and IL-1α/β, whereas IL-4, IL-10 and other anti-inflammatory mediators can reduce CCL3 expression (Kasama et al., 1995). CCL3 is considered to be a pro-inflammatory chemokine and is implemented in several inflammatory disorders, such as asthma, arthritis, pneumonia and psoriasis (Maurer & von Stebut, 2004).

CCL5, or regulated upon activation, normal T cell expressed and secreted (RANTES), binds to the receptors CCR1, CCR3 and CCR5 (Danese & Gasbarrini, 2005), which are expressed by different immune cells, such as T cells, monocytes and granulocytes (reviewed in (Murdoch & Finn, 2000)). CCL5 is secreted by many different immune and non-immune cells, such as macrophages, platelets, eosinophils, fibroblasts, endothelial cells, epithelial cells and endometrial cells (Levy, 2009). CCL5 is a potent chemotactic factor for monocytes, macrophages, T lymphocytes, basophils, eosinophils, NK cells and mast cells (reviewed in (Lukacs et al., 1997; Matsukawa et al., 2001; Rollins, 1997)). CCL5 has been implied to play a role in a variety of diseases, such as asthma, colitis, cancer (O'Hayre et al., 2008) and HIV (Levy, 2009) and its expression is markedly increased at inflammatory sites and in some tumors, but it is rarely expressed in normal adult tissues (Zlotnik & Yoshie, 2000).

1.2.5.2 CXC chemokines

CXCL1, or keratinocyte chemoattractant (KC), is one of the major neutrophil attractants in mouse. It is a functional murine homolog of the closely related human chemokines CXCL8 (IL-8) and CXCL1 (Gro-α). CXCL1 binds to the chemokine receptor CXCR2, which is primarily expressed on neutrophils, (reviewed in (Chintakuntlawar & Chodosh, 2009)) but its expression has also been noted on monocytes and some lymphocytes (reviewed in (Murdoch & Finn, 2000)). It is produced by several different cells, such as monocytes, T cells, NK cells, endothelial and epithelial cells and neutrophils (Mukaida, 2003). CXCL1 production is induced by pro-inflammatory cytokines, endotoxins and viruses (Mukaida, 2003).

CXCL2, or macrophage inflammatory protein (MIP)-2, is the other major neutrophil attractant in mice, and as CXCL1 it binds to the chemokine receptor CXCR2. CXCL2 is secreted by activated macrophages, epithelial cells, astrocytes, mast cells and neutrophils (Armstrong et al., 2004; Biedermann et al., 2000; Mancardi et al., 2003; Nygardas et al., 2000; Zhao et al., 2000). Secretion of CXCL2 can be induced by bacterial cell wall components, such as LPS, and cytokines like IL-1β and TNF-α (Liu et al.,

2000). Little is known about the role of CXCL2 in homeostasis and its constitutive expression.

CXCL8, or IL-8, is a potent neutrophil chemoattractant and activator. Its expression is induced in response to bacterial cell wall components and cytokines, such as TNF-α and IL-1β. CXCL8 is secreted by a variety of cells, including lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells and neutrophils (reviewed in (Witko-Sarsat et al., 2000)). CXCL8 is the main chemokine produced by neutrophils, which in turn activates neutrophils through CXCR2, working in an autocrine loop (reviewed in (Chintakuntlawar & Chodosh, 2009)). CXCL8 has been reported to take part in the pathogenesis of several inflammatory disease, such as Crohn's disease, ulcerative colitis (MacDermott et al., 1998), acute respiratory distress syndrome and asthma (Mukaida, 2003), as well as chronic obstructive pulmonary diseases (Gangur et al., 2002).

1.2.6 Leukocyte recruitment

Leukocyte recruitment is a multi-step process that includes mobilization of cells from the bone marrow into the circulation, rolling on the endothelium, followed by arrest and adhesion to the endothelial cell wall, and ultimately transmigration of the cells into the tissue or lymph node. Leukocytes emigrate from the bone marrow to the circulation where they come in contact with endothelial cells. Rolling of cells on the endothelium depends upon both physical and molecular forces and is dependent on the blood flow rate, their rolling velocity and their proximity with the endothelial cells (reviewed in (Seely et al., 2003)). Rolling is largely mediated by the interactions of selectins, such as L-selectin on leukocytes, E-selectin on endothelial cells and P-selectin on platelets and endothelial cells, with their respective ligands. L-selectin is constitutively expressed by circulating leukocytes, but the expression of E-selectin and P-selectin is induced by chemokines and other mediators produced at the site of infection or inflammation (Kansas, 1996). Most frequently the first contact is between selectins (L-, P- and E-selectins) on the surface of the leukocytes with ligands on high endothelial venule (HEV), inflamed vessels or previously adhered leukocytes (reviewed in (Grailer et al., 2009; Ley et al., 2007)).

The weak molecular interactions during rolling are a prerequisite for the stronger molecular interactions of adherence (reviewed in (Seely et al., 2003)). Adhesion is mediated by a separate set of adhesion molecules, the integrins and their ligands, such as LFA-1 and intercellular adhesion

molecules (ICAMs), resulting in firm adhesion and arrest of leukocytes to the endothelium. Integrins are expressed on cell surfaces and are important for the process of cell adhesion. They are heterodimeric proteins that are made of two different subunits, the α-subunit and β-subunit (Humphries et al., 2006). There are several α- and β-subunits, which, with different combination, make up different adhesion molecules, often classified according to the shared β-integrin. The β2-integrin class shares a common β-subunit (β2-integrin, CD18) that binds to one of three distinct α-subunits (CD11a, CD11b and CD11c). The β2-integrin subclasses are differentially distributed among leukocyte populations. The high affinity bonding of integrins with the complementary surface molecules, i.e. intracellular adhesion molecule (ICAM)-1 or ICAM-2, on endothelial cells is the distinct characteristic of adherence (reviewed in (Seely et al., 2003)). Following adherence, leukocytes pass through the endothelium either through cell-cell junctions or by penetrating the endothelial cell cytoplasm to enter the inflamed tissue (reviewed in (Dejana, 2006)). To complete the process of transmigration a chemoattractant gradient is required, which directs specific leukocyte subsets to the inflamed tissue where their chemokine receptors recognize chemokines presented on the endothelium (reviewed in (Ley et al., 2007)).

Few neutrophils migrate to tissues under normal conditions but monocytes migrate under normal conditions in response to signals to renew resident macrophage populations. During inflammation neutrophils and monocytes are rapidly recruited in response to increased production of inflammatory cytokines and chemokines. The demand for myeloid cells is increased in response to infection or inflammation. Neutrophils are quickly released into the circulation and are soon followed by monocytes (Ueda et al., 2005) The rapid release of myeloid cells from the bone marrow initiates an increase in the production of neutrophils and monocytes at the expense of lymphocyte production (Ueda et al., 2005). This is regulated by several cytokines and growth factors, such as TNF-α, IL-1β, CXCL12 and G-CSF (Ueda et al., 2005). Because of increased demand for neutrophils during inflammation their time for maturation in the bone marrow is decreased resulting in the release of both mature and immature neutrophils into the circulation (Terashima et al., 1996), a phenomenon that used to be called “shift to the left” (Seebach et al., 1997).

1.2.6.1 Neutrophil recruitment

Human neutrophils express the chemokine receptors CXCR1 and CXCR2 which both bind to the well known chemoattractant CXCL8 (IL-8). The equivalent receptor-ligand interaction in mice is mainly represented by CXCR2, but not CXCR1, binding with high affinity to CXCL1 and CXCL2 (Lee et al., 1995). So far the only known ligand for murine CXCR1 is CXCL6 and its role in neutrophil recruitment is minor (Lee et al., 1995). The activation of CXCR2, in combination with G-CSFR, is crucial for rapid neutrophil egress out of the bone marrow in response to infection or inflammation (Lee et al., 1995). The recruitment of neutrophils is either totally or partly dependent on CXCR2 in several inflammatory conditions such as peritonitis, arthritis, parasite infections and ulcerative colitis (Buanne et al., 2007; Del Rio et al., 2001; Garcia-Ramallo et al., 2002; Ness et al., 2003; Wengner et al., 2008). Neutrophils express other chemokine receptors that facilitate their migration. For example, they express CCR1 and migrate in response to CCL3 (Xue et al., 2007). The role of CCR1 in neutrophil recruitment has been demonstrated in mice lacking CCR1 but they exhibit partially impaired neutrophil recruitment during *Toxoplasma* infections (Garcia-Ramallo et al., 2002). In addition, blocking CCR1 during chemically-induced inflammation diminished neutrophil recruitment (Garcia-Ramallo et al., 2002). Although there are other chemokine receptors that can direct neutrophil migration during certain conditions, CXCR1 and CXCR2 are the major regulators of neutrophil recruitment.

1.2.6.2 Monocyte recruitment

Classical monocytes in both mice and humans, along with T and NK cells, express the chemokine receptor CCR2 (Geissmann et al., 2003; Mack et al., 2001). CCR2 binds several chemokines, such as CCL7, CCL8, CCL12 and CCL16, but CCL2 is the most important and best characterized ligand for CCR2 (Lukacs-Kornek et al., 2008). The emigration of monocytes from the bone marrow is CCR2-dependent, as the absence of CCR2, or CCL2 and CCL7 (but not CCL8 or CCL12), causes the retention of monocytes within the bone marrow both during homeostatic and inflammatory conditions (Serbina & Pamer, 2006; Tacke et al., 2007; Tsou et al., 2007). Under inflammatory conditions, the recruitment of classical monocytes to inflamed tissue is also dependent on CCR2 and its ligands. This has been shown in $CCR2^{-/-}$ mice during inflammatory conditions, such as atherosclerosis and in infections with *Mycobacterium tuberculosis* or *Listeriamonocytogenes* (Peters et al., 2001; Serbina et al., 2003b; Tacke et al., 2007). In addition, CCL2 knockout mice ($CCL2^{-/-}$) showed increased susceptibility to *Salmonella typhimurium* and

Listeria (Depaolo et al., 2005; Serbina et al., 2003a) which is probably due to decreased accumulation of $CCR2^{+}$ classical monocytes in blood as a result of decreased recruitment from the bone marrow. Results from another study indicate that decreased availability of $CCR2^{+}$ classical monocytes in the blood of $CCR2^{-/-}$ mice did in part explain the decreased recruitment to the inflamed tissue as demonstrated by equal recruitment of $CCR2^{-/-}$ and $CCR2^{+/+}$ monocytes to the infected organ tissue when they were injected into the blood of *Listeria* infected $CCR2^{-/-}$ mice (Serbina et al., 2003a; Serbina & Pamer, 2006). Nonetheless, after normalizing the monocyte frequency in the blood, the recruitment of monocytes to peritoneum after thioglycolate injection was CCR2-dependent at early time points, although not at later time points (Serbina & Pamer, 2006; Tsou et al., 2007). Furthermore, many infected or inflamed organs induce the production of the CCR2 ligands, in particular CCL2 (Depaolo et al., 2005; Serbina et al., 2003a). This indicates that CCR2 plays a crucial role in monocyte recruitment during several inflammatory conditions.

Non-classical monocytes express low levels of CCR2 and high levels of the fractalkine receptor CX_3CR1, opposite to the expression pattern for these molecules seen on classical monocytes (Geissmann et al., 2003). The non-classical monocytes patrol the blood vessels and their crawling on the endothelium is mediated by CX_3CR1. Due to their close proximity with the endothelium they readily transmigrate to infected tissues, e.g. they are immediately recruited to the peritoneum in response to intra-peritoneal (i.p.) injection of *L. monocytogenes* (Auffray et al., 2007). The different monocyte subsets exhibit different chemokine receptor profile and therefore respond to differently to chemokines (Tacke et al., 2007).

The chemokine receptor CCR5 is weakly expressed on murine monocytes (Mack et al., 2001) and it has been implicated in the migration of monocytes/macrophages into atherosclerotic lesions (Tacke et al., 2007). The lack of CCR5 expression in mice causes impaired monocyte recruitment to the brain during West Nile virus infection but has no effect on monocyte recruitment and clearance of bacteria during *Listeria* infection (Glass et al., 2005). CCR5 binds both the chemokine CCL3 and CCL5, but both these chemokines also bind other receptors, e.g. CCR1. Expression of both CCR5 and CCR1 increases when human monocytes differentiate into macrophages *in vitro*, in contrast to CCR2 expression that decreases (Kaufmann et al., 2001). The role of CCR5 and CCR1 in monocyte recruitment is not fully clear. The chemokines and their ligands most likely have an overlapping function and are differently expressed in response to different inflammatory and infectious conditions.

1.2.7 Inflammation

Inflammation is the body's reaction to harmful stimuli like pathogens, damaged tissue or irritants. It is a defense mechanism that starts the process of eliminating pathogens or toxins and initiates the repair process. Inflammation is a complex sequence of events involving alterations in the inflammatory mediator network, rearrangement of innate immune cell populations and changes in the activation status of these cells, with the final goal of promoting wound repair and reaching homeostasis (Yang et al., 2002). Although inflammation is essentially beneficial, uncontrolled or prolonged inflammation can cause harm and needs to be tightly controlled. The pathology of many chronic conditions, such as autoimmune diseases, arthritis, inflammatory bowel disease and cardiovascular disease is attributed to inflammation.

Inflammation is classified either as acute or chronic inflammation. Acute inflammation is the initial reaction to a harmful insult and is characterized by its immediate onset and short duration. At the onset of inflammation, inflammatory mediators are released by resident cells, especially macrophages and endothelial cells that are already present in the tissue at the time of the insult. Later, newly recruited cells also contribute to the generation of inflammatory mediators. The inflammatory mediators released include cytokines, e.g. TNF-α, IL-1β and IL-6, chemokines, e.g. CCL2, CCL3 and CXCL8 (Seki et al., 2002), eicosanoids, e.g. LTs, TXs, PGs and PGIs (Aderem et al., 1988), NO and matrix metalloproteinases. They are involved in initiating the immune response and further amplify the initial response. They increase vascular permeability and blood flow which in turn permits more fluid, larger molecules (e.g. complement, fibrin and antibodies) and leukocytes to cross the endothelial wall from the blood to the surrounding tissue. Activation of the endothelium increases the expression of adhesion molecules, resulting in better adherence of leukocytes to the vessel wall and resulting in increased diapedesis to the inflammatory loci. The chemokines play a major role in directing the migration of leukocytes to their proposed destination. These events count for the redness, swelling, heat and pain, collectively known as inflammation (Murphy et al., 2008). Beside the cell-derived mediators, there are also acellular factors that play part in the initiation and propagation of the inflammatory response. These include the complement system and the coagulation and fibrinolysis system (Cotran et al., 1998).

Neutrophils provide the first line of defense against invading pathogens. Following their elimination of the pathogen via phagocytosis, a process that releases potentially harmful stimuli, the neutrophils undergo apoptosis. Mononuclear cells follow the infiltration of neutrophils to the tissue where they differentiate into macrophages and clear apoptotic neutrophils via "efferocytosis" (Rossi & Sawatzky, 2008). The clearance of microbes, cellular and tissue debris and the efflux of phagocytes promote the repair of the tissue (Serhan, 2007). The production of anti-inflammatory cytokines, like IL-10, and pro-resolving lipid mediators also plays a role in limiting the inflammatory response which further prevents tissue damage of the host (Saraiva & O'Garra, 2010). Uncontrolled or inappropriate regulation of inflammation or its resolution can result in excessive damage to host tissues and give rise to chronic inflammatory disorders, characterized by excessive generation of inflammatory mediators, leukocyte sequestration and activation, delayed clearance, tissue damage and reduced function (Serhan et al., 2007).

If the initial local inflammation goes out of control with spilling of inflammatory cells and inflammatory mediators (e.g. TNF-α, IL-1β, IL-6 and CXCL8) into the general circulation and spreading to other organs, it can result in a systemic inflammatory response (SIRS) (Bhatia & Moochhala, 2004). When SIRS occurs in response to bacterial infection it is called sepsis. Consequences of SIRS, or sepsis, can be detrimental resulting in multiple organ dysfunction syndrome, organ failure and finally death (Bone et al., 1997).

1.2.7.1 Sepsis

Sepsis is a complex syndrome caused by imbalances in the inflammatory network (reviewed in (Rittirsch et al., 2008)). It presents a major health problem as it is a life-threatening condition associated with high morbidity and mortality, with death occurring in 35-50% of patients in North America (Antonopoulou & Giamarellos-Bourboulis, 2011). Sepsis and sepsis-like hyper-inflammation is initiated after a host is exposed to microbes or microbial products, such as LPS, and is mediated by a number of complex interacting molecular networks, including a large array of mediators, such as lipid metabolites, reactive nitrogen and oxygen metabolites, nucleotides, cytokines and chemokines (Boontham et al., 2003).

Sepsis is a biphasic condition that is initiated with severe systemic inflammation. The hyper-inflammatory phase is characterized by activation of the complement system and excessive production of pro-inflammatory cytokines,

chemokines and eicosanoids (Aderem et al., 1988; Seki et al., 2002). During the early hyper-inflammatory phase, neutrophils and monocytes are activated and migrate to peripheral tissues in response to a chemokine gradient. Activated neutrophils attempt to eliminate the pathogens by phagocytosis and generation of ROS (Nathan, 2006). Following the initial hyper-inflammatory phase a hypo-inflammatory phase develops that can lead to impaired innate immune function (Frazier & Hall, 2008).

Experimental therapies for sepsis aiming to attenuate the initial hyper-inflammatory response have failed, which has lead the research to focus on the anti-inflammatory/immunosuppressive phase, that follows the initial hyper-inflammatory phase (Frazier & Hall, 2008). In addition, it has been recognized that the majority of septic patients succumb during the later phase when they are immune suppressed, but not during the initial inflammatory phase (reviewed in (Rittirsch et al., 2008)). It has therefore been suggested that the host defense failure during sepsis is in fact due to the immune dysfunction or immunosuppression that follows the initial hyper-inflammation (Deng et al., 2006; Docke et al., 1997; Frazier & Hall, 2008; Hotchkiss et al., 2009).

During the phase of immunosuppression the production of anti-inflammatory cytokines becomes predominant (Osuchowski et al., 2006), which in turn inhibits the production of pro-inflammatory cytokines, such as TNF-α and IL-1β (Opal & DePalo, 2000). Increased levels of IL-10 contribute to the deactivation of monocytes as IL-10 inhibits monocytic surface expression of HLA-DR (Volk et al., 1996), which subsequently reduces their ability to induce antigen-specific T cell responses (Volk et al., 1996) and their production of pro-inflammatory cytokines (Docke et al., 1997; Flach et al., 1999; Heagy et al., 2003; McCall et al., 1993; Munoz et al., 1991). Several studies have found a correlation between high levels of IL-10 in septic patients and fatal outcomes, where IL-10 levels remain higher longer and the ratio of IL-10 to TNF-α is higher in non-survivors than in survivors (Gogos et al., 2000; Monneret et al., 2004; van Dissel et al., 1998). In addition, low HLA-DR expression on monocytes is correlated with worse outcome (Venet et al., 2007) and sepsis survivors show a progressive elevation of HLA-DR expression (Monneret et al., 2004). Many aspects of neutrophil functions are also dysregulated in septic patients and despite increased levels of activation markers such as CD11b, ICAM-1, MPO and CD66b on circulating neutrophils (reviewed in (Wang & Deng, 2008)) their adherence and transmigration is impaired (reviewed in (Wang & Deng, 2008)). Decreased neutrophil recruitment during the immune-suppressive phase in septic patients is also in part due to down-regulation of CXCR2 in response to both LPS and high levels of CXCL8 (or CXCL1 and CXCL2 in mice). The failure

of neutrophil migration results in impaired bacterial clearance within the infected tissues (reviewed in (Wang & Deng, 2008)). Together these factors result in weakened host defense and increased risk of secondary infection and death.

1.2.7.2 Endotoxin exposure –experimental models for sepsis

Endotoxin is part of the outer membrane of the cell envelope of gram-negative bacteria. The term endotoxin is often used exchangeably with LPS. LPS consists of a hydrophobic domain called lipid A, a core oligosaccharide and a distal polysaccharide, called O-antigen. LPS, or its lipid A moiety, represents the main biologically active component of endotoxin. It is a potent initiator of immune response in humans and animals and is the most effective microbial mediator implicated in the pathogenesis of sepsis (Opal, 2010). Infusion of LPS initiates a systemic activation of inflammatory pathways that can cause tissue damage and death, where practically any organ or tissue can be affected, depending on the dose and route of exposure (Wagner & Roth, 1999).

Diverse animal models have been used to study the pathophysiology of sepsis but none of them mimic the entire complexity of sepsis (Dejager et al., 2011; Rittirsch et al., 2007). The animal models used to study the pathophysiology of sepsis commonly follow one of three strategies, i.e. administration of exogenous toxins such as LPS, administration of viable pathogens, such as bacteria and viruses and alterations of the endogenous protective barrier of the animal such as in the cecal ligation and puncture (CLP) model. Administration of endotoxin can be either into the circulation resulting in endotoxemia (i.e. appearance of endotoxin in the circulation), or into the peritoneum resulting in severe endotoxin-induced peritonitis. Peritoneal administration of LPS has been shown to result in a rapid (within minutes or hours) appearance of LPS within the circulation (Hirano, 1996).

The advantages of endotoxin exposure models are that they are simple and reproducible, the induced response is acute and they are highly controlled and standardized models. The disadvantages are that the signaling is TLR4-dependent, they do not reflect all complex physiological human responses, the high rapid and transient increase in cytokines differs from human sepsis, and rodents are endotoxin resistant, whereas humans are endotoxin sensitive. In addition, there are different hemodynamic responses compared to human sepsis and the doses and route of administration used have been variable (Dejager et al., 2011). Although endotoxin exposure and other animal models of sepsis do not reproduce

the full clinical complexity and intrinsic heterogeneity of patients they can give some insight into the process of sepsis and using animal models is the best available approach that will remain essential for the development of new therapies for sepsis (Dejager et al., 2011; Rittirsch et al., 2007).

Endotoxin exposure induces the production of pro-inflammatory mediators and generates systemic inflammation. The pro-inflammatory mediators produced include the cytokines TNF-α, IL-1β, and IL-6, and the chemokines CCL2, CCL3, CCL4, CCL12, CXCL1, CXCL2 and CXCL8. These mediators are rapidly induced in serum, liver and lungs (Copeland et al., 2005; Hesse et al., 1988; Kopydlowski et al., 1999). The CXC and CC chemokines are known to exert pro-inflammatory effects in animal models of endotoxin exposure. However, CCL2 was demonstrated to play a role as an anti-inflammatory mediator and to have protective effects in a murine model of endotoxin exposure (Zisman et al., 1997). In addition, CCL2 protected mice from lethal infections by *Pseudomonas aeruginosa* and *S. typhimurium*as the induced chemotaxis of macrophages and enhanced phagocytosis played an important role in bacterial clearance and enhanced survival (Nakano et al., 1994).

The interaction of LPS with CD14 plays a major role in the response to LPS (Wright et al., 1990) as it has been demonstrated that CD14-deficient mice are resistant to high doses of LPS, although, despite their inability to respond they exhibit accelerated clearance of gram-negative bacteria (Haziot et al., 1988; Haziot et al., 1996). LPS binds to the CD14-TLR4-MD2 complex expressed by immune cells, like monocytes, macrophages and granulocytes (Haziot et al., 1993) and induces their secretion of pro-inflammatory mediators and internalization of surface molecules (Khandaker et al., 1998; Lloyd et al., 1995; Lynn et al., 1991). The systemic release of LPS induces a rapid drop in leukocyte numbers due to their immediate accumulation, mainly in lungs (Steinmuller et al., 2006) and liver (Menezes et al., 2009), due to their physical trapping within the microvascular bed (Haslett et al., 1987). In contrast, appearance of LPS in the circulation results in delayed migration of leukocytes to the peritoneum itself (Haziot et al., 1996).

Impaired lung function during systemic inflammation is caused by a rapid expression of pro-inflammatory mediators, pulmonary edema and excessive accumulation of leukocytes (Hirano, 1996). Activated neutrophils become rigid and therefore they sequester within the narrow pulmonary capillaries (Erzurum et al., 1992). The endothelium appears to be the dominant regulator of excessive neutrophil trafficking in the peripheral vasculatures, as leukocyte migration was

dependent on TLR4 expression by endothelial cells but not by neutrophils (Andonegui et al., 2003). The activation of the endothelium was solely through their expression of TLR4 but was independent of CD14 expression (Andonegui et al., 2003). Monocyte emigration following endotoxin exposure is β2 integrin-dependent and mediated through CCL2-CCR2 interactions and results in expansion of alveolar macrophages further contributing to the lung pathology (Steinmuller et al., 2006).

Liver dysfunction is another clinical finding during systemic inflammation resulting from neutrophil sequestration in the liver parenchyma (Ramaiah & Jaeschke, 2007). Accumulation of neutrophils within the liver sinusoids is independent of selectins (Wong et al., 1997) and β2 integrins (Jaeschke & Hasegawa, 2006) but occurs through the interaction of CD44/hyaluronan (HA) and is activated independently of CD14. The neutrophils do not crawl on the endothelium, but simply adhere and few emigrate out of the vasculature (Menezes et al., 2009), a possible mechanism to retain the neutrophils within the circulation where they can trap bacteria using NETs (Clark et al., 2007).

The primary site of infection suffers from reduced leukocyte recruitment during systemic inflammation (e.g. the peritoneum) (Haziot et al., 1996). This may be due to shedding of adhesion molecules and downregulation of chemokine receptors (Khandaker et al., 1998; Lloyd et al., 1995; Lynn et al., 1991). Another explanation is that leukocyte trapping within the microcirculation of lungs and liver, as the physical alterations of leukocytes and endothelial cells precedes the induced endothelial adhesion molecule expression in all tissues, including the peritoneum (Eppihimer et al., 1996). The entrapment of leukocytes causes decreased number of neutrophils circulating in the blood and therefore prevents cell adhesion and recruitment in the periphery. Haziot et al. (2001) also reported that recruitment of neutrophils into peritoneum (primary site) was inhibited by the interaction of LPS with TLR4/CD14 (Haziot et al., 2001). In contrast, Kesteman et al. (2008) reported that LPS induces the migration of splenic neutrophils from red pulp and marginal zone to the white pulp in a CD14-dependent manner. The movement of neutrophils to the white pulp suggests that they may co-localize with T cells and therefore might play a regulatory role in early steps of adaptive immune response (Kesteman et al., 2008). This suggests a differentially regulated neutrophil migration in lymphoid organs and peripheral tissue by a signal transduced by CD14.

1.3 The effects of fish oil on the innate immune system

For over 30 years it has been recognized that fish oil, or n-3 PUFAs, influence the immune system (reviewed in (Calder, 2008; Fritsche, 2007)). With increasing knowledge about the cells of the innate and the adaptive immune systems and their role in homeostasis and disease, elucidation of the mechanisms of the immunomodulatory effects of n-3 PUFAs are of increasing interest. n-3 PUFAs can have diverse effects on the cells of the innate immune system and are considered to exert suppressive or anti-inflammatory effects on the innate immune response. In the following section(s) the effects of fish oil, or n-3 PUFAs, on the innate immune system will be discussed in more detail.

1.3.1 The effects of fish oil on leukocytes and their function

Considerable amount of data exists on the effects of dietary fish oil, or n-3 PUFAs, on human and murine monocytes/macrophages (reviewed in (Fritsche, 2007)). Incorporation of EPA and DHA into leukocyte cell membranes changes their FA composition which in turn can alter their membrane structure and function, increasing membrane fluidity (Calder et al., 1994), affecting formation of functional microdomains (Fan et al., 2003; Stulnig, 2003), protein trafficking (Shaikh & Edidin, 2007), receptor expression (Sanderson & Calder, 1998), intracellular signaling (Miles & Calder, 1998), transcription factor activation (Lo et al., 1999; Novak et al., 2003; Zhao et al., 2004) and gene expression (De Caterina et al., 1994; Renier et al., 1993).

Few studies have examined the effects of dietary fish oil on leukocyte numbers in healthy mice or humans. One such study (Mukaro et al., 2008) reported a decrease in total blood leukocytes in healthy humans, which was mainly due to lower numbers of NK cells but there was also a trend towards a decrease in the number of neutrophils, B cells and T cells. Decreased number of blood neutrophils was also observed in healthy and hyperlipidemic subjects receiving fish oil supplements (Ambring et al., 2006; Kelley et al., 2009). In healthy mice, dietary fish oil did not affect the proportion of total splenic leukocytes but increased the proportion of $CD11b^+$ macrophages and decreased the proportion of NK cells compared with that in mice fed a corn oil diet (Petursdottir & Hardardottir, 2007). Furthermore, in apo-E deficient mice, a diet supplemented with EPA

reduced the number of macrophages found in atherosclerotic lesions when compared with that in mice fed a control diet (Matsumoto et al., 2008).

Results from studies examining the effects of fish oil on leukocyte counts in diverse patient populations vary. Results from a study by Mayer et al. (2003b) showed a trend towards lower numbers of total leukocytes in blood from critically ill patients receiving fish oil for five days, whereas results from a recent study by Barbosa et al. (2010) did not detect any alteration in leukocyte numbers in blood from critically ill patients receiving fish oil for five days. On the other hand, decreased numbers of total leukocytes and neutrophils was reported in alveolar fluid from patients with acute respiratory distress syndrome following four days of enteral feeding with EPA, γ-linolenic acid (GLA) and antioxidants (Gadek et al., 1999).

The n-3 PUFAs have not only been reported to affect leukocyte numbers, but also their activity. Healthy subjects receiving fish oil have been reported to have reduced NK cell activity (Kelley et al., 1998; Thies et al., 2001), although one study failed to demonstrate any effect of fish oil on either the number or the function of NK cells in healthy men (Miles et al., 2006). Patients with ulcerative colitis receiving fish oil extract had decreased NK cell activity and they also had reduced disease activity (Almallah et al., 2000). Decreased NK cell activity was also seen in splenic cells from mice fed fish oil and were infected with influenza virus, but this decrease in activity was suggested to contribute to increased morbidity and mortality in the fish oil group (Schwerbrock et al., 2009).

Several studies have examined the effects ofn-3 PUFAs on surface marker expression on leukocytes. Mayer et al (2003d) reported no effect of short time infusion with lipid emulsion containing fish oil on monocyte expression ofCD11b, CD18, CD49d, CCR5 or CCR2 in healthy volunteers. However, *in vitro* studies using cells from healthy volunteers have shown that n-3 PUFAs can alter their expression of surface markers. Culturing human monocytes, in the presence of n-3 PUFAs decreased the expression ofICAM-1 and LFA-3 in unstimulated monocytes and the expression of ICAM-1, LFA-3, HLA-DR and HLA-DP after activation of the monocytes with IFN-γ (Hughes & Pinder, 1997). Similarly, incubating whole blood cells with fish oil emulsion decreased the expression of CD11b and CD66 on neutrophils and increased the expression of CD62L, but had no effect on monocyte phenotypes (Versleijen et al., 2005).

The effects of fish oil on monocyte activation in critically ill patients has been examined, but without distinguishing between different monocyte

subpopulations. Some (Wang, X. et al., 2009; Weimann et al., 1998; Weiss et al., 2002), but not all (Friesecke et al., 2008), of these studies have shown that fish oil, or n-3 PUFAs, increase HLA-DR expression on monocytes, indicating that n-3 PUFAs may increase the activation level of monocytes in critically ill patients. Neutrophils from hypercholesterolemic mice fed dietary fish oil had less expression of CD11b and CD18 6 h after CLP compared to neutrophils from mice fed soybean oil diet. The expression of CD11b and CD18 on neutrophils from mice fed the fish oil diet had not changed 24 h after CLP, whereas the expression of these molecules on neutrophils from mice fed the soybean oil diet had decreased (Chiu et al., 2009). Supplementation with EPA also prevented a decrease of oxidative potential in neutrophils 48 h after insult in rabbits with multiple trauma, which suggest improved phagoycytosis of bacteria (Koutsostathis et al., 2010).

1.3.2 The effects of fish oil on cytokines

Numerous studies have examined the effects of n-3 PUFAs on cytokine concentrations in the circulation or cytokine production by different cell types, from different organs with different stimulation ex vivo, but the results are inconsistent. The main source of cells in human studies is from the blood, whereas in animal studies cells from peritoneum and spleen are frequently used.

Most of the human studies have used monocytes or endothelial cells stimulated *ex vivo* (reviewed in (Sijben & Calder, 2007)). Many studies have focused on the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and even though a number of reports have demonstrated reduced production of these cytokines by monocytes or endothelial cells from humans consuming n-3 PUFAs (Caughey et al., 1996; Endres et al., 1989; Mayer et al., 2003b; Meydani et al., 1991; Trebble et al., 2003) many others fail to do so (Miles et al., 2004; Rees et al., 2006; Thies et al., 2001). No study though has yet demonstrated increased production of these inflammatory cytokines by endotoxin stimulated monocytes or endothelial cells from healthy subjects receiving dietary fish oil. The discrepancy in the results from studies examining the effects of fish oil on cytokine production cannot be explained by varying doses of n-3 PUFAs used in these studies or the duration of supplementation. As the ability of fish oil to decrease TNF-α production by mononuclear cells has been shown to be influenced by polymorphism in the TNF-α gene (Grimble et al., 2002) it is possible that polymorphism in the cytokine genes may explain the varying results obtained in the studies described above.

A substantial number of animal studies have examined the effects of dietary fish oil on *ex vivo* production of cytokines. These studies have used a variety of cell types from different organs. Murine peritoneal cells are easily harvested and thus ideal for studying *ex vivo* cytokine secretion after dietary treatment. Studies on the effects of dietary fish oil on elicited peritoneal macrophages outnumber studies using resident peritoneal macrophages. It has to be taken into account that prior to the *ex vivo* stimulation, the elicited peritoneal macrophages are already active and the macrophage population has been altered by influx of monocytes. The results from these studies are inconsistent, as dietary fish oil has been demonstrated to increase (Somers & Erickson, 1994; Watanabe et al., 1991), have no effect (Hardardottir & Kinsella, 1992; Tappia & Grimble, 1994; Turek et al., 1991; Watanabe et al., 1991) or to decrease (Bhattacharya et al., 2007; Wallace et al., 2000; Yaqoob & Calder, 1995) LPS-stimulated TNF-α secretion by elicited peritoneal macrophages. In these studies different agents were used to elicit the macrophages, e.g. casein, thioglycolate or complete Freund's adjuvant, which could have different effects on their activation and maturation as well as recruitment of cells into the peritoneum and may in part explain the different results obtained.

There is more consistency in the results from studies examining the effects of dietary fish oil on pro-inflammatory cytokine production by resident peritoneal macrophages, than in those examining the effects of fish oil on elicited peritoneal macrophages. Most of these studies show that dietary fish oil increases LPS-induced TNF-α and IL-1β secretion by resident peritoneal macrophages, compared with that by resident peritoneal macrophages from mice fed a diet rich in n-6 PUFAs (Blok et al., 1992; Chang et al., 1992; Hardardottir & Kinsella, 1991; Lokesh et al., 1990; Petursdottir et al., 2002; Watanabe et al., 1991). The one study that has shownn-3 PUFAs to decrease LPS stimulated TNF-α secretion by resident peritoneal macrophages was performed in rats (Boutard et al., 1994). Results from studies examining the effects of dietary fish oil on *ex vivo* production of the anti-inflammatory cytokine IL-10 are more variable, as one study showed no effect of fish oil on IL-10 secretion by resident peritoneal macrophages (Wallace et al., 1999), another showed increased production of IL-10 (Yaqoob & Calder, 1995) and the third showed decreased production of IL-10 by resident peritoneal macrophages from mice fed fish oil diet compared with that from mice fed a corn oil diet (Petursdottir et al., 2002). The study by Petursdottir et al. (2002) showed that the decreased IL-10 production was because of fewer cells secreting the cytokine which was in contrast to the increased TNF-α secretion by

peritoneal cells from the same study, which was due to increased TNF-α secretion per cell (Petursdottir et al., 2002).

The effects of fish oil on cytokine production by stimulated spleen cells have not been as extensively studied as the effects of fish oil on cytokine production by peritoneal cells. The results from these studies are inconsistent and do not provide a clear picture of whether the effects of fish oil are pro- or anti-inflammatory. When splenocytes from mice fed dietary fish oil or DHA were stimulated with LPS they secreted either more (Albers et al., 2002; Barber et al., 2005) or less (Watanabe et al., 2000; Xi et al., 1998) TNF-α and IL-1β than splenocytes from mice fed corn oil. However, since mixed splenocyte cell preparations were used in these studies, other cells than macrophages might be contributing to the secretion of the cytokines and fish oil could be affecting the different cell populations differently. A study by Petursdottir & Hardardottir (2007) demonstrated that isolated splenic macrophages from mice fed dietary fish oil secreted more TNF-α and IL-10 than splenic macrophages from mice fed a corn oil diet (Petursdottir & Hardardottir, 2007). In contrast, dietary fish oil decreased secretion of both TNF-α and IL-10 by splenic T cells compared with that from mice fed a corn oil diet (Petursdottir & Hardardottir, 2007). These studies indicate that dietary fish oil can have different effects on different types of cells within the same organ.

Compared with the number of human studies examining the effects of n-3 PUFAs on *ex vivo* cytokine production, the number of studies examining the effects of n-3 PUFA on circulating cytokines in healthy subjects is more limited. Fish oil supplementation or dietary intervention does not seem to affect circulating cytokine concentrations in healthy individuals. A recent study on healthy, middle aged, individuals receiving high doses of n-3 PUFAs (1.5 g/day) for 12 weeks showed no effect on serum concentrations of several inflammatory markers (TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, CCL2, CCL3, CCL5, CCL11 and CXCL8 (IL-8)) (Pot et al., 2009) and dietary fish oil had no effect on plasma concentrations of IL-6 in young healthy men (average 26 years) (Yusof et al., 2008). However it has been suggested that fish oil may have beneficial effects on pro-inflammatory mediators in low grade inflammation. DHA supplementation for 91 days decreased circulating levels of IL-6 and GM-CSF, but had no effect on TNF-α, IL1-β, IL-2, IL-10 or CXCL8 in hypertriglyceridemic men, compared with the baseline levels and the levels in a placebo group receiving olive oil (Kelley et al., 2009). Overweight premenopausal women receiving fish oil high in DHA had decreased IL-6 levels in plasma compared to baseline, but

there was no difference when compared with the control group receiving linoleic/oleic acid supplementation (Browning et al., 2007). It has been suggested that low grade inflammation is prevalent in older people (Fagiolo et al., 1993). A study by Tsitouras et al (2008) showed a trend towards decreased plasma levels of IL-6 in a mixed population of elderly (>60) men and women receiving a diet supplemented with sardine oil (Tsitouras et al., 2008) and another study by Ciubotaru et al.(2003) showed that menopausal women receiving fish oil had decreased plasma levels of IL-6 compared with baseline and the control group that received safflower oil. In contrast fish oil supplementation did not affect TNF-α or IL-6 levels in menopausal women with type 2 diabetes compared with a control group receiving placebo (Kabir et al., 2007) nor in obese men compared with men receiving high oleic sunflower oil (Jellema et al., 2004).

During the course of acute inflammation, brought on by endotoxin or bacteria, there is an increase in the production of inflammatory mediators, including cytokines. A number of studies show that fish oil can decrease circulating levels of pro-inflammatory cytokines during acute inflammation. Mice fed dietary fish oil had lower plasma concentrations of TNF-α, IL-1β and IL-6 following LPS administration compared with that in mice fed safflower oil, whereas plasma levels of IL-10 were not affected (Sadeghi et al., 1999). In addition, parenteral nutrition containing fish oil decreased serum TNF-α and IL-6concentrations in burned rats compared with that seen in rats receiving n-6 PUFA-rich parenteral nutrition (Hayashi et al., 1998). Furthermore, mice fed dietary fish oil had lower serum concentrations of IL-12 after secondary infection with *L. monocytogenes* (Cruz-Chamorro et al., 2011) and lower lung mRNA expression of TNF-α and IL-6 seven days after influenza infection compared with control mice (Schwerbrock et al., 2009). Two studies have examined the effects of fish oil supplementation on cytokine levels in volunteers receiving endotoxin infusion. Healthy volunteers that received fish oil infusion 2 days prior to endotoxin challenge had reduced plasma levels of TNF-α compared with subjects not receiving fish oil, but no effects were seen on IL-6 (Pluess et al., 2007). In healthy volunteers that received fish oil supplementation for 3-4 weeks prior to endotoxin challenge there was no effect on serum levels of either TNF-α or IL-6 (Michaeli et al., 2007).

There are increasing data from clinical trials on the effects of fish oil on cytokines in surgical and critically ill patients. Patients with acute lung injury showed reduced levels of TNF-α and IL-6 in alveolar fluid following enteral EPA, DHA and GLA supplementation for 7 days (Pacht et al., 2003). Due to

the mixture of nutrients in the formula it cannot be determined whether the effects in the treatment group were due to the fish oil or other factors. Patients that received parenteral fish oil emulsion following radical colorectal cancer resection showed a greater decline in IL-6 levels in serum between day 1 and 8 than patients receiving parenteral soybean oil emulsion (Liang et al., 2008). Similarly, fish oil emulsions given to patients following abdominal surgery decreased TNF-α and IL-6 serum levels at post-operative days 6 and 10, respectively (Wachtler et al., 1997). In addition, Weiss at al. (2002) reported lower levels of IL-6 in serum on days 0 ,1 and 3 in patients receiving fish oil starting 1 day before and lasting to 5 days after abdominal surgery, compared with that in patients not receiving fish oil (Weiss et al., 2002). Furthermore, a decrease in IL-6 serum levels was observed in critically ill patients with manifested SIRS that received fish oil for 5 days (Barbosa et al., 2010). Neither Weiss et al. (2010) nor Barbosa et al. (2010) observed an effect of the fish oil on cytokine secretion by endotoxin-stimulated whole blood cells or endotoxin stimulated monocytes. In contrast, in a study by Mayer et al. (2003c) fish oil had no effect on cytokine serum levels in septic patients but monocytes from those patients generated less TNF-α, IL-1β, IL-6 and IL-10 after endotoxin stimulation. A recent study by Friescke et al. (2008) reported that fish oil supplementation for 7 days had no effect on IL-6 serum levels in ICU patients with SIRS compared with patients who did not receive fish oil.

1.3.3 The effects of fish oil on chemokines

Only a limited number of studies have examined the effects of n-3PUFAs on chemokines, with the majority of the data coming from *in vitro* and *ex vivo* studies. The majority of *in vitro* studies using human cell lines have shown decreased mRNA or protein levels of chemokines following incubation with n-3 PUFAs. Incubating HK-2 epithelial cells with EPA or DHA decreased LPS-induced expression of mRNA for CCL2 and CCL2 secretion compared with that when the cells were incubated without n-3 PUFA (Li et al., 2009). Similarly, monocytic THP-1 cells incubated with either EPA or AA expressed less CCL2 mRNA and secreted less CCL2 after stimulation with LPS than cells incubated with saturated FAs (myristic acid or palmitic acid), with DHA being less effective than either EPA and AA (Wang, S. et al., 2009a). EPA also attenuated cytokine-induced upregulation of CCL2 expression in a human umbilical vein endothelial (HUVEC) cells (Matsumoto et al., 2008) and in Caco-2 cells that had been differentiated into small intestinal enterocytes (Ramakers et al., 2007).

However, EPA upregulated CCL8, CCL15 and CXCL12 in the differentiated Caco-2 cells compared with that in cells incubated with AA, and had no effect on CXCL8 expression (Ramakers et al., 2007). In contrast to the results showing that n-3 PUFA reduce CCL2 expression and secretion by different cell lines, Tull et al. (2009) observed no difference in mRNA expression of CCL2, CXCL1 or CXCL2 nor in secretion of CCL2, CCL3, CCL5 and CXCL8 when incubating human endothelial cells with or without EPA. Furthermore, Shaw et al. (2007) demonstrated increased CCL2 expression by HUVEC cells after incubation with EPA and LA compared with those incubated with AA or DHA. The four studies reporting decreased CCL2 expression or secretion had longer incubation times (from 24 h to 22 days) with the FAs and longer stimulation times (16-24 h) (Li et al., 2009; Matsumoto et al., 2008; Ramakers et al., 2007; Wang, S. et al., 2009a). In the other two studies, the HUVEC cells were incubated with the FAs for 24 h and the stimulation was only during the last 4 h (Shaw et al., 2007; Tull et al., 2009). That could, in part, explain the lack of effect of the FAs in the study by Tull et al. (2009) but it is unlikely to be the cause for the increase in CCL2 expression observed in the study by Shaw et al. (2007).

One research group has published two studies comparing unoxidized EPA and oxidized EPA since the FAs readily undergo auto-oxidation in the body and oxidation might thus provide some of the effects observed by the FAs. These two studies (Chaudhary et al., 2004; Mishra et al., 2004) demonstrated that oxidized EPA inhibited cytokine-stimulated CCL2 and CXCL8 expression by HUVEC cells, human microvessel endothelial cells and human glomerular endothelial cells and CCL2 but not CXCL8 expression by mesangialcells compared with vehicle-treated cells. However, unoxidized EPA had no effect on CCL2 or CXCL8 expression by any of these cells (Chaudhary et al., 2004; Mishra et al., 2004). In these studies, the cells were only incubated with FAs for 1 h, after which they were washed off and the cells stimulated for 3 or 5 h, which could explain the lack of effect of the unoxidized EPA. Similarly, unoxidized DHA and AA had no effect on CCL2 and CXCL8 expression in HUVEC cells in contrast to oxidized DHA which decreased both CCL2 and CXCL8 expression (Mishra et al., 2004). Oxidized AA also decreased CCL2 expression, but to a lesser extent than the n-3 PUFAs, but increased the expression of CXCL8 (Mishra et al., 2004).

Several studies have examined the effects of dietary fish oil on *ex vivo* chemokine expression in humans and rodents. Dietary fish oil decreased mRNA levels for CCL2, without an effect on CCL2 protein secretion by

adherence-activated mononuclear cells from healthy humans (Baumann et al., 1999), whereas endotoxin-stimulated monocytes from healthy volunteers receiving fish oil based lipid emulsion showed reduced production of CXCL8(Mayer et al., 2003d). In addition, the increase in CCL2 mRNA expression in the intestinal mucosa in senescence-accelerated mice was ameliorated in mice receiving n-3 PUFAs (Matsunaga et al., 2009). Furthermore, dietary fish oil decreased deoxinivalenol (DON)-induced expression of CCL2, CCL7, and CXCL2 (Kinser et al., 2005) and phytohemagglutinin (PHA)-induced secretion of CCL2 and CCL5 by splenocytes from autoimmune prone mice, without affecting CCL3 expression (Venkatraman & Meksawan, 2002). However, secretion of CCL2 by splenocytes from the control mice fed the fish oil diet was slightly higher compared with that by splenocytes from control mice fed the control diet (Venkatraman & Meksawan, 2002).

Results from studies examining the effects of dietary fish oil on chemokine levels in serum or other fluids are contradicting. Fish oil reduced serum levels of CCL2 in healthy individuals after 7 weeks of supplementation (Elvevoll et al., 2008). In contrast, dietary fish oil did not affect serum levels of CCL2, CCL3, CCL5 orCXCL8 in healthy middle-aged individuals (Pot et al., 2009) nor did a Mediterranean diet high in n-3 PUFAs affect serum levels of CCL2 in healthy individuals (Ambring et al., 2006). However, in patients with cystic fibrosis, dietary fish oil decreased serum concentrations of CXCL8. In rats fed atherogenic diet, dietary fish oil decreased plasma concentrations of CCL2 and CCL5 (El Seweidy et al., 2005) and in mice fed a diet high in saturated fat (HSF) with a 1:1 ratio of n-6:n-3PUFAs there was a tendency towards lower CCL2 plasma concentrations than in mice fed either HSF n-6 diet with no EPA or DHA or HSF diet with n-6:EPA+DHA ratio of 20:1 (Wang, S. et al., 2009b). Feeding EPA alone decreased plasma concentrations of CCL2 in type 2 diabetic mice (Hagiwara et al., 2006), whereas there was no effect of dietary EPA on CCL2 serum levels in mice with choroidal neovascularization (Koto et al., 2007) or in mice with diabetic nephropathy, although they had lower levels of urinary CCL2 (Hagiwara et al., 2006). Together these results indicate that fish oil, or n-3 PUFAs, may attenuate the immune response in individuals suffering from low grade or chronic inflammation by decreasing the production of certain chemokines, which could lead to decreased influx of leukocytes into tissue. Dietary fish oil also attenuated CCL2 and CCL4 tissue levels in kidneys from rats with obstructive renal injury (Peake et al., 2011) and in *fat-1* mice, that are genetically altered to synthesize EPA and

DHA from AA *de novo*, there was a reduction in CCL2, CCL7 and CCL12 in colon during acute and chronic colitis and reduced CCL3 and CCL4 during acute colitis (Gravaghi et al., 2011).

Knowledge of the effects of fish oil on chemokines is important for understanding of the mechanism by which fish oil exerts its effect on the immune system. During infection or acute inflammation there is a massive transport of leukocytes between compartments of the body which is directed by chemokines. Fish oil based lipid emulsion given to mice prior to LPS challenge reduced CXCL2 generation in lungs when LPS was given intratracheally and CXCL2 serum levels when it was given i.p. (Schaefer et al., 2007). In addition, *fat-1* mice had lower CXCL2 levels in bronchoalveolar lavage (BAL) fluid 4 h after induction of acute lung injury (ALI) compared to control mice although CXCL2 levels in fat-1 mice were higher before and 24 h after the induction of ALI (Mayer et al., 2009). Furthermore, CXCL1 levels in BAL fluid from fish oil fed mice were higher 16 h after infection with *P. aeruginosa* (Tiesset et al., 2009). Results from these two animal models of lung infection indicate slower reduction in the neutrophil chemoattractants CXCL1 and CXCL2 after lung infection in the mice receiving or synthesizing n-3 PUFAs, compared with those having less of these FAs. Similar effects of dietary fish oil were seen in hypercholesterolemic mice with polymicrobial sepsis where dietary fish oil decreased CCL2 levels in peritoneal fluid 6 h after CLP-induced sepsis, but by 24 h the difference had diminished (Chiu et al., 2009). In contrast, there was no effect of dietary fish oil on CXCL2 levels in BAL in rats following intravenous injection with *Salmonella* endotoxin (Mancuso et al., 1997b). The timing and route of administration of the n-3 PUFAs was shown to be critical in a study (Tsou et al., 2008) where the effect of n-3 PUFAs on CCL2 levels in peritoneal fluid of rats with polymicrobial sepsis was studied. The rats receiving parenteral fish oil emulsion after CLP operation had higher CCL2 levels in peritoneal fluid than rats that received soybean oil based emulsion. On the other hand, dietary fish oil supplementation for 10 days prior to CLP operation had no effect on the CCL2 levels postoperatively (Tsou et al., 2008).

Although fish oil, or n-3 PUFAs, has been implicated in the beneficial effects in many immune related disorders and some infections, it has been shown that it might also have detrimental effects in certain infections (Schwerbrock et al., 2009). Whether those effects are mediated through the effects of fish oil on chemokine secretion has yet to be elucidated. A study by Fritsche et al. (2005) demonstrated detrimental effects of dietary fish oil

on *L. monocytogenes* infection in mice. Although fish oil had no effect on circulating CCL2 levels in normal mice infected with *L. monocytogenes* it increased its levels in neutrophil-depleted mice (Fritsche et al., 2005). Another study (Cruz-Chamorro et al., 2011) failed to show any effect of dietary fish oil on CCL3 levels in mice following secondary infection with *L. monocytogenes.* Dietary fish oil reduced mRNA for CCL3 in lungs 7 days after influenza virus infection, whereas no effects were seen on the expression of CCL5 (Schwerbrock et al., 2009).

1.3.4 The effects of fish oil on leukocyte recruitment

Leukocyte recruitment is in part mediated by the expression of adhesion molecules and chemokine receptors on leukocytes and endothelial cells. n-3PUFAs can affect expression of adhesion markers on monocytes (Hughes et al., 1996; Mayer et al., 2003d), macrophages (Miles et al., 2000) and endothelial cells (De Caterina et al., 1995; De Caterina et al., 1994; De Caterina & Libby, 1996; Matsumoto et al., 2008). Incubating human endothelial cells with DHA, but not EPA, reduced expression of vascular cell adhesion molecule (VCAM)-1, E-selectin and ICAM-1 in response to TNF-α, IL-1, IL-4, or bacterial endotoxin *in vitro* (De Caterina et al., 1995; De Caterina et al., 1994; De Caterina & Libby, 1996). In contrast, Matsumoto et al. (Matsumoto et al., 2008) reported decreased expression of cytokine-induced VCAM-1 and ICAM-1 mRNA in HUVEC cells following incubation with EPA. Monocytes from healthy humans receiving fish oil also expressed lower levels of ICAM-1 when stimulated *ex vivo* with IFN-γ (Hughes et al., 1996). In addition, although there was no effect of a fish oil based lipid emulsion on expression of several adhesive molecules (CD11b, CD18, CD49d and CCR2) by human monocytes, adhesion to endothelial cells and transmigration was reduced following cytokine stimulation *ex vivo* (Mayer et al., 2003d). Furthermore, thioglycolate-elicited macrophages from mice fed dietary fish oil expressed lower levels of ICAM-1 and macrophage scavenger receptor A (MSR-A) type I and type II, compared with thioglycolate-elicited macrophages from mice fed dietary coconut oil or safflower oil (Miles et al., 2000).

Several studies report suppressive effects of fish oil supplementation on chemotaxis of human monocytes and neutrophils towards various chemoattractants, such as LTB_4 and bacterial peptides *ex vivo* (Schmidt et al., 1989; Schmidt et al., 1992; Sperling et al., 1993) although others do not show any effect (Healy et al., 2000; Hill et al., 2007; Mukaro et al., 2008; Schmidt et al., 1996). Only one study (Gorjao et al., 2006) reports an

increase in chemotactic response of neutrophils following fish oil supplementation. In this study humanssupplemented their diets with fish oil rich in DHA and the chemotactic responses of neutrophils to complement C_5 *ex vivo* was examined. The different results obtained by Gorjao et al. (2006) compared with results from the other studies that have examined the effects of fish oil on neutrophil chemotaxis cannot be explained by their use of a fish oil rich in DHA. The studies by Healy et al. (2000) and Hill et al. (2007) used fish oils containing high amount of DHA, compared with the amount of EPA. In the study by Schmidt et al. (1996) the lack of effect on monocytes could be explained by the low dose of EPA and DHA. Thus, it could be speculated that EPA might promote the anti-chemotactic properties of fish oil, but not DHA, as the use of fish oil high in DHA resulted in either increased or no effects on neutrophils chemotaxis. However, the mechanism by which n-3 PUFAs alter chemotaxis is unclear but could possibly be in part due to their effect on cell surface marker expression, such as integrins, selectins and chemokine receptors.

A recent study by Tull et al. (2009) revealed a critical step in neutrophil recruitment that was affected by fish oil. They demonstrated that incubating HUVEC cells with EPA did not affect the number of neutrophils adhering to the endothelium, although it reduced the number of neutrophils transmigrating through the endothelium. Instead of adhering to the endothelium the neutrophils reverted back to rolling form of adhesion. Full transmigration of neutrophils was demonstrated to be independent of gene expression of endothelial adhesion molecules, cytokines and chemokines but instead was dependent on PGD_2-mediated secondary signaling, which is necessary for full activation and transmigration of neutrophils. EPA is a precursor of PGD_3that competes with PGD_2 for binding to the PGD_2 receptor (PD-1) on neutrophils and inhibits PGD_2-mediated secondary signaling and thereby the transmigration of neutrophils through the endothelium (Tull et al., 2009). Additionally, recently discovered bioactive lipid mediators that derive from EPA and DHA, such as Rvs, PDs and maresins, have also been shown to reduce neutrophil transmigration in several disease models (reviewed in (Serhan, 2010)) and might in part explain some the effects of fish oil on neutrophil recruitment.

Most studies examining the effects of dietary, intravenous or intraperitoneal n-3 PUFAs on leukocyte recruitment in response to inflammation show that n-3 PUFAs decrease leukocyte recruitment. Fish oil supplementation decreased the number of macrophages in atherosclerotic plaques in patients suffering from cardiovascular disease (Thies et al., 2003) and EPA, added to a Western type

diet, decreased the number of F4/80 positive macrophages in atherosclerotic lesions in apo-E deficient mice (Matsumoto et al., 2008). In the latter study EPA also decreased the number of macrophages infiltrating into the peritoneal cavity following thioglycolate administration (Matsumoto et al., 2008). Feeding rats n-3PUFAs following cardiac transplantation decreased macrophage infiltration during cardiac allograft vasculopathy (CAV) and subsequently alleviated the development of CAV (Yin et al., 2008). Furthermore, intravenous administration of n-3PUFAs to rats suppressed neutrophil adherence and transmigration into the gut in response to ischemia–reperfusion injury (Byrne et al., 2011) and dietary fish oil attenuated macrophage infiltration into the intestinal mucosa of mice with intestinal inflammation (Matsunaga et al., 2009). Dietary fish oil also attenuated macrophage infiltration into kidneys of rats with obstructive renal injury (Peake et al., 2011) and intraperitoneal administration of EPA reduced the number of macrophages in the glomeruli of mice with type 2 diabetic nephropathy (Zhang et al., 2006). Results from these studies indicate that n-3 PUFAs have suppressive effects on macrophage infiltration during low grade or chronic inflammation which might be beneficial in diseases such as arteriosclerosis and obesity, where excessive infiltration of macrophages contribute to the pathology of the disease.

Whether the effects of fish oil, or n-3 PUFAs, are beneficial or detrimental in infections has been debated (reviewed in (Anderson & Fritsche, 2002)). Mice fed a diet enriched with EPA and DHA had greater influx of neutrophils into BAL fluid 16 and 24 h after infection with *P. aeruginosa* than mice fed a control diet, although there was no effect of the n-3 PUFAs diet on macrophage recruitment into BAL fluid (Tiesset et al., 2009). The increase in neutrophil influx was reported to contribute to better survival of the mice receiving the n-3 PUFA rich diet (Tiesset et al., 2009). On the contrary, in a model of LPS-induced lung infection, mice receiving fish oil based lipid emulsion had reduced neutrophil invasion into the alveolar space at 4 and 24 h post LPS administration and the reduced neutrophil invasion into the lungs was thought to ameliorate the LPS-induced tissue damage (Schaefer et al., 2007). In contrast to the beneficial effects of n-3 PUFAs in infection reported in some studies, there are others reporting detrimental effects of n-3 PUFAs in certain infectious diseases (Fritsche et al., 2005; Schwerbrock et al., 2009). Before being infected with influenza virus, mice fed dietary fish oil had a lower number of neutrophils in lungs. Following infection, neutrophil infiltration into lungs increased in both the fish oil and the control group, but the total neutrophil number remained lower in mice fed the fish oil diet resulting in increased morbidity and mortality (Schwerbrock et al., 2009). Furthermore, dietary fish oil had no effect on neutrophil influx into the peritoneum

of mice the first 24 h following i.p. administration of *L. monocytogenes* and the detrimental effects of dietary fish oil on host resistance to *L. monocytogenes* was reported to be neutrophil independent (Fritsche et al., 2005). Influx of innate immune cells, in particular neutrophils and monocytes, to the site of infection is of utmost importance in the host response to infection to eliminate the invading pathogen. The effects of n-3 PUFAs on leukocyte recruitment during infection could in part be mediated by their effects on inflammatory mediators, such as cytokines, chemokines and eicosanoids, or because of alterations in expression of adhesion molecules on either leukocytes or endothelial cells. The mechanism by which dietary fish oil alters leukocyte recruitment is not completely understood and further investigation is needed.

1.3.5 The effects of fish oil on critically ill patients

The immune modulating properties of n-3PUFAs have lead to their incorporation into therapeutic regimens of critically ill patients in an attempt to modulate the dysregulation of the immune system that often occurs in these patients. The effects of n-3 PUFAs in sepsis have been studied both in animal models and in humans. Whether incorporation of fish oil, or n-3 PUFAs, into the therapeutic regiment of critically ill patients is beneficial is yet to be determined (Calder, 2009).

The delivery of nutrition to critically ill patients can be either through enteral or parenteral route. Interpreting the effects of n-3 PUFAs in enteral nutrition is difficult because of the inclusion of other nutrients in the formulas, such as arginine or GLA and antioxidants which may contribute to the overall effects of the enteral formulas. Three clinical trials using a formula containing EPA, GLA and antioxidants in critically ill patients have been completed (Gadek et al., 1999; Pontes-Arruda et al., 2006; Singer et al., 2006). Two of these studies only included patients with acute lung injury (Gadek et al., 1999; Singer et al., 2006) but one included patients with sepsis and septic shock (Pontes-Arruda et al., 2006). Patients with acute respiratory distress syndrome receiving the enteral formula had a more marked decrease in the number of leukocytes and neutrophils in alveolar fluid after 4 days of treatment compared with patients fed a high-fat, low-carbohydratecontrol diet (Gadek et al., 1999). An ancillary study from the same trial reported a lower concentration of CXCL8 and a trend towards lower concentrations of IL-6, LTB_4 and TNF-α in alveolar fluid from patients receiving the enteral diet containing EPA and GLA compared with those receiving the control diet (Pacht et al., 2003). The patients in the EPA and GLA group also had improved oxygenation of haemoglobin, decreased

duration on ventilation support, fewer organ failures and spent less time in the intensive care unit (ICU) on average than patients in the control group (Gadek et al., 1999). Similar results were shown for patients with acute lung injury (Singer et al., 2006) and ventilated patients with severe sepsis and septic shock (Pontes-Arruda et al., 2006) receiving enteral nutrition containing EPA and GLA, i.e. improved oxygenation, reduced time in ventilation and shorter time in ICU compared with patients receiving a high-fat, low-carbohydrate control diet. No effect was seen on mortality in the patients with acute lung injury (Singer et al., 2006),a trend towards decreased mortality in the patients with acute respiratory distress syndrome (Gadek et al., 1999) and in the ventilated patients with severe sepsis and septic shock the enteral formula containing EPA and GLA decreased 28-day mortality (Pontes-Arruda et al., 2006). The effects of other enteral formulas containing fish oil and additional immunomodulating nutrients, such as arginine, glutamine or antioxidant vitamins have been studied in critically ill patients (reviewed in (Calder, 2007b)). A meta-analysis on enteral nutrition studies by Marik and Zaloga (2010) divided the studies according to the nutrients included in the formula and the patient population. They concluded that using fish oil without arginine might reduce mortality, secondary infection and length of hospital stay in septic, SIRS and acute respiratory distress syndrome (ARDS) patients, whereas combining arginine with fish oil showed no benefits in patients with trauma, burns or in the surgical ICU (Marik & Zaloga, 2010).

As enteral administration of nutrients is often unfeasible or impossible in critically ill patients, parenteral administration of n-3 PUFAs is often used. Parenteral infusion of n-3 PUFAs allows for their rapid incorporation into leukocytes and other cells while bypassing the process of digestion and absorption, which can be impaired in these patients. Septic patients receiving parenteral n-3 PUFAs containing lipid emulsion for 5 days tended to have lower numbers of leukocytes in blood and lower concentration of C-reactive protein in serum (Mayer et al., 2003b). Neutrophils from the patients receiving the n-3 PUFA emulsion produced more LTB_5 after *ex vivo* stimulation than neutrophils from septic patients receiving n-6 PUFA lipid emulsion. In another study by Mayer et al. (2003c), monocytes from septic patients receiving n-3 lipid infusion produced less TNF-α, IL-1β, IL-6 and CXCL8 throughout the infusion period, than monocytes from septic patients receiving n-6 lipid infusion which showed elevated production of these cytokines during the first 2 days of infusion. These studies indicate that n-3 lipid infusion can modulate inflammatory responses that may be

associated with clinical outcome. One study that included several different patient groups, including patients with abdominal sepsis, severe head injury and multiple trauma, demonstrated that critically ill patients receiving more than 0.05g fish oil/kg/day had lower infection rates and shorter ICU and hospital stay compared with those receiving less fish oil and more patients survived when 0.1-0.2 g/kg/day of fish oil were administered (Heller et al., 2006). Patients with severe acute pancreatitis receiving n-3 PUFA supplemented fat emulsion for 5 days were reported to have better gas exchange and reduced requirement for continuous renal replacement therapy but there were no effects of the n-3 fat emulsion on inflammatory markers, number of infections or length of ICU or hospital stay (Wang et al., 2008). A recent study by Barbosa et al. (2010) demonstrated greater reduction in plasma concentration of IL-6, less reduction in plasma concentration of IL-10, improved gas exchange and a trend towards shorter hospital stay in septic ICU patients receiving parenteral fish oil. On the other hand, a study by Friesecke et al. (2008) found no effect of fish oil, when given to ICU patients over 7 days, on serum IL-6 concentrations, HLA-DR expression of monocytes, duration of mechanical ventilation requirement, bleeding events, incidences of nosocomial infections, length of ICU stay or mortality. It should be noted that the study started after the initial inflammatory response was already resolving which might possibly explain the lack of effect of fish oil on the outcomes measured.

Several studies have investigated the effects of parenteral lipid emulsions on surgical patients, who, unlike critically ill patients, can start receiving n-3 PUFAs prior to surgery allowing accumulation of n-3 PUFAs in cell membranes and in plasma before insult. Many of those studies only examined the effects of the parenteral lipid emulsions on clinical outcome, but an increasing number of studies are now reporting effects, or no effects, on inflammatory mediators. Patients receiving fish oil based lipid emulsions have altered FA composition, towards increased EPA content at the expense of AA, in blood leukocytes (Mayer et al., 2003c; Morlion et al., 1996; Wachtler et al., 1997). Lipid emulsions containing EPA given to surgical patients have also been shown to result in more production of eicosanoids derived from EPA, and accordingly less production of eicosanoids derived from AA, by blood monocytes after *ex vivo* stimulation with the calcium ionophore A23187 (Grimm et al., 2006; Koller et al., 2003; Morlion et al., 1996; Wachtler et al., 1997). Patients that received fish oil based lipid emulsions 5 days post surgery had lower TNF-α concentrations in plasma at day 6 and lower IL-6 concentrations at day 10 compared with

patients receiving soybean based lipid emulsion (Wachtler et al., 1997). Similarly, patients receiving fish oil based lipid emulsions perioperatively (one day prior to and 5 days after) had lower plasma IL-6 concentrations at days 0, 1 and 3 compared with patients in the control group (Weiss et al., 2002). Monocytes from patients receiving fish oil did not downregulate expression of HLA-DR, whereas at day 3 and 5 monocytes from the control group had reduced expression of HLA-DR. Although, this study reported no effects of fish oil on infection rate or mortality, there was a trend towards shorter post-operative stay in ICU among patients receiving fish oil compared with those not receiving fish oil (Weiss et al., 2002). A study by Liang et al. (2008) demonstrated a greater decrease in serum IL-6 levels between post-operative day 1 and day 8 in patients receiving fish oil for 7 days following colorectal cancer resection (Liang et al., 2008). In the same study they observed a higher ratio of CD4+ to CD8+ cells in the circulation, which is thought to be an indicator of a better cell-mediated immune response. Patients receiving the fish oil emulsions tended to spend shorter time in the hospital but infectious complications and mortality were similar in the two groups. Two studies reported shorter length of hospital stay in patients receiving fish oil based emulsions after surgery (Grimm et al., 2006; Wichmann et al., 2007). Several studies only examined the effects of fish oil on clinical outcomes. One of those studies reported that patients receiving fish oil lipid emulsions perioperatively (before and after surgery) had decreased need for mechanical ventilation, shorter hospital stay, less need for readmittance to ICU and had lower mortality rate. On the other hand, when they only received the fish oil postoperatively no difference was observed except that patients from the fish oil group had a lower rate of readmittance to intensive care (Tsekos et al., 2004). Another study where patients were given intravenous infusion of lipid emulsions for 5 days after a gastrointestinal surgery demonstrated similar results, i.e. no effects on the length of intensive care or hospital stay, although patients with increased risk of sepsis that received fish oil spent less time in the ICU (Heller et al., 2006). Additionally, Heller et al. (Heller et al., 2006) reported that patients receiving fish oil had accelerated normalization of liver and pancreatic functions. Results from a study by Berger et al. (Berger et al., 2008) showed no difference in inflammatory markers or clinical outcomes in patients receiving fish oil after abdominal aorta aneurysm repair surgery, but there was a tendency towards a shorter ICU and hospital stay. Many of the above studies indicate possible beneficial effects of parenteral supplementation of n-3 PUFAs in post-operative patients undergoing

selective surgery, which might be mediated through the effects of the n-3 PUFAs on the generation of inflammatory eicosanoids (Grimm et al., 2006; Wachtler et al., 1997) and cytokines (Liang et al., 2008; Wachtler et al., 1997; Weiss et al., 2002) or by counteracting the downregulation of antigen presenting cell activity (Weiss et al., 2002). The influence of fish oil on inflammatory processes, immune function and clinical endpoints in surgical and critically ill patients is unclear and further studies are needed before supplementation of n-3 PUFAs can be recommended those patients.

1.3.6 The effects of fish oil on animal models of endotoxin exposure

The effects of fish oil on inflammatory responses have been studied in variety of models of acute inflammation. However, a limited number of studies have investigated the effects of fish oil, or n-3 PUFAs, on endotoxin exposure in animals. Fish oil, either supplemented in the diet or given intravenously, improved survival in endotoxemic guinea pigs when compared with safflower oil diet or infusion (Mascioli et al., 1988; Mascioli et al., 1989). A recent study also demonstrated better survival in mice fed dietary fish oil prior to a two-hit i.p. LPS challenge compared with mice fed a diet high in saturated fat (Vijay-Kumar et al., 2011). The same study also reported less reduction in body weight and blood glucose levels and lower levels of serum IL-1β (Vijay-Kumar et al., 2011). Less LPS-induced reduction in diaphragm-specific force generation has also been reported in rats following gavage with EPA simultaneously with i.p. administration of LPS (Supinski et al., 2010). In addition, dietary fish oil has been shown to decrease plasma levels of PGE_2, TXB_2, 6-keto-$PGF_{1\alpha}$, IL-6 and IL-10, but to increase TNF-α levels in mice following i.p. administration of LPS, compared with that in mice fed a safflower oil diet (Chavali et al., 1998). An increase in serum levels of TNF-α in mice fed a fish oil containing diet was also seen in a study by Chang et al. (1992), when comparing the fish oil diet to either a coconut oil diet or a low fat diet (Chang et al., 1992). In contrast, in a study by Sadeghi et al. (1999) endotoxemic mice fed dietary fish oil had lower plasma levels of TNF-α, IL-1β and IL-6 than mice fed dietary olive oil or safflower oil but similar levels of plasma IL-10. Schaefer et al. (Schaefer et al., 2007) demonstrated that serum levels of TNF-α as well as CXCL2 were also decreased in mice receiving parenteral fish oil emulsion for 3 days prior to i.p. administration of a low dose of LPS (2 µg/mouse) compared with that in mice receiving soybean oil emulsion. Although leukocyte numbers in blood from the mice receiving fish oil were

slightly lower than in those receiving saline, the difference was not statistically significant. In addition it was found that the fish oil emulsion decreased influx of leukocytes into the lungs 4 h following intratracheal (i.t.) administration of LPS with a trend towards a decrease in leukocyte numbers also at 24 h (Schaefer et al., 2007). MPO levels in BAL fluid were decreased in mice receiving the fish oil emulsion 24 h following LPS administration, the concentration of CXCL2 was lower at both 4 and 24 h, whereas TNF-α was lower at 4 h but not 24 h following LPS administration, compared with that in the soybean oil group (Schaefer et al., 2007). Similarly, *fat-1* mice had reduced influx of leukocytes 24 and 48 h following i.t. administration of LPS compared with that in wild type mice (Mayer et al., 2009). *Fat-1* mice also had higher levels of leukocytes in blood 4 h following LPS compared with that in wild type mice but by 24 h the leukocyte numbers in blood from *fat-1* mice reached similar levels as in the wild type mice. Following LPS administration MPO levels were lower and CXCL2, PGE_2 and TXB_2 were reduced but TNF-α levels were similar in BAL fluid from *fat*-1 mice compared with that in in BAL fluid from wild-type mice (Mayer et al., 2009).

In some studies LPS was administered intravenously (i.v.) to pigs (Carroll et al., 2003; Murray et al., 2000) or rats (Mancuso et al., 1997a; Mancuso et al., 1997b; Pscheidl et al., 2000; Pscheidl et al., 1992; Sane et al., 2000; Vollmar et al., 2002). In one of the two studies in pigs, Murray et al. (2000) showed no effect of enteral nutrition with EPA or EPA and GLA given prior to i.v. administration of LPS, on pulmonary compliance, edema, hemorrhage or leukocyte influx into lungs, compared with that in pigs receiving corn oil prior to LPS administration. In the other study Caroll et al. (2003) reported decreased serum levels of TNF-α, IFN-γ, cortisol and corticosteroid-binding globulin in nursery pigs receiving fish oil compared with that in pigs receiving corn oil. In rats, fish oil emulsion improved blood flow to the intestines, spleen and liver following i.v. administration of LPS (Pscheidl et al., 2000; Pscheidl et al., 1992). Rats fed the fish oil emulsion also had improved intestinal perfusion and glucose tolerance as well as increased lactate clearance (Pscheidl et al., 1992). In addition, there were fewer bacteria observed in mesenteric lymph nodes and livers of rats in the fish oil group compared with that in rats fed the soybean oil diet, which was suggested to be due to improved bacterial killing (Pscheidl et al., 2000). EPA ethyl ester emulsion decreased plasma levels of TXB_2 and decreased lung edema in rats following bolus injection of LPS i.v. but had no effect on neutrophil count in lungs or blood when compared with rats not receiving EPA (Sane et al., 2000). Fish oil enriched diet did not protect

against LPS associated liver dysfunction, although it did prevent systemic hypotension, leukocytopenia and metabolic acidosis in rats receiving i.v. LPS administration (Vollmar et al., 2002). In the rats fed the fish oil enriched diet, serum concentrations of IL-6 were higher and there was a tendency towards improved survival compared with that in rats receiving unsupplemented chow (Vollmar et al., 2002). Rats fed dietary fish oil 3 weeks prior to i.v. administration of *Salmonella enteritidis* endotoxin had attenuated endotoxin-induced systemic hypotension and lung permeability compared with that in rats fed a corn oil diet (Mancuso et al., 1997a). The fish oil diet also ameliorated endotoxin-induced acute lung injury apparently by reducing the production of pro-inflammatory eicosanoids in BAL fluid and reducing accumulation of pulmonary neutrophils (Mancuso et al., 1997b).

The studies reviewed in this section indicate that fish oil may improve survival following endotoxin exposure and have beneficial effects on pulmonary function. They also consistently show that fish oil reduces concentrations of PGE_2, TXB_2 and TXB_4, but its effects on other biochemical parameters are somewhat inconsistent. That may in part be because of the great variability in the experimental setup in the studies reviewed, including the different animals or strains of animals used, the different serotypes and dosages of LPS used, the different timing and route of administration of LPS, as well as the different dosage of fish oil used and the different controls that it was being compared with.

Only a few of these studies examined the influx of leukocytes to blood or lungs following endotoxin exposure, demonstrating that fish oil reduced influx of leukocytes to these sites. However, no studies examined the effects of fish oil on leukocyte phenotypes or immune cell recruitment following endotoxin exposure. Understanding the effects of dietary fish oil on immune cell phenotypes and their recruitment during the initial phase of acute inflammation may provide an understanding of the mechanism by which fish oil can have beneficial effects in sepsis.

2 AIMS

Dietary fish oil, or n-3 PUFAs, may have beneficial effects in sepsis. However, the mechanism by which n-3 PUFAs have their beneficial effects is not clear. In sepsis there is a systemic inflammatory response mediated by a number of complex interacting molecular networks, including eicosanoids, cytokines, and chemokines. n-3 PUFAs have been shown to affect eicosanoid and cytokine production but their effects on chemokines are less known. Chemokines are key participants in the early inflammatory response in sepsis, instigating a widespread inflammatory cell recruitment and activation. Thus, the potential benefits of n-3 PUFAs in sepsis might involve effects on production of chemokines and/or expression of chemokine receptors, and the recruitment of specific populations of immune cells to the sites of infection or inflammation is very important.

The primary goal of this project was to determine the effects of dietary fish oil on immune cell subpopulations, immune cell chemokine receptor expression and chemokine concentrations in normal mice and mice with severe endotoxin-induced peritonitis.

Specific aims were:

A. To determine the effects of intraperitoneal LPS administration in mice on:

1. blood, peritoneal, spleen and bone marrow cell populations
2. chemokine concentrations in serum and peritoneal fluid
3. surface marker and chemokine receptor expression on blood, peritoneal, spleen and bone marrow cells

B. To determine the effects of dietary fish oil on:

1. blood, peritoneal, spleen and bone marrow subpopulation phenotype in normal mice and mice with endotoxin-induced peritonitis.
2. chemokine concentrations in serum from normal mice and mice with endotoxin-induced peritonitis.
3. chemokine receptor expression on blood cells from normal mice and mice with endotoxin-induced peritonitis.
4. chemokine receptor expression on bone marrow, spleen and peritoneal cells from normal mice and mice with endotoxin–induced peritonitis.
5. chemokine production by mouse peritoneal macrophages following stimulation with LPS ex vivo.

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: 0507-1502a) and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Female C57BL/6 mice weighing 18-20 g were purchased from Taconic Europe (Ejby, Denmark). Mice were housed five to eight per cage in a humidity (45-55%) and temperature (23-25°C) controlled environment with a 12 h light and dark cycle. Mice were acclimatized for one week prior to initiation of the experiment. They were randomly assigned to receive either a control (C) diet (D07121302; Research Diets Inc., New Brunswick, NJ) or a fish oil (FO) diet (D07121303; Research Diets Inc.) for a 6-week period. 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 by the authors (see Table 3).

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 the safflower oil (Welch, Holme & Clark CO Inc., Newark, NJ). Arachidonic acid (AA) ethyl ester (Nu-Check-Prep, Elysian, MN) (0.5 g/kg) was added to the control diet to adjust for the AA content in the fish oil 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 4**. The diets were aliquoted into daily portions and were stored under an atmosphere of nitrogen at -20°C to prevent oxidation.

All mice were provided fresh food daily and consumed water and food ad libitum. Weight of the mice was monitored weekly throughout the experiment and did not differ between the two dietary groups. The mice were fed the experimental diets for four to six weeks. Female C57BL/6 mice receiving standard laboratory chow were used for preliminary experiments and time curves. Three batches of diets were used; for experiments 1-4, for experiments 5-6, and for experiments 7-8.

Table 3. Composition of the control and fish oil diets as provided by the manufacturer

	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
High Oleic Sunflower, 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
α-Vitamin E Acetate[e]	0.15	0.15
t-BHQ	0.03	0.03

[a] Refined, Bleached, Deodorized

[b] United States Pharmacopeia

[c] Containing the following (g/kg mineral mix): sodium chloride, 259; magnesium oxide, 41.9; magnesium sulfate $7H_2O$, 257.6, chromium KSO_4 $12H_2O$, 1.925; cupric carbonate, 1.05; sodium fluoride, 0.2; potassium iodate, 0.035; ferric citrate, 21; manganous carbonate, 12.25; ammonium molybdate $4H_2O$, 0.3; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose 399.105.

[d] Containing the following (g/kg vitamin mix): retinyl palmitate, 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 highlighted

Table 4. Fatty acid composition of the control and fish oil diets as provided by the manufacturer[a]

	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

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

3.2 Analysis of hepatic phospholipid fatty acids

Hepatic total lipids were extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959) after perfusing the livers using saline with 0.1% ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO). Briefly, 0.2g of frozen liver samples were mechanically homogenized in 0.8 ml of 0.9% NaCl. Three ml of methanol (MeOH)/chloroform ($CHCl_3$) (2/1, v/v) (Merck, Darmstadt, Germany) solvent containing 0.005% of the antioxidant butylated hydroxytoluene (BHT) was added to the liver homogenate. The extraction medium was agitated for 30 minutes at room temperature and after centrifuging at 1700 rpm for 15 minutes the solution became biphasic. The lower phase, $CHCl_3$ and the total lipids, was transferred to a pyrex tube, where $CHCl_3$ was evaporated under a stream of nitrogen (N_2). The remaining lipids were dissolved in $CHCl_3$ containing BHT and the phospholipids were separated on thin layer chromatography (TLC) plates (Adsorbosil H, Alltech, Deerfield, IL). The samples were placed on a TLC plate which was placed in a glass tank containing 100 ml of the mobile phase $CHCl_3$/MeOH (8/1, v/v)) for 30-60 minutes. The phospholipid bands were scraped off the TLC plate, transferred to a pyrex tube, 2 ml 14% boron-trifluoride (BF_3) /methanol (Merck) added and then flushed with N_2. The phospholipid FAs were methylated at 110°C for 45 minutes. The fatty acid methyl esters (FAME) were extracted with a mixture of 1.5 ml of distilled water and 1.5 ml hexane (Merck), vortexed thoroughly and centrifuged at 1700 rpm for 10 minutes at room temperature. The upper phase, containing hexane and the FAMEs, was transferred to a pyrex tube and hexane was dried under a stream of N_2. The FAMEs were dissolved in 125µl isooctane (Merck), transferred to gas chromatography (GC) vials, sealed and stored at -30°C until analysis was performed. The FAMEs were analyzed using a HP Series II 5890 A GC (Hewlett Packard Co/Agilent, Palo Alto, CA, USA) equipped with a Chrompack CP-Wax 52CB capillary column (25 m × 0.25 mm i.d. × 0.2 µm film thickness). The oven was programmed to provide an initial temperature of 90°C for 2 minutes, then rising by 30°C per minute to 165°C, followed by 3°C per minute to 225°C, after which the temperature was held isothermal for 6 minutes. The injector and detector temperatures were maintained at 235°C and 250°C, respectively. Hydrogen was used as the carrier gas. Identification of specific FAMEs was detected by comparing their retention times with that of commercial standards (Sigma-Aldrich, Nu-Chek-Prep, Elysaian, MN.

3.3 Endotoxin administration and sample collection

Mice were injected i.p. with 0.5 mg/kg LPS (E. coli, serotype 055:B5; Fluka, Sigma Aldrich) in PBS (Gibco BRL, Invitrogen, Paisley, UK). The LPS-PBS solution was prepared in the final concentration of 0.1mg/ml LPS, mice were weighed and received appropriate volume of LPS-PBS solution according to their body weight (approx. 10-12.5µg LPS/mouse). At indicated time points 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 and peritoneal fluid, liver, spleen and bone marrow were collected.

3.3.1 Blood collection and serum preparation

For flow cytometry analysis blood was collected into EDTA-K_2 coated tubes (S--Monovette; Sarstedt, Nümbrect, Germany). For serum preparation, blood was collected into Eppendorf tubes, allowed to clot for 30 minutes at room temperature and then centrifuged at 3000 rpm for 10 min and the serum collected. Serum used in cell cultures (autologous serum) was heat inactivated for 45 minutes at 56°C and serum from mice within the same dietary group was pooled together and 10% v/v was added to the culture medium. Serum for cytokine and chemokine analysis was stored at -70°C until analyzed with ELISA.

3.3.2 Collection of peritoneal cells and peritoneal fluid

Peritoneal lavage (fluid and cells) was obtained by injecting 3 ml of cold phosphate buffered saline PBS without calcium or magnesium into the peritoneal cavity, massaging gently and collecting the abdominal fluid. Cells were spun down and the fluid collected and stored at -70°C until analyzed with ELISA. The peritoneal cells were washed with PBS and resuspended in Dulbecco‘s Modified Eagle Medium (DMEM; Gibco) with Gluta-MAX-I (2mM), penicillin (100U/ml) and streptomycin (100 µg/ml). Peritoneal cells were counted using Trypan blue (Invitrogen) and Countess automated cell counter (Invitrogen).

3.3.3 Collection of splenocytes and bone marrow cells

Spleens were aseptically removed from mice postmortem and placed in 4 ml of cold DMEM medium. Bone marrow cells were collected from right femur and tibia from each mouse, skin and muscle were removed, the

femurs and tibiae were cut at the diaphyses and bone marrow cells were flushed out by repeated injections of DMEM medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) using a 1-ml syringe and a 25 gauge needle.

Spleens were passed through a cell strainer (70µm) (BD Bioscience, San Jose, CA) to obtain a single cell suspension. Spleen and bone marrow cells were washed by centrifugation and red blood cells were lysed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA in PBS). After washing and debris removal, cells were resuspended in 5ml DMEM medium and counted using Trypan blue and Countess automated cell counter.

3.4 Activation of resident peritoneal macrophages ex vivo

The concentration of peritoneal cells was adjusted to 1x106 cells/ml and cultured in a volume of 250 µl/well on a 48-well flat bottom plate at 37°C with 5% CO2. After 2 h culturing, non-adherent cells were discarded and the adherent cells washed twice with PBS and incubated in 250 µl DMEM medium supplemented with 50 µl autologous serum per ml medium. Adherent macrophages were stimulated with 1 µg/ml LPS (E. coli 055:195; Difco laboratories, Detroit, MI) for 24 h. For cytokine measurements, the cells were spun down and the supernatant collected and stored at -70°C until analyzed with ELISA. For intracellular staining, Brefeldin A (eBioscience, San Diego, CA) was added to the cell culture (3 µg/ml) for the last 6 h of the 24 h LPS stimulation. Cells receiving no LPS were used as a negative control. After culturing, the supernatants were collected and remaining cells treated with cold PBS containing 20 mM EDTA for 15 minutes. Adherent cells were then scraped off the plate. Cells were centrifuged and resuspended in 250 µl FACS staining buffer (PBS with 0.5% bovine serum albumin (BSA), 2mM EDTA, 0.1% NaN3).

3.5 Flow cytometric analysis of blood, peritoneal, spleen and bone marrow cells

Fifty µl of blood or 0.3 x 10^6 peritoneal, spleen and bone marrow cells were pre-incubated with mixture of 2% normal rat: normal mouse serum (1:1) (AbD Serotec, Kidlington, UK). The cells were stained with fluorochrome-labeled monoclonal antibodies (mabs) against CD11b, F4/80, CD115, CD62L, CD43, GR-1 (clone RB6-8C5) (eBioscience), Ly6G (clone 1A8, BD Bioscience), Ly6C (AbD Serotec), CXCR2 or with unlabelled CCR2 (a

generous gift from Dr. M. Mack, Regensburg University Medical Center, Regensburg, Germany) combined with biotinylated rat anti-mouse IgG and Strepdavidin-FITC (eBioscience). Peritoneal, spleen and bone marrow cells were additionally stained with PE-Cy7-labeled mabs against B220, CD90.2 and NK1.1 (eBioscience) for exclusion of lymphocytes. After staining, red blood cells in the blood samples were lysed with FACS Lysing Solution (BD Bioscience) for 15 minutes. All cells were washed two times. Appropriate isotypic controls were used to set the analysis gates and quadrants. Following staining and washing, the samples were suspended in 200 µl FACS staining buffer and 10,000 cells collected on FACScalibur (BD Biosciences) and data analyzed using Cell Quest software (BD Biosciences) and FlowJo software (Tree Star, Inc., Ashland, OR).

For determination of total blood cell numbers and numbers of cell subpopulations 50 µl of blood was precisely measured into Trucount tubes (BD Biosciences) and stained with monoclonal antibody mixture (Ly6G-FITC for neutrophils, CD115-APC for monocytes, CD62L-PE to discriminate between monocyte subpopulations and B220-PE-Cy7, NK1.1-PE-Cy7 and CD90.2-PE-Cy7 for total lymphocytes). After 15 minutes incubation with the antibodies the sample was suspended in FACS lysing solution (1000 µl) and raw data collected within FACScalibur 3 h of antibody labeling.

For determination of intracellular staining, the cultured peritoneal macrophages were incubated (1×10^6/ml in 100 µl volume) with anti-mouse CD11b-APC, B220-PE-Cy7, NK1.1-PE-Cy7 and CD90.2-PE-Cy7 mabs for cell surface staining. The cells were washed and fixed with 4% formaldehyde on ice for 10 minutes. Following a second wash the cells were permeabilized with a buffer containing 0.1% saponin in FACS staining buffer for 10 minutes on ice. Following the third wash cells were suspended in blocking solution (i.e. the permeabilizing buffer with 20% normal mouse and normal rat serum in a 1:1 ratio). The cells were stained with mixture of either anti-mouse CCL2-FITC and IL-10-PE or CCL3-PE and TNF-α-FITC (eBioscience) for 30 minutes. Cells were then washed two times, first with a wash buffer containg 0.1% saponin and then withstaining buffer and respuspended in the staining buffer. Cells were analyzed on FACScalibur as described above. The intracellular staining for CCL2 and IL-10 was not successful.

3.6 Cytokine and chemokine ELISA

Cytokines (TNF-α, IL-1β, IL-10, IL-33 and G-CSF) and chemokines (CCL2, CCL3, CCL5, CXCL1 and CXCL2) were measured in serum, peritoneal fluid and supernatants from peritoneal macrophage cultures using Duo set ELISA kits (R&D Systems). Detection limits for TNF-α, IL-10. G-CSF and CCL5 was 15.6 pg/ml, 7.8 pg/ml for IL-1β, IL-33, CXCL1 and CXCL2, 3.9 pg/ml for CCL3 and 1.9 pg/ml for CCL2. The samples were diluted to fit the appropriate standard curves. The results were expressed as pg/ml.

3.7 Statistical analysis

All data are expressed as mean values ± standard error of the mean (SEM). One-way ANOVA and Tukey's post hoc test was used to determine the effects of LPS administration with time. Unpaired Student's t test was used to determine if the differences between the two dietary groups were statistically significant. Interactions between LPS treatment and diets were determined by two-way ANOVA and Tukey's post hoc test. Statistical analysis was performed using SPSS software, version 17 (SPSS Inc, Chicago IL, USA). Differences were considered significant if $P<0.05$.

4 RESULTS

4.1 The effects of intraperitoneal LPS administration on leukocyte populations, cytokine and chemokine concentrations

4.1.1 Alterations in blood leukocyte populations following intraperitoneal administration of LPS

Characterization of the endotoxin induced-peritonitis model used in this study was a necessary prerequisite for being able to assess the effects of dietary modification on the model. Peritonitis was induced by LPS administration into peritoneum. In blood, the administration of LPS into the peritoneum of mice caused a decrease in the number of leukocytes (**Fig. 4A**). The leukocyte count remained low for 24 h but at 48 h the blood leukocyte count had increased to levels similar to that seen in blood from mice prior to LPS administration (**Fig. 4A**).

Mouse blood leukocyte populations were distinguished according to their size and granularity (**Fig. 4B**) and by their expression or lack of expression of several surface markers (**Figs. 4C and 4E**). Lymphocytes were excluded from the analysis of monocytes and neutrophils by their expression of the lymphocyte markers B220, NK1.1 and CD90.2 (**Fig. 4C, gate L**). These cells were small and with low granularity (**Fig. 4B**).

Mouse blood monocytes were identified as cells expressing both CD11b (Mac-1) (**Fig. A1C**) and CD115 (a receptor for monocyte colony stimulating factor (M-CSF)) and lacking expression of the lymphocyte markers B220, NK1.1 and CD90.2 (**Fig.4C, gate M**). They were larger than lymphocytes and with low granularity (**Fig. 4B**). Two major subpopulations of mouse monocytes were detected; classical monocytes expressing high levels of the surface marker Ly6C and the chemokine receptor CCR2 but low levels of CD43 (**Fig.4C, MI**) and non-classical monocytes expressing low levels of both Ly6C and CCR2 but high levels of CD43 (**Fig. 4C, MII**). The classical monocytes also expressed CD62L (**Fig. A2A and A2B**) and were larger and more granulated than the non-classical monocytes (**Fig. 4C**).

Two h after administration of LPS, monocytes had almost disappeared from the blood and few monocytes were detected in blood for the first 24 h after administration of LPS (**Figs. 4B and 1D**). Forty-eight h after administration of LPS the number of monocytes in blood had increased to levels similar to that seen prior to LPS administration (**Figs.4B and 4D**).

However, some of the monocytes present in blood 48 h after LPS administration were larger and slightly more granular than the majority of the monocytes seen in blood prior to LPS administration (**Fig. 4B**). Classical and non-classical monocytes were in equal proportions in blood from mice that had not received LPS (**Fig. A3**). In contrast, 24 h after administration of LPS the few monocytes that were detected in the circulation were all classical monocytes (**Fig. A3**). However, 48 h after administration of LPS the non-classical and the classical monocytes were again in almost equal proportions (**Fig. A3**).

Neutrophils were identified by their expression of the granulocyte marker Ly6G which is specifically expressed by neutrophils and not by monocytes (**Fig. 1E**). Before administration of LPS the neutrophils were smaller than the monocytes and highly granular (**Fig. 1B**). Three h after LPS administration the granularity of the neutrophils had decreased and 8 h after LPS administration they had divided into two populations, N1 and N2, which were less granular than the neutrophils present in blood prior to LPS administration, although N2 population was even less granular than N1 (**Fig. 4B**). The less granular N2 population consisted of larger neutrophils than the more granular N1 neutrophil population (**Fig.4B**). At 24 h only one population of neutrophils was visible, but at 48 h they had again divided into two populations with the N2 neutrophils being less granular than the N2 neutrophils present in blood 8 and 12 h after administration of LPS (**Fig. 4B**). Both neutrophil populations, N1 and N2, expressed Ly6G at all time points (**Fig. 4E**). The neutrophil population present in blood prior to LPS administration expressed CXCR2 whereas the neutrophil populations present in blood 3-12 h following administration of LPS did not express CXCR2 (data not shown). Twenty four and 48 h after LPS administration the neutrophils expressed CXCR2 similar to the neutrophils present in blood prior to LPS administration (data not shown and **Fig. 4E**, respectively). The less granular neutrophil population (N2) expressed the same surface markers and chemokine receptors as the more granular neutrophil population (N1) (**Table 5**). There was no difference in the expression of CXCR2 between the two neutrophil populations at 48 h (**Fig. 4E**), but the less granular neutrophil population (N2) expressed higher levels of CD11b and Ly6G (**Table 5**).

Neutrophils accounted for approximately 15% of blood leukocytes in healthy mice (**Fig. 4F**). There was a transient decrease in both the number and the proportion of neutrophils in blood following i.p. administration of LPS (**Fig.4F**). At 8 and 12 h following administration of LPS, the number of

neutrophils was similar to that prior to LPS administration (0.2 x 10^6/ml) whereas at 48 h the number of neutrophils was more than two fold higher (0.45 x 10^6/ml) than in blood of mice that did not receive LPS (**Fig. 4F**). On the other hand, the proportion of neutrophils in blood transiently increased, peaking at 12 h following LPS administration. The decrease in the proportion of neutrophils at 24 and 48 h (**Fig. 4F**), while their numbers were increasing, was due to an increase in the number of other cell populations, such as monocytes (**Fig. 4D**) and lymphocytes (**Fig. A4**).

Table 5. Expression of surface markers on N1 and N2 neutrophil populations in blood 48 h following LPS administration.

	N1	N2
CD11b	+	++*
CD115	-/low	-/low
Ly6G	+	++*
Ly6C	-/low	-/low
CCR2	-	-
CCR5	-	-
CXCR2	+	+
CXCR3	-	-
L-selectin (CD62L)	+	+
CD43	-/low	-/low
CD90	–	–
B220 (CD45R)	–	–
NK1.1	–	–

* Different from control, P <0.05**.**

4.1.2 Peritoneal cell populations prior to and following intraperitoneal administration of LPS

At the site of onset, the peritoneum, injection of LPS resulted in a rapid decrease in the number of peritoneal cells (**Fig. 5A**). The number of peritoneal cells remained low until 24 h after LPS administration. At 48 h following LPS administration the number of peritoneal cells had increased from what it was at 24 h but was only about half the number of cells present in the peritoneum prior to LPS administration (**Fig. 5A**).

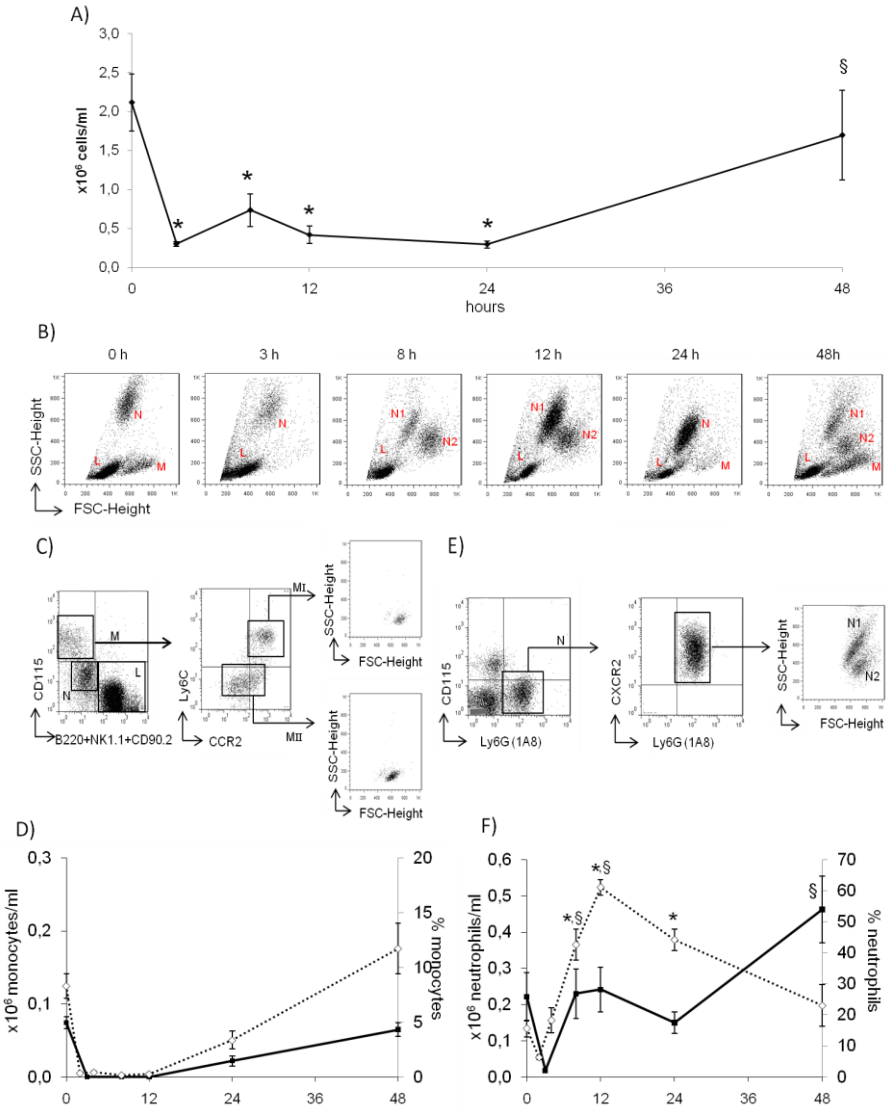


Figure 4. Effects of intraperitoneal LPS administration on mouse blood leukocytes.

(A) Total blood cell numbers prior to and following i.p. administration of LPS. (B) Dot plot showing forward and side scatter of leukocytes in blood prior to and following LPS administration. (C) Monocytes were identified as cells expressing CD115 and not the lymphocyte markers B220, NK1.1 and CD90.2, and their subsets were defined as (MI) $Ly6C^{hi}CCR2^{hi}$ (classical monocytes) and (MII) $Ly6C^{low}CCR2^{low}$ (non-classical monocytes). (D) Number (black line) and proportion (dashed line) of monocytes in blood prior to and following LPS administration. (E) Neutrophils were identified as $CD115^{med}CXCR2^{+}Ly6G^{+}$ cells. (F) Number (black line) and proportion (dashed line) of neutrophils in blood prior to and following LPS administration. Mice either received LPS (0.5 mg/kg) or not (0 h). They were anesthetized at indicated time points and blood collected into EDTA-K2 coated monovette tubes. Total cell count and leukocyte population cell counts were determined with TruCount and flow cytometry. The data was analyzed using FlowJo software. The values on the graphs are means ± SEM, n=5-8 per time point. * Different from 0 h, § different from previous time point, $P < 0.05$. Dot plots are representative of data from 3 independent experiments.

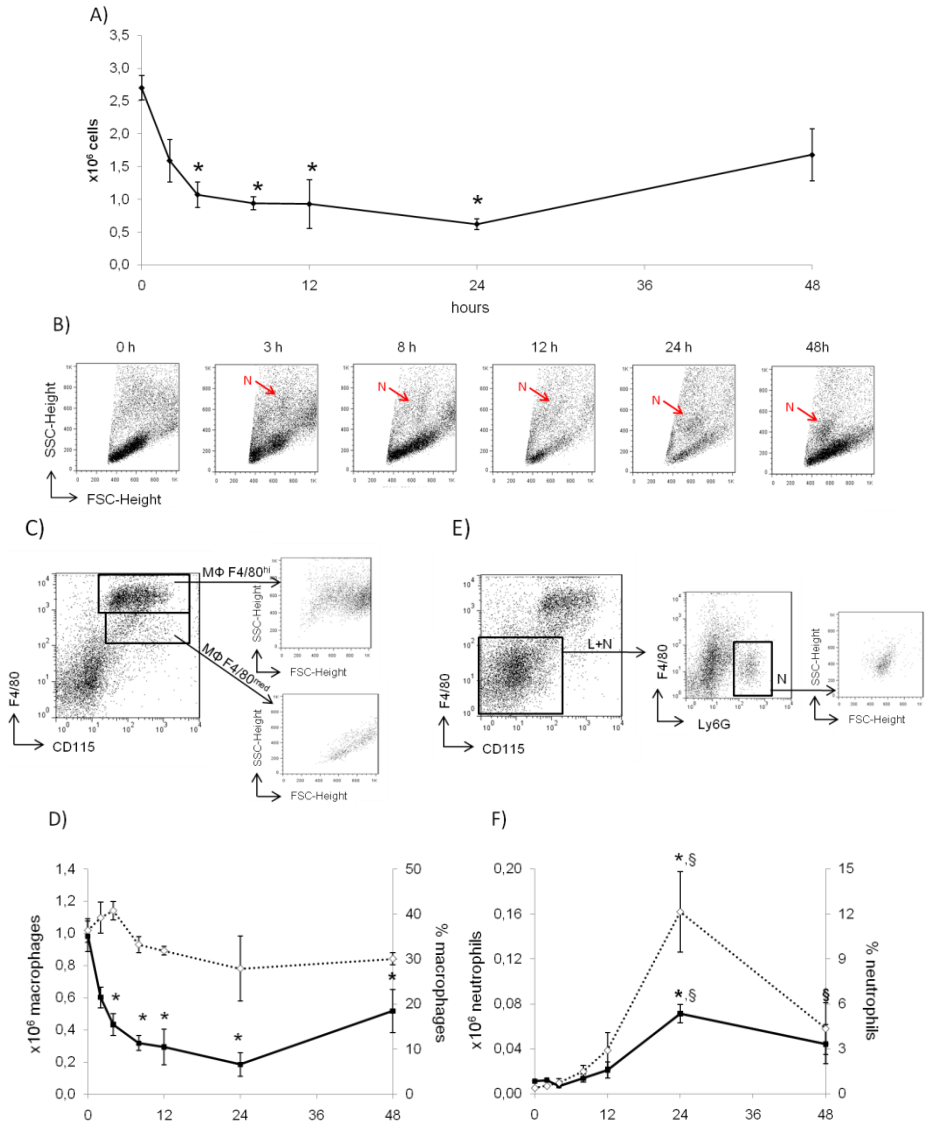


Figure 5. Effects of intraperitoneal LPS administration on mouse peritoneal cells.

(A) Total mouse peritoneal cell numbers prior to and following administration of LPS. (B) Dot plot showing forward and side scatter of leukocytes in peritoneum prior to and following LPS administration. (C) Macrophages were identified as cells expressing F4/80 and CD115 and not the lymphocyte markers B220, NK1.1 and CD90.2. (D) Number (black line) and proportion (dashed line) of macrophages in peritoneum prior to and following LPS administration. (E) Neutrophils were identified as F480^{-}CD115^{-}Ly6G^{+} cells. (F) Number (black line) and proportion (dashed line) of neutrophils in peritoneum from mice prior to and following LPS administration. Mice either received LPS (0.5 mg/kg) or not (0 h). Peritoneal lavage was collected at indicated time points postmortem. Cell count was determined with Countess automated cell counter and leukocyte populations determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values on the graphs are means ± SEM, n=5-8 per time point. * Different from 0 h, § different from previous time point, P <0.05. Dot plots are representative of data from 3 independent experiments.

Lymphocytes, identified by their expression of B220, CD90.2 and NK1.1, constituted around 70% of the cells in the peritoneal lavage from untreated mice (data not shown). Following LPS administration the changes in lymphocyte numbers correlated with the changes in total peritoneal cells (Fig. A5). Lymphocytes were excluded from the analysis of macrophages and neutrophils by their expression of the lymphocyte markers and lack of CD115and F4/80 (**Fig. 5C**).

Macrophages were identified by their expression of CD11b (data not shown) or F4/80 and CD115 (**Fig. 5C**). They could be divided into two subpopulations according to the level of expression of F4/80 and CD11b with the $CD11b^{hi}F4/80^{hi}$ cells being more granulated than the $CD11b^{med}F4/80^{med}$ macrophages (**Fig. 5C**). Macrophages constituted about 30% of the cells in the peritoneum of untreated mice (**Fig. 5D**). There was a decrease in the number of macrophages following administration of LPS into the peritoneum and their numbers did not start to increase until after 24 h following LPS administration (**Fig. 5D**). CD115 was not detected on macrophages in the peritoneum 2 to 24 h after administration of LPS, but 48 h after LPS administration the macrophages in the peritoneum all expressed CD115 (**Fig. 5C**).

Neutrophils were identified by expression of Ly6G and lack of expression of F4/80 and CD115 (**Fig. 5E**). Few neutrophils were detected in the peritoneum of untreated mice but their numbers started to increase 12 h after administration of LPS (**Figs. 5B and F**). The number of neutrophils in the peritoneum increased from 12-24 h, when their numbers started to decrease again (**Fig. 5F**).

4.1.1 Bone marrow cells prior to and following intraperitoneal administration of LPS

Since leukocytes originate and are recruited from the bone marrow during infection or inflammation it was of interest to characterize the changes in leukocytes in the bone marrow following LPS administration. In bone marrow, LPS administration led to a rapid decrease in the number of total bone marrow cells and a slower decrease in their numbers for the duration of the experiment (**Fig. 6A**). Forty eight h following LPS administration the number of total bone marrow cells was only one third of that present in the bone marrow prior to LPS administration (**Fig. 6A**). The bone marrow cells detected 48 h after LPS administration were more homogenous in size and granularity (**Fig. 6B**) as well as expression of several surface markers than the cells present in bone marrow prior to LPS administration (data not shown).

Lymphocytes, identified by their expression of B220, NK1.1 or CD90.2, accounted for around 40% of total bone marrow cells prior to LPS administration (Fig. A6). Following LPS administration the number of lymphocytes decreased steadily and by 48 h they were only one third of the number of lymphocytes present in bone marrow prior to LPS administration (**Fig. A6**). Monocytes and neutrophils were distinguished from the lymphocytes by their lack of expression of the lymphocyte markers B220, NK1.1 and CD90.2 and by their expression of CD11b, with monocytes expressing lower levels of CD11b than neutrophils (**Figs. 6C and 6E**).

Monocytes in the bone marrow were identified by their expression of CD11b (**Fig. 6C**), CD115 (Fig. A7) and CCR2 (**Fig. 6C**) and lack of expression of Ly6G and CXCR2 (data not shown). The monocyte population in the bone marrow was homogenous, with a phenotype of classical monocytes expressing CCR2 (**Fig. 6C**), with high expression of Ly6C and CD62L and low expression of CD43 (data not shown). The number of monocytes in bone marrow decreased rapidly following LPS administration and remained low until 24 h after LPS administration (**Fig. 6D**). At 48 h following LPS administration their number had increased slightly but was only half of that present in the bone marrow prior to LPS administration (**Fig. 6D**). Following LPS administration there was a downregulation of CD115 on bone marrow monocytes, but they still expressed CCR2 and CD11b (data not shown). Prior to LPS administration the monocytes were homogenous in size and granularity but 48 h following LPS administration they had become larger and more granular and some were similar in size and granularity to the neutrophils (**Fig. 6B**).

Neutrophils constituted around 30% of total bone marrow cells prior to administration of LPS (**Fig. 6F**). They were identified by their expression of CXCR2 (Fig. A8) or Ly6G (**Fig. 6E**). The number of neutrophils in bone marrow decreased rapidly in the first 3 h following LPS administration and remained low at 24 h after administration of LPS (**Fig. 6F**). At 48 h following LPS administration the number of neutrophils had increased slightly and accounted for around 40% of total bone marrow cells (**Fig. 6F**).

4.1.2 Splenic cell populations prior to and following intraperitoneal administration of LPS

The spleen serves as a reservoir of monocytes that can readily migrate to the site of infection or inflammation. Three h following administration of LPS, the number of cells in spleen was increased compared with that in mice not administered LPS, but 8 h after LPS administration their numbers had decreased again, reaching similar numbers as before administration of LPS (**Fig. 7A**).

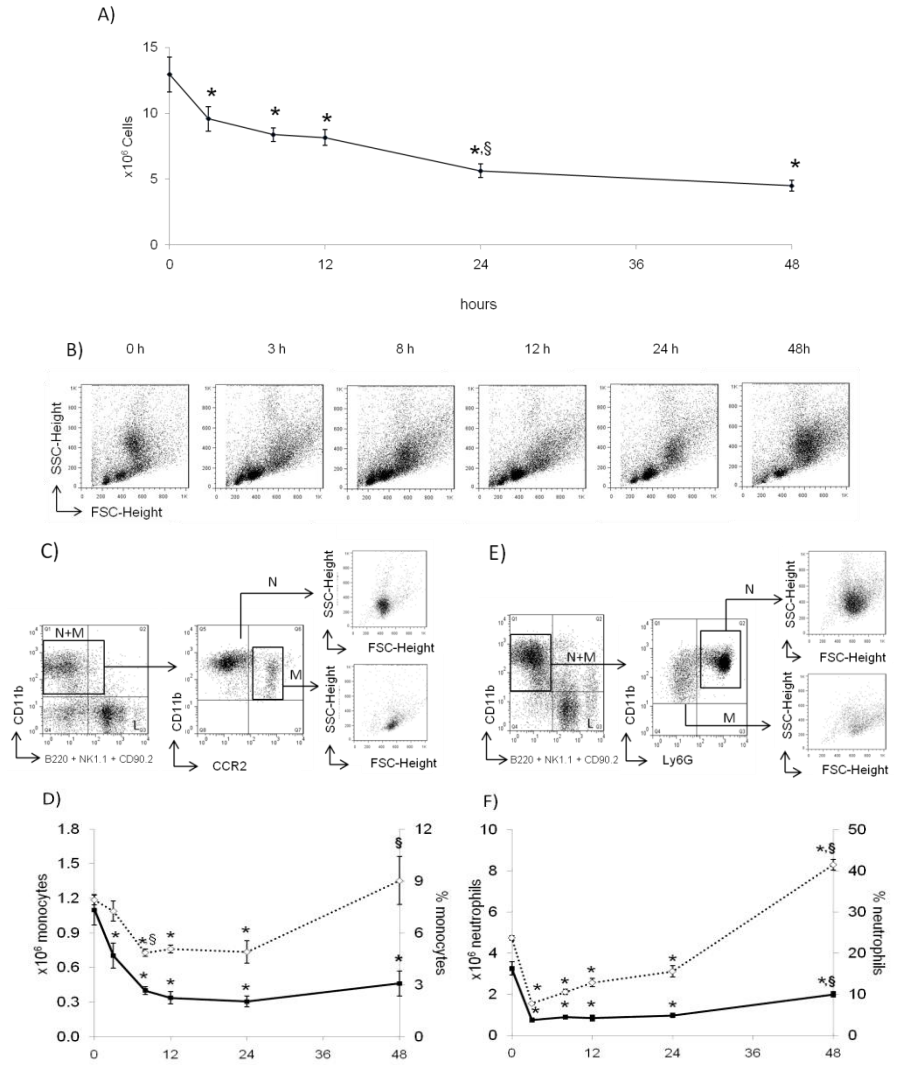


Figure 6. Effects of intraperitoneal LPS administration on mouse bone marrow cells.

(A) Total mouse bone marrow cell numbers prior to and following administration of LPS. (B) Dot plot showing forward and side scatter of leukocytes in bone marrow prior to and following LPS administration. (C) Monocytes were identified as cells expressing CD11b and CCR2 and not the lymphocyte markers B220, NK1.1 and CD90.2. (D) Number (black line) and proportion (dashed line) of monocytes in bone marrow prior to and following LPS administration. (E) Neutrophils were identified as $CD11b^+$ $Ly6G^+$ cells. (F) Number (black line) and proportion (dashed line) of neutrophils in bone marrow from mice prior to and following LPS administration. Mice either received LPS (0.5 mg/kg) or not (0 h). Bone marrow was collected from right femur and tibiae at indicated time points postmortem. Cell count was determined with Countess automated cell counter and leukocyte populations determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values on the graphs are means ± SEM, n=5-8 per time point.* Different from 0 h, § different from previous time point, $P < 0.05$. Dot plots are representative of data from 3 independent experiments.

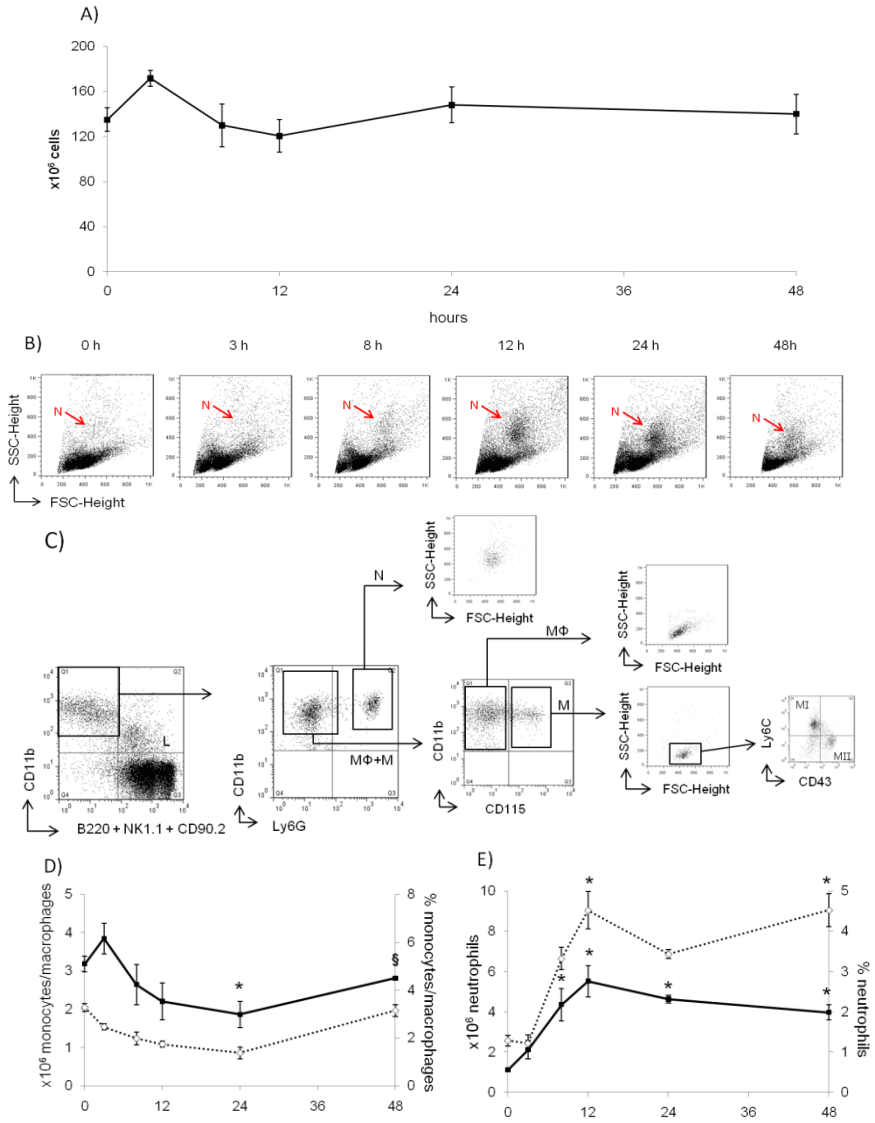


Figure 7. Effects of intraperitoneal LPS administration on mouse spleen cells.

(A) Total mouse spleen cell numbers following administration of LPS. (B) Dot plot showing forward and side scatter of leukocytes in spleen prior to and following LPS administration. (C) Neutrophils were identified as cells expressing CD11b and Ly6G, monocytes were identified as cells expressing CD11b and CD115 and not Ly6G and macrophages were identified as cells expressing CD11b and not Ly6G, CD115 nor the lymphocyte markers B220, NK1.1 and CD90.2. (D). Number (black line) and proportion (dashed line) of macrophages/monocytes in spleen prior to and following LPS administration. (E) Number (black line) and proportion (dashed line) of neutrophils in spleen from mice prior to and following LPS administration Mice either received LPS (0.5 mg/kg) or not (0 h). Spleens were collected at indicated time points postmortem. Cell count was determined with Countess automated cell counter and leukocyte populations determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values on the graphs are means ± SEM, n=5-8 per time point. * Different from 0 h, § different from previous time point, P <0.05. Dot plots are representative of data from 3 independent experiments.

Lymphocytes, identified by their expression of B220, CD90.2 and NK1.1, were over 90% of the cells in spleen (Fig. A9). They were excluded from analysis of monocytes, macrophages and neutrophils which all expressed CD11b (**Fig. 7C**).

Macrophages and monocytes were identified according to their expression of CD11b and F4/80 (**Fig. A10**) and lack of expression of Ly6G (**Fig. 7C**). Undifferentiated monocytes were distinguished from macrophages by their expression of CD115 (**Fig. 7C**). The monocytes were divided into classical monocytes ($Ly6C^{hi}$ and $CD43^{low}$) and non-classical monocytes ($Ly6C^{low}$ and $CD43^{hi}$) (**Fig. 7C**). After administration of LPS monocytes downregulated their expression of CD115 making it difficult to distinguish between them and macrophages. The number of macrophages/monocytes in spleen was increased first after injection with LPS but then decreased and remained low at 24 h after LPS administration but had increased slightly by 48 h (**Fig. 7D**).

Neutrophils were identified according to their expression of CD11b, Ly6G (**Fig. 7C**) and CXCR2 (**Fig. A11**). There was a steady increase in neutrophil numbers in the spleen for 12 h after LPS administration, followed by a slight decline in their numbers (**Fig. 7E**). At 48 h after administration of LPS the neutrophil numbers were more than threefold higher than that in spleen from mice not injected with LPS (**Fig. 7E**).

4.1.3 Chemokine and cytokine concentrations in serum prior to and following intraperitoneal administration of LPS

Chemokine and cytokine concentrations in serum and the kinetics of their appearance and disappearance from serum were determined in order to gain insight into whether they are involved in the chemotaxis of the monocytes/macrophages and neutrophils to blood. Intraperitoneal administration of LPS induced a rapid (within 4 h) increase in serum concentrations of the chemokines CCL2, CCL3, CCL5 (**Fig. 8A**), CXCL1 and CXCL2 (**Fig. 8B**) and the cytokines TNF-α, IL-6 (**Fig. 8C**), IL-10 and G-CSF (**Fig. 8D**). TNF-α and CXCL1 peaked one h following administration of LPS; CCL2, CCL3 and CXCL2 at 2 h; and CCL5 at 4 h following administration of LPS (**Figs. 8A-C**). The concentration of TNF-α and CXCL2 declined rapidly and reached basal levels at 3 h, whereas the levels of the other chemokines and cytokines reached basal levels between 8 and 12 h, with the exception of CCL5 that reached basal levels at 24 h following administration of LPS (**Figs. 8A-C**). The decline in IL-6 levels was slower

than that for TNF-α, and did not reach levels close to basal levels until 8 h following administration of LPS. IL-10 and G-CSF levels did not peak until 8 h after LPS administration, with the levels of the former declining more rapidly and reaching basal levels at 24 h, whereas G-CSF declined more slowly and reached levels close to basal levels at 48 h following administration of LPS (**Fig. 8D**).

The cytokine IL-33 was not detected in serum at any time point.

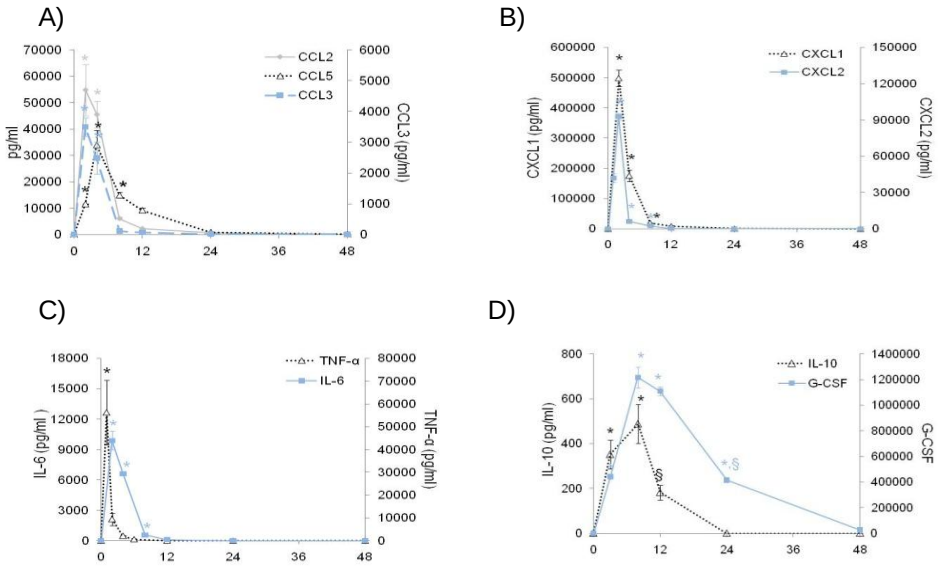


Figure 8. Effects of intraperitoneal LPS administration on mouse serum chemokine and cytokine concentrations

Concentrations of CC chemokines (A), CXC chemokines (B) and cytokines (C-D) in serum following administration of LPS**.** Mice either received LPS (0.5 mg/kg) or not (0 h). They were anesthetized at indicated time points and blood collected. Blood was allowed to clot for 30 minutes, then spun down and serum removed and stored at -80°C. Serum chemokine and cytokine concentrations were measured with ELISA. The values on the graphs are means ± SEM, n=3-5 per time point. * Different from 0 h, **§ different from previous time point, P <0.05.**

4.1.4 Chemokine and cytokine concentrations in peritoneal fluid prior to and following intraperitoneal administration of LPS

The chemokines and cytokines secreted by and involved in the chemotaxis of monocytes/macrophages and neutrophils were also examined in the peritoneal fluid. Intraperitoneal administration of LPS induced a rapid increase in the concentrations of chemokines in peritoneal fluid which peaked between 2 and 4 h and had reached levels close to basal levels by 12 h (**Figs. 9A and 9B**). Only CCL3 and CCL5 were detectable in peritoneal fluid 48 h after LPS administration (**Fig. 9A**).

IL-33 and TNF-α were detected in peritoneal fluids prior to LPS administration whereas IL-6 and G-CSF were not (**Figs. 9C and 9D**). IL-6 and TNF-α levels peaked 2 h following LPS administration and declined rapidly, reaching basal levels by 12 h after administration of LPS (**Fig. 9C**). IL-33 levels peaked 4 h after administration of LPS and reached baseline levels at 12 h, whereas G-CSF levels peaked at 8 h after LPS administration and reached basal levels at 48 h (**Fig. 9D**).

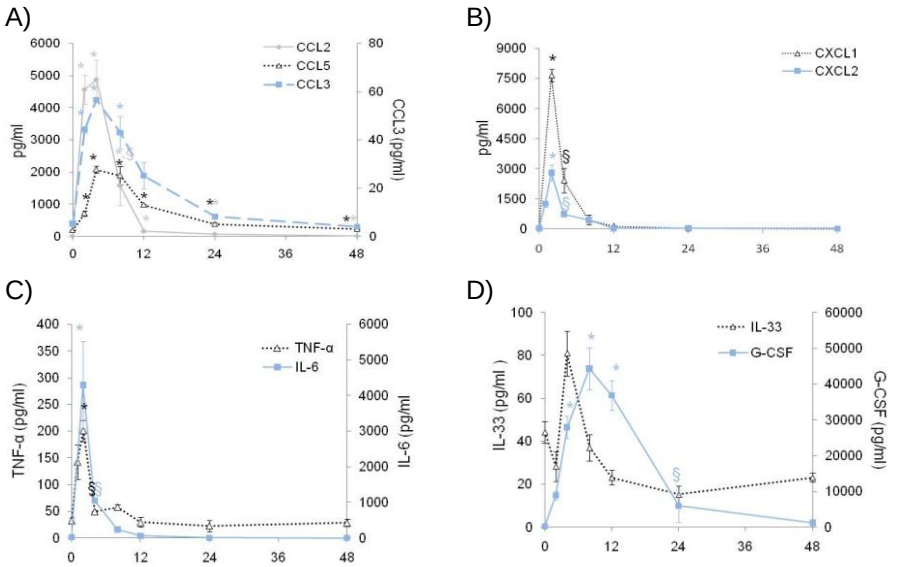


Figure 9. Effects of intraperitoneal LPS administration on mouse peritoneal fluid chemokine and cytokine concentrations.

Concentrations of CC chemokines (A), CXC chemokines (B) and cytokines (C-D) in peritoneal fluid following administration of LPS. Mice either received LPS (0.5 mg/kg) or not (0 h). Peritoneal lavage was collected at indicated time points postmortem. Cells were spun down and peritoneal fluid was aliquoted into Eppendorf tubes and stored at -80°C. Chemokine and cytokine concentrations in peritoneal fluid were measured with ELISA. The values on the graphs are means ± SEM, n=3-5 per time point.* Different from 0 h, § different from previous time point, P <0.05.

4.2 Effects of dietary fish oil on monocytes, macrophages and cytokines and chemokines involved in monocyte/macrophage recruitment

4.2.1 Mouse weight and food intake

There was no difference in the average daily food intake of the mice receiving either control or the fish oil diet. In addition, the initial body weight of the mice, relative weight gain throughout the experiment or weight loss following LPS administration did not differ between the two dietary groups.

4.2.2 Liver phospholipid fatty acid composition

FA composition of hepatic phospholipids from mice fed the control and the fish oil diets is shown in Table 6. The effectiveness of the diet was observed by increased (n-3):(n-6) PUFA ratio in hepatic phospholipids of livers from mice fed the fish oil diet, compared with that in hepatic phospholipids of livers from mice fed the control diet.

Table 6. Fatty acid composition of liver phospholipids from healthy mice fed control and fish oil diets[a,b].

	Control diet	Fish oil diet
Fatty acid	mol%	
16:0	22.0±1.2	23.0±0.8
18:0	18.4±1.0	16.4±0.9
18:1	10.3±1.2	11.5±0.3
18:2 (n-6)	9.5±0.2	7.3±0.7*
20:4 (n-6)	24.7±0.6	10.3±0.4*
20:5 (n-3)	-	7.9±0.8*
22:5 (n-3)	-	1.01±0.03*
22:6 (n-3)	11.2±1.0	19.1±0.9*
(n-3)/(n-6) PUFA[c]	0.3±0.02	1.5±0.12*

[a] Only selected fatty acids are listed
[b] Values are means ± SEM, n=4. * Different from control (P<0.05)
[c] (n-3)/(n-6): Σ20:5; 22:5; 22:6 / 18:2; 20:3; 20:4; 22:4

4.2.3 Effects of dietary fish oil on peritoneal cells

When the effects of dietary fish oil on mouse peritoneal macrophages were examined, it became apparent that healthy mice fed dietary fish oil had fewer peritoneal cells than healthy mice fed the control diet (**Fig. 10**). As shown in section 4.1.2 (**Fig. 5A**), LPS administration decreased the number of cells in the peritoneum. In mice fed the fish oil diet, the decrease in total peritoneal cell numbers at 3 h after LPS administration was less striking than in mice fed the control diet and the number of peritoneal cells was higher than in mice fed the control diet (**Fig. 10**). At 48 h after LPS administration the number of peritoneal cells had increased again but mice fed the fish oil diet had fewer peritoneal cells than mice fed the control diet and fewer peritoneal cells than healthy mice fed the fish oil diet (**Fig. 10**).

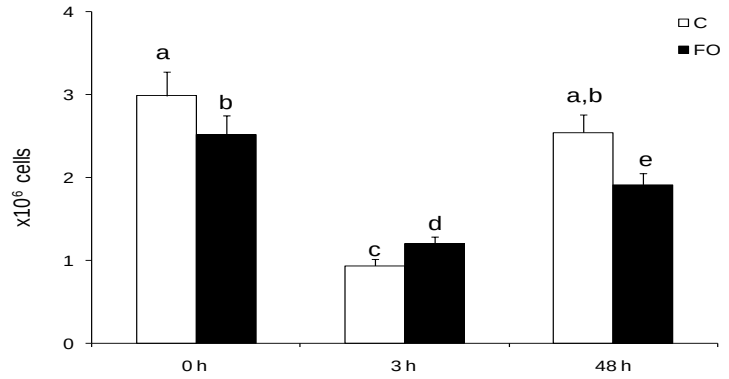


Figure 10. Effects of dietary fish oil on the number of peritoneal cells prior to and 3 and 48 h after intraperitoneal administration of LPS.

Mice were fed either a Western type diet (open bars) or a Western type diet with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with 0.5 mg/kg LPS and sacrificed either 3 or 48 h later. Peritoneal cells were harvested postmortem, washed and counted with Countess automated cell counter. Values are means ± SEM, n=16 for healthy mice and mice with endotoxin-induced peritonitis (48 h), n=12-13 for mice with endotoxin-induced peritonitis (3 h). Means without a common letter differ, $P < 0.05$.

The effects of fish oil on the number of total peritoneal cells were in part due to its effects on peritoneal macrophages. Healthy mice fed dietary fish oil had fewer residential macrophages than healthy mice fed the control diet (**Fig. 11**). Three h after LPS administration the number of peritoneal macrophages had decreased in mice from both dietary groups with no difference between the two groups but a tendency towards higher numbers of macrophages in mice fed the fish oil diet compared with the number of macrophages in mice fed the control diet **(Fig. 11)**. At 48 h following LPS administration the number of peritoneal macrophages had increased in both dietary groups with a trend towards fewer macrophages in mice

fed the fish oil diet compared with that in mice fed the control diet (**Fig. 11**). Forty eight h after LPS administration the number of macrophages had reached the same number as before LPS administration in mice fed the fish oil diet, whereas mice fed the control diet had fewer macrophages 48 h after LPS administration than prior to LPS administration (**Fig. 11**).

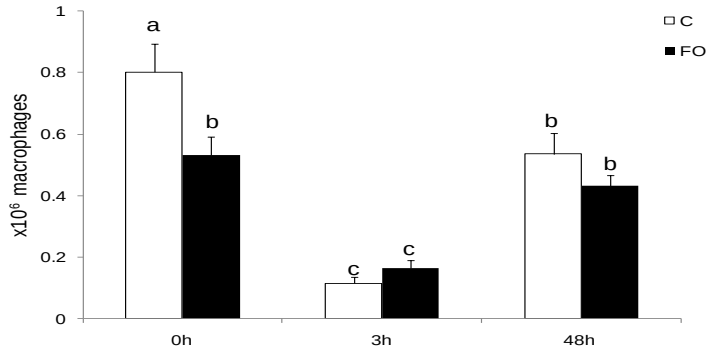


Figure 11. Effects of dietary fish oil on the number of peritoneal macrophages prior to and3 and 48 h after intraperitoneal administration of LPS.

Mice were fed either Western type diet (open bars) or Western type diet with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with 0.5 mg/kg LPS and sacrificed at either 3 or 48 h. Peritoneal cells were harvested postmortem, washed and counted with Countess automated cell counter. Macrophages were identified by their expression of F4/80 and CD11b using flow cytometry. Values are means ± SEM, n=15-17 for healthy mice and mice with endotoxin-induced peritonitis (48h), n=10-12 for mice with endotoxin-induced peritonitis (3h). Means without a common letter differ, P <0.05.

4.2.4 Effects of dietary fish oil on chemokine and cytokine concentrations in peritoneal fluid

As dietary fish oil altered the number of total peritoneal cells and in particular the number of peritoneal macrophages, the question arose whether those effects could be due to its effects on chemokines and cytokines. Therefore, the concentrations of chemokines and cytokines in peritoneal fluid were measured before and after LPS administration. Prior to LPS administration the chemokines CCL2 and CCL3 were not detected in peritoneal fluid, but the cytokines TNF-α and IL-6 were detected and there was no difference in their concentration between the two dietary groups (**Table 7**). Mice fed dietary fish oil had a higher concentration of the chemokine CCL3 and a trend towards a higher concentration of TNF-α in peritoneal fluid 3 h after LPS administration when compared with that in mice fed the control diet. There was no difference in the concentrations of CCL2 or IL-6 in mice fed the different diets, 3 h after LPS administration. At 48 h after LPS administration there was a higher concentration of CCL2, IL-6 and a trend towards higher concentration of TNF-α in peritoneal

fluid from mice fed the fish oil diet compared with that in peritoneal fluid from mice fed the control diet (**Table 7**).

Table 7. Effects of dietary fish oil on chemokine and cytokine concentrations (pg/ml) in peritoneal fluid from mice prior to and 3 and 48 h following administration of LPS

	0h		3h		48h	
	Control	**Fish oil**	**Control**	**Fish oil**	**Control**	**Fish oil**
CCL2	N/D	N/D	4344 ±284 [a]	4675 ±373 [a]	10 ±0.78 [b]	20 ±3.51 [c]
CCL3	N/D	N/D	25.0 ±1.35[a]	30 ±2.01 [b]	N/D	N/D
TNF-α	602.5±78.1 [a]	603.9±69.4 [a]	795 ±79.8 [b]	932 ±131 [b]	357±53.2 [c]	424±39.5 [c]
IL-6	89.7 ±9.79[a]	84.0 ±9.77[a]	17370±2344[b]	17863±1587[b]	100 ±3.59[a]	192 ±40.4[c]

Values are means ± SEM, n=12-15 for chemokines, n=8-11 for cytokines. Means without a common letter in a row differ, P <0.05**.**

4.2.5 Effects of dietary fish oil on CCL3 and TNF-α secretion by cultured resident peritoneal macrophages *ex vivo*

Resident macrophages are present in the peritoneum at the onset of inflammation or infection and secrete cytokines and chemokines that initiate the following inflammatory response. It was therefore of interest to examine whether these cells had the potential to secrete some of the chemokines involved in recruitment of monocytes and neutrophils to the peritoneum and the cytokines involved in inducing the inflammatory response. Of the chemokines measured in peritoneal fluid and serum only antibodies against CCL2 and CCL3 were commercially available and IL-10 and TNF-α were chosen as they are the major anti-inflammatory and pro-inflammatory cytokines produced by macrophages. Staining for IL-10 and CCL2 was not successful. Culturing resident peritoneal macrophages ex vivo showed that CCL3 and TNF-α were produced by the same cells, as shown by intracellular staining following stimulation with LPS for 24 h (**Fig. 12**). Dietary fish oil decreased the proportion of resident peritoneal macrophages that secreted TNF-α and CCL3 upon stimulation with LPS ex vivo when compared with the proportion of TNF-α and CCL3 secreting resident peritoneal macrophages from mice fed the control diet (**Fig. 13A**).

However, the mean fluorescent intensity (MFI) of CCL3 expression, or the mean CCL3 production, per cell from the peritoneum of mice fed the fish oil diet was higher than the MFI of CCL3 expression by peritoneal cells from mice fed the control diet (**Fig. 13B**). There was no difference in MFI for TNF-α in peritoneal cells from mice fed the different diets (**Fig. 13C**).

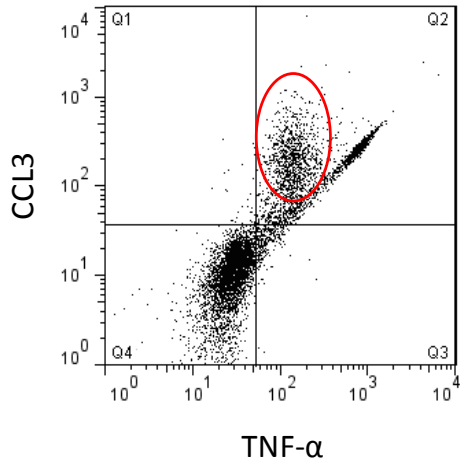


Figure 12. Representative dot plot from flow cytometric analysis of intracellular expression of CCL3 and TNF-α by resident peritoneal macrophages from a mouse fed the control diet.

Peritoneal cells were harvested in cold PBS postmortem. Peritoneal cells ($1x10^6$ cells/ml) from mice fed the control diet and the fish oil diet were stimulated with LPS (2 µg/µl) for 24 h. Brefeldin A was added to the culture for the last 8 h of the stimulation. Intracellular cytokine secretion was measured with flow cytometry and analyzed using the CellQuest software.

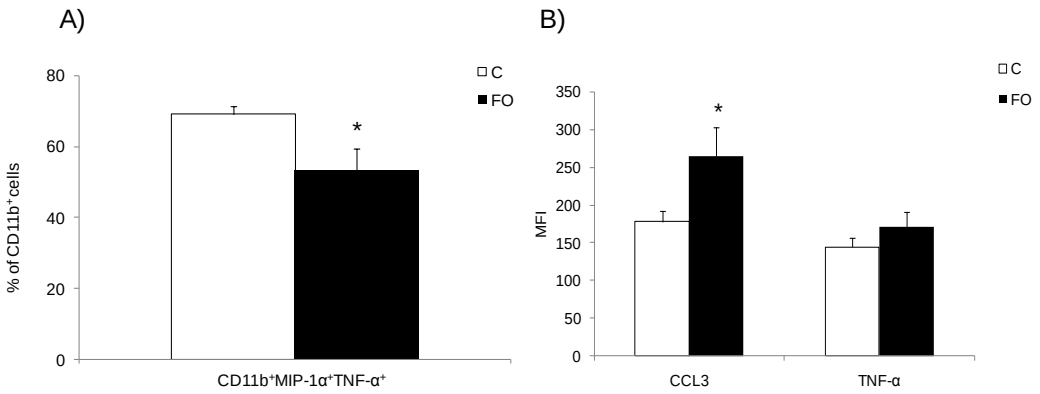


Figure 13. Effects of dietary fish oil on LPS-induced CCL3 and TNF-α secretion by resident peritoneal macrophages.

(A) Proportion of CCL3/TNF-α secreting cells (B) mean fluorescent intensity (MFI) of CCL3 and TNF-α on peritoneal cells. Peritoneal cells were harvested in cold PBS postmortem. Peritoneal cells ($1x10^6$ cells/ml) from mice fed the control diet and the fish oil diet were stimulated with LPS (2 µg/µl) for 24 h. Brefeldin A was added to the culture for the last 6 h of the stimulation. Intracellular cytokine secretion was measured with flow cytometry and analyzed using the Cell Quest software. Values are means ± SEM, n=11-16. * Different from control, $P < 0.05$.

4.2.6 Effects of dietary fish oil on CCL3 and TNF-α concentration in supernatants from cultured resident peritoneal macrophages

When resident peritoneal macrophages were stimulated with LPS ex vivo, macrophages from mice fed the fish oil diet secreted more CCL3 than macrophages from mice fed the control diet (**Fig. 14A**). Similar trend was observed for TNF-α secretion (**Fig. 14B**). These results are in accordance with results presented in the previous section (4.2.5) demonstrating that dietary fish oil increased secretion of CCL3, with a similar trend for TNF-α.

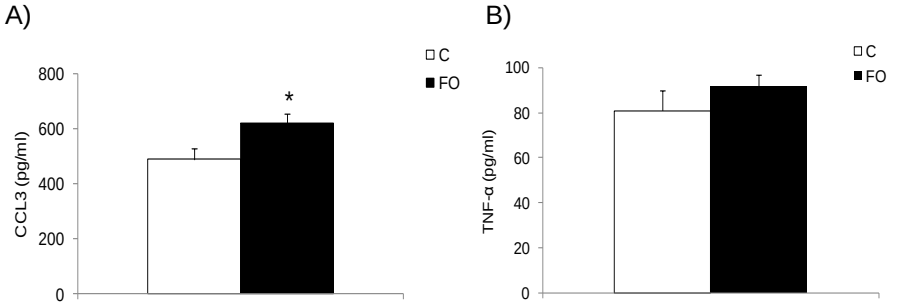


Figure 14. Effects of dietary fish oil on CCL3 and TNF-α secretion by resident peritoneal macrophages.

Concentration of CCL3 (A) and TNF-α (B) in supernatants from resident peritoneal macrophages after stimulation with LPS *ex vivo* for 24 h. Peritoneal cells were harvested in cold PBS postmortem. Peritoneal cells ($1x10^6$ cells/ml) from mice fed the control diet and the fish oil diet were stimulated with LPS (2 µg/µl) for 24 h. The cell culture supernatant was collected and cytokines measured with ELISA assay. Values are means ± SEM, n=17 for CCL3, n=8-10 for TNF-α. * Different from control, P <0.05.

4.2.7 Effects of dietary fish oil on the number of total leukocytes and leukocyte populations in blood in healthy mice and mice with severe endotoxin-induced peritonitis

Peritoneal administration of LPS results in systemic activation of the immune system and therefore it was of interest to examine the effects of dietary fish oil on leukocyte populations in blood. Administration of LPS resulted in a decrease in the number of leukocytes in blood as early as 2 h after administration with the number of leukocytes remaining low at 24 h but higher 48 h after administration of LPS (**Fig. 15**). There was no difference between the two dietary groups in the number of blood leukocytes in healthy mice or mice with severe endotoxin-induced peritonitis (**Fig. 15**). In the mice fed the FO diet the number of blood leukocytes was lower (P<0.05) 48 h after LPS administration than prior to LPS administration.

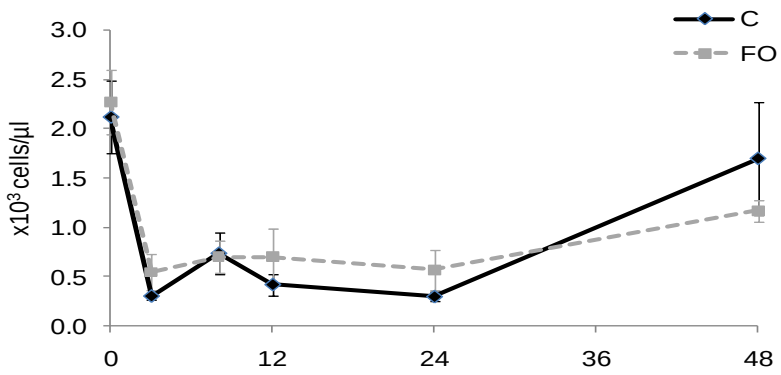


Figure 15. Effects of dietary fish oil on total number of leukocytes in mouse blood prior to and at different time points following intraperitoneal administration of LPS.

Mice were fed a Western type diet (black line) or a Western type diet supplemented with 2.8% fish oil (dashed line) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed at indicated time points. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and counted using TruCount and flow cytometry. Values are means ± SEM, n=3-5.

There was no difference in the proportions of monocytes, neutrophils or lymphocytes in the circulation between the two dietary groups, before and 48 h after LPS administration (Fig. A12 and A13, respectively). There was also no difference in the number of monocytes in blood from mice in the two dietary groups prior to or after administration of LPS (**Fig. 16**).

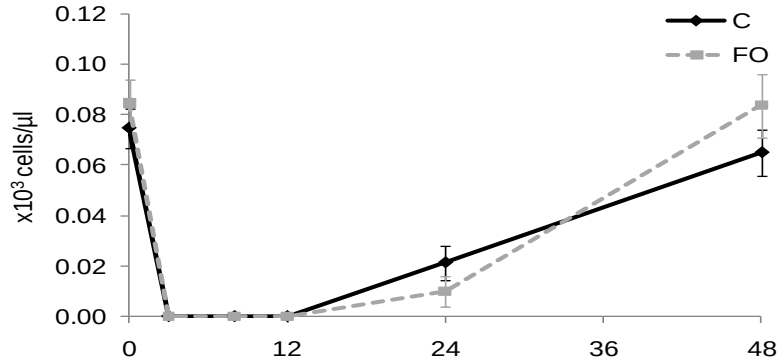


Figure 16. Effects of dietary fish oil on total number of monocytes in mouse blood prior to and at different time points following intraperitoneal administration of LPS.

Mice were fed a Western type diet (black line) or a Western type diet supplemented with 2.8% fish oil (dashed line) for 6 week. Mice were injected with LPS (0.5 mg/kg) and sacrificed at indicated time points. Blood was collected via axillary bleeding into EDTA-K2 containing tubes and red blood cells lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and counted using TruCount and flow cytometry. Values are means ± SEM, n=3-5.

4.2.8 Dietary fish oil altered the proportion of classical monocytes in blood of healthy mice and mice with severe endotoxin-induced peritonitis

Although dietary fish oil had no effect on the number of total monocytes in blood, when examining its effects on the subpopulations of monocytes it became apparent that it altered the proportion of monocyte subpopulations. Healthy mice fed the fish oil diet had a lower proportion of classical monocytes in blood than mice fed the control diet (**Fig. 17**). On the other hand, 48 h after LPS administration the mice that had been fed the fish oil diet had a tendency towards a higher proportion of classical monocytes in the circulation than mice fed the control diet (**Fig. 17**). Importantly, when comparing the change in the proportion of classical monocytes upon injection with LPS (48 h), there was a significant increase (~60%) in the mice fed the fish oil diet, whereas there was only a slight increase (~15%, not statistically significant) in the proportion of classical monocytes in the mice fed the control diet (**Fig. 17**).

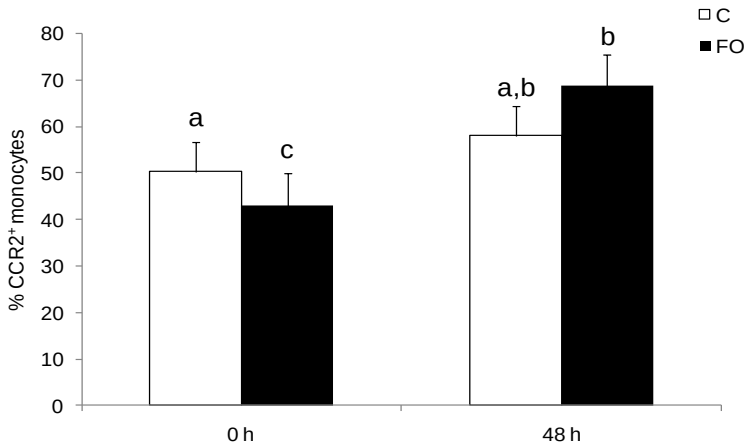


Figure 17. Effects of dietary fish oil on the proportion of classical monocytes in blood prior to and 48 h after LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells were lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and analyzed with flow cytometry. The results are expressed as proportion of classical monocytes of total monocytes in blood. Values are means ± SEM, n=17-20 for healthy mice, n=8 for mice with endotoxin-induced peritonitis. Means without a common letter differ, $P < 0.05$.

4.2.9 Dietary fish oil altered cell numbers in spleen in healthy mice and mice with severe endotoxin-induced peritonitis

Healthy mice fed the fish oil diet had a trend towards a higher total cell number in spleen compared with that in mice fed the control diet (**Fig. 18**). On the other hand, 48 h after LPS injection there was a tendency towards lower (not statistically significant) total cell number in spleen from mice fed the fish oil diet than in mice fed the control diet (**Fig. 18**). When comparing the change in the total cell number in spleen within the same dietary group prior to and following LPS administration, there was no difference in the total cell number in spleen between healthy mice and mice with severe endotoxin-induced peritonitis fed the control diet, whereas the mice with endotoxin-induced peritonitis fed the fish oil diet had a lower total cell umber in spleen than healthy mice fed the fish oil diet (**Fig. 18**).

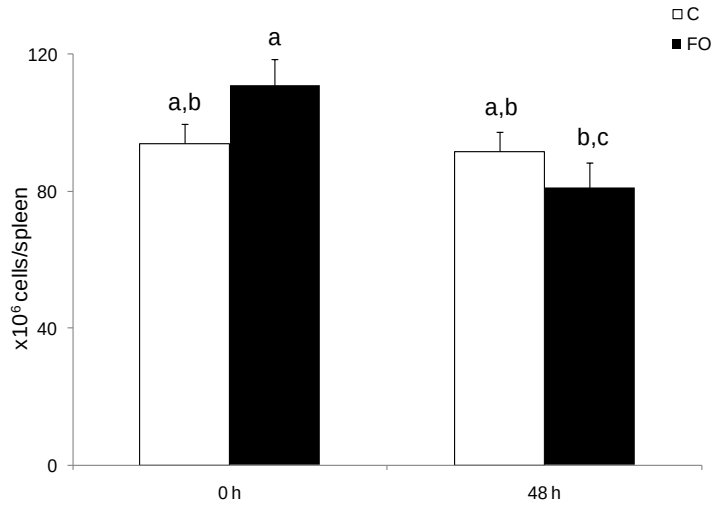


Figure 18. Effects of dietary fish oil on the number of cells in spleen ofmice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5mg/kg) and sacrificed at 48 h. Spleens were collected postmortem and passed through a cell strainer. After the red blood cells were lysed, cells were washed and debris removed and the cells were then resuspended in DMEM and counted using a Countess automated cell counter. Values are means ± SEM, n=15 for healthy mice, n=11 for mice with endotoxin-induced peritonitis. Means without a common letter differ, $P < 0.05$.

4.2.10 Dietary fish oil increased the number and proportion of classical monocytes in spleen of mice with severe endotoxin-induced peritonitis

As previously mentioned, the spleen serves as a reservoir of undifferentiated monocytes which resembles blood monocytes. Therefore, it was of interest to examine the effects of fish oil on monocytes and monocyte subpopulations in spleen. The number of total monocytes in spleen was similar from healthy mice and mice with severe endotoxin-induced peritonitis 48 h following LPS administration and did not differ between the two dietary groups (Fig. A14). However, examination of the subpopulations of monocytes demonstrated a higher number (Fig. A15) and a higher proportion (**Fig. 19**) of classical monocytes (expressing Ly6C, CCR2 and CD62L but not CD43) in spleens from mice with endotoxin-induced peritonitis (48 h) fed the fish oil diet compared with that in mice endotoxin-induced peritonitis fed the control diet (48 h), which is in accordance with what was seen in blood. However, there was no effect of dietary fish oil on the number (data not shown) or the proportion of classical monocytes in spleen from healthy mice (**Fig. 19**).

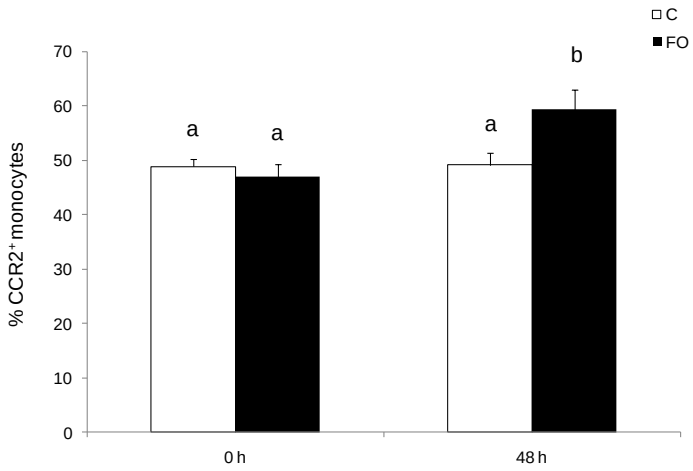


Figure 19. Effects of dietary fish oil on the proportion of CCR2$^+$ monocytes in spleen of mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Spleens were collected postmortem and passed through a cell strainer. After the red blood cells were lysed, cells were washed and debris removed and the cells were then resuspended in DMEM and counted using a Countess automated cell counter. Monocytes and monocyte subpopulations were identified by antibody staining and analysis by flow cytometry. Values are means ± SEM, n=15 for healthy mice, n=11 for mice with endotoxin-induced peritonitis. Means without a common letter differ, P <0.05.

4.2.11 Dietary fish oil decreased the number of CCR2$^+$monocytes in bone marrow from mice with severe endotoxin-induced peritonitis

As leukocytes, such as monocytes, originate from the bone marrow and are recruited from the bone marrow during infection or inflammation, the effects of dietary fish oil on leukocyte and monocyte numbers in the bone marrow were examined. There were fewer bone marrow cells in mice with severe endotoxin-induced peritonitis (48 h) from both dietary groups than in healthy mice but there was no difference in the number of bone marrow cells between the two dietary groups (Fig. A16).

There was no difference in the number of CCR2$^+$ cells in bone marrow from healthy mice or mice with endotoxin-induced peritonitis fed the different diets (**Fig. 20**). However, the decrease in the number of CCR2$^+$ cells in bone marrow following LPS administration was more prominent in mice fed fish oil diet than in mice fed the control diet (**Fig. 20**). Dietary fish oil did not affect the proportion of CCR2$^+$ cells in bone marrow in healthy mice or mice with endotoxin-induced peritonitis (Fig. A17).

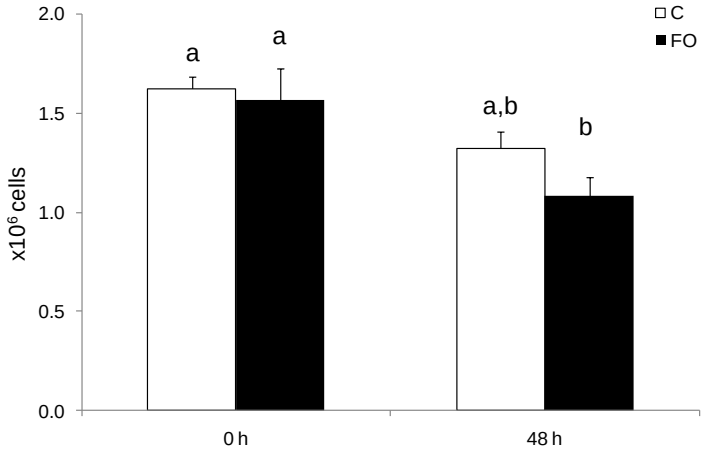


Figure 20. Effects of dietary fish oil on the number of CCR2$^+$ cells in bone marrow from mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed at 48 h. Bone marrow was flushed from right femur and tibiae with DMEM supplemented with 10% FBS. After red blood cells were lysed, cells were washed, debris removed and the cells resuspended in DMEM with 10% FBS. Cells were counted using Countess automated cell counter. The results are expressed as the number of CCR2$^+$ monocytes in bone marrow collected from right femur and tibiae. Values are means ± SEM, n=16 for healthy mice, n=13 for mice with endotoxin-induced peritonitis. Means without a common letter differ, P <0.05.

4.2.12 Dietary fish oil altered CCL2 concentrations in serum of healthy mice and mice with severe endotoxin-induced peritonitis

CCL2 is the chemokine required for recruiting CCR2+ inflammatory monocytes from the bone marrow to the blood and determines their frequency in the circulation (Serbina & Pamer, 2006; Sunderkotter et al., 2004; Tacke et al., 2007). Thus, serum concentrations of CCL2 in mice fed the control and the fish oil diets, before and after administration of LPS, were determined. Healthy mice fed the fish oil diet had lower serum levels of CCL2 than mice fed the control diet (**Fig. 21A**). In contrast, administration of LPS caused a more pronounced increase inCCL2 levels in serum from mice fed the fish oil diet followed by a slower reduction compared with that in mice fed control diet (P<0.05) (**Fig. 21B and C**).

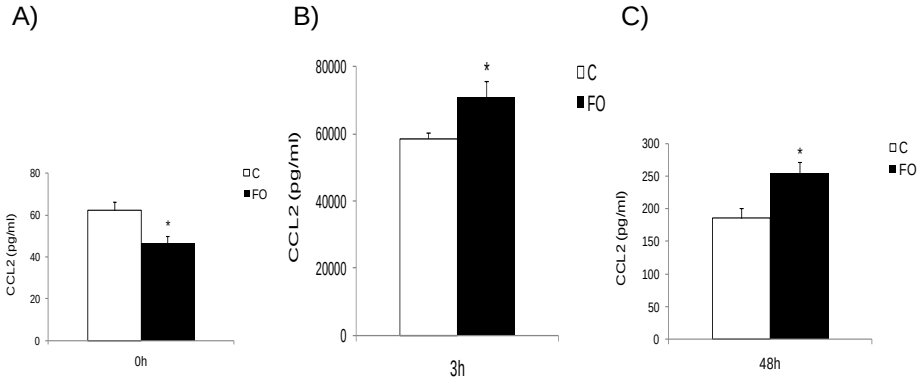


Figure 21. Effects of dietary fish oil on CCL2 concentrations in serum from mice prior to and 3 and 48 h following endotoxin administration.

Concentration of CCL2 in serum from mice prior to (A) and 3 h (B) and 48 h (C) following endotoxin administration. Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Mice either received an LPS (0.5 mg/kg) injection or not and were sacrificed at 0, 3 or 48 h. Blood was collected into Eppendorf tubes and allowed to clot for 30 minutes before centrifugation (3000 rpm, 25°C, 10 minutes). Serum was collected and stored at -80°C. CCL2 concentration in serum was measured with ELISA assay. The results are expressed as pg/ml serum. Values are means ± SEM, n=16-20 for healthy mice, n=11 for mice with endotoxin-induced peritonitis (48 h), n=17-18 for mice with endotoxin-induced peritonitis (3h). *Different from control at indicated time point, P <0.05.

4.3 Effects of dietary fish oil on neutrophils and chemokines involved in neutrophil recruitment

4.3.1 Effects of dietary fish oil on blood neutrophils in healthy mice and mice with severe endotoxin-induced peritonitis

Dietary fish oil did not alter the proportion of neutrophils in blood prior to or 2-48 h following LPS administration (Fig. A18). Dietary fish oil did not affect the number of total neutrophils in healthy mice or mice with severe endotoxin-induced peritonitis at 3, 8 or 48 h following LPS administration. However, at 12 and 24 h following administration of LPS there was a trend (not statistically significant) towards a higher number of neutrophils in blood in mice fed the fish oil diet compared with that in mice fed the control diet (**Fig. 22**).

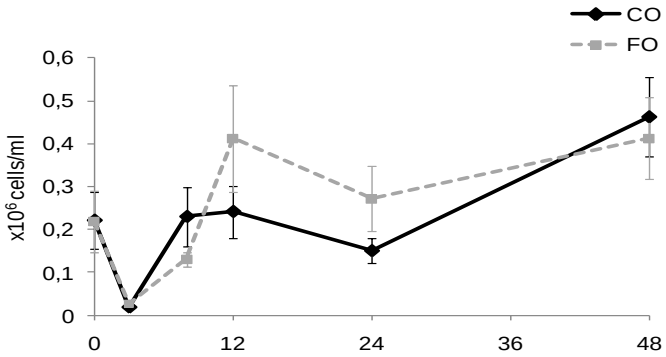


Figure 22. Effects of dietary fish oil on the number of neutrophils in blood prior to and following intraperitoneal administration of LPS.

Mice were fed a Western type diet (black line) or a Western type diet supplemented with 2.8% fish oil (dashed line) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed at indicated time points. Blood was collected via axillary bleeding into EDTA-K2 containing tubes and red blood cells were lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and counted using TruCount and flow cytometry. Values are means ± SEM, n=3-5.

As shown earlier (section 4.1.1) two populations of neutrophils were present in blood at 8, 12 and 48 h following LPS administration, N1 resembling the population present in blood prior to LPS administration and N2 consisting of less granular neutrophils. Forty eight h following LPS administration mice fed dietary fish oil had a larger proportion of the less granular neutrophils (N2) compared with mice fed the control diet (**Figs. 23A and 23B**). After 12 h the less granular neutrophils (N2) expressed higher levels (higher MFI) of Ly6G, and there was also a trend (not statistically significant) for increased expression of Ly6G on the more granular neutrophils (N1) in blood from mice fed the fish oil diet compared with that in blood from mice fed the control diet (Fig. A19).

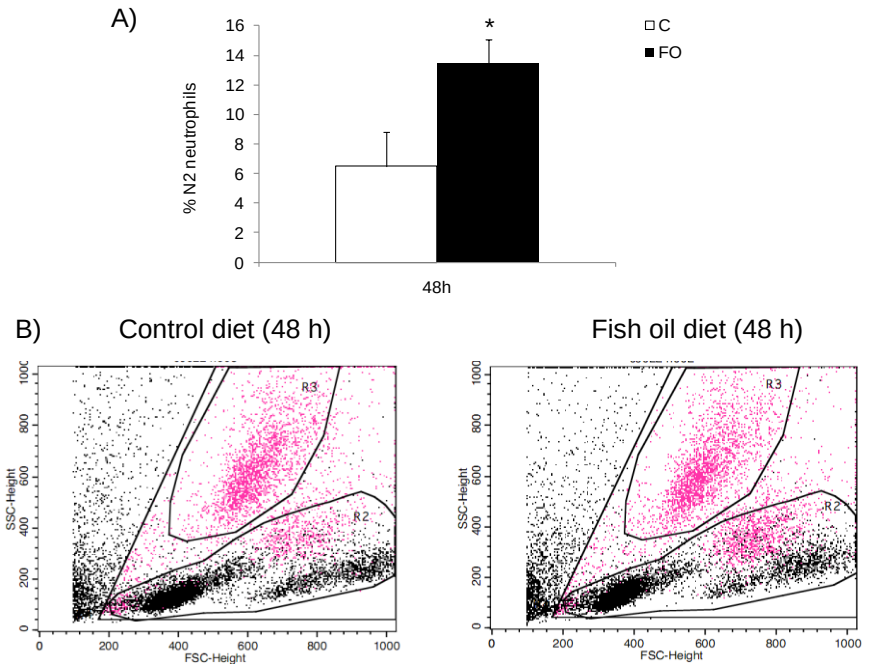


Figure 23. Effects of dietary fish oil on the proportion of a specific neutrophil population (N2) in blood from mice 48 h following intraperitoneal administration of LPS.

The proportion of hypo-granulated N2 neutrophils of total blood cells 48 h after administration of LPS (0.5 mg/kg LPS) (A). Representative forward side scatter of blood cells from mice fed the control and fish oil diet 48 h after administration of LPS (0.5 mg/kg LPS) (B). Mice were fed a Western type diet (open bar) or a Western type diet supplemented with 2.8% fish oil (black bar) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells were lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and analyzed with flow cytometry. The results are expressed as proportion of a specific neutrophil population of total blood cells. Values are means ± SEM, n=8. *Different from control, P <0.05. One of two duplicate experiments is shown for (A).

Dietary fish oil did not affect either the number (Fig. A20A) or the proportion (Fig.A20B) of neutrophils in bone marrow, neither in healthy mice nor in mice with endotoxin-induced peritonitis (2 to 48 h). Dietary fish oil did not affect the proportion or number of cells expressing CXCR2 or CXCR2 expression by each cell (data not shown).

4.3.2 Effects of dietary fish oil on serum concentrations of the cytokines G-CSF and IL-33 and the chemokines CXCL1, CXCL2 and CCL3 in mice with severe endotoxin-induced peritonitis

Murine CXCL1 and CXCL2 are potent neutrophil chemoattractants that bind to the chemokine receptor CXCR2 and recruit neutrophils to the site of infection or inflammation (Chintakuntlawar & Chodosh, 2009). CCL3 has also been implicated to play a role in the recruitment of neutrophils (Reichel et al., 2009). Having observed the effects of dietary fish oil on neutrophil numbers and the proportion of their subpopulations in blood (described in section 4.2.12) it was therefore of interest to examine the effects of dietary fish oil on serum levels of chemoattractants involved in recruitment of neutrophils. Dietary fish oil did not affect serum levels of CXCL1 in healthy mice or mice with severe endotoxin-induced peritonitis (3 and 48 h) mice (data not shown). CXCL2 and CCL3 were not detected in blood from healthy mice but mice with endotoxin-induced peritonitis (3 h) that were fed the fish oil diet had lower serum concentrations of CXCL2 (**Fig. 24A**) and higher serum concentrations of CCL3 than mice with endotoxin-induced peritonitis (3 h) that were fed the control diet (**Fig. 24B**). The effects of serum concentrations of the cytokines G-CSF and IL-33 were examined as G-CSF stimulates the release of neutrophils from the bone marrow and IL-33 prevents the downregulation of CXCR2 on neutrophils during acute inflammation and thus enhances neutrophil migration to site of infection (Alves-Filho et al., 2010). There was a trend towards a higher concentration of G-CSF in serum from mice fed the fish oil diet 3 h after administration of LPS than in serum from mice fed the control diet (**Fig. 24C**). IL-33 was not detected in serum from healthy mice or mice with endotoxin-induced peritonitis.

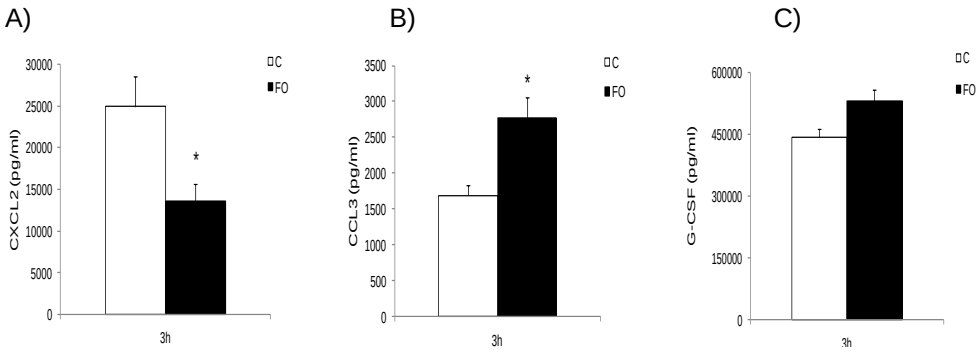


Figure 24. Effects of dietary fish oil on serum concentrations of CXCL2 (A), CCL3 (B) and G-CSF (C) in mice 3h following intraperitoneal administration of LPS.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed at 3 h later. Blood was collected into Eppendorf tubes, allowed to clot for 30 minutes and centrifuged (3000 rpm, 25°C, 10 min). Serum was collected and stored at -80°C. Chemokine concentration in serum was measured with ELISA assay. The results are expressed as pg/ml. Values are means ± SEM, n=8-9 for CXCL2, 16-17 for CCL3 and 3 for G-CSF. * Different from control, P <0.05.

4.3.3 Effects of dietary fish oil on peritoneal neutrophil populations in healthy mice and mice with severe endotoxin-induced peritonitis

The number of total neutrophils was similar in peritoneum from healthy mice fed the fish oil diet and in peritoneum from those fed the control diets (**Fig. 25A**). However, the time curve for the effects of LPS on the number of peritoneal neutrophils differed between the two dietary groups. In mice fed the fish oil diet the influx of neutrophils into the peritoneum was delayed by 24 h whereas in mice fed the control diet their number started to increased already after 8 h following LPS administration (**Fig. 25A**). At 48 h after administration of LPS, there was a tendency towards more (not statistically significant) neutrophils being present in peritoneum of mice fed the fish oil diet compared with that in peritoneum of mice fed the control diet (**Fig. 25A**) and the proportion of neutrophils in peritoneum of mice fed the fish oil diet was greater in mice fed the fish oil diet than in those fed the control diet (**Figs. 25B and 25C**). In other experiments with fewer time points and higher n the difference in neutrophil numbers in the peritoneum was significant (Fig. A21).

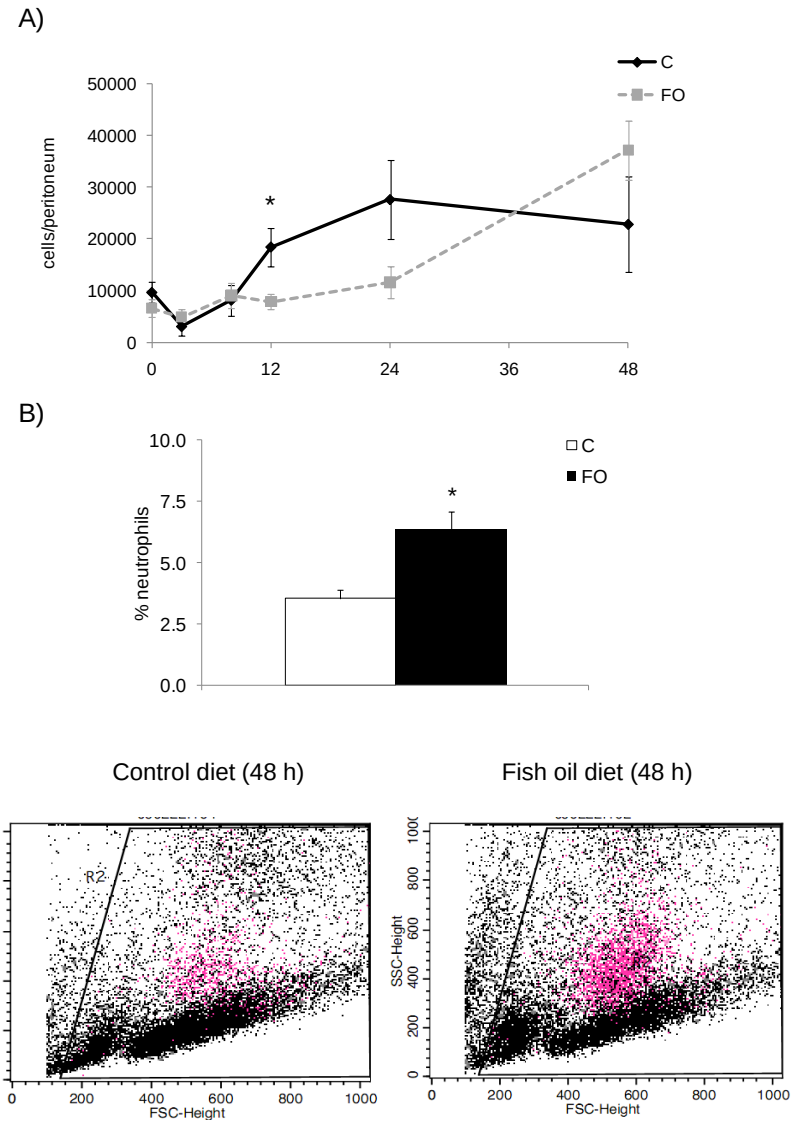


Figure 25. Effects of dietary fish oil on the number and proportion of neutrophils in peritoneum.

Number of neutrophils in peritoneum from mice fed a Western type diet (black line) or a Western type diet supplemented with 2.8% fish oil (dashed line) for 6 weeks, followed by LPS administration or not (A). Proportion of neutrophils in peritoneum from mice fed a Western type diet (open bar) or a Western type diet supplemented with 2.8% fish oil (black bar) for 6 weeks and injected with LPS (48 h) (B). Representative forward and side scatter of peritoneal cells from mice fed control or fish oil diet 48 h after administration of LPS (C). Peritoneal cells were collected in cold PBS. Cells were washed and resuspended in DMEM. Cells were stained with monoclonal antibodies and analyzed with flow cytometry. The results are expressed as total number of neutrophils or the proportion of neutrophils in peritoneum. Values are means ± SEM, n=15.* Different from control. (A) P=0.05 for diet*time interaction.(B) P<0.05.

4.3.4 Effects of dietary fish oil on concentrations of the neutrophil chemoattractants CXCL1, CXCL2 and CCL3 in peritoneal fluid from mice with severe endotoxin-induced peritonitis

Dietary fish oil did not affect the concentrations of CXCL1 and or CXCL2 in peritoneal fluid from mice with severe endotoxin-induced peritonitis (3 h) (data not shown). However, as shown in **Table 6** mice with endotoxin-induced peritonitis (3 h) fed the fish oil diet had a higher concentration of CCL3 in peritoneal fluid than mice fed the control diet.

5 DISCUSSION AND CONCLUSION

The study presented in this thesis examined the effects of dietary fish oil on monocyte and neutrophil populations in blood, peritoneum, bone marrow and spleen in healthy mice and mice with severe endotoxin-induced peritonitis as well as the effects of dietary fish oil on several cytokines and chemokines involved in the recruitment of immune cells. The results show that dietary fish oil decreased the concentration of CCL2 as well as the proportion of classical monocytes in blood from healthy mice. In contrast, dietary fish oil increased the concentration of CCL2 and the proportion of classical monocytes both in blood and spleen of mice with endotoxin-induced peritonitis. The increase in the proportion of classical monocytes in blood and spleen from mice with endotoxin-induced peritonitis that were fed fish oil was accompanied with a more pronounced decrease in monocyte numbers in bone marrow from mice fed the fish oil diet compared with that in mice fed the control diet.

In addition, there was a delay in the peritoneal recruitment of neutrophils during the early stages (<24 h) of endotoxin-induced peritonitis in mice fed dietary fish oil, although at 48 h mice fed dietary fish oil had increased proportion and number of neutrophils in peritoneum compared with mice with endotoxin-induced peritonitis that were fed the control diet. Mice with endotoxin-induced peritonitis that were fed dietary fish oil also had a higher proportion of the less granulated neutrophil population (N2) in blood 48 h following LPS administration. Dietary fish oil increased the concentration of CCL3 in serum and peritoneum in mice with endotoxin-induced peritonitis (3 h), but in contrast decreased the concentration of CXCL2 in serum compared with that in mice with endotoxin-induced peritonitis fed the control diet.

Furthermore, dietary fish oil decreased the number of resident peritoneal macrophages that secrete CCL3 and TNF-α*ex vivo*, but the secretion of CCL3 by each cell was higher and there was a tendency towards higher secretion of TNF-α by cells from mice fed the fish oil diet compared with cells from mice fed the control diet. The results also demonstrated that CCL3 and TNF-α were secreted by the same cells.

5.1 The effects of intraperitoneal LPS administration on leukocyte populations and cytokine and chemokine concentrations

5.1.1 The effects of intraperitoneal LPS administration on neutrophils

The results from the present experiments on the effects of i.p. LPS administration on leukocyte populations show that i.p. LPS administration caused a rapid decrease in blood neutrophils. Blood neutrophil numbers rapidly increased again and at 8 h following LPS administration they had divided into two separate populations that differed in size and granulation. LPS administration did not result in rapid infiltration of neutrophils into the peritoneum as observed with other inflammatory stimuli, such as zymosan (Rao et al., 1994), but caused a delayed neutrophil recruitment to the peritoneum, with the neutrophils only starting to infiltrate the peritoneum at 12 h after administration of LPS. The delayed neutrophil recruitment to the peritoneum following i.p. administration of LPS is in accordance with a previous report by Haziot et al. (Haziot et al., 2001). The rapid decrease in blood neutrophils upon LPS administration can be explained by their marginalization to the endothelium as well as their recruitment to liver and lungs as that has been shown by others to be the fate of blood neutrophils in response to systemic inflammation (Andonegui et al., 2003; Erzurum et al., 1992; McAvoy et al., 2011). The rapid sequestration of neutrophils in liver and lungs is in part due to mechanical entrapment that leads to a profound neutropenia and reduced leukocyte trafficking in the peripheral circulation (Andonegui et al., 2003; Erzurum et al., 1992; McAvoy et al., 2011), as seen in this study. The decreased availability of neutrophils in the circulation is thought to contribute, in part, to the reduced migration of neutrophils to the peritoneum at the beginning of the immune response. The fast recovery of neutrophils in blood in the present study was probably due to a rapid release of neutrophils from the bone marrow, as the number of neutrophils in the bone marrow decreased rapidly in the first 3 h following LPS administration and their numbers remained low throughout the experiment.

Neutrophils are differentially recruited to different organs, i.e. liver, lungs and peritoneum, following endotoxin exposure. Recruitment of neutrophils to liver and lungs following endotoxin exposure is independent of CD14 (Andonegui et al., 2003). On the contrary, interaction of LPS with CD14 and a subsequent signal through TLR4 on neutrophils has been shown to

prevent their infiltration into the peritoneum (Haziot et al., 2001). Additionally, expression of the integrins CD11b/CD18 differentially affects neutrophil recruitment. Neutrophil migration to lungs and liver is independent of CD11b/CD18, whereas their migration to the peritoneum is CD11b/CD18-dependent (Conlan & North, 1994; Mercer-Jones et al., 1997), especially at the beginning of the immune response, as it was shown that the recruitment of neutrophils to the peritoneum became CD11b/CD18 independent 18 and 24 h post infection (Mercer-Jones et al., 1997; Winn & Harlan, 1993). Thus, different mechanisms seem to be involved in the recruitment of neutrophils at different time points during the immune response. The recruitment of neutrophils has also been shown to be differentially regulated in response to infection with different pathogens (Conlan & North, 1994; Mercer-Jones et al., 1997). Recruitment of neutrophils to the peritoneum in response to gram-negative bacteria was CD18-dependent (Conlan & North, 1994; Mercer-Jones et al., 1997; Winn & Harlan, 1993), whereas recruitment of neutrophils to the peritoneum in response to pathogens like *L. monocytogenes* was CD18-independent (Conlan & North, 1994). In the present study involvement of CD11b/CD18 in the recruitment of neutrophils to peritoneum was not determined. However, in the present study neutrophil recruitment into the peritoneum appeared to be, at least in part, dependent on CXCR2 expression, as the neutrophils did not start to migrate into the peritoneum until 12 h after LPS administration, at a time when blood neutrophils had begun to up-regulate CXCR2 on their surface. These results are in accordance with previous studies showing that LPS induces internalization of CXCR2 by blood neutrophils (Khandaker et al., 1998) and that CXCR2 is critical for neutrophil recruitment into the peritoneum following LPS administration (Goncalves & Appelberg, 2002). Furthermore, down-regulation of CXCR2expression has been linked to impaired neutrophil migration in sepsis (Rios-Santos et al., 2007). Expression of CXCR2 on blood neutrophils returned to pre-LPS levels 24 h after LPS administration when serum levels of the chemokines CXCL1 and CXCL2 had reached levels close to basal levels. Unlike the delayed recruitment of neutrophils to the peritoneum, recruitment of neutrophils to the spleen was rapid and occurred within the first 12 hours. As neutrophil recruitment to the spleen is thought to be independent of CXCR2 expression (Kesteman et al., 2008) that may explain the rapid recruitment of neutrophils to the spleen compared with their delayed recruitment into the peritoneum in the present study.

Prior to LPS administration, blood neutrophils were highly granular and expressed both Ly6G and CXCR2. However, administration of LPS resulted in degranulation of the neutrophils. The less granular neutrophil

population (N2) that was present in blood at 8, 12 and 48 h following LPS administration expressed more CD11b than the more granular neutrophils. The increase in CD11b expression on the less granular neutrophils (N2) may reflect their activation status as increased CD11b expression is a hallmark of neutrophil activation (Kishimoto et al., 1989; Liu et al., 2005). Activated neutrophils rapidly mobilize their secretory vesicles, which serve as intracellular reservoirs for CD11b, and the secretory vesicles fuse with the cellular membrane elevating the CD11b content of the neutrophil membranes (Dahlgren et al., 2001). Thus, this new population of neutrophils that appears later in the inflammatory response, and has to our knowledge not been identified previously, may be the result of degranulation caused by activation.

5.1.2 The effects of intraperitoneal LPS administration on monocytes/macrophages

The results from the present experiments on the effects of i.p. LPS administration on monocyte and macrophage populations showed that i.p. LPS administration resulted in immediate disappearance of both classical and non-classical monocytes from blood as well as a rapid reduction in macrophages in peritoneum and classical monocytes in bone marrow. Similar to the reduction in neutrophils in the circulation, the disappearance of monocyte populations is probably due to their rapid marginalization and infiltration to lungs and liver (O'Dea et al., 2009). When monocytes first reappeared in the circulation following LPS administration (at 24 h), only the classical $CD62L^{+}CCR2^{+}$ monocytes were present. The lack of non-classical monocytes in blood at 24 h may be due to the absence of non-classical monocytes in the bone marrow. However, the appearance of non-classical monocytes in the circulation at 48 h indicates that they developed from the less mature classical monocytes. This is in accordance with previous findings that classical $CCR2^{+}$ monocytes are recruited from bone marrow to the blood where they develop into non-classical $CCR2^{-}$ monocytes (in 1-2 days) (Serbina & Pamer, 2006; Tsou et al., 2007). Monocyte recruitment from the bone marrow was likely caused by an increase in the chemokine CCL2 in blood, as CCL2 has been shown to be the main chemokine responsible for the emigration of monocytes from bone marrow (Serbina & Pamer, 2006). The rapid decrease in monocytes in bone marrow seen in the present study following LPS administration coincided with the rapid increase in CCL2 levels in serum with a decline in

the recruitment levels only starting when serum levels of CCL2 had returned to baseline levels.

Splenic monocytes resemble blood monocytes (Swirski et al., 2009) and can be readily recruited to peripheral tissues in response to insult or inflammation (Swirski et al., 2009). Therefore, it would have been interesting to analyze monocyte subpopulations in the spleen following administration of LPS. However, CD115 expression on monocytes was downregulated following LPS administration making it impossible to distinguish the monocytes from the splenic macrophages.

The rapid decrease in total number of cells in peritoneum following LPS administration in the present study was mainly due to a decrease in the number of macrophages and lymphocytes. The reduction in peritoneal macrophages and lymphocytes following LPS administration is in accordance with results from others showing a rapid egress of both macrophages (Cao et al., 2005) and lymphocytes (mainly B1 cells) from the peritoneum (Ha et al., 2006; Rajakariar et al., 2008) upon stimulation with bacteria or bacterial components. The macrophages and lymphocytes were shown to adhere to the mesothelium and eventually migrate to the lymphatics (Cao et al., 2005; Ha et al., 2006; Rajakariar et al., 2008). In this study, the destination of the emigrated macrophages and lymphocytes could not be determined.

To evaluate the course of the systemic inflammation induced by a low dose (0.5 mg/kg) LPS, cytokine and chemokine concentrations in both serum and peritoneal fluid were determined at various time points. Low dose LPS administration induced a rapid systemic as well as local (in the peritoneum) production of the major pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) and chemokines (CCL2, CCL3, CCL5, CXCL1 and CXCL2), which is in accordance to what has been seen previously in experimental models of endotoxin exposure (Steinmuller et al., 2006). Increase in chemokine concentrations in peritoneum and blood could not explain the decrease in cell numbers observed in blood and peritoneum. However, the rapid emigration of cells (i.e. monocytes and neutrophils) from bone marrow to blood during the first hours following LPS administration could be due to the high concentration of chemokines in serum during that time.

5.2 The effects of dietary fish oil on monocytes and macrophages, and the cytokines and chemokines involved in monocyte/macrophage recruitment

5.2.1 The effects of dietary fish oil on peritoneal cells in healthy mice and mice with severe endotoxin-induced peritonitis

In the present study dietary fish oil altered the number of peritoneal exudate cells both in healthy mice and mice with severe endotoxin-induced peritonitis, mainly through its effect on the number of peritoneal macrophages. The decreased number of peritoneal cells in healthy mice fed dietary fish oil may be due to a decrease in the recruitment of monocytes into the peritoneum, where they differentiate into resident macrophages. Dietary fish oil has been shown to increase the number of $CD11b^+$ macrophages as well as total cells in spleen when compared with that in spleen from mice fed a corn oil diet (Petursdottir & Hardardottir, 2007). Their results showing an increase in the number of total splenocytes in mice fed the fish oil diet are in accordance with results from the present study showing that the cell numbers in spleen from mice fed the fish oil diet were higher than in spleen from those fed the control diet. However, in contrast to their results showing an increase in $CD11b^+$ macrophages in spleen from mice fed the fish oil diet, in the present study fish oil had no effect on the number or the proportion of $CD11b^+$ cells or $CD11b^+CCR2^+$ classical monocytes. The difference between two studies could be due to several factors such as different type of mouse strain and difference in diet, especially the proportion of fish oil and a different control diet. These results indicate that dietary fish oil may differently affect the recruitment of monocytes to different tissues, such as peritoneum and spleen, under normal conditions.

Resident peritoneal macrophages are thought to develop from non-classical monocytes that are recruited from blood to the peritoneum and to some extent by local proliferation (Gordon & Taylor, 2005). Their recruitment to the peritoneum under normal conditions seems to be independent of CCL2, as the number of resident peritoneal macrophages in CCL2-defecient mice was comparable to that in wild-type mice (Lu et al., 1998). In the present study CCL2 was not detected in peritoneal fluid in healthy mice making it unlikely that the effects of dietary fish oil on the number of resident peritoneal macrophages is mediated by an effect on CCL2. In the present study the number of total monocytes in blood was

similar in healthy mice from the two dietary groups despite the decreased serum concentrations of CCL2 in mice fed the fish oil diet. However, the higher proportion of the more mature non-classical monocytes in blood from mice fed dietary fish oil may indicate that they are less efficiently recruited into tissues, such as the peritoneum, and instead accumulate in the blood which in turn could explain the lower number of resident peritoneal macrophages in mice fed fish oil compared with that in mice fed the control diet.

Mice fed dietary fish oil had lower number of macrophages than mice fed the control diet prior to LPS administration and the alterations in both total cell numbers and the number of macrophages in peritoneum following administration of LPS were less pronounced in mice fed the fish oil diet than in mice fed the control diet. The effects of fish oil on the changes in the number of peritoneal macrophages could not be explained by alterations in the concentration of the chemokine CCL2 as the number of macrophages, as well as the number of total cells, decreased in the peritoneum with increasing levels of CCL2 in peritoneal fluid. Therefore the tendency towards higher numbers of macrophages in mice 3 h following LPS administration indicates that fewer macrophages egress from the peritoneum of mice fed the fish oil compared with that in mice fed the control diet during the early stages of endotoxin-induced peritonitis. However, mice fed the fish oil diet had a slower reduction in CCL2 levels, as observed by the higher concentrations of CCL2 in peritoneal fluid at 48 h compared with that in mice fed the control diet, but in contrast they had lower number of macrophages, as well as total cell numbers at this time point. The less egression of macrophages from the peritoneum of mice fed the fish oil diet during the first hours following LPS administration may be explained by a decrease in the eicosanoids PGD_2 and PGE_2 as egression of macrophages and lymphocytes (mainly B1 cells) has been shown to be dependent on these eicosanoids (Cao et al., 2005; Ha et al., 2006; Rajakariar et al., 2008; Tajima et al., 2008) and these eicosanoids are decreased in mice receiving fish oil (Chavali et al., 1998).

5.2.2 The effects of dietary fish oil on cytokine production by resident peritoneal macrophages

The results from the current experiments examining the effects of dietary fish oil on CCL3 production by resident peritoneal macrophages *ex vivo* show that the increased concentration of CCL3 in the supernatant from LPS-stimulated resident peritoneal macrophages was due to increased

CCL3 production by each cell although there were fewer resident peritoneal macrophages secreting CCL3 in mice fed the fish oil diet than in mice fed the control diet. Therefore, despite the lower number of cells present at the time of insult in mice fed the fish oil diet compared with that in mice fed the control diet, the increased concentration of CCL3 in peritoneal fluid from mice fed dietary fish oil 3 h following LPS administration could be due to increased production of CCL3 by activated cells. The results also demonstrated, for the first time, that the pro-inflammatory mediators CCL3 and TNF-α are secreted by the same cells. Although there was not a statistically significant increase in the concentration of TNF-α in either peritoneal fluid or supernatant from stimulated resident peritoneal macrophages from mice fed the fish oil diet compared with that from mice fed the control diet, there was a trend towards an increase in TNF-α concentrations in both the peritoneal fluid and the supernatant. No previous studies have examined the effects of dietary fish oil on CCL3 concentration in peritoneal fluid in models of endotoxin exposure or infectious models in animals and only one study has examined the effects of dietary fish oil on CCL3 secretion *ex vivo* (Venkatraman & Meksawan, 2002). In that study PHA-stimulated splenocytes from mice fed dietary fish oil secreted more CCL3 than splenocytes from mice fed a control diet when the mice from both dietary groups received low levels of vitamin E. These results are similar to those obtained in the present study although different cell types were stimulated in these two studies, i.e. T cells (Venkatraman & Meksawan, 2002) and macrophages (the present study). However, it was suggested that vitamin E could counteract the effects of fish oil on CCL3 concentration, since T cells secreted less CCL3 when mice fed fish oil received high levels of vitamin E than when mice received low levels of vitamin E (Venkatraman & Meksawan, 2002). Surprisingly, there was no effect of dietary fish oil on CCL3 levels by PHA-stimulated splenocytes in autoimmune prone mice, independent of vitamin E levels (Venkatraman & Meksawan, 2002).

Several studies have reported an increase in TNF-α secretion by resident peritoneal macrophages from mice fed dietary fish oil (Blok et al., 1992; Hardardottir & Kinsella, 1991, 1992; Petursdottir et al., 2002; Watanabe et al., 1991). Although the present study did not reveal a statistically significant difference in TNF-α levels in supernatants from *ex vivo* stimulated resident peritoneal macrophages from mice fed the fish oil diet, the same trend was observed. The less pronounced effects of fish oil on TNF-α production from resident peritoneal macrophage stimulated *ex*

vivo in the present study compared with that in the study by Petursdottir et al. (2002) may be explained by different content of fish oil in the experimental diets used in the two studies. In the present study the fish oil diet contained only 2.8% fish oil, whereas in the study by Petursdottir et al. (Petursdottir et al., 2002), and others (Blok et al., 1992; Hardardottir & Kinsella, 1991, 1992; Watanabe et al., 1991), the fish oil diet contained much higher proportion of fish oil, or up to 20%.

5.2.3 The effects dietary fish oil on monocyte subpopulations in healthy mice

The results from the present study on the effects of dietary fish oil on blood monocyte subpopulations and CCL2 concentrations in serum showed that healthy mice fed fish oil diet had a lower proportion of classical monocytes in blood and decreased concentration of CCL2 in serum compared with healthy mice fed the control diet. No other study has examined the effects of fish oil on monocyte subpopulations in healthy mice. The lower proportion of classical monocytes in blood from healthy mice fed the fish oil diet compared with that in healthy mice fed the control diet may be the result of lower serum levels of CCL2, which is in accordance with other studies demonstrating that CCL2 is an important regulator of classical monocyte emigration from bone marrow (Serbina & Pamer, 2006). Lower levels of CCL2 in healthy mice fed the fish oil diet are consistent with results from other studies that have previously shown that n-3 PUFAs decrease CCL2 levels in serum in low grade or chronic inflammation both in animals (El Seweidy et al., 2005; Wang, S. et al., 2009b) and humans (Elvevoll et al., 2008). High serum levels of CCL2 have been implicated in the pathogenesis of chronic diseases characterized by infiltration of monocytes, such as atherosclerosis (Ballantyne & Nambi, 2005) and arthritis (Klimiuk et al., 2005) and obesity (Sartipy & Loskutoff, 2003). Several studies have shown that dietary fish oil has beneficial effects in cardiovascular disease (Calder, 2004; Singh et al., 1997; Wang et al., 2006; Yokoyama et al., 2007), which could in part be mediated by the effects of fish oil on lowering CCL2 levels in serum and subsequently decreasing the proportion of $CCR2^+$ classical monocytes in blood. A decrease in classical monocytes in blood could be the cause of the lower number of macrophages seen in atherosclerotic plaques from patients receiving fish oil supplements (Thies et al., 2003) and the fewer macrophages seen in atherosclerotic lesions in Apo-E deficient mice fed a Western type diet supplemented with 5% EPA (Matsumoto et al., 2008).

Classical monocytes have been shown to play a key role in plaque formation and development where they infiltrated into lesions and differentiated into macrophages (Swirski et al., 2007). Accordingly, Apo-E deficient mice lacking CCR2 were shown to have decreased lesion formation (Boring et al., 1998).

5.2.4 The effects of dietary fish oil on monocyte subpopulations in mice with severe endotoxin-induced peritonitis

The results from the present study show that dietary fish oil increased the proportion of classical monocytes in blood and spleen in mice following endotoxin exposure and increased the concentration of CCL2 in serum. To our knowledge no other studies have examined the effects of fish oil on monocyte subpopulations in either mice or humans following endotoxin exposure. In accordance with the results from the present study, Mayer et al. (2003a) showed a trend towards a lower number of total leukocytes in blood from critically ill patients receiving fish oil for five days. Another study reported a decrease in the number of total leukocytes and neutrophils in alveolar fluid from patients with ARDS following four days of enteral feeding with EPA, DGLA and antioxidants (Gadek et al., 1999). In contrast, one study reported no alteration in blood leukocyte numbers in critically ill patients receiving fish oil (Barbosa et al., 2010).

Although no previous studies have examined the effects of fish oil on different monocyte subpopulations, there are studies that have examined the effects of dietary fish oil on monocyte activation in critically ill patients. Three studies have reported that fish oil preserved HLA-DR expression on monocytes in critically ill patients as compared with lowered HLA-DR expression on monocytes in patients receiving the control diet (Wang, X. et al., 2009; Weimann et al., 1998; Weiss et al., 2002). However, one study failed to see an effect of fish oil on HLA-DR expression on monocytes (Friesecke et al., 2008), which may be because the effect of fish oil on HLA-DR expression on monocytes was examined after the initial inflammatory response had started to resolve. Loss of HLA-DR expression on monocytes (Volk et al., 1996), as well as decreased production of pro-inflammatory cytokines following *ex vivo* stimulation (Docke et al., 1997; Flach et al., 1999; Heagy et al., 2003; McCall et al., 1993; Munoz et al., 1991) has been shown to be an indicator of monocyte deactivation.

Deactivation of monocytes is becoming recognized as one of the key features of host defense failure during sepsis (Docke et al., 1997). n-3PUFAs

have been shown in some studies to have beneficial effects in patients with sepsis (Barbosa et al., 2010; Heller et al., 2006; Mayer et al., 2003c) but the mechanism by which they act is unknown. The present study attempted to shed light on the mechanism by which n-3 PUFAs may be beneficial in sepsis by investigating the effects of dietary fish oil on circulating classical monocytes in mice following endotoxin exposure. The classical monocytes have also been referred to as inflammatory monocytes (Geissmann et al., 2003; Sunderkotter et al., 2004) and they secrete large amounts of pro-inflammatory molecules, such as TNF-α and NO (Geissmann et al., 2003; Nahrendorf et al., 2007; Serbina et al., 2008; Serbina & Pamer, 2006; Sunderkotter et al., 2004). Our finding that there was a significant increase in the proportion of these specific monocytes 48 h following endotoxin exposure in mice fed the fish oil diet indicates that dietary fish oil may have beneficial effect following infection or inflammation by counteracting the deactivation of monocytes in the immunosuppressive state.

The increase in the proportion of circulating classical monocytes in mice fed the fish oil diet upon endotoxin exposure may be due to increased recruitment of these cells from the bone marrow in response to CCL2. Consistent with that is the increase in CCL2 levels in blood following endotoxin exposure in mice fed the fish oil diet compared with that in mice fed the control diet, as well as the more prominent decrease in the number of $CCR2^{+}$ cells in bone marrow from mice fed the fish oil diet. When monocytes are recruited from the bone marrow they exhibit a phenotype of classical monocytes and in the circulation they can serve as precursors for the more mature non-classical monocytes (Sunderkotter et al., 2004). The more prominent decrease in the number of monocytes in bone marrow following endotoxin exposure from fed the fish oil diet compared with that in mice fed the control diet may be due to faster monocyte emigration from the bone marrow in response to higher CCL2 levels observed in mice fed the fish oil diet. This is consistent with the results in the present study showing higher proportion of classical monocytes in blood 48 h following LPS administration in mice fed the fish oil diet compared with that in mice fed the control diet. As the spleen can serve as a reservoir for undifferentiated monocytes that resemble their blood counterparts (Swirski et al., 2009) it was of interest to see if fish oil affected monocyte populations in spleen. The results show that mice with endotoxin-induced peritonitis fed dietary fish oil had a higher number of classical monocytes in spleen compared with that in healthy mice fed the fish oil diet and mice with endotoxin-induced peritonitis fed the control diet, indicating that the spleen may serve as a reservoir for monocytes in blood.

The higher levels of CCL2 in blood from mice with endotoxin-induced peritonitis fed the fish oil diet compared with that in mice fed the control diet in the present study is not in accordance with results from Fritsche et al. (2005). In that study dietary fish oil had no effect on serum levels of CCL2 in mice 24 h after a challenge with *L.monocytogenes*, except when the mice were depleted of neutrophils. Mice depleted of neutrophils and fed the fish oil diet had higher levels of CCL2, which was suggested to be because of increased bacterial load due to neutrophil depletion. The inconsistency between these two studies could be due to several factors, such as different mouse strains used and different type of insult, i.e. endotoxin vs. bacteria. The increased levels of CCL2 in peritoneal fluid (48 h) are in accordance with results showing that rats receiving fish oil infusion had higher levels of CCL2 in peritoneal fluid following CLP operation than rats receiving soy bean oil infusion (Tsou et al., 2008). In contrast to results from the present study, hypercholesterolemic mice fed dietary fish oil had lower levels of CCL2 in peritoneal fluid at 6 h but not 24 h following CLP than mice fed soybean oil diet (Chiu et al., 2009). The discrepancy between the results from that study and the present study may be due to the difference in the cholesterol levels in the mice in the two studies as well as the type of insult, i.e. endotoxin vs. CLP.

5.3 The effects of dietary fish oil on neutrophils and chemokines involved in neutrophil recruitment in healthy mice and mice with severe endotoxin-induced peritonitis

The results from the current study on the effects of dietary fish oil on neutrophil populations in blood and peritoneum showed that dietary fish oil increased the proportion of less granulated neutrophils (N2) in blood and increased the proportion and number of neutrophils in peritoneum from mice fed dietary fish oil 48 h following LPS administration. The infiltration of neutrophils into the peritoneum was delayed in mice fed the fish oil diet compared with that in mice fed the control diet, revealed by the decreased number of neutrophils in the peritoneum at 12 h and a trend towards a decrease at 24 h. However, by 48 h there were more neutrophils in the peritoneum of mice from the fish oil group than in the peritoneum of mice in the control group. The tendency towards a higher number of neutrophils in blood from mice fed the fish oil diet at 12 and 24 h following LPS administration compared with that in mice fed the control diet, indicates their accumulation in the circulation reflecting their delayed infiltration into

the peritoneum. These results are in accordance with recent findings by Yates et al. (2011) demonstrating that DHA, but not EPA, inhibited the adhesion of neutrophils to the endothelium by reducing surface expression of E-selectin on endothelial cells following cytokine stimulation. They concluded that this was due to disruption in the intracellular translocation mechanism. These results are also in accordance with findings by Tull et al. (2009) reporting that neutrophil migration across the vascular endothelium is reduced by n-3 PUFAs. They concluded that supplementing endothelial cells with EPA generated PGD_3 that antagonized the PGD_2-mediated neutrophil response (Tull et al., 2009). Interestingly, they also showed that the initial activation and adherence of the neutrophils was not affected by EPA, but in the absence of the PGD_2-mediated signals the neutrophils did not transmigrate but instead reverted back to the rolling form (Tull et al., 2009). The increase in the proportion of the less granulated neutrophils seen in blood from mice fed the fish oil diet in the present study may, therefore, be a result of the neutrophils reverting back to the circulation after initially adhering to the endothelium following their activation. This suggestion is supported by the higher CD11b levels of the less granulated neutrophils, but activated neutrophils have been shown to increase CD11b levels on their surface upon mobilizing their granules (Sengelov et al., 1993).

The increase in neutrophil recruitment to the peritoneum in mice fed dietary fish oil 48 h following LPS administration indicates an alternative mechanism of neutrophil recruitment during the later phase of endotoxin-induced peritonitis. The late phase (48 h) increase in neutrophil migration to the peritoneum in the mice fed the fish oil diet may be dependent on a different mechanism than the early (12 and 24 h) neutrophil migration to the peritoneum in the control mice. Whether this difference is due to a difference in chemokines not measured in this study or a difference in the expression of adhesion molecules remains to be determined.

Although several studies have examined the effects of dietary fish oil on neutrophil recruitment *in vivo*, most of them have examined the effects of neutrophil recruitment into lungs. As previously mentioned, the recruitment of neutrophils to lungs is differently regulated than the recruitment of neutrophils to the peritoneum following LPS administration. Only one other study has examined the effects of dietary fish oil on neutrophil recruitment to the peritoneum and shown that dietary fish oil had no effect on neutrophil numbers in peritoneum 24 h following infection with *L. monocytogenes* (Fritsche et al., 2005). These results are not in accordance with the results

from the present study as there were fewer neutrophils in the peritoneum 12 h following LPS administration and a trend towards fewer neutrophils in the peritoneum 24 h following LPS administration in mice fed the fish oil diet. That there was a difference in the results from the two studies is not surprising as it has been shown that the mechanism of neutrophil recruitment to the peritoneum in response to *L.monocytogenes* is different from the mechanism of neutrophil recruitment in response to *E. coli* (or LPS), the former being CD11b/CD18-independent but the latter CD11b/CD18-dependent (Conlan & North, 1994).

The effects of dietary fish oil on the chemokines measured could not explain the effects of dietary fish oil on neutrophil recruitment in the present study. The higher levels of CCL3 in the peritoneum of mice fed dietary fish oil 3 h following LPS administration did not lead to increased recruitment of neutrophils to the peritoneum. On the contrary, at the following time points (12 and 24 h) there was a trend towards decreased number of neutrophils in the peritoneum. The increase in neutrophil proportion and numbers was not evident until 48 h following LPS, at a time point where none of the chemokines involved in neutrophil recruitment were detected in the peritoneal fluid. The effect of dietary fish oil on neutrophil numbers in blood following LPS administration are unlikely due to the decreased levels of CXCL2 and increased levels of CCL3 in serum observed 3 h following LPS administration, as the number and proportion of neutrophils in bone marrow were similar in the two dietary groups, indicating a similar emigration rate in both dietary groups. It is more likely due to the effect of dietary fish oil on neutrophil recruitment through the endothelium as discussed above.

5.4 Summary and conclusion

In summary, the results demonstrate on one hand that dietary fish oil lowers serum levels of CCL2 and the proportion of classical (inflammatory) monocytes in blood from healthy mice. These results may indicate that under normal conditions dietary fish oil can generate a lower inflammatory status, as CCL2 is a pro-inflammatory chemokine that increases dramatically during inflammation and the classical monocytes that are recruited by CCL2 during inflammation produce high levels of pro-inflammatory cytokines like TNF-α and IL-1 (reviewed in (Geissmann et al., 2003; Serbina et al., 2008; Serbina & Pamer, 2006; Sunderkotter et al., 2004)). As low grade inflammation has been suggested to play a pathogenic role in disorders like chronic obstructive pulmonary disease, coronary atherosclerosis and Alzheimer's dementia and healthy

asymptomatic individuals with high baseline values of blood inflammatory markers, such as C reactive protein, are at increased future risk for coronary disease, malignancy and Alzheimer's dementia (Federico et al., 2007; Koenig et al., 1999), reduction in the baseline inflammatory levels in healthy individuals may be beneficial.

On the other hand, dietary fish oil increased serum levels of CCL2 and CCL3 early in endotoxin-induced peritonitis (3h) as well as the proportion of classical (inflammatory) monocytes and the number and proportion of neutrophils at later stages of endotoxin-induced peritonitis (48 h). These results indicate that dietary fish oil may enhance the immune response at later stages in infections. An increase in the proportion of classical monocytes may perhaps counteract the monocyte deactivation that occurs normally in sepsis during the immunosuppressive state (Williams et al., 1998). Furthermore, the increased neutrophil recruitment later in the inflammatory response might counteract the neutrophil impairment also observed during the immunosuppressive state in sepsis (reviewed in (Wang & Deng, 2008)). The beneficial effects of an increase in the proportion of $CCR2^+$ classical monocytes during infection is supported by studies showing that CCR2 knockout mice have impaired monocyte migration to the peritoneal cavity and a reduced Th1 type cytokine response (Boring et al., 1997) and are more susceptible to infections (Goodyear et al., 2010). Thus, the effect of dietary fish oil to increase the proportion of classical (inflammatory) monocytes and increase the number of neutrophils at a late stage of the inflammatory response may be of benefit in sepsis where a substantial share of the morbidity and mortality observed is due to the consequences of sepsis-associated immunosuppression (Sinistro et al., 2008) and may in part explain the beneficial effects seen in some studies using n-3 PUFAs in sepsis (Barbosa et al., 2010; Heller et al., 2006; Lanza-Jacoby et al., 2001).

5.5 Limitations of the study

Although animal models, such as mice, are invaluable tools for *in vivo* research of the immune system, there are significant differences between mice and men (reviewed in (Mestas & Hughes, 2004)) and, therefore, the results from the current study cannot be extrapolated directly to humans. Rodents are more sensitive to dietary n-3 PUFAs than humans, and appear to accumulate more n-3 PUFAs in the immune cell membranes (Fritsche, 2007); therefore, there might be differences in the responses to n-3 PUFAs between mice and humans. In an attempt to minimize this difference

between mice and humans, the amount of n-3 PUFAs in the fish oil diet was limited so that the accumulation of n-3 PUFAs in cell membranes would not exceed that observed in human immune cells (Fritsche, 2007).

Although endotoxin-induced peritonitis is a simple and easily reproducible model that is widely used to study the pathophysiology of sepsis, it does not reflect the complex physiological responses to live pathogens. The transient increase in cytokines differs in models of endotoxin exposure and septic patients. In addition, rodents are resistant to endotoxin challenges, whereas humans are sensitive to endotoxin (Dejager et al., 2011). Despite the limitations of the mouse model used in these studies, these data may provide some insight into the processes of sepsis in humans. Furthermore, the use of animal models is a valuable tool for immunological research and remains essential for increased understanding and possible advances in development of new therapies for sepsis (Dejager et al., 2011; Rittirsch et al., 2007).

The diet used in the present study has been modified to mimic a Western type diet, i.e. with FA composition similar to that consumed by average humans living in Western societies. The experimental diet provides an additional 1.4 en% of EPA+DHA which represents about 3 g of n-3 LC-PUFA per 2000 kcal diet in humans, which is generally thought to be safe for human consumption. The menhaden fish oil used in the diet as the n-3 PUFA source contained a small amount of AA so the same amount of AA was added to the control diet. The fish oil was free of vitamins A and D, ensuring that both the experimental diet and the control diet provided equal amounts of vitamins A, D and E. This is important since these vitamins have been shown to affect immune function (Dawson et al., 2006; Hsieh & Lin, 2005; Imazeki et al., 2006; Venkatraman & Chu, 1999).

5.6 Future tasks

The work presented in this thesis raised some thoughts on possible future tasks. Identification of surface markers that can discriminate between the different neutrophil populations (N1 and N2) seen following LPS administration would allow for further examination of the two populations as they could then be separated and their functions examined. It would also be of interest to further examine the lymphocyte populations and subpopulations in this model of severe endotoxin-induced peritonitis and the effects of dietary fish oil on these populations.

Determining the effects of dietary fish oil on CD11b/CD18 expression on neutrophils at different time points following LPS administration would also be of interest as it has been shown that neutrophil recruitment to the peritoneum in response to gram negative bacteria or LPS is CD11b/CD18 dependent early in the immune response, although not later in the immune response. As this study showed that dietary fish oil had different effects on neutrophil recruitment during the early and late immune responses it would be of interest to examine if these effects could be in part mediated by the effects of dietary fish oil on these surface molecules. In addition, the effects of fish oil on leukocyte infiltration, especially neutrophil and monocyte infiltration, into lungs, liver and lymph nodes could further expand our understanding on the effects of fish oil during endotoxin-induced peritonitis. Furthermore, examining the efflux of macrophages out of the peritoneum and determining their final destination would be of interest.

It would also be interesting to compare the effects of dietary fish oil on leukocyte recruitment to different tissues in response to challenges with endotoxin and live gram-positive, gram-negative or viral infections.

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Appendix

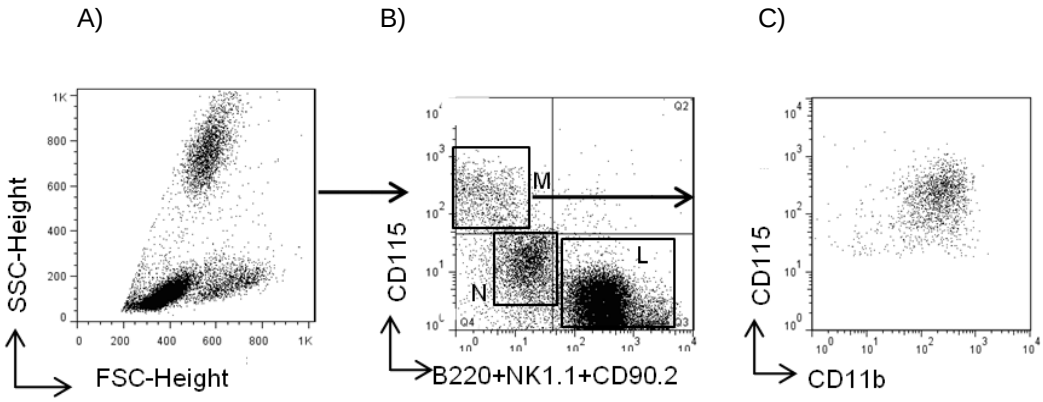

Figure A1. Identification of mouse blood monocytes.

Dot plot showing (A) forward and side scatter of leukocytes in blood prior to LPS administration. (B) Monocytes (M) were identified as cells expressing CD115 and not the lymphocyte (L) markers B220, NK1.1 and CD90.2. Neutrophils (N) were identified as cells lacking CD115 and the lymphocyte markers. (C) All monocytes expressed both CD115 and CD11b. Blood was collected into EDTA-K2 coated monovette tubes. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed using FlowJo software. Dot plots are representative of data from 3 independent experiments.

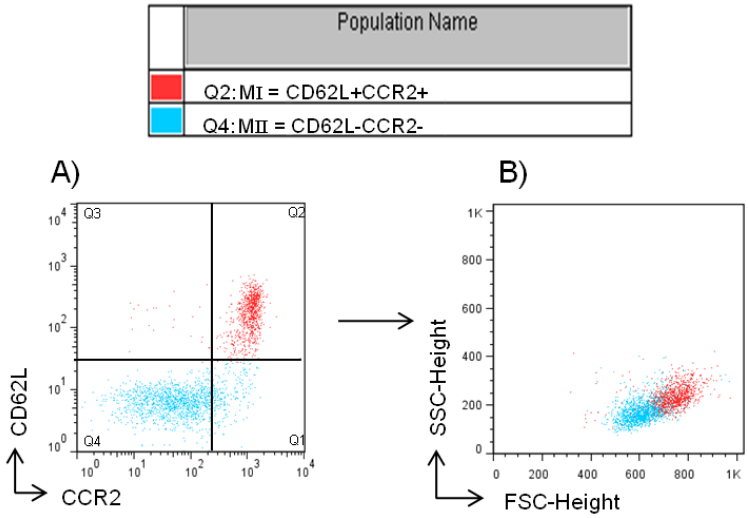

Figure A2. Identification of blood monocyte subpopulations.

Dot plot showing (A) CD62L and CCR2 expression and (B) forward side scatter of monocyte subpopulations prior to LPS administration. Classical monocytes were identified as monocytes expressing CCR2 and CD62L (Q2 - red) and non-classical monocytes were identified as monocytes lacking the expression of CCR2 and CD62L (Q4 - blue). The mice were sacrificed and blood was collected into EDTA-K2 coated monovette tubes. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed using FlowJo software. Dot plots are representative of data from 3 independent experiments**.**

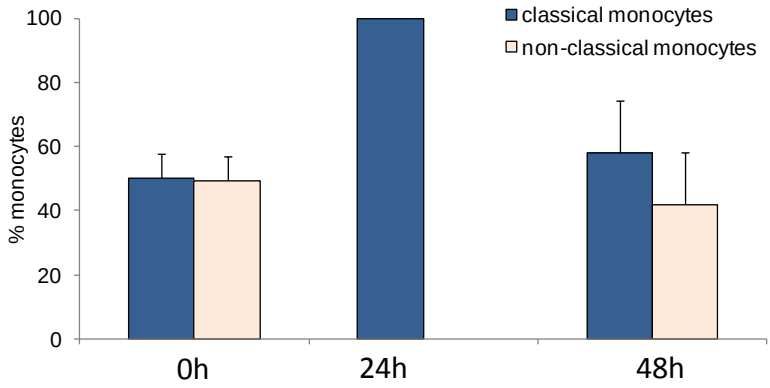

Figure A3. Distribution of blood monocyte subpopulation prior to and 24 and 48 h following LPS administration

Proportion of classical and non-classical monocyte subpopulation of total monocytes before (0h) and 24 and 48 h following LPS administration (0.5mg/kg). The mice were sacrificed at indicated time points and blood collected into EDTA-K2 coated monovette tubes. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed using FlowJo software. The values are means ± SEM, n=16 for 0 h, n=6 for 24 h and n=8 for 48 h.

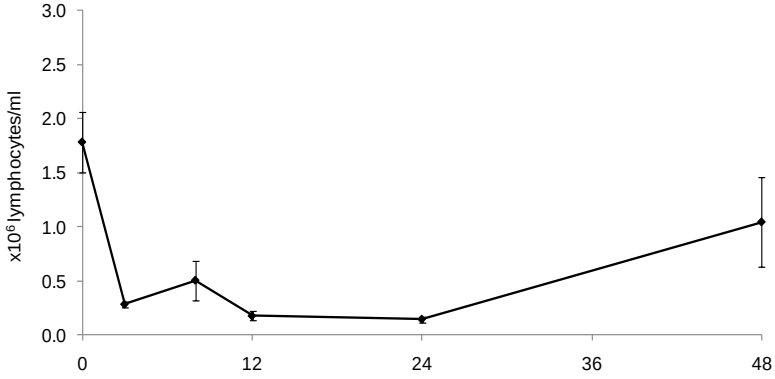

Figure A4. Effects of intraperitoneal LPS administration on mouse blood lymphocytes.

Blood lymphocyte numbers prior to (0 h) and following i.p. administration of LPS (0.5mg/kg). The mice were sacrificed at indicated time points and blood collected into EDTA-K2 coated monovette tubes. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed using FlowJo software. The values are means ± SEM, n=5-8 per time point.

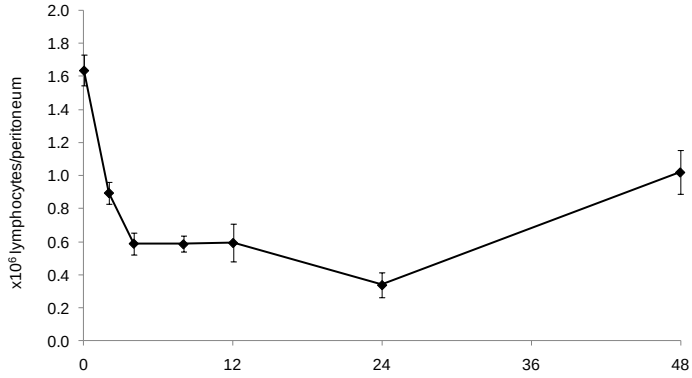

Figure A5. Effects of intraperitoneal LPS administration on mouse peritoneal lymphocytes.

Peritoneal lymphocyte numbers prior to (0 h) and following i.p. administration of LPS (0.5mg/kg). The mice were sacrificed at indicated time points and peritoneal lavage was collected. Total cell count was performed with Countess automated cell counter. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values are means ± SEM, n=5-8 per time point.

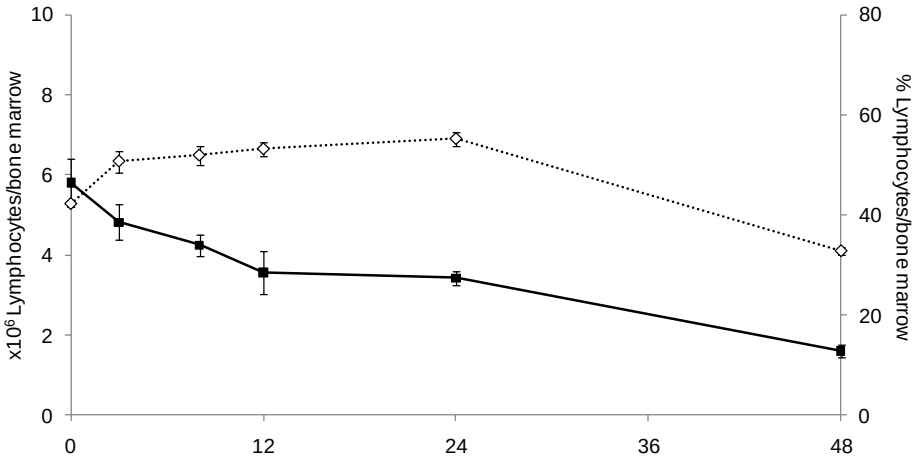

Figure A6. Effects of intraperitoneal LPS administration on mouse bone marrow lymphocytes.

Bone marrow lymphocyte numbers (solid line) and proportion (dotted line) prior to (0 h) and following i.p. administration of LPS (0.5mg/kg). The mice were sacrificed at indicated time points and bone marrow was collected from right femur and tibiae at indicated time points. Cell count was performed with Countess automated cell counter. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values are means ± SEM, n=5-8 per time point.

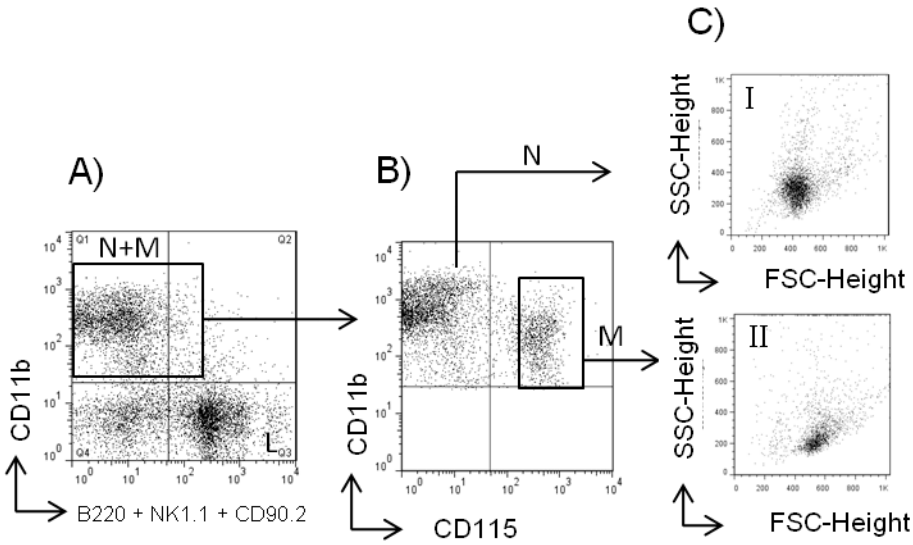

Figure A7. Identification of mouse bone marrow monocytes.

(A) Lymphocytes (L) were excluded as cells expressing B220, NK1.1 and CD90.2, (B) monocytes (M) were identified as cells expressing both CD11b and CD115, whereas neutrophils only expressed CD11b but not CD115. (C) Forward side scatter of (I) neutrophil and (II) monocyte populations prior to LPS administration. Mice were sacrificed and bone marrow collected from right femur and tibiae at indicated postmortem. Leukocyte populations determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Dot plots are representative from three independent experiments.

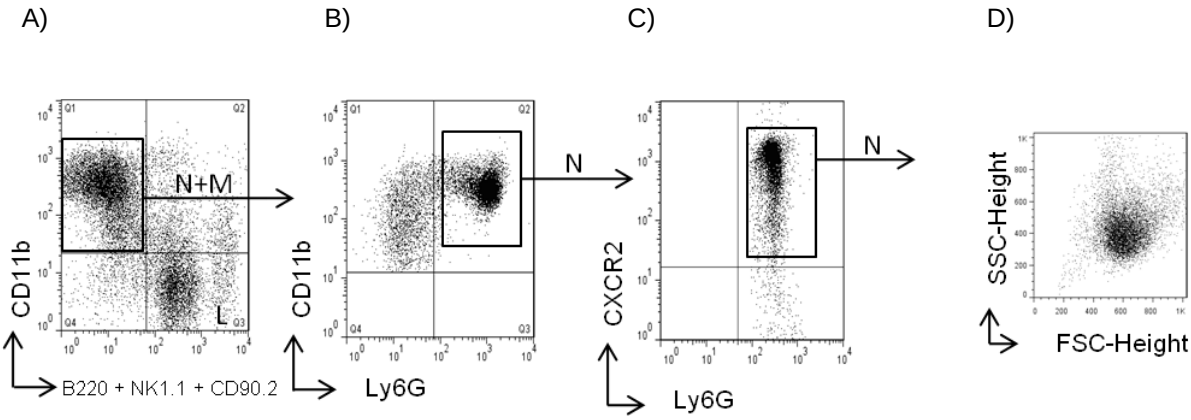

Figure A8. Identification of mouse bone marrow neutrophils.

A) Lymphocytes (L) were excluded as cells expressing B220, NK1.1 and CD90.2, (B) neutrophils (n) were identified as cells expressing both CD11b and Ly6G, whereas monocytes expressed only CD11b but not Ly6G and (C) neutrophils expressing Ly6G also expressed CXCR2. (D) Forward side scatter of neutrophils 48 h following LPS administration. Mice received LPS (0.5mg/kg) and were sacrificed48 h later. Bone marrow was collected from right femur and tibiae. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Dot plots are representative from three independent experiments.

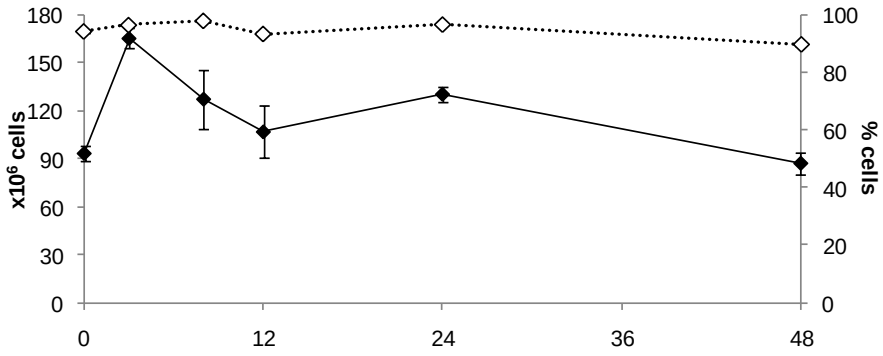

Figure A9. Effects of intraperitoneal LPS administration on mouse spleen lymphocytes.

Spleen lymphocyte numbers (solid line) and proportion (dotted line) prior to (0 h) and following i.p. administration of LPS (0.5mg/kg). The mice were sacrificed at indicated time points and spleen was collected. Cell count was preformed with Countess automated cell counter and leukocyte populations determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. The values are means ± SEM, n=5-8 per time point.

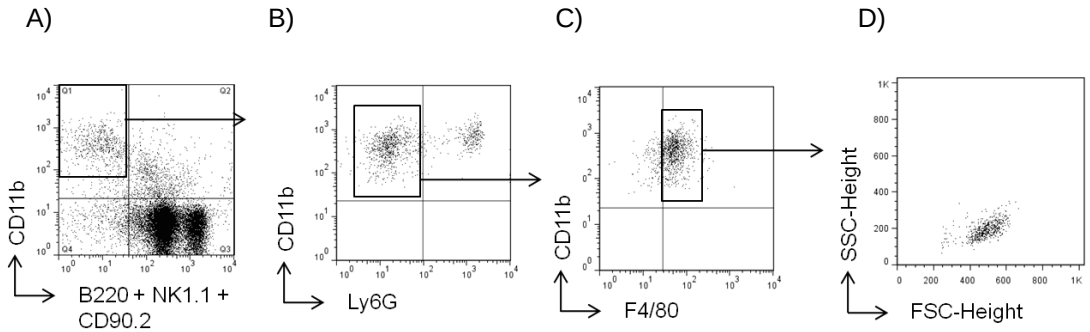

Figure A10. Identification of mouse spleen macrophages/monocytes

(A) Lymphocytes (L) were excluded as cells expressing B220, NK1.1 and CD90.2, (B) monocytes/macrophages (M) were identified as cells expressing CD11b but lacking Ly6G, neutrophils (N) expressed Ly6G. (C) F4/80 expression on $CD11b^+$ monocyte/macrophages. (D) Forward side scatter of $CD11b^+$ $F4/80^+$ macrophages/monocytes prior to LPS administration. Mice were sacrificed and spleens collected. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Dot plots are representative of data from three independent experiments

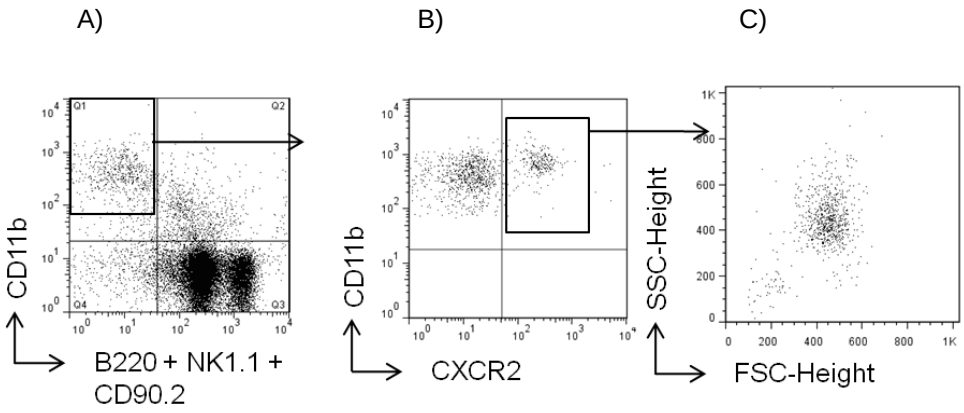

Figure A11. Identification of mouse spleen neutrophils.

(A) Lymphocytes (L) were excluded as cells expressing B220, NK1.1 and CD90.2, (B) neutrophils (N) were identified as cells expressing CD11b and CXCR2, (C) Forward side scatter of $CD11b^{+}$ $CXCR2^{+}$ neutrophils prior to LPS administration. Mice were sacrificed and spleens collected. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Dot plots are representative of data from three independent experiments.

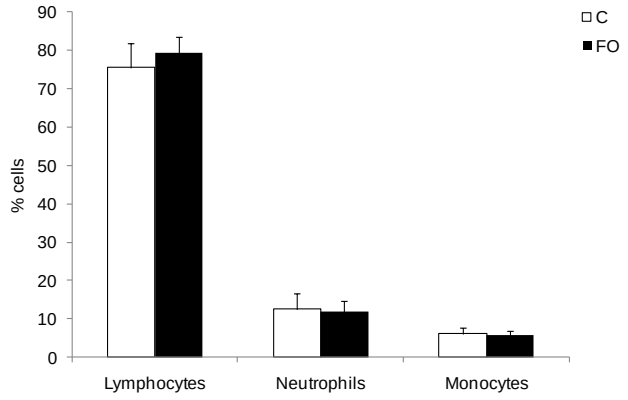

Figure A12. Effects of dietary fish oil on the proportion of leukocyte population in healthy mice.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Mice were anesthetized and blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells lysed using FACS lysing solution. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Values are means ± SEM, n=28-29.

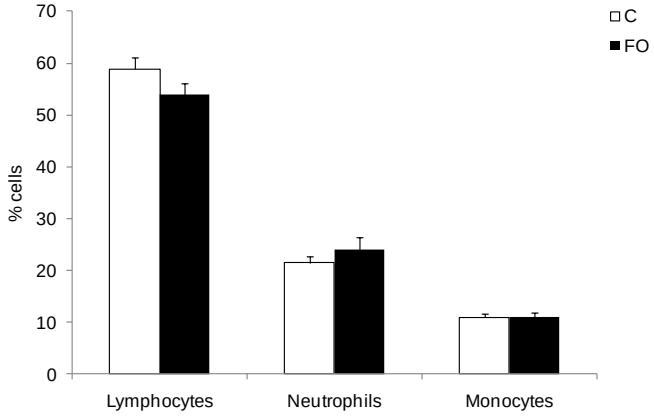

Figure A13. Effects of dietary fish oil on the proportion of leukocyte population 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Mice received LPS (0.5 mg/kg) and were anesthetized 48 h later. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells lysed using FACS lysing solution. Leukocyte populations were determined with antibody staining and flow cytometry. The data was analyzed with FlowJo software. Values are means ± SEM, n=17.

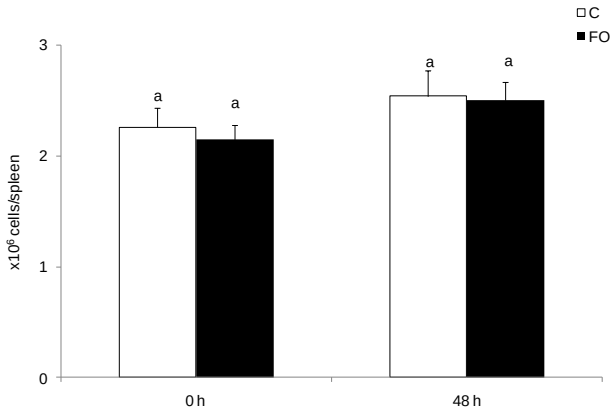

Figure A14. Effects of dietary fish oil on the number of totalmonocytes in spleen of mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Spleen was collected and passed through a cell strainer, the red blood cells were lysed and cells counted using a Countess automated cell counter. Monocytes were identified by antibody staining and flow cytometry. The data was analyzed with FlowJo software. Values are means ± SEM, n=15 for healthy mice, n=11 for mice with endotoxin-induced peritonitis. Means without a common letter differ, P <0.05.

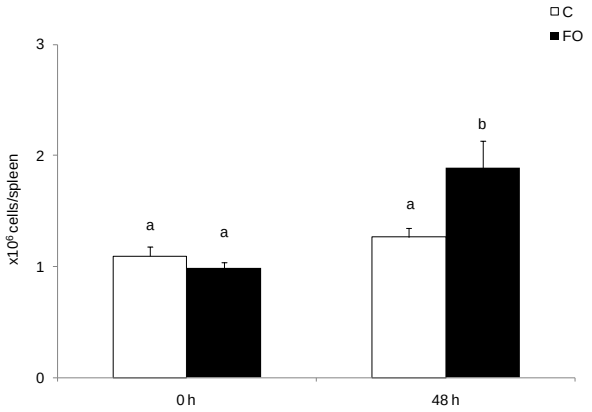

Figure A15. Effects of dietary fish oil on the number of CCR2^{+} monocytes in spleen of mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Spleen was collected and passed through a cell strainer, the red blood cells were lysed and cells counted using Countess automated cell counter. Monocytes and monocyte subpopulations were identified by antibody staining and flow cytometry. The data was analyzed with FlowJo software. Values are means ± SEM, n=15 for healthy mice, n=11 for mice with endotoxin-induced peritonitis. Means without a common letter differ, P <0.05.

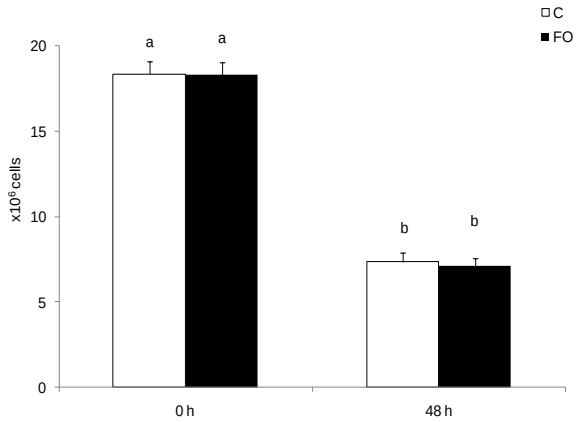

Figure A16. Effects of dietary fish oil on the number of bone marrow cells from mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed at 48 h. Bone marrow was flushed from right femur and tibia, red blood cells lysed, and cells counted using Countess automated cell counter. Values are means ± SEM, n=16. Means without a common letter differ, P <0.05.

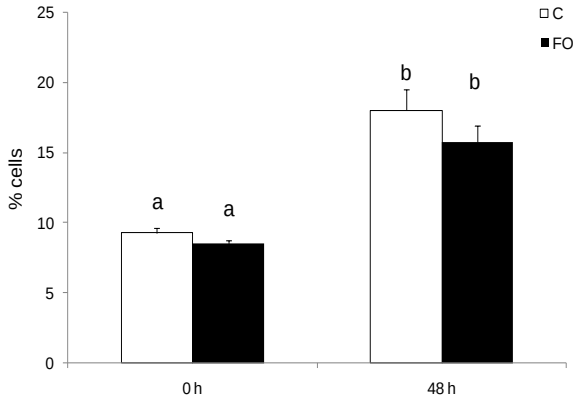

Figure A17. Effects of dietary fish oil on the proportion of CCR2$^+$ cells in bone marrow from mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (open bars) or a Western type diet supplemented with 2.8% fish oil (black bars) for 6 weeks. Half of the mice in each dietary group were injected with LPS (0.5 mg/kg) and sacrificed at 48 h. Bone marrow was flushed from right femur and tibia, red blood cells lysed and cells counted using Countess automated cell counter. Monocytes and monocyte subpopulations were identified by antibody staining and flow cytometry. The data was analyzed with FlowJo software. Values are means ± SEM, n=16 for healthy mice, n=13 for mice with endotoxin-induced peritonitis. Means without a common letter differ, P <0.05.

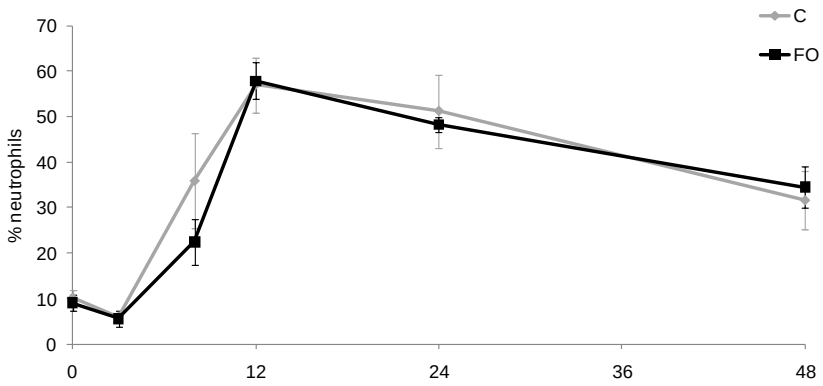

Figure A18. Effects of dietary fish oil on the proportion of neutrophils in blood prior to and following intraperitoneal administration of LPS.

Mice were fed a Western type diet (black line) or a Western type diet supplemented with 2.8% fish oil (dashed line) for 6 weeks. Mice were either injected with LPS (0.5 mg/kg) or not (0 h) and sacrificed at indicated time points. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells were lysed using FACS lysing solution. Cells were stained with monoclonal antibodies and counted using TruCount and flow cytometry. Values are means ± SEM, n=3-5.

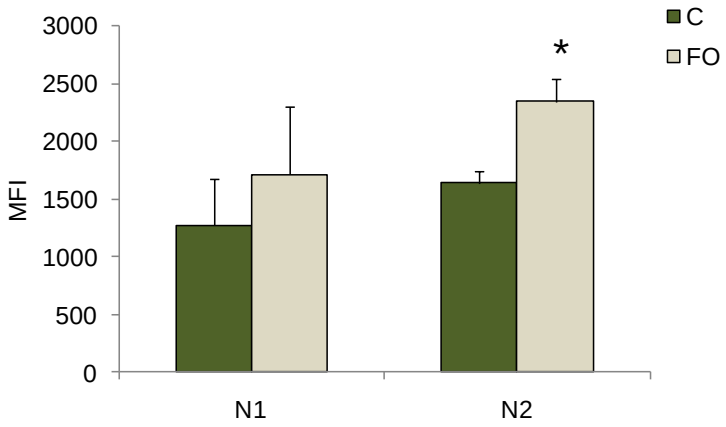

Figure A19. Effects of dietary fish oil on the expression on Ly6G on the neutrophil populations, N1 and N2 12 h following LPS administration.

Mice were fed a Western type diet (green bar) or a Western type diet supplemented with 2.8% fish oil (light green bar) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed 12 h later. Blood was collected via axillary bleeding into EDTA-K_2 containing tubes and red blood cells were lysed using FACS lysing solution. Cells were stained with monoclonal antibodies analyzed with flow cytometry. Values are means ± SEM, n=5. * Different from control, $P < 0.05$

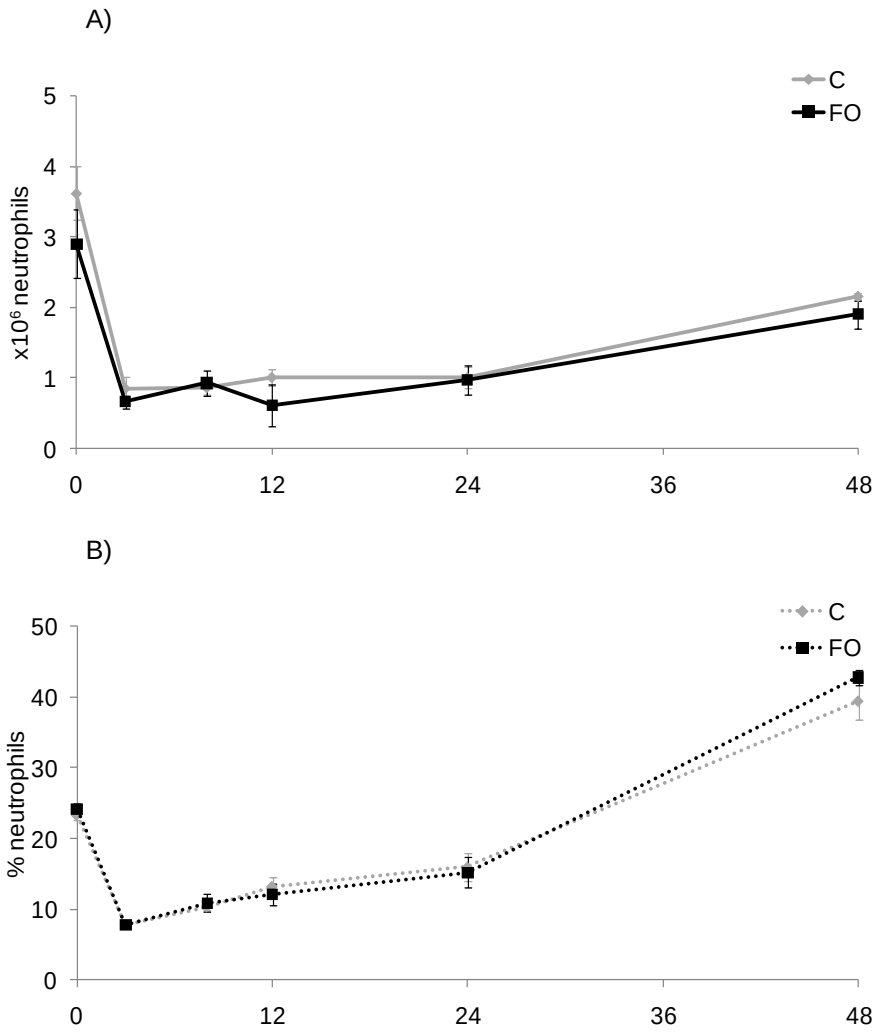

Figure A20. Effects of dietary fish oil on the (A) number and (B) proportion of neutrophils in bone marrow from mice prior to and 48 h following LPS administration.

Mice were fed a Western type diet (gray lines) or a Western type diet supplemented with 2.8% fish oil (black lines) for 6 weeks. Mice were either injected with LPS (0.5 mg/kg) or not (0 h), and sacrificed at indicated time points. Bone marrow was flushed from right femur and tibia, red blood cells lysed, and cells were counted using Countess automated cell counter. The results are expressed as (A) number of neutrophils or (B) proportion of neutrophils of total cells in bone marrow collected from right femur and tibiae. Values are means ± SEM, n=3-5per time point.

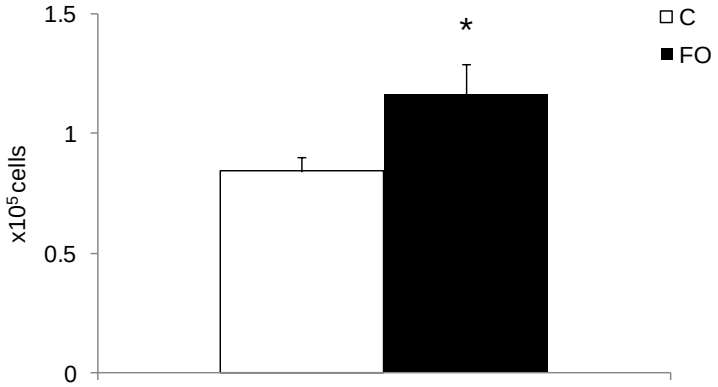

Figure A21. Effects of dietary fish oil on the number of neutrophils in peritoneum fro mice 48 h following LPS administration.

Mice were fed a Western type diet (open bar) or a Western type diet supplemented with 2.8% fish oil (black bar) for 6 weeks. Mice were injected with LPS (0.5 mg/kg) and sacrificed 48 h later. Peritoneal lavage was collected and cell counted using Countess automated cell counter. Neutrophil populations were determined with antibody staining and flow cytometry. The values are means ± SEM, n=11-14. * Different from control, P <0.05