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

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HÁSKÓLI ÍSLANDS

# Áhrif ómega-3 fitusýra á sérhæft ónæmissvar í vakamiðlaðri lífhimnubólgu

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

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# Ágrip

Auk þess að stuðla að hindrun bólgu gætu ómega-3 fitusýrur mögulega stuðlað að hjöðnun bólgu 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. Mýsnar voru bólusettar með mBSA og ónæmisglæði undir húð og lífhimnubólga framkölluð með innsprautun á mBSA í kviðarhol og mýsnar aflífaðar á mismunandi tímapunktum í bólguferlinu. Miltum var safnað, þau frystiskorin og litað fyrir B-frumum í kímstöðvum, plasmafrumum, $IgM^+$ frumum, $IgG^+$ frumum og B1 frumum. Að auki voru sneiðar úr milta litaðar fyrir T-frumum, makrófögum og eosinofilum. Niðurstöðurnar sýna að tveimur dögum eftir framköllun lífhimnubólgu voru kímstöðvar milta í þeim músum sem fengu fiskolíu í fæði stærri en kímstöðvar milta í músum sem fengu viðmiðunarfæði. Hinsvegar reyndist ekki vera munur á milli fæðuhópa í fjölda plasmafrumna í milta eða fjölda $IgG^+$ frumna. Þær mýs sem fengu fiskolíu í fæði höfðu hinsvegar fleiri $IgM^+$ frumur í milta og þá aðallega í rauðu miltiskvikunni. Niðurstöður tengds doktorsverkefnis sýndu að mýs sem fengu fiskolíu í fæði höfðu meira af BSA sértæku IgM í sermi og fleiri B1 frumur í kviðarholi miðað við mýs sem fengu viðmiðunarfæði en enginn munur var á milli fæðuhópa í magni BSA sértæks IgG í sermi eða fjölda B2 frumna í kviðarholi. Niðurstöðurnar benda því til að fiskolía í fæði hafi ekki áhrif á hinar hefðbundnu B2 frumur heldur leiði til auknins fjölda og virkni B1 frumna í kviðarholi músa með vakamiðlaða bólgu. Sterkara B1 frumusvar getur leitt til virkari bólguhjöðnunar og betri vörn gegn endurteknum sýkingum.

# Abstract

Omega-3 fatty acids may, in addition to their anti-inflammatory effects, may have a pro-resolving action that may be of therapeutic use in a variety of chronic inflammatory conditions. 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 where one group was fed a control diet and the other the same diet with 2.8% fish oil. The mice were immunized subcutaneously with mBSA and adjuvant, whereafter peritonitis was induced by intraperitoneal mBSA injection. The mice were euthanized at different time points during the course of the inflammation, the spleen harvested, cryosectioned and stained for germinal center B-cells, plasma cells, $IgM^+$ cells, $IgG^+$ cells and B1 cells. In addition, spleen sections were stained for T-cells, macrophages and eosinophils. The results show that 2 days after induction of peritonitis the germinal centers in spleen of mice fed the fish oil diet were larger than the germinal centers in spleen of mice fed the control diet. There was, however, no difference between the groups in the number of plasma cells in spleen or the level of $IgG^+$ cells. Mice fed the fish oil diet had more $IgM^+$ cells in spleen, mainly in the red pulp, than mice fed the control diet. Results from a related PhD project showed that mice fed the fish oil diet had higher levels of BSA-specific IgM antibodies in serum and more B1-cells in the peritoneum compared with that in mice fed the control diet; whereas there was no difference in the levels of BSA-specific IgG antibodies in serum and the number of B2-cells in the peritoneum. We conclude that in this murine model of antigen-induced peritonitis, dietary fish oil does not have an effect on the conventional B2-cell immune response but leads to an increase in the B1-cell immune response, which may aid in the clearance of inflammatory residues and thereby to a more effective return to homeostasis and possibly better protection against secondary infection.

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## List of abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ALA | α-linolenic acid |
| ANOVA | Analysis of variance |
| APCs | Antigen presenting cells |
| AT-Rv | Aspirin triggered resolving |
| BCR | B-cell receptor |
| 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 |
| MHC | Major histocompatibility complex |
| 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 |
| TCR | T-cell receptor |
| TGF | Transforming growth factor |
| ($T_H$) | T helper |
| TM | Thioglycolate medium |
| TNF | Tumor necrosis factor |

# 1 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 (1) .

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-cells and white blood cells capable of killing or eliminating pathogens.

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

### 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, commonly called pattern recognition receptors (2). 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-cells 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 (3).

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. Recently there has been some evidence that eosinophils also contribute to the adaptive immune response by supporting plasma cell survival (4).

### 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- and T-cells. The B-cells mature fully 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, lymph nodes, 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 regard to the way they recognize pathogens. Each lymphocyte is only capable of recognizing one specific form of antigen; however, because of rearrangement of the gene segments that make up the antigen receptor chains, the receptor reservoir of 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, expressing antigen receptors recognizing the antigen. There are two types of effector T-cells. Activated $CD8^+$ T-cells are cytotoxic T-cells that fight viruses or intracellular bacteria by killing host cells. Activated $CD4^+$ T-cells are helper T-cells that recruit other cells of the immune system. Activated $CD4^+$ T-cells can be divided into several subtypes, such as T-helper 1 ($T_H1$), $T_H2$, $T_H17$ 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. This process, for the adaptive immune system to become effective, takes about 4-6 days. After activation some plasma cells migrate to the bone marrow and continue their 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 antigen that led to their activation in the first place and are more easily activated if a second infection from the same antigen 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).

### 1.1.3 B cells

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 BCR is a membrane bound immunoglobulin (Ig) and when it has

bound an antigen, with the help of a T helper cell, it differentiates into an effector cell, called a plasma cell, and starts to secrete the BCR, most often called antibody, to combat the pathogen. Secretion of antibodies is the main function of plasma cells. There are five classes of Igs 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.

Activation of B-cells occurs when the innate immune cells are unable to dispose of antigens that enter the body and antigen specific antibodies are needed to completely clear the antigen. B-cells can be activated to differentiate into antibody producing plasma cells both with and without T-cell help and the manner of activation is dependent on the type of antigen. Generally, B-cell response to protein antigens require T-cell help, termed thymus dependent response (TD-response) and the less effective response to polysaccharides and other polymers that does not require help from T-cells, termed thymus independent response (TI-response).

Activation of a B-cell by a TD-antigen and a T-helper cell requires a second signal along with the signal that is generated by the cross-linking of its BCR. Upon recognition, the BCR binds to an antigen, engulfs it and degrades it into smaller peptide fragments. The B-cells express major histocompatibility complex (MHC) molecule along with the peptide attached on its surface where it can bind to a TCR on T-cells. CD40L on the T-cells binds to CD40 on the B-cells by which the B-cells receive the necessary second signal. The combined signaling of the BCR and CD40, along with IL-4 that is produced by the activated T-cells, pushes the B-cells to differentiate into antibody secreting plasma cells and memory cells. In the case of a TI-antigen the second signal that is needed for the B-cells to become activated, normally supplied by T-cells, is supplied by the antigen itself or by repeated binding of the same epitopes on the antigen in question (1).

In the case of a TD-response the B- and T-cells meet at the T-zone border in peripheral lymphoid organs, such as lymph nodes and the spleen (reviewed in (5)). There, B-cells present peptides to T-cells and at the same time receive help from the T-cells. The B cells become activated and move to the B cell follicles where they start forming a germinal center (GC). There mature activated B-cells undergo various activation-induced changes, such as hypermutation and affinity maturation, where B-cells which higher affinity for the antigen are selected and class switching, most commonly from IgM/IgD into IgG, and thereby altering the effector part of the antibody. Fully activated B-cells become plasma cells. The formation of GCs is not only crucial for the maturation of B-cells into plasma cells but also for the formation of the previously mentioned memory cells. Thus, in the case of a TD-antigen the formation of GCs is necessary to form an accurate response.

Apart from the classical B-cells, also called follicular B-cells or B2-cells, other types of B-cells can be found (6, 7). These are marginal zone (MZ) B-cells, B1-cells and regulatory B-cells. The MZ B-cells reside in the marginal zone of the spleen and do not re-enter the circulation. They are sometimes classified as B2-cells since they develop around the same time as the follicular B-cells and from the same precursor cell type (1), but most of the time they are grouped as innate-like B-cells with the B1-cells. B1-cells, like MZ B-cells, are not thought to be found in B-cell follicles. These cells can be distinguished from B2-cells because they are, unlike B2-cells, $CD5^+$ and have high levels of IgM and

little IgD. B1-cells are about 5% of total B-cells in mice and are localized mainly in the peritoneal and pleural cavities where they are the main B-cell population (8).

B1-cells are considered to be the source of natural IgM in the blood (9). Natural IgM is so named because it is continuously produced in the body without any external stimulation. This natural IgM is polyspecific and can bind to self as well as non-self antigens. It is thought to have a protective role by binding to pathogens, immune complexes and residues of inflammation, thus aiding in clearance and return to homeostasis (7, 10). In the mucosa of the gut and the respiratory tract the B1 cells are known to produce IgA that is, like B1-derived IgM, polyspecific and thereby contributes to the first line of defense (11). The ability of B1-cells to recognize and respond to self antigens can, of course, have harmful, as well as beneficial effects. In autoimmune disorders certain self neoepitopes may become accessible to natural antibodies that are not accessible under normal circumstances and B1-cells have thus, been considered to be linked to several autoimmune disorders as reviewed in (12, 13) but the mechanism varies or remains unclear.

B1-cells can be classified as B1a- or B1b-cells based on the expression of CD5, $CD5^+$ cells being the B1a-cells and B1b-cells being $CD5^-$. Although little is known about the different roles of these two subpopulations of peritoneal B1-cells, recent studies show that B1a-cells spontaneously secrete protective natural antibodies while some B1b-cells are capable of producing antigen-specific IgM, IgG3 and IgA antibodies (reviewed in (7)).

## 1.2 The spleen

The spleen is a secondary (peripheral) lymphoid organ, where lymphocytes meet potential antigens. The spleen, along with being a lymphoid organ, also filters the blood and disposes of dying red blood cells (RBCs), which is reflected in its anatomical structure. It is mainly composed of red pulp, which is a destruction site for RBCs and it has recently been shown that the red pulp contains half of the body's monocyte reserves (14). In between the red pulp are patches of white pulp, which contain the lymphoid tissue. The white pulp consists of central arterioles that are surrounded by a periarteriolar lymphoid sheath (PALS), containing T-cells. The PALS is where APCs and T-cells come into contact. The follicles running alongside the PALS contain mostly B-cells and, as discussed above, when activated form GCs. Surrounding the follicles is the marginal zone which contains mostly macrophages but also some MZ B-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, heat and loss of function. 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 tissue scarring and/or impaired function of the inflamed tissue.

### 1.3.1 Induction of inflammation

The first cells to respond to infection are the cells that reside at the infection site, i.e. the macrophages, the dendritic cells and the mast cells. When these cells detect infection they start producing various chemokines and cytokines which induce the inflammatory response (15). Tumor necrosis factor (TNF)-α is one of the main cytokines 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 and can, therefore, be harmful if produced in excess. CXCL8 (also named IL-8) is a chemokine that attracts neutrophils, basophils and T-cells to the infected site. Chemokines also act on the leukocytes 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 activates lymphocytes and increases antibody production. IL-12 activates natural killer cells and promotes differentiation of $CD4^+$ T-cells into $T_H1$ cells (1). The lipid derived eicoasanoids, leukotrienes (LT) and prostaglandins (PG), also contribute to the initiation of inflammation (16), out of which $PGE_2$ and $LTB_4$ are the most potent pro-inflammatory mediators (17).

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 (Figure 1). Neutrophils are first to respond and enter the tissue and start to phagocytose invading pathogens and releasing protelytic enzymes. When a neutrophil has reached the end of its lifespan, which is only 8-20 hours, it starts to undergo apoptosis and express signals to to be phagocytosed by the macrophages that have by now entered the affected tissue (18). Uptake of apoptotic neutrophils by macrophages is therefore necessary to recover from the disruption in homeostasis of the affected tissue. But neutrophils are also known to play a different role and are found to promote tissue regeneration by supporting angiogenesis, which is essential for the tissue to effectively reach homeostasis (19). Neutrophils also play a supportive role in the spleen, they are now known to aid MZ B-cells in undergoing class switching, somatic hypermutation and thereby the production of antibodies (20).

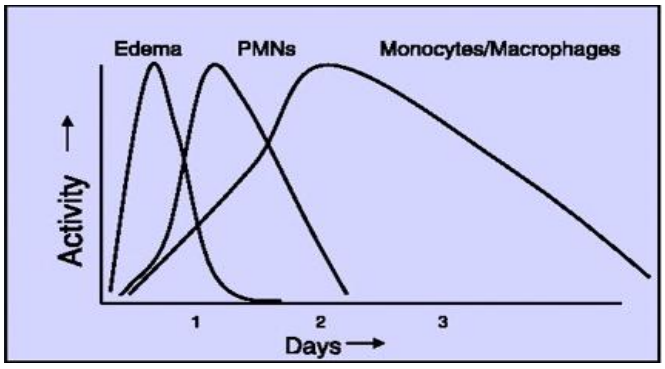


**Figure 1: Cellular events of acute inflammation.** Initiation of inflammation is characterized by early influx of neutrophils into the inflamed cite, followed by an influx of monocytes/macrophages. Figure obtained from Serhan et.al.(21).

### 1.3.2 Resolution of inflammation

Until recently it was believed that the resolution of inflammation was a passive process (22) and only removal of the antigen that caused the inflammation and terminating production of pro-inflammatory mediators could lead to its resolution. Serhan et al. (23) have shown that this is not the case and further studies on the resolution phase have shown that it is a complex process (18, 24) and is in fact an active process that depends on various mediators to be fully effective. Serhan and co-workers have defined a measurement of resolution, the resolution interval (Ri), which is determined as the time from when the neutrophils have reached their highest numbers until 50% of them have been cleared (18) and the resolution phase in total is the time from maximum neutrophil numbers until they have all been cleared from the tissue (21). If the resolution does not function properly, the inflammation can become chronic (25). Although the cells of the innate immune system are the major players in acute inflammation, chronic inflammation 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 (26).

There are several processes that must occur for resolution to be completed and for the tissue to reach homeostasis (reviewed in (21)). First, the antigen must be disposed of and the production of pro-inflammatory mediators terminated. 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 is fully accomplished, the inflamed tissue will again reach homeostasis with little or no damage and can resume its normal function. 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 (27). 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 (28, 29).

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 (30). Recently Ramon et al. showed that 17-HDHA, RvD1 and PD1 are present in the mouse spleen and since the spleen is a major immunological site and plays a big part in the formation of plasma cells, this further suggest a role of

the SPMs in the adaptive immune response. Ramon et al. also showed that 17-HDHA increases *in vitro* plasma cell differentiation in humans (31).

## 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 (32). The murine peritonitis models that have been used to investigate inflammatory 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 (33-35). During the course of zymosan-induced short time peritonitis, lymphocyte numbers did not change, likely because the zymosan-induced peritoneal inflammation is normally resolved within 24–48 hours. Therefore, it was assumed that lymphocytes do not contribute to the course of zymosan-induced peritonitis (36). Rajakariar et al. have, among others, shown that upon induction of zymosan-induced peritonitis, lymphocyte numbers are diminished but when reaching the end of the inflammation the pro-resolution lymphocytes repopulate the peritoneum (33). 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 the proportion of B-cells and a decrease in the proportion of T-cells when compared with immune OVA-challenged mice 24 hours after intrapertioneal (i.p.) challenge (37). In a 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) (38). Therefore, it seems that stimulation of B- and T-cells is dependent on the type of stimulant being used, the injection method and whether the mice are vaccinated with the antigen used prior to the induction of inflammation.

A recent study showed that in case of diffuse peritonitis, caused by commensal gut bacteria, the $CD4^+$ T lymphocytes exerted a net negative effect on the local anti-bacterial defense, and thereby contributed to bacterial dissemination and poor outcome (39), indicating a non-beneficial effect of lymphocytes in peritonitis.

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. (40) introduced a model of antigen-induced 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 not macrophages in the TM-induced peritonitis, indicating an antigen-specific activation of the T-cells.

## 1.5 Polyunsaturated omega-3 fatty acids

PUFAs are classified by the location of the first double bond in their carbon chain. Omega-3 fatty acids have their first double bond located at the third carbon from the methyl end whereas omega-6 fatty acids have their first double bond located at the sixth carbon from their methyl end. Linoleic acid (LA) and ALA are 18 carbon fatty acids that belong to the omega-6 and the omega-3 fatty acids, respectively. They are essential fatty acids that have to be obtained from the diet. Both LA and ALA can be elongated and desaturated in the body as shown in figure 2. However, the conversion of ALA to EPA and DHA is limited in humans (41) and, thus, it is recommended that EPA and DHA are obtained directly from the diet.

Since EPA and DHA have to be obtained directly from the diet there has been a focus over the last years in the medical community on changes in the western diet and its effect on diseases, especially on the increased intake of omega-6 fatty acids at the expense of omega-3 fatty acids. It is generally considered that the ratio of dietary omega-3:omega-6 fatty acids has decreased in the last 100 to 150 years, from being approximately 1:1 to being 1:20-30 (42). This decrease in the consumption of omega-3 fatty acids is possibly linked both to decreased consumption of oily marine products, since that is the major source of omega-3 fatty acids in the diet as shown by Meyer et. al.(43), as well as increased concentration of omega-6 fatty acids in the modern diet. The increase in consumption of omega-6 fatty acids it thought to be due to evolution in agriculture with the introduction of grain feed, which contain high amount of omega-6 fatty acids, into the diet of livestock (reviewed in (44)). Omega-3 fatty acids can also be found in other types of food like plants and some plant oils but there they are in the form of α-linolenic acid (ALA) (45). The omega-6 fatty acids are found in high amount in food that is more commonly consumed today like sunflower oil, nuts, meat and poultry, and although omega-3 fatty acids are also found in meat and poultry it is in much lower amount and the major source (≈71%) continues to be seafood (43).

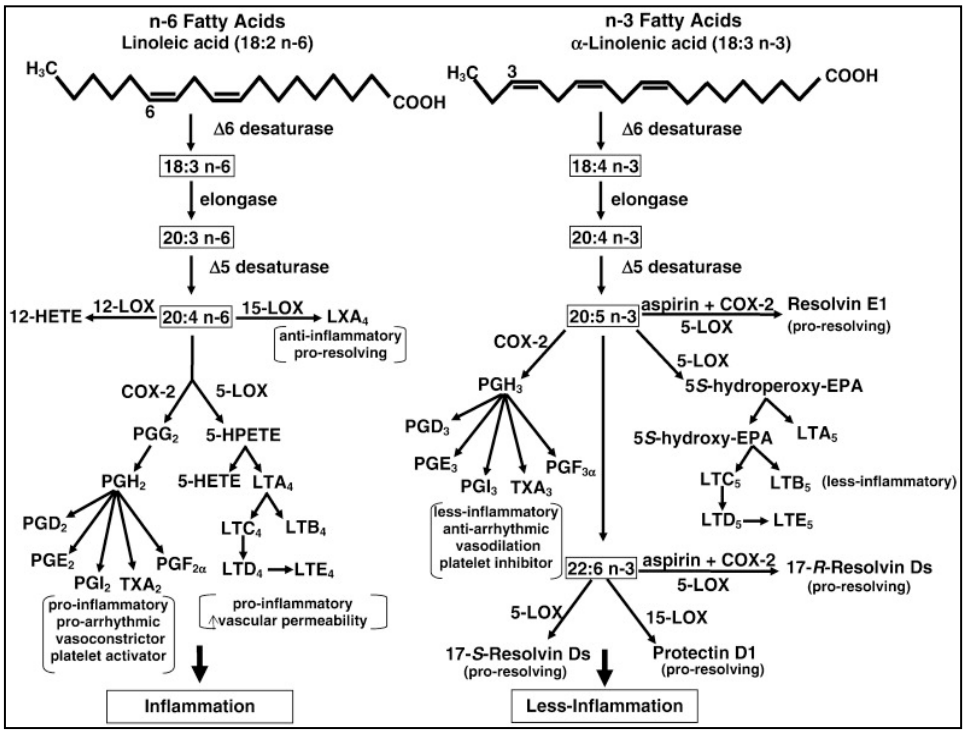


**Figure 2: Elongation and unsaturation of omega-6 and omega-3 fatty acids and subsequent production of eicosanoids and pro-resolution mediators**. Figure obtained from Adkins, Y. and Kelley, D.S. (46).

The omega-6 fatty acid arachidonic acid (AA) and the omega-3 fatty acids EPA and DHA are precursors for various lipid mediators as shown in figure 3. The lipid mediators produced from AA, such as $PGE_2$ and $LTA_2$, are in general more inflammatory than the ones produced from EPA, such as $TXA_3$, $PGI_3$ and $LTB_5$ (46). Increased consumption of omega-3 fatty acids leads to an increase in EPA in cell membranes at the expense of AA and, thus, to decreased production of the pro-inflammatory mediators produced from AA (47). Along with being substrates for the less potent pro-inflammatory mediators, the omega-3 PUFA are also substrates for the previously mentioned pro-resolution mediators Rv, PD and maresins (48) so having more of the omega-3 fatty acids in the body that are able to serve as substrates should, in theory, lead to a less intense inflammatory response.

## 1.6 Omega-3 fatty acids and inflammation

Omega-3 PUFAs and their effects on general health and various diseases have long been an area of interest to the medical community. There are reports on the matter dating at least back to the 1950´s (49) 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 PUFA on acute inflammatory responses, less on resolution of inflammation and very few reports are on their effects on the adaptive immune response.

The functions of PUFA in the cell membrane are to maintain their fluidity, provide proper surroundings for membrane proteins (50) and influence lipid raft formation (51). Fatty acids also serve as energy reservoirs and are used as substrates for production of second messengers and signaling molecules, as ligands for transcription factors and regulators of gene expression (50, 52). The manner in which omega-3 PUFA can affect immune cell function is, therefore, manifold. Reports have shown that increased intake of omega-3 PUFA leads to increased amount of the fatty acids in the cell membrane (53), altering cell signaling pathways and production of lipid mediators (50).

That omega-3 PUFA have beneficial effects on inflammation by reducing the inflammatory response has become quite clear although the specific pathways involved have only partly been recognized (52). A study on the effects of omega-3 PUFAs on the acute inflammatory response done by Kew et al, using human peripheral blood, showed that increased intake is associated with enhanced phagocytosis, both by neutrophils and macrophages, with increased neutrophil oxidative burst, and increased proliferation of lymphocytes which suggest that if more of omega-3 PUFA is incorporated into the cell membranes the more effective they are and more capable of eliminating the cause of inflammation (54). Omega-3 PUFA have also been shown to reduce production of IL-1 and TNF-α by monocytes (55), although other studies have shown an increased TNF-α production by murine resident peritoneal macrophages (56).

The effects of omega-3 PUFA on the resolution phase of inflammation, especially in regard to the previously mentioned SPM production, have been studied to some extent but not in a model mimicking chronic/subacute inflammation, and their role on the adaptive immune response in such a model has not been studied before.

### 1.6.1 Omega-3 fatty acids and B cells

Several *in vitro* studies have demonstrated that omega-3 PUFA reduce proliferation, inflammatory gene expression and secretion of IL-10, TNF-α and IFN-γ by B cells and B cell lines (57-59). In contrast, an *ex vivo* experiment using a high dose of a mixed fish/flaxseed oil diet showed enhanced LPS-induced activation of B cells, which secreted higher levels of IL-6 and IFN-γ and expressed more of CD69, relative to B cells from mice fed a control diet (60). In addition, LPS-stimulated B cells from SMAD knock-out mice fed a diet with high content of DHA had significantly higher CD40 expression and secreted more IL-6 and TNF-α than B cells from mice fed a control diet (61). The SMAD knock-out mice are prone to colitis and the DHA diet resulted in a higher frequency of $B220^{+}$ cells (total B cells) being obtained from mesenteric lymph nodes and Peyer's patches, but not from the spleen, than in mice fed the control diet (61). A higher proportion of peritoneal B cells was also found following infection with *Listeria monocytogenes* in mice fed omega-3 PUFA than in mice fed a control diet (62).

## 1.7 Objective

Over the past years disorders with an inflammatory component have been steadily increasing and the amount of omega-3 PUFA 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 PUFA. The aim of the large project that this thesis is part of is to analyze the effects of dietary fish oil on resolution of inflammation in antigen-induced peritonitis.

Studies have shown that omega-3 PUFA can reduce the magnitude of inflammation by dampening the onset and early stages of inflammation as reviewed in (45). The effects of dietary omega-3 PUFA on the resolution phase have, on the other hand, not been studied as extensively. Furthermore, very few studies have shown the effect of dietary omega-3 PUFA on lymphocytes *in vivo* and to our knowledge there are no studies on their effects on the adaptive immune response in an antigen-induced inflammation.

The aim of this project was to study the effect of dietary omega-3 PUFA on the adaptive immune response during the initiation and resolution phases of antigen-induced inflammation.

The specific aims of the project were to determine the effect of dietary omega-3 PUFA on:

- B cells in the spleen of mice with antigen-induced inflammation
    - This was done by staining spleen sections for the presence of
        - GC B-cells
        - B1-cells
        - Plasma cells
        - $IgG^+$ cells
        - $IgM^+$ cells
        - Eosinophils
- The presence of IgM antibodies against IgG and mBSA immune complexes in serum of mice with antigen-induced inflammation
- The presence of macrophages and mBSA in the spleen of mice with antigen-induced inflammation

# 2 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 for each time point, except for day ten where there were 3 mice in each group. They were fed either a control diet based on typical Western diet alone (control group, C) or supplemented with 2.8% fish oil (fish oil group, FO), starting a week before the first immunization and continuing throughout the experiment.

## 2.2 Induction of peritonitis

Antigen-induced peritonitis was induced by immunizing the mice with 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 immunization, peritonitis was induced by I.p. 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

The spleens 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, 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 of spleen sections

Antibodies used for staining sections from the spleen were against F4/80 (AbD Serotec, Kidlington UK) and MOMA-2 (AbD Serotec) for macrophages, against CD3 (AbD Serotec) for T-cells, against IgG, IgM, IgA and IgE (Southern Biotech, Birmingham, USA) to detect B-cells expressing different Igs, against CD138 (BD Bioscience, San Jose, CA) for detecting plasma cells, against myeloid basic protein (MBP; obtained from Dr. James J. Lee, Mayo Clinic, AZ) and CCR3 (R&D Systems, Minneapolis, MN) for detection of eosinophils, against CD5 and B220 (eBioscience, San Diego, USA) for detection of B1-cells and against BSA (ICL, Portland, OR). For detection of GC B-cells, the sections were stained with peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA). All the antibodies except the antibodies against BSA, IgM and IgG, which were horseradish peroxidase (HRP) labeled, were unconjugated monoclonal rat antibodies, for which a secondary antibody, biotin-labeled anti-rat (BD Biosciences, NJ) was used or were already labeled with biotin. Streptavidin labeled with HRP (AbD Serotec) was then used to bind to the biotin and DAB chromogen substrate

(Dako, Glostrup Denmark) for visualization. The sections were counterstained with hemotoxylin (Sigma), mounted using glycergel (Dako) and analyzed using light microscopy. Appendix A-C contains more detailed information on the staining procedures. One spleen section from each dietary group at all time points was also stained with hemotoxylin and eosin (H&E) to be better able to evaluate the structure of the tissue.

## 2.5 Analysis of samples

All the samples were evaluated using a light microscope. The samples were evaluated blindly by two or more individuals.

Spleen sections stained with anti-PNA were evaluated using a light microscope and the software Axiocam. The number of GCs in each sample was counted and as the spleen sections varied in size, the results were given as GC number/$\mu m^2$. The results were also given as average size of the GCs in each sample.

Spleen sections stained for BSA, IgM and CD138 were evaluated using a light microscope and the staining in the sample given a score according to a predetermined scale between 0 – 4 for four different categories; the number of cells within the follicles, staining intensity within the follicle, staining intensity around follicles (marginal zone) and the level of positive cells in the red pulp. The IgG staining was not given a value but the staining distribution noted.

The staining for eosinophils and B1-cells was not evaluated formally, nor was the staining for T-cells or macrophages.

## 2.6 Enzyme-linked immunosorbent assay (ELISA)

In order to determine whether anti-IgG antibodies were present in the sera of mice following induction of peritonitis, an ELISA plate was coated with purified mouse IgG (AbD Serotec) in several dilutions made in bicarbonate buffer, pH 9.6, ranging from 1,25:1000 to 40:1000, and incubated overnight at 4°C. After the incubation, the plate was washed 4 times with PBS containing 0.05% tween-20 (PBS-T). The samples, diluted in PBS-T from 1:200,000 were incubated for 2 hours and the plate washed as before. HRP-labeled rat anti-mouse IgG antibodies (Southern Biotech), diluted at 1:10,000, were added to the wells and again incubated for 2 hours and the plate washed as before. Substrate buffer (TMB) was added to the wells to produce color. The ELISA method for measuring human rheumatoid factor, used at the Department of Immunology, Landspitali, was also used in order to analyze the presence of antibodies against IgG in sera. There, the plates were coated with rabbit IgG.

Attempts were made to measure mBSA immune complexes where the plates were coated with mouse IgG and the sera applied as described before. Instead of using HRP-labeled anti mouse IgG antibodies, HRP-labeled anti-BSA and anti-IgM antibodies were used.

None of these assays gave positive results.

## 2.7 Statistical methods

Results are expressed as mean +/- standard error of the mean (SEM). Difference between dietary groups was evaluated using a two-way ANOVA, followed by Tukey´s post hoc test using SigmaStat. A t-test was used when comparing the two dietary groups at a single time point. $P < 0.05$ was considered statistically significant. When the P value was between 0.05 and 0.1 it was indicated with (*).

# 3 Results

## 3.1 The effects of dietary fish oil on GCs in spleen

In order to analyze the activation of follicular B cells during peritonitis, spleen section were stained with PNA, which detects GC B-cells, and the average number and size of GCs determined.

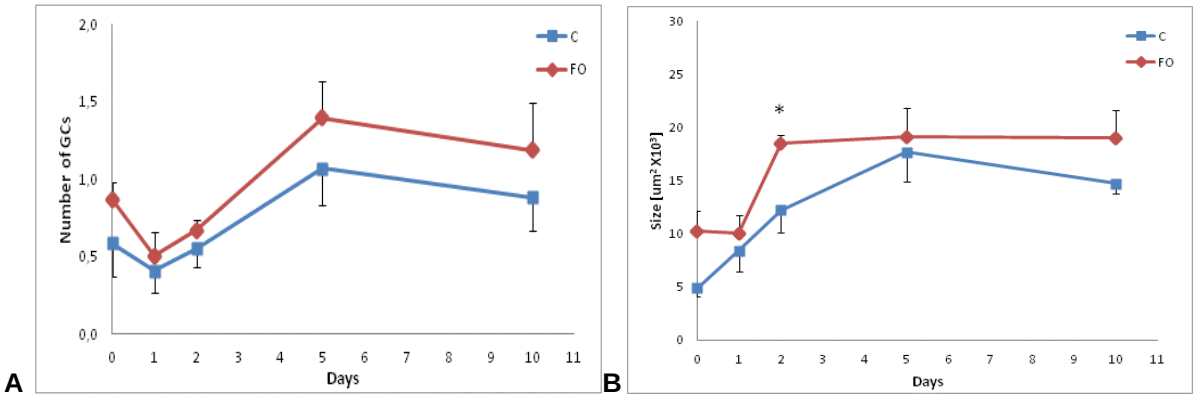


**Figure 3: The effects of dietary fish oil on the number (A) and size (B) of GCs.** Mice were fed a control diet (C, blue line) or a fish oil diet (FO, red line) for 4 weeks and vaccinated with mBSA. Spleens were collected prior to (day 0) or at indicated time points following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-PNA. All sections were analyzed blindly by two persons and both the area and number of GCs measured. The results are shown as number of GCs divided by size of the sample, n= 3-7. * Different from control at the same time point, $P<0.05$. n= 3-7.

In both dietary groups, the number of GCs declined slightly 1 day after induction of peritonitis, but then rose until day 5 (Figure 3A). Dietary fish oil did not affect the average number of GCs in spleen.

The average size of the GCs in spleen from mice fed the C diet did not start to increase until 1 day after induction of peritonitis and reached maximum at day 5 (Figure 3B). The average size of GCs in spleen from mice fed the FO diet did, however, start to increase following peritonitis induction and also reached maximum at day 5 (Figure 3B). In mice fed the FO diet the average size of the GCs in spleen was larger than that in spleen from mice fed the C diet 2 days following induction of inflammation (Figure 3B).

## 3.2 The effects of dietary fish oil on BSA in spleen

In order to determine the level of BSA entering the spleen, spleen sections were stained for BSA and the staining score determined.

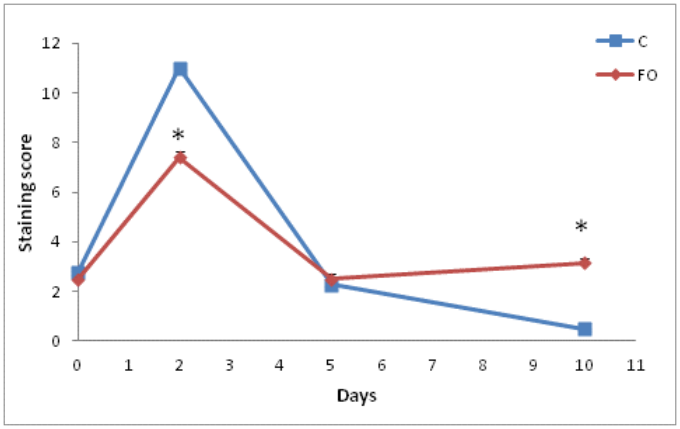


**Figure 4: The effects of dietary fish oil on BSA staining in spleen prior to and following induction of inflammation.** Mice were fed a control diet (C, blue line) or a fish oil diet (FO, red line) for 4 weeks and vaccinated with mBSA. Spleens were collected prior to (day 0) or at indicated time points following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-BSA. The results are shown as the mean staining score. Staining was evaluated blindly and each category given a score according to a predetermined scale from 0 – 4. * Different from control at the same time point, $P<0.05$. n= 3-7.

Prior to induction of inflammation (day 0) there was little BSA in spleen from mice fed the two diets (Figure 4). Preliminary results show similar staining for BSA in spleen one day after induction of inflammation as prior to its induction and indicate no difference in the level of BSA present in spleen from mice in the two dietary groups (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 with the level of mBSA being less in spleen from mice fed the FO diet than in spleen from mice fed the C diet (Figure 4). 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 4). There was more BSA in spleen from mice fed the FO diet 10 days following induction of inflammation than that in spleen from mice fed the C diet (Figure 4).

## 3.3 The effects of dietary fish oil on the number of IgM$^+$ and IgG$^+$ cells in spleen

In order to determine the level of IgM$^+$ and IgG$^+$ cells in the spleen, spleen sections were stained with antibodies against IgM and IgG and the number of IgM$^+$ and IgG$^+$ cells determined.

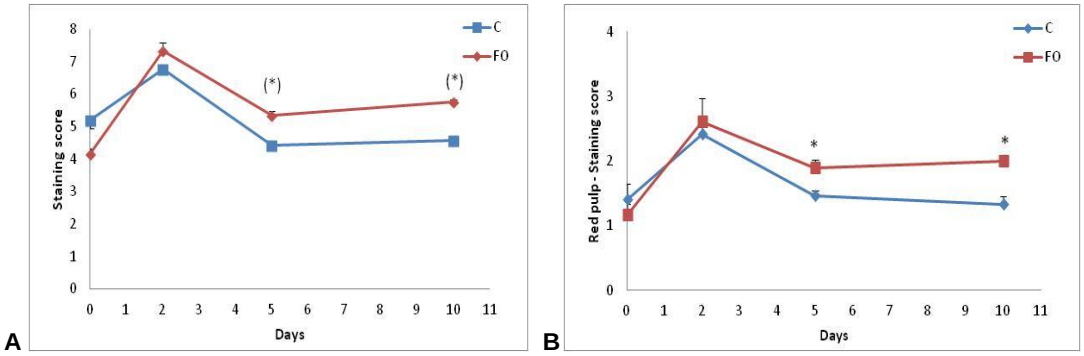


**Figure 5: The effects of dietary fish oil on the amount of IgM$^+$ cells cells in total spleen (A) and red pulp of the spleen (B) prior to and following induction of inflammation.**
Mice were fed a control diet (C, blue line) or a fish oil diet (FO, red line) for 4 weeks and vaccinated with mBSA. Spleens 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-IgM. (A) The overall staining score of the spleen. (B) The staining score of the red pulp of the spleen. The results are shown as the mean staining score. Staining was evaluated blindly and each category given a score according to a predetermined scale from 0 – 4. * Different from control at the same time point. P<0.05. (*) Tendency towards being different from control at the same time point, P<0.1. n= 3-7.

The level of IgM$^+$ cells in spleen had a tendency towards being higher in mice fed the FO diet compared with that in mice fed the C diet at days 5 and 10, with an overall statistical difference between the two dietary groups (P=0.006, two-way ANOVA) (Figure 5). When analysing the red pulp specifically, the level of IgM$^+$ cells was higher at days 5 and 10 in mice fed the FO diet than in mice fed the C diet (Figure 5B), whereas there was no difference between the level of IgM$^+$ cells within or around the follicles (data now shown).

The level of IgG$^+$ cells in spleen was not evaluated numerically, but the staining pattern was documented and showed that there was no staining within the red pulp in the spleen of mice fed the C diet or the mice fed the FO diet. IgG$^+$ cells were noted in the follicles of the spleen and GCs but no difference was observed between the dietary groups (data not shown).

## 3.4 The effects of dietary fish oil on the number of plasma cells in spleen

In order to determine whether the increase in GCs in mice fed the fish oil diet was reflected in the number of plasma cells in spleen, spleen sections were stained with antibodies against CD138 and the number of plasma cells determined on days 5 and 10. Although the average number of $CD138^+$ cells in spleen from mice fed the FO diet were higher at 5 and 10 days following induction of inflammation than in mice fed the C diet, the difference was not statistically significant (Figures 6A and B).

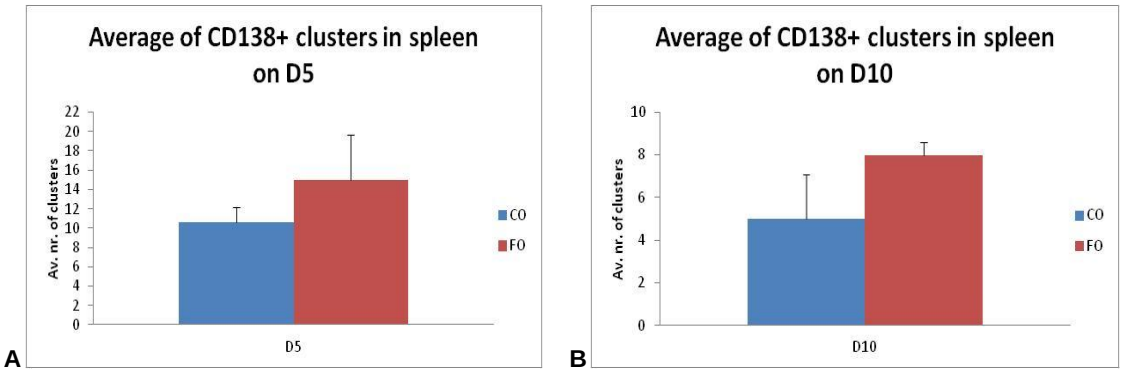


**Figure 6: The average number of $CD138^+$ cells in spleen at day 5 (A) and 10 (B) following induction of inflammation.**
Mice were fed a control diet (C, blue bar) or a fish oil diet (FO, red bar) for 4 weeks and vaccinated with mBSA. Spleens were collected at 5 (A) and 10 (B) days following i.p. injection of mBSA, snap frozen and cryosectioned and the sections fixed and stained with anti-CD138. The results are the mean number of $CD138^+$ clusters. All sections were evaluated blindly by 2 persons. n= 3-7.

## 3.5 The effects of dietary fish oil on the number and location of eosinophils in spleen

As there were more eosinophils in the peritoneum of mice fed the FO diet than in mice fed the C diet 5 and 10 days following induction of peritonitis in the study by Tomasdottir et al. (32) the presence and levels of eosinophils in spleen was determined by staining spleen sections with antibodies against MBP. There was no difference in the level or staining pattern for eosinophils in spleen from mice in the two dietary groups and eosinophils were not detected within the follicles (data not shown). Fewer eosinophils seemed to be present in spleen from an uninflamed mouse than in spleen from inflamed mice, but the distribution of the eosinophils seemed to be similar to that in inflamed mice (data not shown).

## 3.6 The effects of dietary fish oil on B1-cells, macrophages and T-cells in spleen

As there were more B1-cells in the peritoneum of mice fed the FO diet 1, 5 and 10 days following induction of inflammation (Appendix H) and since the present study had shown higher level of $IgM^+$ cells in spleen in mice fed the FO diet than in mice fed the C diet, attempts were made to determine whether B1 cells were present in the spleen. However, as B1-cells in spleen have no specific marker that is not present on other cells and double staining with CD5 and B220 was unsuccessful

Macrophages were distributed all over the spleen and their number was not evaluated. T-cells were observed in the PALS and there was not clear difference between the two dietary groups.

# 4 Discussion

The aim of this study was to analyze the effects of dietary fish oil on adaptive immune response in an antigen-induced inflammation model. This was a part of a larger study in which the aim was to analyze the effects of dietary fish oil on the induction and resolution phases of inflammation, using a murine model of antigen-induced peritonitis. Resolution of inflammation has mainly been investigated in zymosan-induced peritonitis, where zymosan induces a sharp but short-lived inflammation with no participation of the adaptive immune response (33-36). The mBSA-induced inflammation model used in the current study was chosen because it is considered to better reflect the cellular events in a continuous low-grade inflammation, where both the innate and adaptive immune responses participate, like occurs in many chronic inflammatory diseases. In this study the focus was on the B-cell response since B-cells are the majority of the lymphocytes within the peritoneal cavity.

Results from the related Ph.D. project by Valgerdur Tomasdottir showed that upon induction of inflammation the number of total B-cells in the peritoneum increased in both dietary groups but increased more in mice fed the FO diet than in mice fed the C diet (Appendix H). More peritoneal B-cells following consumption of omega-3 fatty acids has been shown in other murine models. Huang et al. (62) showed that mice fed a diet rich in omega-3 PUFA and infected with *Listeria monocytogenes* had higher percentage of peritoneal B-cells compared with that in mice fed a diet rich in omega-6 PUFA, monosaturated fatty acids or saturated fatty acids. Gurzell et al. (61), using SMAD-/- colitis prone mice, also demonstrated higher proportion of B-cells in mesenteric lymph nodes and Peyer´s patches in mice fed DHA enriched diet. That study and the Ph.D. project by Valgerdur Tomasdottir both showed that although B-cell numbers increased locally in mice fed omega-3 PUFA, the increase was not reflected systematically.

In the present study we examined the subpopulations of B-cells in the peritoneum of mice with antigen-induced inflammation, but neither of the previous studies (61, 62) examined the effects of omega-3 PUFA on B-cell subpopulations. To our surprise, we found that the increase in the number of B-cells in the peritoneal cavity of mice fed the FO diet was only reflected in an increase in the B1-cells, but not in the B2-cells (Appendix H). As B1-cells have been shown to travel from the peritoneum to the spleen (63), attempts were made to stain for these cells in splenic cryosections. These attempts were unsuccessful, possibly because B1a-cells, which are more abundant in the peritoneum than B1b-cells, discontinue their expression of CD5 upon activation, but CD5 was one of the markers used to detect the B1 cells in the spleen (63). B1-cells are known to produce natural IgM, that along with regulating pathogenic B-cells by binding to autoreactive IgG, assists in maintaining homeostasis by clearance of apoptotic cells and removal of misfolded proteins (64). Along with their “housekeeping” role, B1-cells may also play an important role in protecting against secondary infections. Rajakariar et al. (33) showed that when mice with zymosan-induced inflammation were injected with live bacteria, B1-cells were the main lymphocytes that re-inhabited the peritoneum of the wild type mice and they showed better survival than the Rag-/- mice, which are lymphocyte deficient (33). This suggests that since mice fed the FO diet in the present study have more B1 cells than mice fed the C diet, they may be

better prepared to fight against secondary infections than mice fed the C diet and also more able to reach a state of homeostasis after the cause of inflammation has been eliminated.

The higher number of $IgM^+$ cells in spleen of mice fed the FO diet in the present study is likely due to the higher number of B1 cells in the peritoneum, as peritoneal B1 cells have been shown to travel to the spleen upon activation (63) and continue to express IgM there. The B2-cell population is more likely to have switched to IgG due to the immunizations and the fact that the $IgM^+$ but not $IgG^+$ cells were found in the red pulp suggests that the $IgM^+$ cells are not B2-cells.

Mice in both dietary groups in the present study produced BSA-specific IgG and IgM antibodies upon induction of peritonitis, with more IgG than IgM being produced in both groups. In mice fed the C diet, serum levels of BSA-specific IgM did not change much upon induction of peritonitis; however, in mice fed the FO diet serum levels of BSA-specific IgM antibodies increased upon induction of peritonitis and were much higher than that in mice fed the C diet (Appendix H). This increase in BSA-specific IgM antibody production is most likely due to the increase in the number of B1-cells, since the B2-cells have been activated and class-switched during the s.c. immunizations. The B1-cells were probably activated by immune complexes formed by BSA-specific IgG antibodies and mBSA; however, it cannot be concluded whether they received T-cell help or not.

Results from the related Ph.D. project by Valgerdur Tomasdottir showed more eosinophils in peritoneum of mice receiving the FO diet than in peritoneum of mice receiving the C diet (32) and others have shown that eosinophils assist in plasma cell survival in the bone marrow (4). Therefore it was of interest to see if the same applied with the spleen as the bone marrow and increased numbers of eosinophils could be seen there. When analyzing the MPO staining it became clear that no eosinophils were present in the follicles of the spleen, where they would likely be if they played a part in sustaining plasma cell survival in the spleen. Some eosinophils were observed in the red pulp of the spleen but their numbers did not differ between the two dietary groups.

Results from the present study show a slight increase in the size of the GCs in spleen of mice fed the FO diet when compared with that in the mice fed the C diet. This was not reflected in higher levels of plasma cells as there was no difference in the levels of $CD138^+$ cells in the spleen of mice fed either diet. Furthermore, there was no difference between the groups in the levels of BSA-specific IgG antibodies in serum (as discussed above). This suggests that dietary FO did not affect B2-cell immune response in this model.

# 5 Conclusion

This project 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 on analysing the adaptive immune response focusing on the B-cells. When combined with results from the Ph.D. project of Valgerdur Tomasdottir the data show that dietary FO increased the number of peritoneal B1-cells, the level of $IgM^+$ cells in red pulp of the spleen and serum levels of BSA-specific IgM antibodies. In contrast, the FO diet did not affect the number of peritoneal B2-cells, the level of $IgG^+$ cells or plasma cells in spleen, the number and size of GCs (except for difference in size on day 2) and serum levels of BSA-specific IgG antibodies.

We conclude that dietary fish oil does not affect the conventional B2-cell immune response but increases the B1-cell immune response in antigen-induced inflammation and may lead to better protection against secondary infection, as well as improvement in reaching homeostasis following antigenic challenge.

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# Appendixes

## A. Staining Protocol for PNA (GC B-cells)

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).

**B. 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).

### C. Staining protocol for $IgG^+$ and $IgM^+$ cells

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 or 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).

**D. Staining protocol for CD138$^{+}$ cells**

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 with avidin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Block with biotin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Unlabeled rat anti-CD138 in 1/10 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

Biotin labeled anti-rat IgG in 1/100 dilution, incubate for 2 hours.

Wash in PBS for 2x5 min.

HRP labeled Streptavidin 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).

**E. Staining protocol for Eosinophils**

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 with avidin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Block with biotin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Rat Anti mouse MBP in 1/500 dilution, incubate for 40 min.

Wash in PBS for 2x5 min.

Biotin labeled anti-rat IgG in 1/100 dilution, incubate for 40 min.

Wash in PBS for 2x5 min.

HRP labeled Streptavidin in 1/500 dilution, incubate for 2 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 $CD3^+$ cells

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 with avidin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Block with biotin blocker (Vector), 10 min.

Wash in PBS for 2x5 min.

Rat Anti-mouse CD3 in 1/100 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

Biotin labeled anti-rat in 1/200 dilution, incubate for 45 min.

Wash in PBS for 2x5 min.

HRP labeled Streptavidin 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).

**G. Staining protocol for F4/80$^+$ cells.**

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.

HRP labeled Streptavidin 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).

## H. Dietary omega-3 fatty acids enhance the B1 but not the B2 cell immune response in mice with antigen-induced peritonitis (Manuscript)

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Running title: Dietary fish oil enhances B1 response

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Key words: Fish oil, antigen-induced inflammation, B1 cells, IgM, peritonitis

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

**Abstract**

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

specific IgG antibodies in mice with mBSA-induced peritonitis. These results indicate that dietary fish oil can enhance the adaptive immune response, specifically the B1 cell response, which may lead to better protection against secondary infection as well as improvement in reaching homeostasis following antigenic challenge.

**1. Introduction**

We have recently shown that dietary fish oil decreases the initiation phase but enhances the resolution phase of antigen-induced inflammation in mice (65). This is an important finding in light of the increase in chronic inflammatory diseases in Western societies in recent decades that have been linked to inadequate resolution of inflammation. Resolution of inflammation is an active process that promotes return to homeostasis following infection or inflammation. It has mainly been investigated in a murine model of zymosan-induced peritonitis, which is a pathogen-driven inflammation (66-68). In contrast, the peritonitis model used in our current and previous (65) studies is an antigen-induced immune response that occurs following immunizations with methylated BSA (mBSA), a harmless molecule, which only becomes immunogenic in the presence of a strong adjuvant (40, 65). The inflammation in this model is induced by the adaptive immune response and mimics the inflammation observed during a flare-up in autoimmune diseases and provides a model that is well suited for investigating, not only the role of dietary fish oil on resolution of inflammation, but also its effects on the lymphocyte response, including antigen-specific antibody production.

B cells play an important role in the protection against extracellular pathogens by secreting antibodies which can neutralize viruses and toxins, activate the complement system and opsonize pathogens for phagocytois. Once B cells recognize their target antigen, they become activated and migrate to the border between the T and B cell zones (reviewed in (5, 69)). Some of these cells can migrate to the medulla of the lymph nodes or into the red pulp of the spleen where they can become short lived low affinity plasmablasts. The majority of the activated B cells migrate to the B cell follicles where they form the germinal center (GC). Within the GCs, the B cells undergo proliferation, class switch recombination, somatic hypermutation and affinity maturation. During the GC reaction, the B cells undergo differentiation to either plasma cells (PCs) or memory B cells. Apart from these conventional B cells, also called follicular B cells or B2 cells, B1 cells and marginal zone B cells have also been identified (reviewed in (70-72)).

In both zymosan- and antigen-induced peritonitis, T and B cells are found in the peritoneum, with B1 cells being in majority (33, 40, 65). B1 cells are innate-like B cells with restricted B cell receptor repertoires, where antibody generation occurs without T-cell help and have limited somatic hypermutation, affinity maturation and class switching (reviewed in (70-72)). Apart from being present in the peritoneal cavity, B1 cells also reside in other cavities, such as the pleural space, and to a lesser extent in lymph nodes, bone marrow and spleen (reviewed in (7)). Peritoneal B1 cells play a key role in early protection against bacterial and viral infections via constitutive production of natural immunoglobulin (Ig)M antibodies and are the major natural antibody-producing B cell population in steady state, contributing to natural IgM antibodies in serum (9, 73) and mucosal tissues of the

respiratory tract (74). Peritoneal B1 cells have also been shown to participate in protection of the gut barrier by producing secretory IgA against commensal bacteria (11, 75). In response to various stimuli, such as live bacteria, lipopolysaccharides, certain carbohydrates, and cytokines, such as IL-5 and IL-10, peritoneal B1 cells migrate to the spleen or mucosa where they differentiate into IgM or IgA secreting cells, respectively (reviewed in (7)). The majority of IgM-secreting B1 cells reside in the bone marrow or spleen (76).

B1 cells have been subdivided into B1a and B1b cells, with B1a cells being the cells that spontaneously produce natural IgM antibodies (reviewed in (77)), whereas B1b cells can be induced to secrete antibodies following antigen specific cross-linking of the B cell receptor. The B1b cells can produce IgM or, following isotype switching, either IgG3 or IgA (78, 79). B1b cells are required for generating long-lasting protective responses against certain parasites and bacteria, although having both B1a and B1b cells has been shown to be important for generation of both natural and acquired bacterial antibody response and thus the greatest level of protection against infection (79). The newly discovered regulatory B cells (B10 cells), defined by their ability to secrete IL-10, have been found amongst the B1a cells (72).

Natural IgM antibodies are polyreactive and the steady state production of natural IgM by B1 cells provides a crucial barrier against pathogen replication before the establishment of specific immune responses (reviewed in (7)). Natural IgM antibodies do not only participate in the first line of defense against infections but also in preventing overstrained inflammatory responses and development of autoimmune diseases, mainly by facilitating uptake of apoptotic cells, as well as in maintenance of tissue integrity and homeostasis (reviewed in (64, 77)).

How dietary omega-3 polyunsaturated fatty acids (PUFA) affect B cell responses *in vivo* is largely unknown. A study in SMAD knock-out mice, which are prone to colitis, showed that a diet with a high content of docosahexaenoic acid (DHA) resulted in a higher frequency of $B220^{+}$ cells (total B cells) being obtained from mesenteric lymph nodes and Peyer‘s patches, but not from the spleen, compared with that in mice fed the control diet (61) and another study showed a higher proportion of peritoneal B cells following infection with *Listeria monocytogenes* in mice fed omega-3 PUFA than in mice fed a control diet (62). The effects of the DHA rich diet on B cell subclasses were not investigated and currently the potential effects of omega-3 PUFA on B cell subclasses are unknown. The aim of the present study was to determine the effects of dietary fish oil on the lymphocyte response in antigen-induced inflammation, focusing on the B cell response and distinguishing between B2 and B1 cells.

## 2. Methods

### *2.1. Animals and diets*

Female C57BL/6 mice weighing 18-20 g were obtained from Taconic Europe (Ejby, Denmark). They were housed 8 per cage with a 12 h light/dark cycle at 23-25°C and 45-55% humidity. All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with NRC´s Guide for the Care and Use of Laboratory Animals. Mice were

allowed to acclimatize for one week and were then divided into two groups which were fed either the control diet (C) or the fish oil diet (FO) starting one week before the immunization protocol and continuing throughout the experiment. All mice were provided fresh food daily and had free access to food and water. The composition of the C diet was based on a typical American diet, i.e. the "US17" diet formulated by Monsanto (St. Louis, MO) and Research Diets Inc (D07121302; Research Diets Inc., New Brunswick, NJ) with minor modification by the authors, as previously described (80). Energy distribution of the diet was as follows: carbohydrate, 44%; fat, 35%; and protein, 21%. The FO diet contained 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA), which was added at the expense of safflower oil (Welch, Holme & Clark Co. Inc., Newark, NJ). To adjust for the arachidonic acid (AA) content of the FO diet, AA ethyl ester (Nu-Check-Prep, Elysian, MN) (0.5 g/kg) was added to the C diet. In brief, the FO diet contained 10.6 g/kg omega-3 polyunsaturated fatty acids (PUFA) (4.0 g/kg EPA and 2.5 g/kg DHA) and the control diet 3.4 g/kg omega-3 PUFA (undetectable levels of EPA and DHA). We have previously shown that healthy mice receiving the FO diet had a higher proportion of n-3 fatty acids and a higher ratio of n-3:n-6 PUFA in hepatic phospholipids than healthy mice receiving the C diet (80).

*2.2 Induction of mBSA-induced peritonitis*

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

*2.3 Collection of serum, peritoneal lavage and spleen*

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

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

Splenic or peritoneal cells ($0.3 \times 10^6$) were incubated with 2% normal rat: normal mouse serum (1:1) (AbD Serotec, Kidlington, UK) for 20 min. Cells were stained with fluorochrome-labeled monoclonal antibodies (mabs) against IgD, B220, CD90.2, NK1.1, CD4, CD5 and CD8 (eBioscience, Vienna, Austria). All samples were washed twice with FACS buffer. Appropriate isotypic controls were used to set the quadrants and evaluate background staining. Samples were suspended in FACS buffer and 10,000 cells collected on FACScalibur (BD biosciences) and data analyzed by FlowJo software (Tree

Star, Inc, Ashland, OR). B cells were identified as $B220^{+}CD90.2^{-}NK1.1^{-}$ cells, which were divided according to their expression of B220, IgD and CD5 into B1 ($B220^{low}IgD^{low}CD5^{+/-}$) and B2 ($B220^{high}IgD^{high}CD5^{-}$) cells. The B1 cell population was divided further into B1a ($CD5^{+}$) and B1b ($CD5^{-}$) subsets. The results are expressed as percentage positive cells, mean fluorescence intensity (MFI) or total number of positive cells.

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

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

*2.6 Immunohistochemical staining of spleen sections*

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

*2.7 Data analysis and statistics*

All data are expressed as mean values ± standard error of the mean (SEM). The nonparametric Mann-Whitney Rank Sum test was used to determine whether differences between the two dietary groups were statistically significant at a single time point. Statistical analysis was performed using SigmaStat software, version 3.2 (Systat software Inc., Chicago, IL, USA). $P < 0.05$ was considered significant. When the P value was between 0.05 and 0.1 it was indicated with (*). Kruskal-Wallis non-parametric ANOVA was used to calculate the overall P values.

**3. Results**

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

B cells constituted around 40% of total peritoneal cells prior to i.p. challenge with mBSA (data not shown). The number of total B cells was the same in both dietary groups prior to administration of mBSA. At 24 h the number of total B cells was higher in the peritoneum of mice fed the FO diet compared with that in mice fed the C diet (Fig. 1A). B1 cells ($B220^{low}IgD^{low}CD5^{+/-}$) constituted around 75% and B2 cells ($B220^{high}IgD^{high}CD5^{-}$) around 25% of the total B cells prior to mBSA administration (data not shown). Mice fed the FO diet had a higher number of B1 cells 24 h and 5 days after administration of mBSA and a tendency towards a higher number (P=0.071) of B1 cells 10 days following mBSA administration than mice fed the C diet (Fig. 1B). There was no difference in the number of B2 cells in peritoneum from mice fed the different diets at any time point (Fig. 1C). B1 cells were divided into B1a cells ($CD5^{+}$), which comprised 2/3 of the total B1 cells and B1b cells ($CD5^{-}$), which comprised 1/3 of the total B1 cells. The number of both B1a and B1b cells was higher in the peritoneum of mice fed the FO diet 24 h and 5 d after induction of inflammation than in mice fed the C diet (data not shown). The proportion of splenic B cells did not change following induction of inflammation and there was no difference in the proportion of B cells in spleen from mice fed the two different diets (data not shown).

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

T cells were only around 10% of the peritoneal lymphocytes prior to peritonitis induction (Fig. 2A). Their number increased sharply following induction of peritonitis with more total T cells (at 5 days) and total $CD4^{+}$ and $CD8^{+}$ T cells (at 12 h and 5 days, and a tendancy at 10 days (P=0.097 and P=0.057, respectively)) being present in the peritoneum of mice fed the FO diet compared with that of mice fed the C diet (Fig. 2A-C). The proportion of total, $CD4^{+}$ and $CD8^{+}$ T cells in the spleen did not change following induction of peritonitis and was not affected by diet (data not shown).

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

Serum levels of BSA-specific IgM antibodies did not change much following induction of inflammation in mice fed the C diet (Fig. 3A). However, in mice fed the FO diet serum levels of BSA-specific IgM antibodies had increased by almost 3 fold 5 days after administration of mBSA and were higher than that in mice fed the C diet (Fig. 3A). There was also a tendency towards higher serum levels of IgM antibodies (P=0.057) in mice fed the FO diet than in mice fed the C diet 10 days after mBSA administration (Fig. 3A). Serum levels of BSA-specific IgG antibodies decreased sharply following induction of inflammation, most likely because of formation of IgG-mBSA immune complexes, and then increased again with no difference between the two dietary groups (Fig. 3B). There was no BSA-specific IgA antibodies detected in serum from mice fed either the C or the FO diet (data not shown).

*3.4 Effects of dietary fish oil on levels of $IgM^{+}$ and $IgG^{+}$ cells in spleen*

Next we determined whether higher serum levels of BSA-specific IgM antibodies in mice fed the FO diet than in mice fed the C diet was reflected in higher levels of $IgM^{+}$ cells in spleen of mice fed the FO diet. Higher level of $IgM^{+}$ cells were detected in the red pulp of the spleen from mice fed the FO diet 5 and 10 days after mBSA administration than in the red pulp of the spleen from mice fed the C diet (Fig.

4A). There was no difference between the two dietary groups in levels of $IgM^+$ cells within the follicles. There was no difference in IgG staining levels between the two dietary groups with IgG staining mainly being observed within the follicles but not in the red pulp (data not shown).

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

We then determined whether higher levels of BSA-specific IgM antibodies in serum of mice fed the FO diet compared with that of mice fed the C diet was reflected in an increase in GC formation or higher level of plasma cells in spleen. There was a slight increase in the average number and size of GCs in spleen following mBSA administration with the only difference between the two dietary groups being that the GCs were larger at 2 days in spleen from mice fed the FO diet compared with that in mice fed the C diet (Fig. 5A-B). There was no difference in the level of CD138 staining (plasma cells are $CD138^+$) at any time point following peritonitis induction in spleen from mice fed the different diets (data not shown).

**4. Discussion**

The results from the present study demonstrate that dietary fish oil affects the B cell response in antigen-induced peritonitis. The increase in the number of total B cells in peritoneum of mice fed the FO diet compared with that in mice fed the C diet, in the present study, is in agreement with results from two other studies. One showed a higher proportion of peritoneal B cells following infection with *Listeria monocytogenes* in mice fed omega-3 PUFA than in mice fed a control diet (62) and the other showed a higher frequency of total B cells in mesenteric lymph nodes and Peyer's patches in SMAD knock-out mice, prone to colitis, that received a diet with a high content of DHA (61). In the latter study the DHA diet did not affect B cell numbers in spleen, which is in agreement with the results from the present study showing no effect of the FO diet on the proportion of splenic B cells. These results demonstrate that a diet with a high content of omega-3 PUFA can affect B cell numbers at the site of immune induction although not systematically. In the present study the higher number of total peritoneal B cells in mice fed the FO diet was due to a higher number of B1 cells, not follicular B2 cells, but neither of the previous studies determined the subclass of the B cells.

B1 cells formed the majority of the peritoneal B cells in the present study, which is in accordance with what has been shown previously (7, 11, 40, 70). The number of B1 cells was higher in mice fed the FO diet than in mice fed the C diet. B1 cells are important for production of natural IgM antibodies during steady-state and these antibodies are important for maintaining tissue homeostasis via clearance of apoptotic and altered cells, inhibition of inflammation, removal of misfolded proteins, and regulation of pathogenic autoantibody-producing B cells (64). In addition, peritoneal B1 cells may play a vital role in protecting against secondary infection as they were the majority of the lymphocytes in the peritoneum of mice that had much better survival than RAG knockout mice following injection of live bacteria after inducing inflammation by zymosan (33). The newly discovered regulatory B10 cells have been observed amongst the B1 population (72). Since these were not analyzed in the present study it cannot be concluded whether the increase in the number of peritoneal B1 cells observed in mice fed the FO diet was because of an increase in the regulatory B10 cell population. As the mice fed the FO

diet in the present study had a higher number of peritoneal B1 cells and higher IgM antibody levels they may be more protected against secondary infections and more competent in reaching homeostasis following antigenic-challenge.

Of the peritoneal B1 cells the majority belonged to the B1a subclass with the number of both B1a and B1b cells being higher in mice fed the FO diet than in mice fed the C diet. Although little is known about the different roles of these two subpopulations of peritoneal B1 cells, recent studies show that B1a cells spontaneously secrete protective natural antibodies while some B1b cells are capable of producing antigen-specific IgM, IgG3 and IgA antibodies (78).

Although dietary fish oil increased the number of peritoneal B1 cells, it did not affect the B2 cell response, as demonstrated by it not affecting peritoneal B2 cell number, serum BSA-specific IgG levels, the level of $IgG^+$ cells in spleen or the number and size (except on day 2) of the GCs in spleen. An efficient B2 immune response against mBSA following the two s.c. immunizations was demonstrated by high levels of BSA-specific IgG antibodies being present in serum prior to peritonitis induction, indicating the formation of BSA-specific plasma cells. BSA-specific memory cells were probably also formed as there was an increase in the number and size of the GCs and the number of peritoneal B2 cells increased following i.p. mBSA injection. There are few reports on the effects of dietary fish oil on antibody response following antigen-challenge *in vivo*. The results from the present study are in agreement with results from a study in mice with an enteric infection by respiratory enteric orphan virus (reovirus), which showed that both reovirus-specific serum IgA and IgG2a antibody responses were similarly induced in mice fed control and DHA-rich diets (81). However, the results from the present study are not in agreement with results from a study showing higher levels of serum influenza-specific IgG antibodies on day 5 but lower levels on day 7 in mice fed a fish oil diet compared with that in mice fed a beef tallow diet (82).

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

As peritoneal B1 cells have been shown to migrate into the spleen and differentiate into antigenic and natural IgM-secreting cells (63, 83, 84) the higher level of $IgM^+$ cells in spleen of mice fed the FO diet than in mice fed the C diet may be the result of the higher number of peritoneal B1 cells in mice fed

the FO diet. That the $IgM^+$ cells are not B2 cells is supported by the finding that the level of $IgG^+$ cells in the spleen did not differ between the two dietary groups.

The higher serum levels of IgM antibodies in mice fed the FO diet in the present study may, in part, be linked to higher numbers of eosinophils in FO fed mice than in mice fed a C diet, shown in our previous study (65), as eosinophils have been shown to be important for priming of B cells and their secretion of antigen-specific IgM antibodies (85).

B1 cells have been shown to induce an M2-like phenotype in peritoneal macrophages, both *in vitro* in co-culture experiments, as well as *in vivo* in transgenic mice overexpressing B1 cells (86). Macrophages co-cultured with B1 cells expressed lower levels of IL-1β, TNF-α and CCL3 and higher levels of IL-10 than macrophages cultured without B1 cells or co-cultured with B2 cells, a phenotype that was also observed for peritoneal macrophages in mice overexpressing B1 cells (86). In the present study, the higher number of peritoneal B1 cells in mice fed the FO diet may have led to an increase in M2-like peritoneal macrophages. Whether it did we do not know as in our previous study we did not determine the M1/M2 phenotypes of the peritoneal macrophages. However, we demonstrated a substantially higher number of $F4/80^{low}$ macrophages expressing CD138 (syndecan-1) in mice fed the FO diet than in mice fed the C diet (65) indicating an increase the number of macrophages linked with resolution of inflammation (87) similar to the M2 macrophage phenotype.

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

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**Figure legends**

Fig. 1. Effects of dietary fish oil on the number of peritoneal total B cells (A), B1 cells (B) and B2 cells (C) prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4-5 weeks. They were immunized twice with mBSA with two weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. Values are means ± SEM, n=3 for the 10 day time point, n=7 for all other time points. * Different from control, $P < 0.05$; (*) different from control, $P<0.1$.

Fig. 2. Effects of dietary fish oil on the number of peritoneal total T cells (A), $CD4^+$ T cells (B) and $CD8^+$ T cells (C) prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4-5 weeks. They were immunized twice with mBSA with two weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points and peritoneal fluid collected. Peritoneal cells were counted using Countess automated cell counter and stained with monoclonal antibodies and analyzed by flow cytometry. Values are means ± SEM, n=3 for the 10 day time point, n=7 for all other time points. * Different from control, $P < 0.05$; (*) different from control, $P<0.1$.

Fig. 3. Effects of dietary fish oil on serum levels of BSA-specific IgM (A) and IgG (B) antibodies prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4-5 weeks. They were immunized twice with mBSA with two weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points, serum collected and antibodies measured by ELISA. Values are in arbitrary units (AU) and are means ± SEM, n=3 for the 10 day time point, n=7 for other time points. * Different from control, $P < 0.05$; (*) different from control, $P<0.1$.

Fig. 4. Effects of dietary fish oil on the level of $IgM^+$ cells in red pulp of the spleen, prior to and at different time points following administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4-5 weeks. They were immunized twice with mBSA with two weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points and spleen collected. Spleen sections were stained with immunohistochemistry and analyzed by light microscope blindly by two individuals. Scoring was performed according to a preformed scoring system, taking into account the number and intensity of the staining. Values are means ± SEM, n=3 for the 10 day time point, n=7 for other time points. * Different from control, $P<0.05$.

Fig. 5. Effects of dietary fish oil on the average number (A) and size (B) of GCs in spleen prior to and at different time points after administration of mBSA. Mice were fed a control diet (C, grey dashed line) or a fish oil diet (FO, black solid line) for 4-5 weeks. They were immunized twice with mBSA with two weeks interval and injected i.p. with mBSA one week later. Mice were sacrificed at indicated time points and spleen collected. Spleen sections were stained with immunohistochemistry and analyzed by light microscope blindly by two individuals. Values are means ± SEM, n=3 for the 10 day time point, n=7 for other time points. * Different from control, $P < 0.05$.

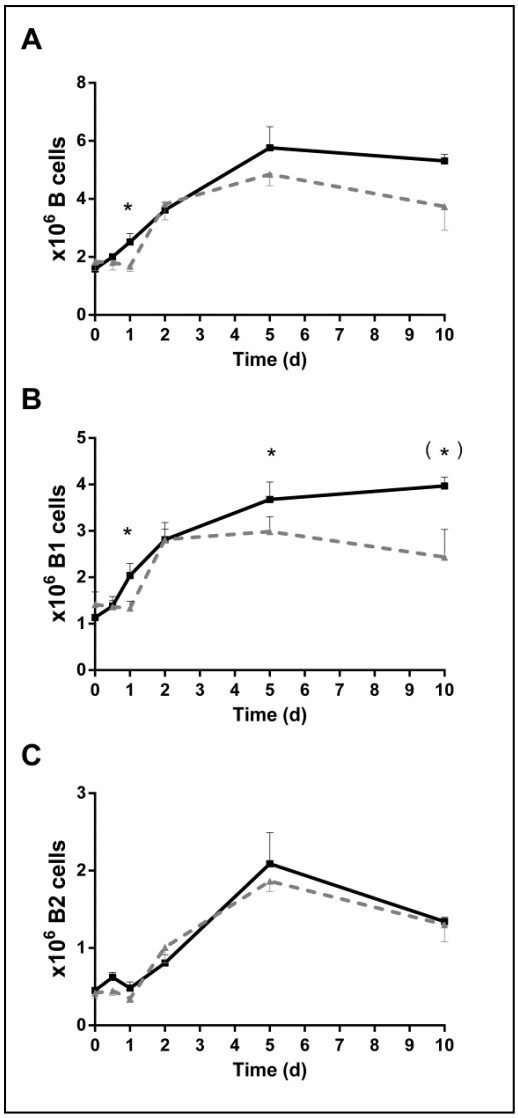


Figure 1

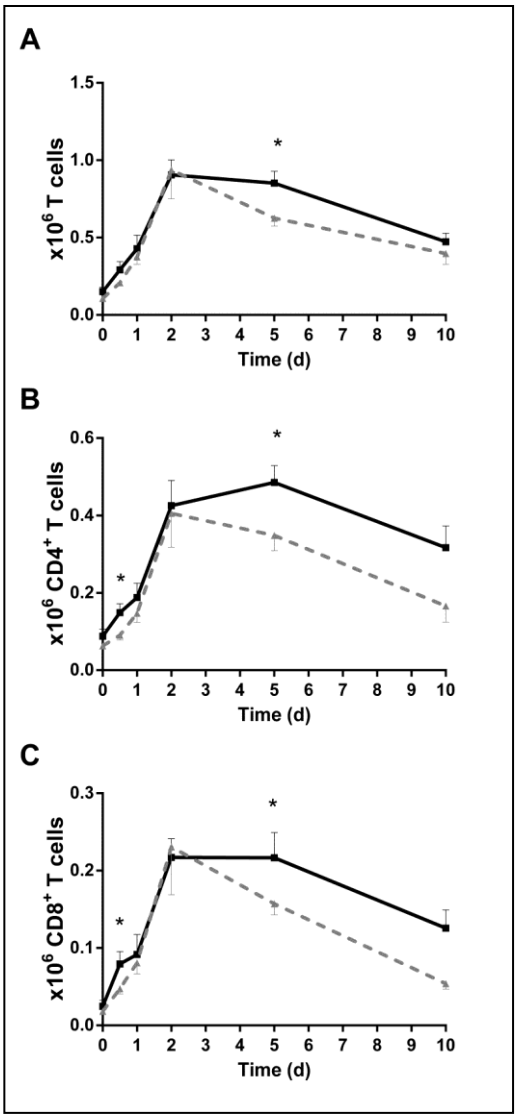


Figure 2

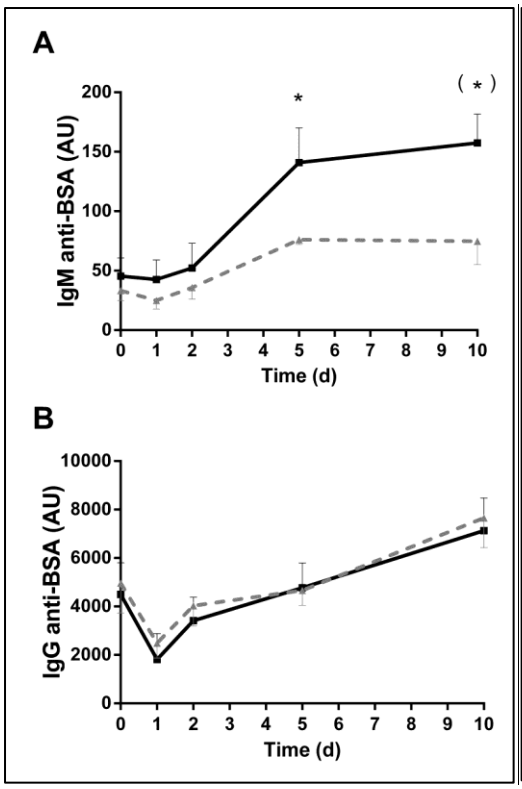


Figure 3

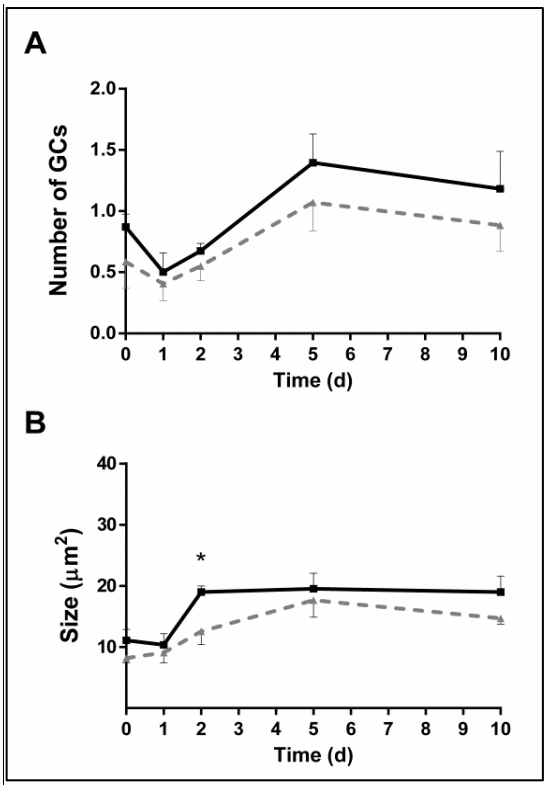


Figure 5

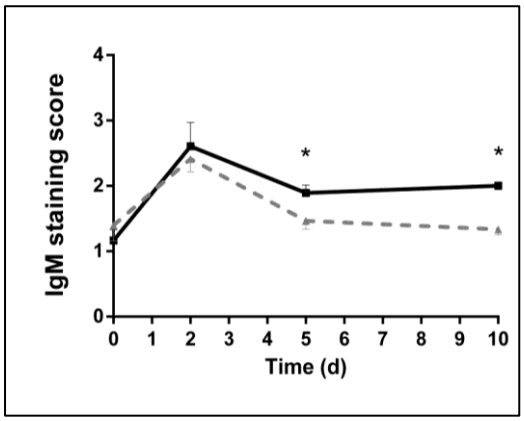


Figure 4