The effects of dietary omega-3 fatty acids on the adaptive immune response in resolution of antigen-induced inflammation

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Áhrif ómega-3 fitusýra á sérhæft ónæmissvar í bólguhjöðnun í vakamiðlaðri bólgu

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Abstract

Omega-3 fatty acids may, in addition to their anti-inflammatory effects, have a pro-resolving action via endogenous lipid mediators that may be of therapeutic use in a variety of chronic inflammatory settings. The aim of this study was to determine the effects of omega-3 fatty acids on the adaptive immune response during the initiation and resolution phases of inflammation in antigen-induced peritonitis. Mice were divided into two groups and fed specific diets for four weeks. One group was fed a control diet and the other the same diet with 2.8% fish oil. After the allotted weeks, peritonitis was induced using mBSA and the mice euthanized at different time points. The spleen and lymph nodes were harvested, cryosectioned and stained for germinal center B-cells and mBSA. Statistical analysis was used to determine if there was a difference between the two groups in germinal center formation and mBSA concentration. The results show a difference in both size and number of germinal centers in the spleen between the dietary groups with the germinal centers in the fish oil group being more numerous and larger. The mBSA staining results show an increase in mBSA concentration in spleen in both groups, reaching maximum concentration at day 2 after intraperitoneal injection of mBSA. There was less mBSA staining in mice fed the fish oil diet on day 2 than in mice fed the control diet. Additional studies are needed before conclusions can be made.

Úrdráttur

Auk þess að stuðla að hindrun bólgu gætu ómega-3 fitusýrur mögulega stuðlað að hjöðnun bólgu með því að vera myndefni fyrir ákveðna lípíð miðlara sem gæti verið hægt að nota í læknisfræðilegum tilgangi í meðferð á ýmsum sjúkdómum þar sem krónísk bólga er hluti af sjúkdómsmyndinni. Markmið rannsóknarinnar var að ákvarða áhrif ómega-3 fitusýra á sérhæft ónæmissvar í upphafi og hjöðnun vakamiðlarar bólgu. Músum var skipt í tvo hópa og gefið sérstakt fæði í fjórar vikur. Öðrum hópnum var gefið viðmiðunarfæði og hinum sama viðmiðunarfæði auk 2,8% fiskolíu. Að fjórum vikum liðnum var lífhimnubólga framkölluð með innsprautun mBSA í kviðarhol og mýsnar aflífaðar á mismunandi tímapunktum í bólguferlinu. Milta og eitlum var safnað, þau frystiskorin og litað fyrir B-frumum í kímstöðvum og fyrir mBSA í milta. Tölfræðipróf voru gerð til að meta hvort munur væri á milli hópanna í myndun kímstöðva og magni mBSA. Niðurstöðurnar sýna að munur var á milli hópa bæði í stærð og fjölda kímstöðva í milta og voru kímstöðvar í fiskolíuhóp bæði stærri og fleiri en í viðmiðunarhóp. Niðurstöður mBSA litunar sýna aukningu í magni mBSA í milta hjá báðum hópum og var mesta magn mBSA á degi 2 eftir innsprautun á mBSA. Magn mBSA í milta á degi 2 var minna í fiskolíuhóp en viðmiðunarhóp. Þörf er á frekari rannsóknum svo hægt sé að draga ályktanir af þessu verkefni.

Acknowledgement

Index

List of figures

List of tables

List of abbreviations

AA	Arachidonic acid
ALA	α-linolenic acid
ANOVA	Analysis of variance
APCs	Antigen presenting cells
AT-Rv	Aspirin triggered resolving
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
GC	Germinal center
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
LA	Linoleic acid
LT	Leukotriene
LX	Lipoxin
mBSA	Methylated bovine serum albumin
MZ	Marginal zone
OVA	Ovalbumin
PALS	Periarteriolar lymphoid sheath
PD	Protectins
PG	Prostaglandin
PLF	Peritoneal lavage fluid
PMN	Polymorphic neutrophil
PNA	Peanut agglutinin
PUFA	Polyunsaturated fatty acid
RBC	Red blood cells
Ri	Resolution interval
Rv	Resolvin
SEM	Standard error of the mean
sIg	Soluble immunoglobulin
SPM	Specialized pro- resolving mediator
TGF	Transforming growth factor
TM	Thioglycolate medium
TNF	Tumor necrosis factor

Introduction

1.1 The immune system

The immune system is composed of many cells and proteins that are there to protect our bodies from harmful agents, such as pathogens, injury and toxins. The responses that our bodies make to different harmful substances are specific to the type of agent that we are being exposed to. The immune response is divided into two segments, the innate response and the adaptive response (3).

The innate response is the first line of defense when a pathogen has entered the body through the skin or mucosal lining of body orifices. It is essential that the first response is fast and effective but it is not as crucial that it is specific to the invading pathogen because its role is mainly to contain the pathogen, not destroying it. Destroying the pathogen and preparing for repeated exposure to the same pathogen is the role of the adaptive response.

For the innate and adaptive responses to complete their job they must perform four main tasks. They have to recognize the danger, contain or eliminate the infection, regulate the immune response and protect the body from recurring disease. These tasks are accomplished by different leukocytes and various proteins.

Immunological recognition is carried out by macrophages, dendritic cells and neutrophils belonging to the innate immune system and lymphocytes belonging to the adaptive immune system.

Immune effector functions are carried out by the complement system, antibodies from B-lymphocytes and white blood cells capable of killing or eliminating pathogens.

Immune regulation and immunological memory is carried out by the cells of the adaptive response.

1.1.1 The innate response

The innate immune system is capable of reacting to many pathogens and containing the infection but it is limited by the number of receptors it has to recognize the pathogens. Ever growing number of receptors have though been discovered the past decade that recognize molecules that are present on many pathogens (4). The innate immune system is therefore not able to react specifically to the invading pathogen but it is able to distinguish self from non-self and react accordingly.

The cells of the innate immune system are monocytes/macrophages, granulocytes, mast cells and dendritic cells. These cells are derived from the common myeloid progenitor in the bone marrow. The first cells to respond to invasion are phagocytic leukocytes that are able to ingest and kill pathogens. The phagocytic cells of the innate immune system are; macrophages, neutrophils and dendritic cells, but neutrophils and macrophages are the most abundant in the initial phase of inflammation.

Although phagocytosis is an important role of these cells, it is not their only role. After they have engulfed the pathogen they digest it and present antigens to the T lymphocytes of the adaptive immune system. Antigen presentation has mainly been attributed to dendritic cells, often referred to as the main antigen presenting cells (APCs) of the immune system, but macrophages are also able to present antigen to T-cells. Recently, neutrophils have also been shown to be able to present antigens to T-cells (5).

The phagocytic cells mentioned above are not the only cells the innate immune system has at its disposal to destroy pathogens as eosinophils and basophils are also among its armory. They are highly granular and their main function is, by use of enzymes and toxins from their granules, to dispose of parasites that invade the body,

1.1.2 The adaptive response

The leukocytes of the adaptive immune system are produced in the bone marrow and are derived from a common lymphoid progenitor. They are divided into two categories, B-lymphocytes and T-lymphocytes. The B-cells fully mature in the bone marrow, but T-cells leave the bone marrow before reaching full maturation and travel to the thymus where they become fully matured. Lymphocytes circulate both in the blood and lymph and can be found anywhere in the body but are most numerous in lymphoid tissues, i.e. spleen, thymus and mucosal lymphoid tissues of body orifices.

The lymphocytes of the adaptive immune system are different from the cells of the innate immune system in regards to the way they recognize pathogens. Each naïve lymphocyte is only capable of recognizing one specific form of antigen but each naïve lymphocyte is different than the next in that each has the ability to recognize different antigen than the other. Because of rearrangement of the three receptor chains, the receptor reservoir of naïve lymphocytes is almost without limit.

When an APC has engulfed a pathogen in an infected tissue it travels to the draining lymph node and presents the antigen of the pathogen to naïve T-cells. So when an APC has found a lymphocyte in the draining lymph node that has a receptor that can bind to that particular antigen it activates the lymphocyte. The B and T-cells have similar receptors on their surface but they do not recognize or respond to antigens in the same manner. The B-cell receptor is a membrane bound immunoglobulin (Ig) and when an antigen, that this receptor is specific for, has bound to the receptor the B-cell, with the help of a T helper cell, differentiates into an effector cell, called a plasma cell, and starts to secrete the same type of Ig as the receptor Ig to combat the pathogen. Soluble Igs (sIgs) are therefore antibodies to a specific pathogen. There are five types of immunoglobulin antibodies that can be found in the body either in soluble form or membrane bound, IgM, IgD, IgA, IgE and IgG and each is different from the other in terms of structure and function. Secretion of antibodies is the main function of effector B-cells.

In mice B-cells can be categorized into three distinct groups. B-2 cells, which are most common, circulate through the body but are found mainly in follicles of lymphoid organs. The marginal zone (MZ) B-cells reside in the marginal zone of the spleen and once there do not re-enter the circulation. B-1 cells, like MZ B-cells, are not found in B-cell follicles. These cells can be distinguished from B-2 cells because they are, unlike B-2 cells, CD5 positive and have high levels of sIgM and little sIgD. B-1 cells are about 5% of total B-cells in mice and are localized mainly in the peritoneal and pleural cavity.

The T-cell receptors are, in contrast to the B-cell receptors, always membrane bound and are not able to bind antigens on their own. The T-cell must have an APC present them small peptide fragments from the pathogen on their MHC to be able to respond. The T-cell then has to recognize both the peptide fragment and the MHC to become activated. There are two types of effector T-cells.

CD8 positive T-cells are cytotoxic T-cells that fight viruses or intracellular bacteria by killing hosT-cells. CD4 positive T-cells are helper T-cells that recruit other cells of the immune system. In fact B-cells can not be activated without assistance from helper T-cells. T-helper cells can be further differentiated into T_H1, T_H2, T_H17 and regulatory T-cells.

After activation most effector lymphocytes leave the lymph node, via efferent lymphatic vessels, and return to the blood stream that eventually carries them to the inflamed tissue where they can start to combat the infection. The process takes about 4-6 days, that is for the adaptive immune system to become effective.

After activation some plasma cells migrate to the bone marrow and continue production of antibodies. In addition, some effector T-cells do not leave the lymph node at all, but remain there to activate more B-cells. When the infection has been eliminated most effector lymphocytes die but some survive and become memory cells. Memory cells remember the pathogen that led to their activation in the first place and are more easily activated if a second infection from the same pathogen occurs. That second response is much faster and more effective than the first so when a second infection occurs it is usually eliminated quickly. Survival of memory cells thus leads to immunological memory.

1.2 The spleen and lymph nodes

The spleen and lymph nodes are secondary (peripheral) lymphoid organs, where lymphocytes meet potential antigens. The structure of the spleen and lymph nodes is very similar and their functions are almost the same. The difference between the two is that the spleen filters the blood whereas the lymph nodes filter the lymph. The spleen, along with being a lymphoid organ, also filters the blood and disposes of dying red blood cells (RBC), which is reflected in its anatomical structure. It is mainly composed of red pulp, which is a destruction site for RBCs. In between there are patches of white pulp which is the immunological site. The white pulp consists of central arterioles that are surrounded by a periarteriolar lymphoid sheath (PALS), containing T-cells. The PALS is where APC and T-cells come into contact. The follicles running alongside the PALS contain mostly B-cells and when activated they form a germinal center. Surrounding the follicles is the marginal zone which contains mostly macrophages but also some MZ B-cells. The red pulp has recently been shown to contain half of the body's monocyte reserves (6).The structure of a lymph node is similar to the white pulp of the spleen, in regards to that it is composed of specific sections for different cell types. The cortex is the B-cell area and the medulla contains T-cells and dendritic cells.

1.3 Inflammation

The role of the inflammatory response is, in general, to protect and rid the body of infections or repair injury. The cardinal signs of acute inflammation are; redness, pain, swelling and heat. These occur as a result of increased blood flow to the inflamed site, increased permeability across blood capillaries and increased movement of leukocytes from the blood stream into the surrounding tissue.

It is of crucial importance that these responses are tightly controlled and regulated as failure to do so may lead to chronic inflammation and scarring of the inflamed tissue and perhaps loss of function of the affected tissue.

1.3.1 Induction of inflammation

The induction of inflammation is dependent on the production of proteins known as chemokines and cytokines. Chemokines and cytokines are small proteins that are produced by many different cell types in the body in response to stimuli.

Cytokines can affect both the cells that produced it and adjacent cells and if it has a long enough half-life in the blood it can affect distant cells. Cytokines bind to specific receptors on cells and that binding leads to a signaling cascade that alters the function of the cell. Chemokines are chemoattractant proteins that stimulate migration and activation of cells.

Since macrophages are the first cells to respond to infection, the focus will be on the chemokines and cytokines they produce and what effects they have on other cells (fig 2.24). Tumor necrosis factor (TNF)-α is produced by macrophages in response to bacterial infection. It increases vascular permeability and promotes expression of adhesion molecules by endothelial cells at the infected site. TNF-α also promotes fever and shock among other things and can therefore be harmful if produced in excess. CXCL8 is a chemokine that attracts neutrophils, basophils and T-cells to the infected site. Chemokines also act on the leukocyte´s adhesion molecules, making them change their conformation and in doing so making them able to cross the endothelial wall and enter the infected site. CXCL8 also, along with CCL2 and the complement peptide C5a, activates neutrophils and macrophages to combat the pathogen and promotes angiogenesis. Interleukin (IL) -1β activates vascular endothelium and lymphocytes and increases access of effector cells to the inflamed site. IL-1β also promotes fever by stimulating production of IL-6. IL-6 also activates lymphocytes and increases antibody production. IL-12 activates natural killer cells and promotes differentiation of CD4 T-cells into T_H1 cells. The lipid derived eicoasanoids, leukotrienes (LT) and prostaglandins (PG), also contribute to the initiation of inflammation (7), out of which PGE_2 and LTB_4 are the most potent pro-inflammatory mediators (8).

The cellular events of acute inflammation are characterized by an early influx of neutrophils into the affected tissue and monocyte infiltration later in the process. When the monocytes have entered the tissue they differentiate into macrophages that start clearing the tissue of dead neutrophils and other cellular debris (Figure 1).
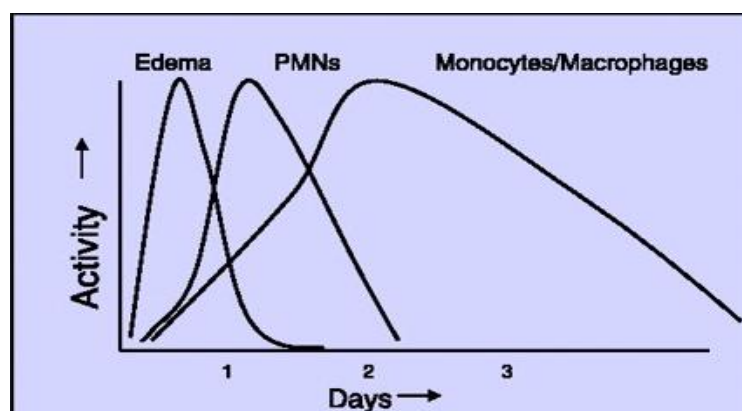

Figure 1. Cellular events of acute inflammation. Initiation of inflammation is characterized by early influx of neutrophils into the inflamed cite, followed by an influx of monocytes/macrophages. (Figure used from Serhan et.al. (9))

1.3.2 Resolution of inflammation

Until recently it was believed that the resolution of inflammation was a passive process (9) and only removal of the antigen that caused the inflammation could lead to its resolution. Serhan et al. (10) have shown that this is not the case and further studies on the resolution phase have shown that it is a complex process (11, 12) and is in fact an active process that has many players. The resolution interval (Ri) is determined as the time from when the neutrophils have reached their highest numbers until 50% of them have been cleared (12) and the resolution phase in total is the time from maximum neutrophil numbers until they have all been cleared from the tissue (13). Although cells of the innate response are the major players in acute inflammation, chronic inflammation, which is common in many diseases, is mainly due to the adaptive immune response and the cells of the innate response signal the cells of the adaptive response, therefore the first steps of inflammation determine the end (14).

There are several processes that must occur for resolution to be completed and for the tissue to reach catabasis. First the antigen must be disposed of and production of pro-inflammatory mediators halted. Secondly, there must be a release of factors that hinder leukocyte trafficking to the inflamed site. The third process is clearance of leukocytes from the inflamed site, either by apoptosis of inflammatory cells followed by phagocytosis or they simply leave the inflamed site via the lymphatics, and return to the circulation. Lastly, production of pro-inflammatory molecules in the inflamed tissue must stop. If this process is completed there will be little or no damage to the previously inflamed tissue. As most processes in the body, this process is tightly controlled with anti-inflammatory and pro-resolution mediators.

Anti-inflammatory mediators are for example the anti-inflammatory cytokines IL-4 that regulates diverse B- and T-cell responses and IL-10 that inhibits expression of some pro-inflammatory cytokines. IL-13 affects monocytes and macrophages, inhibiting production of pro-inflammatory cytokines and transforming growth factor (TGF)-β mediates matrix synthesis and apoptosis. These mediators are released in response to phagocytosis of apoptotic cells (15). Lipoxins (LX) are lipid derived mediators that can have anti-inflammatory roles among other functions. For example, LXA_4 and LXB_4 limit infiltration of neutrophils to the inflamed site by causing the dilated veins to constrict again and return to their normal state (16, 17).

New lipid derived mediators have been found that are actively biosynthesized in the resolution phase of an infection from essential omega-3 polyunsaturated fatty acids (PUFA). There are three families of these specialized pro-resolving mediators (SPM), named resolvins (Rv), protectins (PD) and maresins. Each of these novel SPMs are potent agonists that control the magnitude and duration of the inflammation and stimulate uptake of apoptotic neutrophils (18).

1.3.3 Resolvins

Rvs were originally identified using a systems approach with LC-MS-MS on inflammation exudates and they are made from the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As seen in figure 2 there are a E-series Rvs and a D-series Rvs, the name depending on which fatty acid is used in for their synthesis (18). An alternative pathway for production of Rvs is the

cyclooxygenase (COX) -2 pathway, which is aspirin dependant and produces an aspirin triggered form of Rvs (AT-Rv) (19).
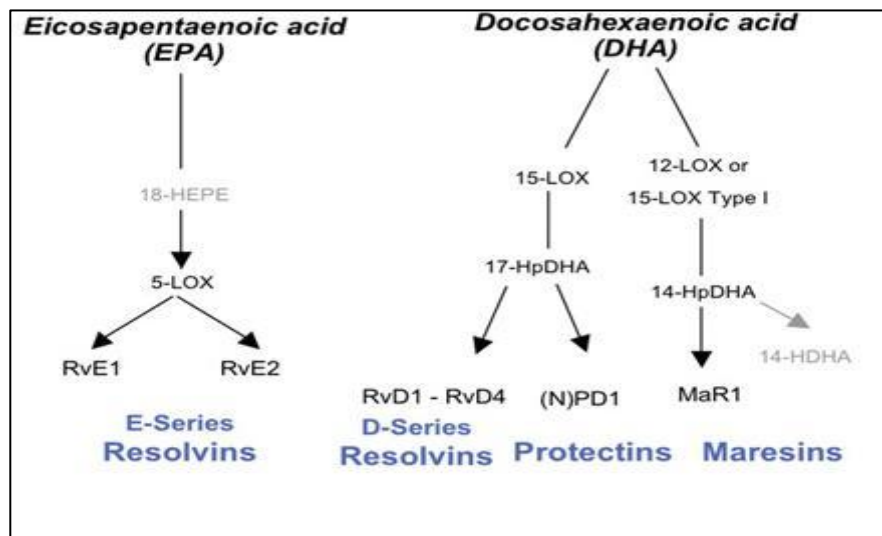

Figure 2. Production pathways for resolvins, protectins and maresins.
Production of Rv, PD and maresins from eicoasapentaenoic and docosahexaenoic acids. Figure modified by author from Fredman and Serhan (2).

The pro-resolution and anti-inflammatory capabilities of Rvs are blocking the production of pro-inflammatory mediators and inhibiting leukocyte recruitment by stopping infiltration of polymorphic neutrophils (PMN) (2). In an experiment carried out by Sun et al. (11) using purified synthetic RvD1 and AT-RvD1 they found a dose-dependent decrease in PMN infiltration with a maximal inhibition of ~35% occurring at the 10- to 100-ng dose. Interestingly the AT-RvD1 proved to be more potent at the 10 ng dose than RvD1 although they had similar effects on total PMN inhibition (11).

1.3.4 Protectins
PDs were identified along with Rvs and as seen in figure 2 are also made from DHA. They are also potent in stopping PMN infiltration (18). The PDs differ from Rvs in that they have a conjugated triene containing structure and since there is only one type of fatty acid used for their production there is only the D-series. Unlike Rvs, PDs act on the glial cells of the nervous system and are, in fact, also made by the glial cells, hence the term neuroprotectin (18).

1.3.5 Maresins
Recently maresins were added to the SPM family by Serhan et al. (20). The maresins are macrophage derived autocoids that stimulate phagocytosis by macrophages and, like resolvins and protectins, stop PMN infiltration (20).

1.4 Murine models of peritonitis
Several murine models of peritonitis have been used to investigate inflammatory responses. In healthy uninflamed mice, macrophages and lymphocytes, in equal proportions, comprise almost 95% of cells in the peritoneum, with the remainder of the cells being mostly mast cells. The murine peritonitis

models that have been used to investigate inflammatoary responses differ in the kind of inflammatory response they induce and what types of immune cells they activate. Zymosan has been widely used to induce peritonitis and study the inflammatory response. During the course of zymosan peritonitis, lymphocyte numbers do not change, likely because the zymosan-induced peritoneal inflammation is normally resolved within 24–48 hours. Therefore it is assumed that lymphocytes do not contribute to the course of zymosan-induced peritonitis (21). In a murine model where ovalbumin (OVA) was used to induce peritonitis to investigate oral tolerance, which is a T-cell mediated response, the results showed that there was no change in total lymphocyte count in the peritoneum in OVA tolerant mice, but the subpopulations changed. There was an increase in B-cells and a decrease in T-cells when compared with immune OVA-challenged mice 24 hours after i.p. challenge (22). In another murine model, of allergic inflammation, where ragweed extract was used to induce peritonitis, an increase was seen in T-cells (and NK cells) in the peritoneal lavage fluid (PLF) (23). Therefore, it seems that stimulation of B- and T-cells is dependent on the type of stimulant being used. In case of bacterial-induced peritonitis there was an increase in gamma/delta T-cells in the peritoneal cavity (24). When looking at the role of the adaptive immune system in murine peritonitis one also has to ask, are there any non-beneficial effects of getting the adaptive immune system involved? A recent study showed that in case of diffuse peritonitis, caused by commensal gut bacteria, the CD4^{+} T lymphocytes exert a net negative effect on the local anti-bacterial defense, and thereby contribute to bacterial dissemination and poor outcome (25).

Thioglycollate medium (TM)-induced peritonitis is frequently used for studying the inflammatory response, especially when studying macrophages. However, the inflammatory response induced by TM may not be suitable for studying chronic inflammatory responses. Cook et al. (26) introduced a model of antigen-induced murine peritonitis that may be more suitable for studying chronic inflammatory responses. They induced peritonitis by injecting an antigen, methylated bovine serum albumin (mBSA), into the peritoneum and compared the inflammatory response to that obtained using TM as a stimulant. They found that there was a small increase in lymphocytes on day 4 after the i.p. challenge in the mBSA-induced peritonitis model. The macrophages from day 4 in the mBSA-induced peritonitis were able to stimulate allogeneic T-cells but macrophages from day 4 in the TM-induced peritonitis were not able to do so. This further suggests that the mBSA-induced peritonitis model is more suitable as a model for studying chronic inflammatory diseases then the TM model (26).

1.5 Polyunsaturated omega-3 fatty acids

EPA and DHA are omega-3 PUFAs derived from α-linolenic acid (ALA). ALA is an essential fatty acid and has to be obtained from the diet but EPA and DHA can be synthesized from ALA. However, the conversion of ALA to EPA and DHA is limited in humans (27) and thus EPA and DHA may have to be obtained from the diet.
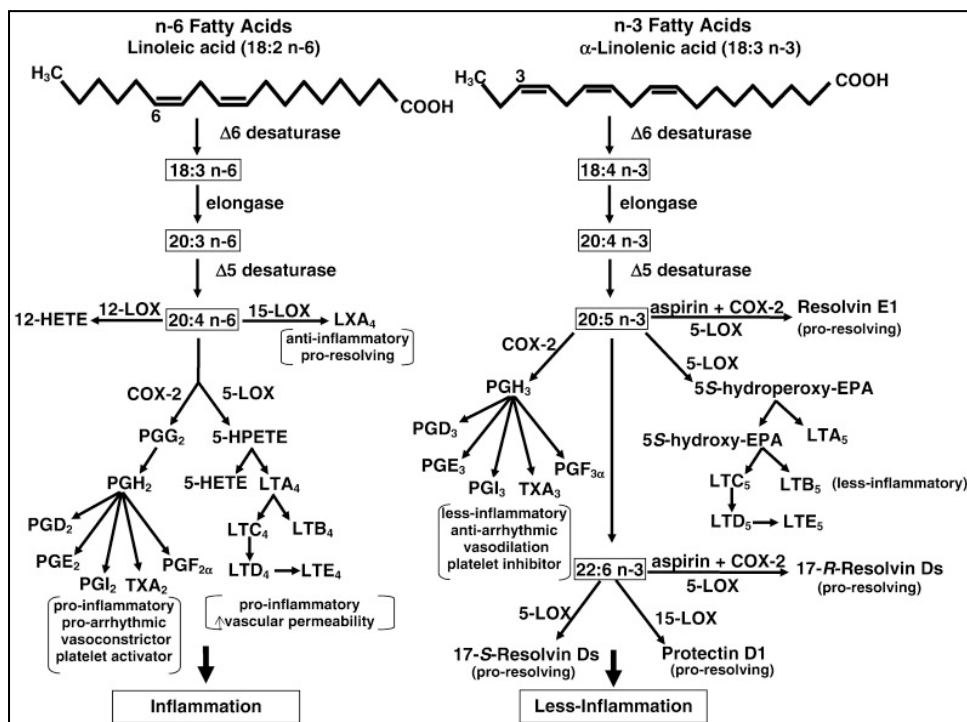
Figure 3. Elongation and unsaturation of omega-6 and omega-3 fatty acids and subsequent production of eicosanoids and pro-resolution mediators
Figure from Adkins, Y. and Kelley, D.S.(1).

Fatty acids are classified as omega-3 because the first double bond in their carbon chain is located at the third carbon from the methyl end (28). Omega-6 fatty acids are another group of polyunsaturated fatty acids derived from linoleic acid (LA) that have the first double bond located at the sixth carbon from their methyl end. Both LA and ALA can be elongated and desaturated as shown in figure 3. The longer and more unsaturated omega-3 and omega-6 fatty acids EPA, DHA and arachidonic acid (AA) are precursors for various lipid mediators as shown in figure 3. The lipid mediators produced from the omega-6 fatty acid AA are in general more inflammatory than the ones produced from EPA (1). Increased consumption of omega-3 fatty acids leads to an increase in EPA in cell membranes at the expence of AA and to decreased production of the strong pro-inflammatory mediators produced from AA, such as prostaglandin (PG) E_2, thromboxane (TX) A_2 and leukotriene (LT) B_4. On the other hand, EPA is a precursor for TXA_3, which is compared to TXA_2 a weak platelet aggregator and a weak vasoconstrictor, an increase in PGI_3 which in contrast to PGI_2 produced from AA is a weak pro-inflammatory mediator and LTB_5 which is a weak pro-inflammatory mediator compared to LTB_4 (29). The omega-3 fatty acids are also substrates for the previously mentioned potent pro-resolution mediators resolvins, protectins and maresins (30).

1.6 Omega-3 fatty acids and inflammation

The subject of omega-3 PUFAs and their effects on general health and various diseases has long been an area of interest to the medical community. There are reports on the matter dating at least back to the 1950´s (31) and there has been much progress in understanding the mechanisms involved since then. The research focus has mainly been on the effects of omega-3 PUFAs on acute inflammatory responses, less on resolution of inflammation and very few reports are on their effects on the adaptive immune response.

17

To investigate in what manner omega-3 PUFAs affect the immune system we must consider the role of PUFAs in the body. Along with being substrates for various lipid mediators as previously mentioned, PUFAs are also an important part of cell membranes and to be able to affect cell behavior they must be incorporated into the cell membrane or taken into the cell. Reports have shown that increased intake of omega-3 PUFAs leads to increased amount of the fatty acids in the cell membrane (32). PUFAs function in the cell membrane by maintaining their fluidity, providing proper surroundings for membrane proteins (33) and influencing lipid raft formation (34). Fatty acids in the also serve as energy reservoirs and are used as substrates for production of second messengers and signaling molecules, as ligands for transcription factors and regulate gene expression (33, 35) The manner in which omega-3 PUFAs can affect immune cell function is therefore many fold. Increased intake of omega-3 PUFAs can alter the composition of cell membranes, alter cell signaling pathways and production of lipid mediators (33).

That omega-3 PUFAs have beneficial effects on inflammation by reducing the inflammatory response has become quite clear although the specific pathways involved have only partly been recognized (35). Studies on the effects of omega-3 PUFAs on the acute inflammatory response have shown that increased intake leads to enhanced phagocytosis, both by neutrophils and macrophages, and increased neutrophil oxidative burst (36). Omega-3 PUFAs have also been shown to reduce production of IL-1 and TNF-α by monocytes (37). There are reports showing that omega-3 PUFAs in the diet led to production of less potent pro-inflammatory PGs and LTs (Figure. 3) (38). The effects of omega-3 PUFAs on the resolution phase of inflammation, especially in regards to the previously mentioned SPM production, have been studied to some extent but if and in what other way the omega-3 PUFAs may affect the resolution phase has not been studied in much detail. The effects of omega-3 PUFAs on the adaptive immune response have not been studied as extensively, as discussed in chapter 1.4 and the main focus has been on their effects on the T-cell population (39).

1.7 State of the art and proficiency

Omega-3 PUFA decrease production of pro-inflammatory lipid mediators like PGE_2 and LTB_4 that are derived from the omega-6 PUFA, AA, and cause an overall decrease in the production of pro-inflammatory cytokines and an increase in secretion of anti-inflammatory cytokines (38). In addition, the omega-3 PUFA, EPA and DHA, serve as substrates for the recently identified lipid mediators, resolvins and protectins, which play a significant role in the resolution phase of inflammation. Thus, n-3 PUFA are potentially potent anti-inflammatory agents and may in addition, have a pro-resolving action that may be of therapeutic use in a variety of chronic inflammatory settings.

1.8 Objective

Studies have shown that omega-3 PUFA can reduce the magnitude of inflammation by dampening the early stages and onset of inflammation. The effects of omega-3 PUFAs on the resolution phase or the adaptive immune response have, on the other hand, not been much studied.

Over the past years disorders with an inflammatory component have been steadily increasing and the amount of omega-3 PUFAs in the diet in Western societies have been decreasing. With this in

mind we set forth the hypothesis that the increase in disorders with inflammatory components may partly be due to incomplete or failed resolution of inflammation because of insufficient intake of omega-3 PUFAs.

This project is a part of a larger study for which the aim is to analyze the effects of dietary fish oil on the resolution of inflammation in antigen-induced peritonitis. The aim of this diploma project was to study the adaptive immune response during the initiation and resolution phases of antigen-induced inflammation.

The specific aims of the project were to determine:

- the number and size of germinal centers in spleen from healthy mice and mice with antigen-induced inflammation
- the levels of mBSA in spleen from healthy mice and mice with antigen-induced inflammation

Materials and Methods

2.1 Mice and diet

Female C57BL/6 mice weighing 18-20 g were purchased from Taconic Europe (Ejby, Denmark). All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Mice were housed five or eight per cage in humidity (45-55%) and temperature (23-25°C) controlled environment with a 12 h light and dark cycle. Mice were acclimated for one week prior to initiation of the experiments. All mice consumed water and food ad libitum. Seven mice were in each dietary group. They were fed for 4 weeks either a control diet based on typical Western diet alone (control group, C) or supplemented with 2.8% fish oil (fish oil group, FO).

2.2 Induction of peritonitis

Antigen-induced peritonitis was induced by vaccinating the mice with methylated bovine serum albumin (mBSA; Sigma, St Louis, MO) in complete Freund´s adjuvant (Sigma) and again in incomplete Freund´s adjuvant (Sigma) two weeks later. Seven days after the last vaccination peritonitis was induced by intraperitoneal injection of mBSA. The mice were euthanized with an overdose of isoflurane at different time points, i.e. before, and 1, 2, 5 and 10 days after mBSA injection, to monitor the induction and resolution phase of the inflammation. This part of the project was completed before this study commenced.

2.3 Preparation of the spleen and lymph nodes

The spleen and lymph nodes were harvested and snap frozen in OCT compound (Sakura Finetek, Europe B.V.) and stored at -80°C before they were cryosectioned. Seven µm thick sections were cut from the spleen and 5 µm thick sections from the lymph nodes, air dried over night and then fixed for 10 minutes in acetone, air dried again for 30 minutes and then stored at -80°C until staining.

2.4 Staining protocols

Antibodies that were used when staining sections from the spleen were anti-F4/80 (AbD Serotec, Oxford UK) and anti-MOMA-2 (AbD Serotec) for macrophages, anti-CD-3 (AbD Serotec) for T-cells, anti-peanut agglutinin (PNA) (Vector Laboratories Inc., Burlingame, CA, USA) for germinal center B-cells and anti-BSA (ICL, Portland USA) to detect BSA in the spleen. All the antibodies were monoclonal rat antibodies conjugated to biotin except anti-BSA that was directly labeled with horseradish peroxidase (HRP) and anti-CD3 that was unlabeled. In the case of anti-CD3 staining a biotin labeled anti-rat secondary antibody (BD, NJ USA) was used. Streptavidin labeled with HRP (AbD Serotec) was then used to bind to the biotin and DAB chromogen substrate (Dako, Glostrup DK) for visualization. Appendix A contains more detailed information on the staining procedures. One spleen section from each dietary group at all time points was also stained with H&E to better evaluate the structure of the tissue.

2.5   Analysis of samples

2.5.1   Germinal center analysis

Spleen sections stained with anti-PNA were evaluated using a light microscope and the software Axiocam that showed the number of germinal centers (GC) in each sample and as well as the average size of the GCs. The samples were evaluated blindly. The spleen sections varied in size, therefore the results for the number of GCs are shown as the average number of GCs divided by the size of the sample.

2.5.2   BSA analysis

Spleen sections stained for BSA were evaluated using a light microscope and the staining in the sample given a score according to a predetermined scale from 0 – 4 depending on the BSA quantity. Since the staining within a sample was not always equal, each sample was evaluated by 4 different categories; distribution of positive cells within a follicle, intensity of staining within a follicle, staining around follicles, and distribution of positive cells in the red pulp. The scoring was performed blindly by two individuals.

2.5.3   Statistical methods

Results are presented as means with error bars representing the standard error of the mean (SEM).

Difference between dietary groups and different time points were evaluated using a two-way ANOVA, followed by a Tukey´s post hoc test using SigmaStat. A t-test was used when comparing the two groups at a single time point. $P < 0.05$ was considered statistically significant.

Results

3.1 The effects of dietary fish oil on germinal centers in spleen.

Frozen sections from the spleen of mice in each dietary group were stained for GCs using anti-PNA and the average number of GCs (Figure 3) and their average size analyzed (Figure 4).
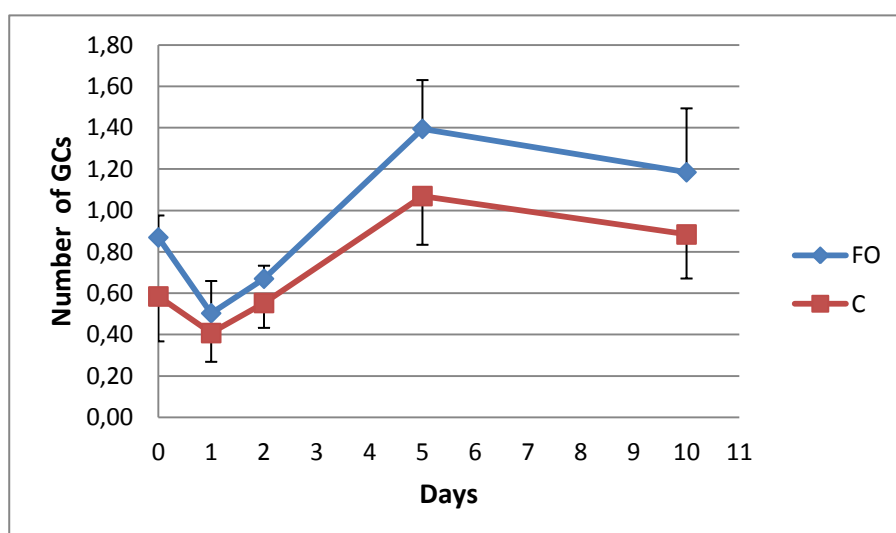

Figure 4. The effects of dietary fish oil on the number of GCs. The effects of dietary fish oil on the number of GCs in spleen at indicated time points following induction of inflammation. Mice were fed a control diet (C, red line) or a fish oil diet (FO, blue line) for 4 weeks and vaccinated with mBSA. Spleens were collected prior to (day 0) or at indicated time points following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-PNA. The results are shown as number of GCs divided by size of the sample, n= 3-7.

There were more germinal centers in spleen from mice fed the two diets five days after induction of inflammation than either before or one day following induction of inflammation (Figure 4). Dietary fish oil did not affect the average number of GCs in spleen, although there was a tendency towards more GCs in spleen from mice fed the fish oil diet than in spleen from mice fed the control diet (P=0,076; Figure 4).

The average size of the GCs in spleen from mice fed the two diets was larger 5 days after induction of inflammation than prior to or one day after induction of inflammation (Figure 5). In mice fed the fish oil diet the average size of the GCs of spleen were larger than that in spleen from mice fed the control diet at all time points but the difference was only statistically different on day 2 (Figure 5).
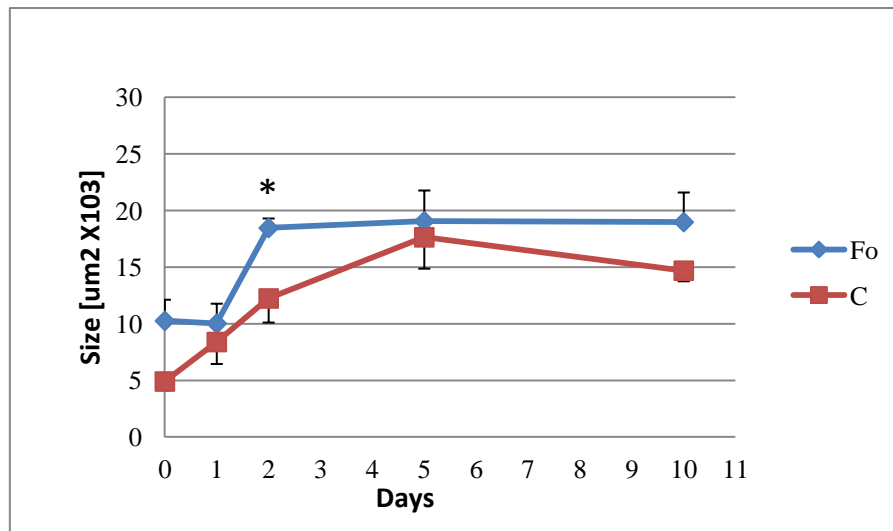
Figure 5. The effects of dietary fish oil on average size of GCs in spleen.
The effects of dietary fish oil on the average size of GCs in spleen at indicated time points following induction of inflammation. Mice were fed a control diet (C, red line) or a fish oil diet (FO, blue line) for 4 weeks and vaccinated with mBSA. Spleens were collected prior to (day 0) or at indicated time points following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-PNA. μm^2 x 10^3 * Different from control at the same time point. $P<0.05$, n= 3-7.

3.2 The effects of dietary fish oil on BSA in spleen

Frozen sections from the spleen of mice in each dietary group were stained for BSA and the staining score determined (Figure 6).
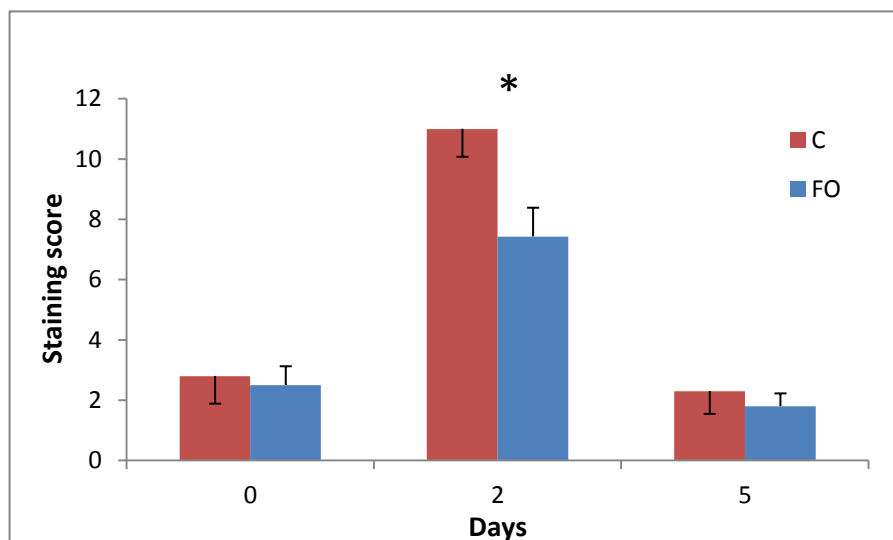
Figure 6. The effects of dietary fish oil on BSA staining in spleen. The effects of dietary fish oil on the amount of BSA in spleen at indicated time points following induction of inflammation. Mice were fed a control diet (C, red column) or a fish oil diet (FO, blue column) for 4 weeks and vaccinated with mBSA. Spleens were collected prior to (day 0) or at indicated time points following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-BSA. Staining was evaluated blindly given a score according to a predetermined scale from 0 – 4. * Different from control at the same time point. $P<0.05$, n= 5-7.

Prior to induction of inflammation (day 0) there was little BSA in spleen from mice fed the two diets (Figure 6). Preliminary results indicate that one day after induction of inflammation there was little increase in the amount of BSA present in spleen from mice in the two dietary groups compared with that in mice prior to induction of inflammation (data not shown). However, two days after induction of inflammation there was more BSA in spleen from mice fed the two diets than prior to induction of inflammation (Figure 6). Five days after induction of inflammation the amount of BSA in spleen from mice fed the two diets was similar to that prior to induction of inflammation (Figure 6). In mice fed the fish oil diet the increase in BSA in spleen was less than that in spleen from mice fed the control diet (Figure 6).

Discussion

In this study we used a murine model of BSA-induced peritonitis to investigate the effects of dietary omega-3 PUFA on the formation of germinal centers and the presence of BSA in the spleen.

Injecting mBSA into the peritoneal cavity led to an increase in both number and size of GCs in the spleen as seen in figures 4 and 5. The two immunizations of the mice with mBSAshould have led to the induction of both memory B-cells and plasma cells. The memory B-cells are most likely the source of the B-cells entering the GCs. This is supported by a rapid increase in serum concentration of mBSA specific IgG antibodies seen in the mice after i.p. injection of mBSA (Tómasdóttir et al. unpublished manuscript), which can only be produced by memory B-cells. The results from the present study show that dietary omega-3 PUFA increased the number of GCs in the spleen (Figure 4) and also increased the size of the GCs (Figure 5). This increase in both size and number of GCs in spleen from mice fed the fish oil diet would be expected to result in an increase in concentrations of serum mBSA specific IgG antibodies but the data from Tómasdóttir et al. show no difference between dietary groups in the concentration of mBSA specific IgG antibodies in the serum (unpublished manuscript). In addition, Tómasdóttir et al. showed no difference in the number of B2 cells in the peritoneum following mBSA injection (unpublished manuscript), but the B2-cells are the conventional B-cells which form GCs. Hence, the consequence of the higher number and size of GCs in the spleen of mice fed the fish oil diet remains unanswered.

Although there was no difference in serum concentrations of mBSA specific IgG antibodies between the dietary groups, mice fed the fish oil diet had higher concentrations of mBSA specific IgM antibodies in serum compared with that in mice fed the control diet (Tómasdóttir et al., unpublished manuscript). As IgM antibodies are the hallmark of a primary activation of B-cells it is unlikely that the mBSA specific IgM antibodies have been produced by the memory B-cells induced by immunizations with mBSA. These antibodies are most likely the product of either one of the non-conventional B-cells, the B1-cells that are present in the peritoneal cavity or the MZ B-cells present in the marginal zone in the spleen. When non-conventional B-cells are stimulated they produce IgM antibodies and as they do not need T-cell help these antibodies appear very early following stimulation. This could, therefore, explain the rapid increase in serum IgM anti-mBSA antibody levels following the i.p. mBSA injection observed by Tómasdóttir et al. (unpublished manuscript). The raised number of B1-cells in the peritoneal cavity of mice fed the fish oil diet following the i.p. mBSA injection compared with that of mice fed the control diet (Tómasdóttir et al. unpublished manuscript) may explain the higher mBSA specific IgM antibody levels observed in the mice fed the fish oil diet. In addition, MZ B-cells can produce memory cells that are located in the marginal zone and are quick to respond to stimuli so those memory cells may be the source of the mBSA specific IgM antibodies in serum of mice in the fish oil group. Staining protocols to detect IgG and IgM in spleen were set up and will be used to further analyze these results.

The inflammation in the peritoneum resulting from the i.p. injection of mBSA is likely to lead to macrophages ingesting the mBSA as immune complexes formed by mBSA binding to mBSA specific antibodies. Following immune complex formation, the macrophages will probably use their Fc and C3b

receptors for the phagocytosis since they have no other receptors for binding the mBSA. The mBSA should be carried to draining lymph nodes and spleen, either directly in the lymph fluid and blood, or by macrophages. mBSA was present in spleen in only trace amounts before i.p. injection of mBSA (Figure 6), but already on day 1 mBSA could be observed within cells surrounding blood vessels in the red pulp of the spleen (data not shown). There was a further increase in the levels of mBSA in the spleen on day 2 with mBSA being present not only in the red pulp but also in the marginal zone and the B-cell follicles (Figure 6). Our data is in agreement with results using zymosan-induced peritonitis, as macrophages containing zymosan started to appear in spleen at 24 hours after i.p. injection (40) which is the same time mBSA was detected in the spleen in this study. Macrophages containing zymosan were most abundant in the marginal zone of the spleen (40) whereas the first appearance of mBSA was inside cells around blood vessels in the red pulp of the spleen.

There was a significantly less mBSA staining in spleen from mice fed the fish oil diet on day 2 than in spleen from mice fed the control diet. As antigens are known to drive B-cell activation and subsequent GC formation, these data were inconsistent with the data showing that both the size and number of GCs were higher in spleens from mice in the fish oil group than that in spleen from mice in the control group. The less BSA staining in spleen from mice fed the fish oil diet than that in spleen from mice fed the control diet suggests that the phagocytes in the fish oil group are less capable of ingesting mBSA immune complexes than phagocytes from mice in the control group or less effective in migrating from the inflamed site to the spleen. The formation of mBSA immune complexes may also be less effective in mice fed the fish oil diet than in mice fed the control diet. However, as the concentration of IgG anti-BSA antibodies was similar in serum from mice fed the different diets, the difference would have to lie in a difference in complement activation of the immune complexes.

One possible way to shed a light on these results will be to analyze the number of macrophages in the spleen from mice fed the two diets and determine the extent of their infiltration into spleen following i.p. mBSA injections, since they are the most likely cells to be carrying the mBSA to the spleen. Macrophages in spleen from mice fed the different diets have been stained but the results remain to be analyzed. A protocol has been set up for staining with anti-MOMA-2 antibodies that detect macrophages at different maturation stages and in different tissues. The marker F4/80 will also be used as it is more effective i staining macrophages in the red pulp of the spleen, whereas MOMA-2 is more effective in staining macrophages in T-cell areas and macrophages containing apoptotic bodies. Apoptotic bodies within macrophages are called tangible bodies and are most commonly found in the GCs in spleen (reference). It will also be useful to do a double staining for mBSA with MOMA-2 or F4/80 to determine what type of macrophages have ingested the mBSA and whether there is much free mBSA in the spleen.

To further investigate the adaptive immune response, a protocol using anti-CD3 has been set up for staining T-cells in spleen but due to lack of time the staining has not been analyzed. The analysis of T-cells will cast light on the number of T-cells in spleen following i.p. injection of mBSA as well as whether there is a difference in the T-cell numbers between the dietary groups.

Apoptosis of neutrophils is a known marker for resolution of inflammation and these apoptotic neutrophils are cleared by macrophages that in some instances travel to the draining lymph nodes and

spleen. In order to determine the level of macrophages containing apoptotic neutrophils, the spleen will be stained for apoptosis using the TUNEL method.

Conclusion

This study is a part of a larger study in which the effects of dietary fish oil on the resolution of inflammation is being investigated. In this study the emphasis was laid on the analysis of the adaptive immune response. The main findings are that dietary fish oil led to more frequent and larger GCs in spleen, although it did not result in higher levels of mBSA specific IgG antibodies in serum. In addition, the amount of mBSA in spleen was less in mice fed the fish oil diet compared with that in mice fed the control oil diet. These data are non-conclusive and additional studies are needed before conclusions can be made.

The next steps will be to do additional staining for various markers in the spleen, such as IgG, IgM, CD3 and TUNEL staining for apoptosis to obtain data for further illustration of the cellular events in spleen during the course of both the induction and resolution phase of the inflammation. Further studies to investigate cellular events in the draining lymph nodes will also be performed since other studies of antigen-induced peritonitis have shown that immune cells in the draining lymph nodes are the first to respond to the antigen.

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Appendixes

A. Staining Protocol for F4/80

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

Block avidin with avidin blocker (Vector), incubate for 10 min.

Wash in PBS for 5 min.

Block biotin with biotin blocker (Vector) incubate for 10 min.

Wash in PBS for 2x5 min.

Biotin labeled anti-F4/80 in 1/250 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

Streptavidin linked HRP in 1/500 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

B. Staining Protocol for PNA

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

Block avidin with avidin blocker (Vector), incubate for 10 min.

Wash in PBS for 5 min.

Block biotin with biotin blocker (Vector) incubate for 10 min.

Wash in PBS for 2x5 min.

Biotin labeled anti-PNA in 1/200 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

Streptavidin linked HRP in 1/500 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

C. Staining Protocol for BSA

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

HRP linked anti-BSA in 1/100 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

D. Staining Protocol for CD3

Dip in PBS solution

Block Peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

Block avidin with avidin blocker (Vector), incubate for 10 min.

Wash in PBS for 5 min.

Block biotin with biotin blocker (Vector) incubate for 10 min.

Wash in PBS for 2x5 min.

Unlabeled anti-CD3 in 1/100 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

Biotin labeled anti-rat in 1/1000 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

Streptavidin linked HRP in 1/500 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0.037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

E. Staining protocol for MOMA-2

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

Block avidin with avidin blocker (Vector), incubate for 10 min.

Wash in PBS for 5 min.

Block biotin with biotin blocker (Vector) incubate for 10 min.

Wash in PBS for 2x5 min.

Unlabeled anti-MOMA-2 in 1/1500 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

Biotin labeled anti-rat in 1/1000 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

Streptavidin linked HRP in 1/500 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

F. Staining protocol for IgM

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

HRP linked anti-IgM in 1/1000 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

G. Staining protocol for IgG

Dip in PBS solution

Block peroxidase and alkaline phosphatase using dual enzyme blocker (Dako), incubate for 5 min.

Wash in PBS for 5 min.

HRP linked anti-IgG in 1/500 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

DAB chromogen, to develop HRP, incubate for ≈10 min.

Wash in water.

Dip in hematoxylin for 5 sec for counterstaining.

Dip in ammoniak (0,037M) 10 times.

Rinse in water

Cover with glycergel (Dako).

H. Statistical analysis of PNA staining results

Statistical analysis was done on the PNA results using two-way ANOVA. Results for the average numbers of GCs are shown in table 1.

Table 1: Two-way ANOVA results on average number of GCs.

Source of Variation	DF	P
Time	4	**0.001**
Diet	1	0.076
Time x Diet	4	0.969

To further analyze the two way ANOVA a Tukey´s post hoc test was performed on the results. The test showed that there was a statistical difference (P<0.050) in average number of GCs between day 1 and day 5, and between day 2 and day 5.

A Two-way ANOVA was also performed on the average size og GCs. The results are shown in table 2.

Table 2: Two-way ANOVA results on average size of GCs.

Source of Variation	DF	P
Time	4	**<0.001**
Diet	1	**0.017**
Time x Diet	4	0.86

The Tukey´s post hoc test results on the average size of the GCs showed that there was a statistical difference (P<0.050) between day 1 and day 5, and between day 2 and day 5. There was also a statistical difference (P=0,017) between dietary groups.

A t-test done on the data from day 2 showed a statistical difference (P= 0.04) between the dietary groups.

A two-way ANOVA was performed on the BSA staining score. The results are shown in table 3.

Table 3: Two-way ANOVA results on BSA staining score.

Source of Variation	DF	P
Time	2	<0.001
Diet	1	0,046
Time x Diet	2	0,112

A Tukey´s post hoc test on the BSA staining score showed that there was a statistical difference (P<0.001) between day 0 and day 2, and between day 5 and day 2. There was also a statistical difference (P=0,046) between dietary groups.

A t-test done on the data from day 2 showed a statistical difference (P= 0.02) between the dietary groups.