The effects of fatty acids on secretion of cytokines and chemokines by natural killer cells *in vitro*

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ABSTRACT

The effects of fatty acids on secretion of cytokines and chemokines by natural killer cells *in vitro*

Introduction: Resolution of inflammation was previously thought to be a passive process but is now considered to be an active biochemical process. As persistent inflammation is associated with several diseases, it is desirable to seek substances that might aid its resolution. Dietary fish oil, rich in omega-3 fatty acids, has been shown to enhance resolution of antigen-induced peritonitis in mice as well as to increase natural killer (NK) cell count early in the inflammation. Moreover, NK cells have been shown to be necessary for resolution of antigen-induced inflammation in mice as the resolution is hampered by their depletion. However, it is not known whether omega-3 fatty acids affect resolution through the increase in NK cell count and/or by affecting NK cell activity.

Aim: The aim of this study was to determine whether the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) affect the activity of human NK cells, namely their secretion of cytokines and chemokines *in vitro*.

Methods: Human NK cells, isolated from peripheral blood, were seeded in 96 well plates, cultured with or without 50 μM of arachidonic acid (AA), EPA or DHA and stimulated with cytokines to produce cytokines. The effects of the fatty acids were determined by measuring the concentration of tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin-17 (IL-17), IL-13, IL-10, CC chemokine ligand 3 (CCL3) and IL-8/CXC chemokine ligand 8 (CXCL8) in the culture supernatants by enzyme-linked immunosorbent assay (ELISA).

Results: Culturing NK cells with the omega-3 fatty acids EPA and DHA decreased their secretion of TNF-α and IL-13, compared with secretion of these cytokines by NK cells cultured without fatty acids. DHA additionally decreased secretion of GM-CSF. EPA and DHA did not affect secretion of IFN-γ, IL-10 or CCL3. Culturing NK cells with the omega-6 fatty acid AA did not affect their secretion of the cytokines tested for.

Conclusion: The results indicate that the omega-3 fatty acids EPA and DHA affect NK cell activity, namely by decreasing their secretion of the pro-inflammatory cytokines TNF-α and GM-CSF. This might be contributive to the effects that omega-3 fatty acids have been shown to have on the resolution of inflammation, as these cytokines are known to enhance survival of neutrophils and therefore delaying resolution of inflammation.

ÁGRIP

Áhrif fitusýra í rækt á seytun náttúrulegra drápsfrumna á boðefnum og flakkboðum

Inngangur: Áður fyrr var talið að bólguhjöðnun væri óvirkt ferli en nú er litið á hana sem virkt lífefnafræðilegt ferli. Í ljósi þess að viðvarandi bólga liggur að baki mörgum sjúkdómum þykir eftirsóknarvert að leita efna sem gætu stuðlað að hjöðnun hennar. Ómega-3 fitusýrur finnast í ríkulegu magni í fiskolíu. Sýnt hefur verið að fiskolía í fæði músa eykur fjölda náttúrulegra dráps (NK) frumna snemma í vaka miðlaðri bólgu og eykur sömuleiðis hjöðnun hennar. Þá hefur verið sýnt fram á mikilvægi NK frumna í hjöðnun vaka miðlaðrar bólgu, þar sem fækkun þeirra hefur hamlandi áhrif á bólguhjöðnunina. Það er hinsvegar ekki vitað hvort að áhrif ómega-3 fitusýra á bólguhjöðnun séu vegna áhrifa þeirra á fjölda NK frumna og/eða vegna áhrifa á virkni NK frumnanna.

Markmið: Markmið verkefnisins var að ákvarða hvort að ómega-3 fitusýrurnar eikósapentaensýra (EPA) og dókósahexaensýra (DHA) hefðu áhrif á virkni NK frumna í rækt, nánar tiltekið á seytun þeirra á boðefnum og flakkboðum.

Aðferðir: NK frumum einangruðum úr útvefjablóði manna var sáð í 96 holu bakka, þær ræktaðar með eða án 50 μM af arakídónsýru (AA), EPA eða DHA og örvaðar með boðefnum til boðefnaframleiðslu. Áhrif fitusýranna voru ákvörðuð með því að mæla styrk TNF-α, GM-CSF, IFN-γ, IL-17, IL-13, IL-10, CCL3 og IL-8/CXCL8 boðefna og flakkboða í frumuflotinu með ELISA aðferð.

Niðurstöður: NK frumur sem voru ræktaðar með ómega-3 fitusýrunum EPA og DHA seyttu minna af boðefnunum TNF-α og IL-13, samanborið við NK frumur sem voru ræktaðar án fitusýra. Ræktun með DHA leiddi auk þessa til minni seytunar NK frumna á GM-CSF. Ræktun NK frumna með EPA og DHA hafði ekki áhrif á seytun þeirra á IFN-γ, IL-10 eða CCL3. Ræktun NK frumna með ómega-6 fitusýrunni AA hafði ekki áhrif á seytun þeirra á þeim boðefnum sem prófað var fyrir.

Ályktun: Niðurstöður verkefnisins benda til þess að ómega-3 fitusýrurnar EPA og DHA hafi áhrif á virkni NK frumna með því að draga úr seytun þeirra á bólguboðefnunum TNF-α og GM-CSF. Þetta gæti verið þáttur í þeim áhrifum sem ómega-3 fitusýrur hafa sýnt á bólguhjöðnun, þar sem vitað er að þessi boðefni geta stuðlað að lifun neutrófíla og þannig seinkað bólguhjöðnun.

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ADCC	Antibody-dependent cell cytotoxicity
ANOVA	Analysis of variance
APC	Antigen presenting cell
BCR	B-cell receptor
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD62L	L-selectin
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CX_3CL	CX_3C chemokine ligand
CX_3CR	CX_3C chemokine receptor
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEVs	High endothelial venules
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
KIRs	Killer cell immunoglobulin-like receptors
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein

NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear factor-kappa B
NK cells	Natural killer cells
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PG	Prostaglandin
PE	Phycoerythrin
PMN	Polymorphonuclear cell
PRRs	Pattern recognition receptors
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
SEM	Standard error of the mean
SI	Secretion index
SPMs	Specialized pro-resolving mediators
TBS	Tris buffered saline
TCR	T-cell receptor
TGF	Transforming growth factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

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1 INTRODUCTION

1.1 The immune system

The immune system is an effective defense system of the body, composed of various molecules, organs and effector cells. The cells, called leukocytes, are divided into myeloid and lymphoid lineages. A common myeloid progenitor (CMP) gives rise to the myeloid lineage while a common lymphoid progenitor (CLP) gives rise to the lymphoid lineage, both of which derive from a common progenitor in the bone marrow called pluripotent hematopoietic stem cell (figure 1). The main purpose of the immune system in whole is to defend the body against pathogens and the damage they can cause by initiating an immune response.

The immune response is generally divided into two parts, i.e. the innate immune response and the adaptive immune response. The innate system reacts rapidly on exposure to various pathogens while the response of the adaptive system is specific and takes longer to develop. Although differing in many features, these systems share the same purpose; to protect the body from pathogens and other harmful substances (Murphy, Travers, Walport, & Janeway, 2012; Parham, 2014).

1.1.1 The innate immune system

Physical and chemical barriers, e.g. epithelial surfaces and various antimicrobial agents secreted by cells of the epithelia, provide the body's first line of defense against infections. Once these barriers are overcome by a pathogen, innate immune responses are rapidly initiated in order to eliminate the pathogen and clear an infection.

The innate immune system comprises miscellaneous cells, including monocytes, macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, basophils and natural killer (NK) cells, which will be discussed in more detail later (figure 1). Monocytes circulate in the blood before maturing into macrophages, which are resident in virtually all tissues. Thus, when a pathogen invades the body it generally encounters macrophages. The macrophages express various germ-line encoded receptors, e.g. mannose and glucan receptors, scavanger receptors and Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) and allow them to distinguish the invading pathogen from the body's own cells. Activation of macrophages through these pattern recognition receptors (PRRs) triggers phagocytosis and production of cytokines and chemokines, small proteins which induce inflammation and attract monocytes and neutrophils to the infected tissue from the bloodstream (Murphy et al., 2012; Parham, 2014).

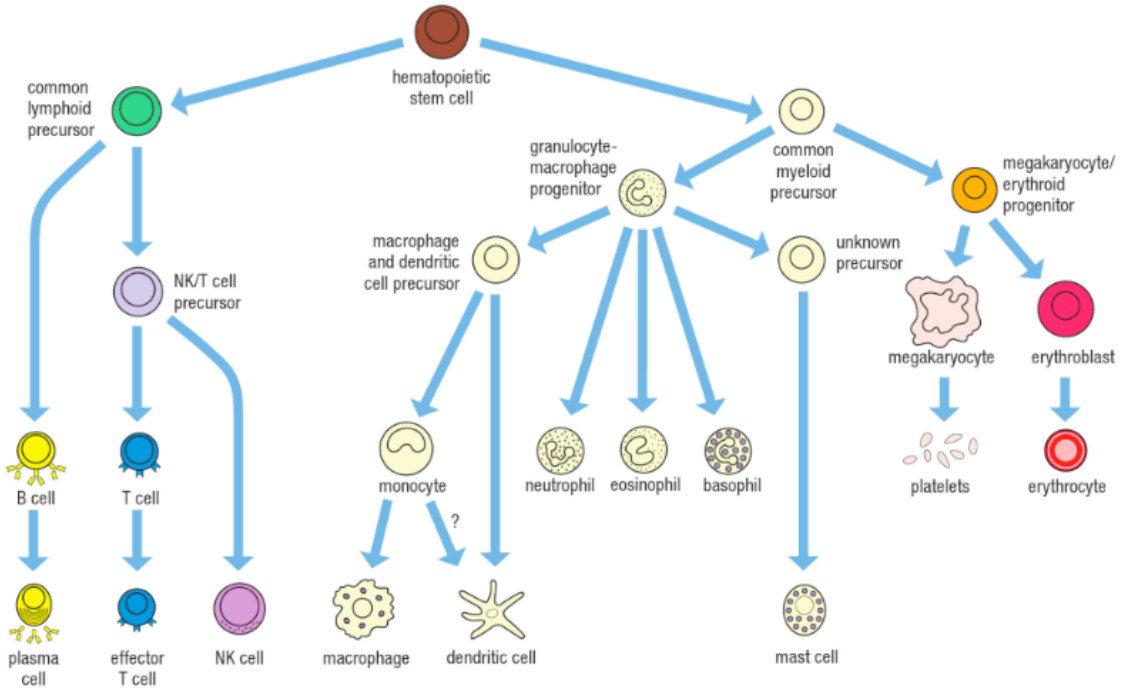

Figure 1: Leukocytes derive from hematopoietic stem cells in the bone marrow

The cells of the immune system, called leukocytes, derive from a common progenitor in the bone marrow called pluripotent hematopoietic stem cells. They are divided into two lineages; myeloid and lymphoid. A common myeloid progenitor (CMP) gives rise to the myeloid lineage while a common lymphoid progenitor (CLP) gives rise to the lymphoid lineage. Majority of the cells of the innate immune system, including monocytes, macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils and basophils, are a part of the myeloid linage. Natural killer (NK) cells are an exception from this, as they are a part of the innate immune system but derive from the CLP like lymphocytes, which are a part of the adaptive immune system (Figure adapted from Parham, 2014).

Neutrophils, eosinophils and basophils are collectively called polymorphonuclear cells (PMNs) or granulocytes, owing to prominent granules in their cytoplasm. The neutrophils are phagocytic and once recruited from the bloodstream, their primary role is to ingest and eliminate pathogens at the site of infection. Basophils and eosinophils possess less phagocytic activity but are thought to be involved in the body's defence against parasites, which cannot be ingested by phagocytosis due to size. They also play a role in allergic responses and so do mast cells (Murphy et al., 2012; Parham, 2014).

Mast cells residing in tissues are, along with macrophages, first responders to infection and tissue trauma. Upon sensing of pathogens or broken cells, perivascular mast cells release various mediators such as histamine, cytokines and chemokines and thus contribute to inflammation responses (Nathan, 2002).

DCs are yet another type of phagocytic cells. They express many PRRs and utilize them to detect pathogens in the tissue they reside in. A conventional immature DC that encounters a

pathogen in an infected tissue will internalize it upon recognition. This stimulates maturation of the DC, which then migrates to a nearby lymphoid organ where it presents antigens to antigen specific T cells of the adaptive immune system and activates them. DCs thus call for adaptive responses when needed and thereby link the innate immune system to the adaptive immune system (Murphy et al., 2012; Parham, 2014).

1.1.2 The adaptive immune system

Most infections are successfully cleared by the innate immune response. However, when the innate immune response fails to eliminate a new infection, the adaptive immune system comes to the fore. The response by this specific system takes longer to develop, but is more efficient in clearing the infection once it has developed. The adaptive immune response is initiated in secondary lymphoid tissues, e.g. lymph nodes, where lymphocytes encounter their antigen. Lymphocytes bare highly variable antigen receptors, i.e. each cell has one type of receptor with unique antigen specificity, generated by rearrangement of gene segments. This makes the lymphocytes specific for the pathogen whose antigen their specific receptor recognizes. Prior to recognition and activation by antigen in lymphoid tissue, lymphocytes circulate through the body. These cells derive from the CLP (figure 1) and are of two types, i.e. T lymphocytes and B lymphocytes.

After migration from bone marrow to maturate in the thymus, T lymphocytes (T cells) circulate the body through peripheral lymphoid tissues in search for an antigen their T-cell receptor (TCR) is specific for. TCR recognizes an antigen bound to major histocompatibility complex (MHC) molecules on the surface of an antigen presenting cell (APC), e.g. DC. To become activated, however, a T cell also requires a second signal through co-stimulatory molecules present on the APC's surface. Activation of a T cell induces its proliferation and differentiation into an effector T cell. Effector T cells are of three main categories; cytotoxic $CD8^+$ T cells, $CD4^+$ helper T cells, which influence the activity of other cells, and $CD4^+$ regulatory T cells which participate in controlling immune responses.

B lymphocytes, or B cells, go through maturation in the bone marrow before circulating secondary lymphoid tissues. They become activated when their antigen specific B-cell receptor (BCR) binds to its antigen and receive signals from helper T cells. Upon activation, B cells proliferate and differentiate into effector cells called plasma cells. These cells produce antibodies, a secreted form of the BCR, which bind to their specific antigens and neutralize them.

Under normal circumstances, the adaptive immune response successfully eliminates an infection when needed. In addition, it can provide immunological memory, which accelerates the recognition and termination of a pathogen upon second exposure (Murphy et al., 2012).

1.2 NK cells

As mentioned before, NK cells are a part of the innate immune system. However, unlike other cells of the innate system, the NK cells derive from the CLP and are thus a part of the lymphoid lineage along with the antigen-specific B and T lymphocytes of the adaptive immune system. As the receptors on NK cells are invariant and not antigen specific, being germ-line encoded, NK cells are classified as a part of the innate immune system (Murphy et al., 2012).

Bone marrow is thought to be the primary site of NK cell development, although the process is not known in detail. After development NK cells become widespread in the body, throughout lymphoid and non-lymphoid tissues, but in most tissues they represent a minor fraction of total lymphocytes (Gregoire et al., 2007). In the steady state they are present at high frequency in the circulation and can be rapidly recruited to tissues in the case of inflammation (Bernardini, Gismondi, & Santoni, 2012). NK cells have been shown to participate in the early control against virus infection and play a part in tumor immunosurveillance (Vivier et al., 2011).

NK cells possess different effector functions, i.e. cytotoxicity and cytokine production. The cytotoxicity is mediated by two pathways. One involves NK cell release of cytoplasmic granules containing perforin and granzymes while the other involves engagement of ligands, such as FasL, on the NK cell by death receptors on a target cell. Both pathways lead to apoptosis of the target cell (Smyth et al., 2005). The cytokine effector function involves production of cytokines that can influence immune responses. Activation of the cytotoxicity effector function is regulated by interactions between activating and inhibitory receptors on the NK cell surface with ligands on the target cell. The inhibitory receptors, e.g. killer cell immunoglobulin-like receptors (KIRs) and the CD94-NKG2A receptor, recognize MHC class I molecules on target cells and can thus prevent the killing of normal host cells while malignant or virus infected cells with reduced expression of those molecules may be killed. Preponderant stimulation of activation receptors by ligands on a target cell can, however, trigger its killing. NKG2D and natural cytotoxicity receptors (NCR), such as NKp46 (CD335), are examples of activating NK cell receptors (Lanier, 2005; Smyth et al., 2005; Yokoyama, Kim, & French, 2004). Target cell killing can also be triggered through an antibody-dependent cell cytotoxicity (ADCC) where CD16 (FCγRII), an activating low affinity Fc receptor on NK cell surface, recognizes IgG antibody bound to the target cell's surface (Murphy et al., 2012). In addition to trigger the killing of a target cell, the stimulation through the activation receptors can lead to production of inflammatory cytokines by the NK cells. Cytokine production can also be stimulated by binding of monocyte or macrophage derived cytokines, e.g. interleukin-12 (IL-12), IL-15 and IL-18, to constitutively expressed receptors on the NK cells (Cooper, Fehniger, Turner, et al., 2001; Fehniger et al., 1999). Binding of IL-2 to an IL-2 receptor on NK cells can promote their proliferation, cytotoxicity and cytokine secretion to some degree (Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008).

Phenotypically, NK cells are defined by their expression of CD56 and lack of CD3 expression (Robertson & Ritz, 1990). Distinct subsets of NK cells have been described, differing in

phenotype, function, anatomical features, homing capabilities and tissue distribution (Cooper, Fehniger, & Caligiuri, 2001). Human NK cells are commonly divided into $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. These subsets have different expression of various receptors and adhesion molecules (figure 2). The $CD56^{bright}$ NK cells have low expression of CD16 and KIRs while expressing high levels of CD94-NKG2A, opposite to the $CD56^{dim}$ NK cells. The $CD56^{bright}$ NK cells additionally express the chemotactic receptor CC chemokine receptor 7 (CCR7) and the adhesion molecule L-selectin (CD62L), which are involved in trafficking to secondary lymph nodes through high endothelial venules (HEVs). By contrast, $CD56^{dim}$ NK cells express the chemotactic receptors CXC chemokine receptor 1 (CXCR1) and CX_3C chemokine receptor 1 (CX_3CR1) which can bind e.g. CXC chemokine ligand 8 (CXCL8), also called IL-8, and CX_3C chemokine ligand 1 (CX_3CL1), respectively**.** Both subsets express the intermediate affinity IL-2 receptor (IL-2Rβ and IL-2Rγ), which both IL-2 and IL-15 signal trough, but the $CD56^{brigh}$ cells additionally express the high affinity IL-2 receptor, IL-2Rα. The $CD56^{dim}CD16^{+}$ NK cells make up around 90% of the NK cells in peripheral blood and spleen while most NK cells in lymph nodes and tonsils are $CD56^{bright}CD16^{-}$. The $CD56^{dim}$ NK cells express perforin and are more cytotoxic than the $CD56^{bright}$ NK cells which readily produce cytokines in response to stimulation with cytokines while lacking perforin (Cooper, Fehniger, & Caligiuri, 2001; Gregoire et al., 2007; Vivier et al., 2008).

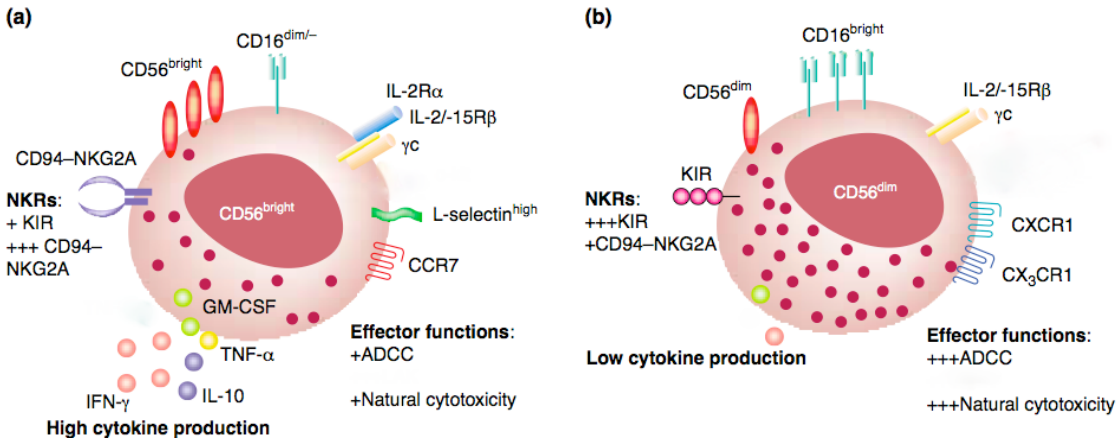

Figure 2: Differences in effector functions and expression of receptors and adhesion molecules of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets

The subsets of human NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells, differ in expression of various receptors and adhesion molecules and in effector functions. ADCC, antibody-dependent cell-mediated cytotoxicity; CCR7, CC chemokine receptor 7; CXCR1, CXC chemokine receptor 1; CX_3CR1, CX_3C chemokine receptor 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interefon-γ; TNF-α, tumor necrosis factor-α; IL-10, interleukin-10; KIR, killer cell immunoglobulin-like receptor; NKRs, NK receptors. Figure adapted from (Cooper, Fehniger, & Caligiuri, 2001).

1.2.1 Cytokines and chemokines secreted by NK cells

NK cells are known to secrete several immunoregulatory cytokines and chemokines. Tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-13, IL-10, CC chemokine ligand 3 (CCL3), also called macrophage inflammatory protein-1α (MIP-1α), and IL-8 are examples of those cytokines and chemokines, which NK cells readily produce in response to stimulation by cytokines such as IL-12, IL-15 and IL-18. Different conditions and combinations of these cytokines affect which cytokines are primarily released (Bluman, Bartynski, Avalos, & Caligiuri, 1996; Cooper, Fehniger, Turner, et al., 2001; Fehniger et al., 1999).

TNF-α, GM-CSF and IFN-γ are pro-inflammatory and fall under type 1 cytokines that favor cellular immune responses. TNF-α is a key mediator in acute inflammation and all three are known to enhance neutrophil survival (Bhatnagar et al., 2010; Murphy et al., 2012). Type 2 cytokines favor humoral immune responses. Among them are the IL-13 and IL-10 cytokines which NK cells are known to secrete. IL-10 is an anti-inflammatory cytokine that, for example, inhibits the production of TNF-α and GM-CSF (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). CCL3 and IL-8 are pro-inflammatory chemoattractants. These and other chemokines secreted by NK cells may influence the recruitment of other leukocytes during immune responses (Robertson, 2002). IL-8 has a role in inflammation as it helps attract neutrophils, and other cells, to the site of matter (Murphy et al., 2012).

1.3 Inflammation

Acute inflammation is a part of the induced phase of the innate immune response and is characterized by pain, redness, heat and swelling. Its role is to prevent or combat infection, and promote the repair of injured tissues (Murphy et al., 2012; Serhan, 2014). The inflammatory response is induced when macrophages and other immune cells in tissue recognize PAMPs or damage-associated molecular patterns (DAMPs) from necrotic cells, e.g. through TLRs (Portou, Baker, Abraham, & Tsui, 2015; Soehnlein & Lindbom, 2010). This activates the cells and leads to their production of inflammatory mediators, including chemokines such as IL-8, cytokines such as TNF-α and IL-6, and lipid mediators such as pro-inflammatory prostaglandins (PGs) (Lawrence & Gilroy, 2007; Murphy et al., 2012). The cytokines cause dilation of local small blood vessels, increasing local blood flow, and activation of their endothelial cells leading to expression of cell-adhesion molecules. The permeability of local blood vessels is also increased, allowing plasma proteins and fluid to enter the tissue and cause edema. In the case of infection, further spread of the pathogen in blood is prevented by inducing clotting in microvessels at the site (Murphy et al., 2012).

The endothelial changes, induced by pro-inflammatory cytokines such as TNF-α and IL-1β, help neutrophils and other leukocytes to extravasate into the tissues. The leukocytes are guided

by chemokines such as IL-8 and CCL3, whose role is to recruit more phagocytic cells to the site of infection. TNF-α, IL-1β and IL-6, also cause fever and induce the production of acute-phase response proteins, including mannose-binding lectin and C-reactive protein (Murphy et al., 2012). PGI_2, PGE_2 and leukotriene B_4 (LTB_4), eicosanoid lipid mediators rapidly produced by monocytes and macrophages in the inflammation process, also contribute to the induction of edema and migration of neutrophils to the tissue (Dennis & Norris, 2015; Lawrence & Gilroy, 2007; Serhan, 2014). Once neutrophils have migrated to the site of inflammation they start to phagocytose and digest infectious particles by releasing reactive oxygen species (ROS) and degradative enzymes of intracellular granules into the phagosome. When an infection has been terminated by inflammatory responses, neutrophils die by apoptosis and resolution of the inflammation is triggered (Mocsai, 2013).

These responses of acute inflammation are important in the protection against infection and promoting the repair of the injured tissues. However, the resolution of the inflammation is also of great importance as it avoids excessive damage of the tissue, promotes its healing and prevents the development of chronic inflammatory diseases (Lawrence & Gilroy, 2007; Serhan, 2014; Soehnlein & Lindbom, 2010).

1.4 Resolution of inflammation

Resolution of inflammation is the process of returning back to homeostasis after inflammatory responses, by ceasing further infiltration of leukocytes and clearing debris including apoptotic neutrophils from the inflamed tissue (Soehnlein and Lindbom, 2010). The resolution process was previously thought to be passive (Serhan, Chiang and Dyke, 2008). Now, however, it is considered to be an active biochemical and metabolic process, requiring signals that prevent further neutrophil infiltration and promote the clearance of apoptotic cells. This active process of resolution is considered to depend, in part, on lipid-mediators (Serhan, Chiang and Dyke, 2008; Soehnlein and Lindbom, 2010).

Early in inflammation, pro-inflammatory PGs and LTs are released. Later, PGE_2 and PGD_2 promote the synthesis of anti-inflammatory and pro-resolving lipid mediators, e.g. by stimulating leukocytes to produce a functional enzyme for their production (Levy, Clish, Schmidt, Gronert, & Serhan, 2001; Serhan, Chiang, & Van Dyke, 2008). Lipoxins, resolvins, protectins and maresins are examples of these mediators, collectively called specialized pro-resolving mediators (SPMs), which reduce the inflammation, stimulate its resolution and promote tissue regeneration (Serhan, 2014).

SPMs play a role in the stimulation of the key histological event in the resolution of inflammation, i.e. the reduction of neutrophils in the inflamed tissue. This is achieved by ceasing neutrophil infiltration to the inflamed tissue, while recruiting monocytes and stimulating macrophages to ingest apoptotic neutrophils (Serhan et al., 2008). The generation of lipoxins in

inflamed tissue decreases the recruitment of neutrophils. Additionally, while stopping neutrophils from migrating to the inflamed tissue, lipoxin A_4 stimulates the chemotaxis of monocytes and macrophage efferocytosis of apoptotic neutrophils. Resolvin E1 and protectin D1 also increase macrophage phagocytic activity and thus promote the resolution process (Schwab, Chiang, Arita, & Serhan, 2007). Resolvins and protectins also have a role in ceasing neutrophil influx by upregulating the expression of CCR5 on apoptotic neutrophils, leading to the clearance of its ligands CCL3 and CCL5 (Ariel et al., 2006; Soehnlein & Lindbom, 2010). Resolvins and protectins also reduce pro-inflammatory mediators such as PGs and LTB_4 (Serhan, 2014). Maresins, biosynthesized by macrophages, similarly promote resolution by decreasing neutrophil infiltration and stimulate macrophage efferocytosis of apoptotic neutrophils (Deng et al., 2014; Serhan et al., 2012).

Lipoxins are derived from arachidonic acid (AA), an omega-6 polyunsaturated fatty acid (PUFA). Resolvins, protectins and maresins, on the other hand, are biosynthesized from omega-3 PUFAs (Lopez-Vicario et al., 2015; Serhan et al., 2008). E-series resolvins derive from eicosapentaenoic acid (EPA) while D-series resolvins and maresins derive from docosahexaenoic acid (DHA) (Serhan, 2014). It has therefore been assumed that dietary omega-3 PUFAs might be important for the resolution of inflammation. Previous study by the research group has shown that dietary omega-3 PUFAs reduce the inflammatory response and enhance the resolution phase of the inflammation in a murine model of antigen-induced inflammation (Tomasdottir, Vikingsson, Freysdottir, & Hardardottir, 2013).

1.5 Polyunsaturated fatty acids

Fatty acids are dietary constituents that have various roles within the body (Calder, 2011). Structurally, a fatty acid consists of a hydrophobic hydrocarbon chain with a carboxyl group at the end. The hydrocarbon chain can vary in length and it may be saturated, monounsaturated or polyunsaturated. PUFAs are fatty acids that have two or more double bonds in their hydrocarbon chain. PUFAs with the first double bond between the third and fourth carbon from the methyl end of the hydrocarbon chain are called omega-3 fatty acids (denoted n-3). In the same way, fatty acids with the first double bond at the sixth carbon from the methyl end are called omega-6 fatty acids (denoted n-6).

Omega-3 and omega-6 PUFAs cannot be synthesized in humans, because of our inability to form double bonds beyond the 9^{th} carbon from the carboxyl end of a fatty acid. This makes two fatty acids dietary essentials, i.e. the PUFAs α-linolenic acid (ALA; 18:3, n-3) and linoleic acid (LA; 18:2, n-6) (Calder, 2013b; Fritsche, 2007). In the body, ALA can be lengthened and desaturated to produce EPA (20:5, n-3) and DHA (22:6, n-3) (figure 2). However, this conversion from ALA to EPA and DHA is inefficient in humans so it may be important to get

EPA and DHA from the diet (Surette, 2008). On the other hand, LA is the precursor of AA (20:4, n-6) (see figure 3).

Cell membrane-derived AA is a substrate of cyclooxygenase and lipoxygenase enzymes for the synthesis of eicosanoids, i.e. lipid mediators including PGs and LTs. Eicosanoids possess a range of activities and play an important role in mediating and regulating inflammation, as has been mentioned previously. Eicosanoids derived from AA are generally pro-inflammatory and synthesized rapidly by e.g. monocytes and macrophages in inflammation (Soehnlein & Lindbom, 2010). EPA is also a substrate for the synthesis of eicosanoids. However, eicosanoids derived from EPA differ in structure from those derived from AA and possess less biological activity (Calder, 2011).

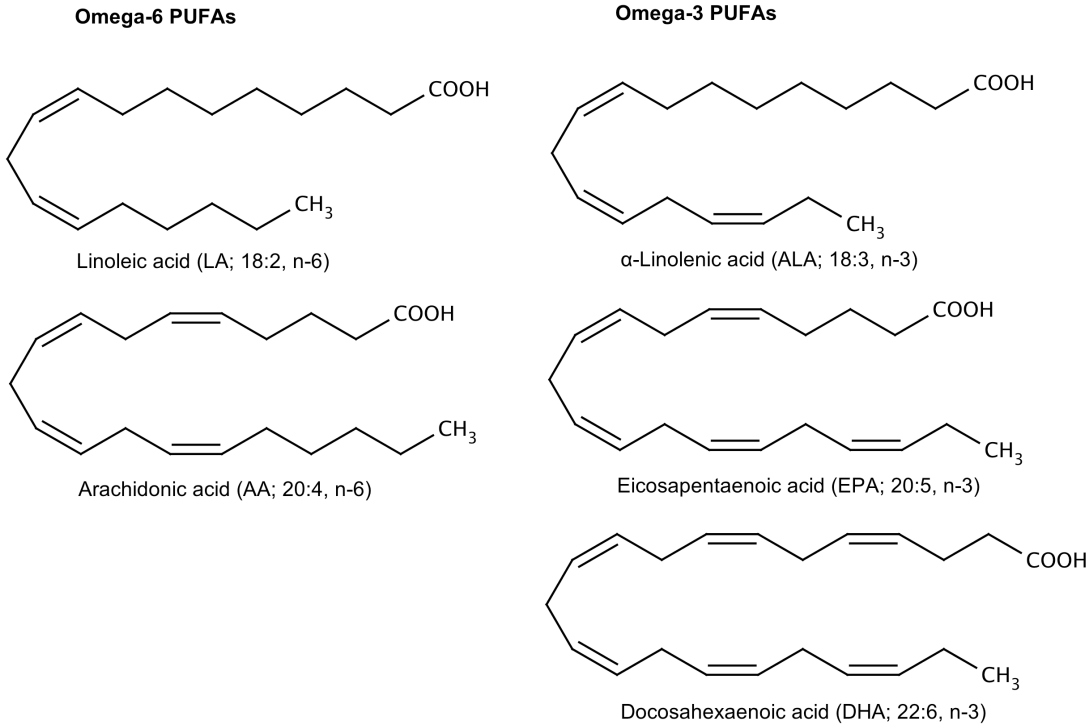

Figure 3: Omega-6 and omega-3 PUFAs

Linoleic acid (LA; 18:2, n-6) and α-linolenic acid (ALA; 18:3, n-3) are essential omega 6 and omega-3 PUFAs, repectively. LA is a precursor for arachidonic acid (AA; 20:4, n-4) in the body while ALA can be desaturated and lengthened to produce eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3). The 20 carbon fatty acids, AA and EPA, are substrates for synthesis of different eicosanoids in the body.

PUFAs are key components of phospholipids in cell membranes and necessary for maintenance of fluidity of the membrane as well as for a variety of other cellular functions. Upon ingestion, these fatty acids are distributed to just about every cell in the body (Stulnig, 2003).

Oral administration of the omega-3 PUFAs EPA and DHA can affect the fatty acid composition of cell membranes, i.e. increase their incorporation at the expense of AA and thereby reduce the synthesis of pro-inflammatory eicosanoids such as PGE_2 and LTB_4. This interference with AA metabolism represents a key anti-inflammatory activity of omega-3 PUFAs (Calder, 2011). In addition, EPA and DHA are substrates for the synthesis of other lipid mediators, i.e. some of the SPMs mentioned before, which participate in the resolution of inflammation. The bulk of the studies investigating the immunomodulatory effects of omega-3 PUFAs have used fish oils, containing both EPA and DHA (Weldon, Mullen, Loscher, Hurley & Roche, 2007).

1.6 The effects of omega-3 PUFAs on the immune system

As mentioned, omega-3 PUFAs have been shown to affect inflammatory responses and are thought to have an anti-inflammatory activity. Various effects of the omega-3 PUFAs EPA and DHA on inflammatory responses have been reported, e.g. decrease in leukocyte chemotaxis, expression of adhesion molecules and production of pro-inflammatory eicosanoids and inflammatory cytokines (Calder, 2013b). A few possible mechanisms of how the omega-3 PUFAs modulate immune cell function on molecular level have been proposed. Their modulation of membrane order and fluidity as well as effects on lipid mediators and eicosanoids are there among. They can also affect gene expression, by altering signaling pathways or by interacting with nuclear receptors (Calder, 2013b; Shaikh & Edidin, 2008). Numerous studies have examined the effects of omega-3 PUFAs on various cells of the immune system.

1.6.1 The effects of omega-3 PUFAs on cytokine production

Omega-3 PUFAs have been shown to decrease production of pro-inflammatory cytokines, likely by decreasing activation of nuclear factor κB (NF-κB), a transcription factor, and thus the expression of inflammatory cytokine genes (Calder, 2013a). As an example, in a study on the individual effects of EPA and DHA on cytokine expression and NF-κB activation in lipopolysaccharide (LPS) stimulated human THP-1 monocyte-derived macrophages, both EPA and DHA reduced the production of TNF-α, IL-1β and IL-6, and down-regulated NF-κB/DNA binding (Weldon, Mullen, Loscher, Hurley, & Roche, 2007). In another study, where the effects of DHA on pro-inflammatory cytokine production by bone marrow derived DCs stimulated with TLR ligands were examined, DHA inhibited the production of IL-12 cytokine family (IL-12p70, IL-23 and IL-27) through inhibition of NF-κB (Kong et al., 2010).

1.6.2 The effects of omega-3 PUFAs on NK cells

The effects, and the underlying mechanisms, of PUFAs on NK cell activity are not known in detail (Shaikh and Edinin, 2008). In a study on the effects of PUFAs on human blood lymphocytes *in vitro,* incubation with EPA, DHA or γ-linolenic acid (GLA; 18:3, n-6) reduced the cytotoxic activity of NK cells and the omega-3 fatty acids showed greater suppression than the omega-6 acid (Purasiri, McKechnie, Heys, & Eremin, 1997). In the same study, incubation with the fatty acids also resulted in a decrease in cytokine production by the lymphocytes, which were isolated from blood by centrifugation on a density gradient. Previously, another study on human peripheral blood lymphocytes had also shown EPA and DHA to suppress NK cell activity in a cytotoxicity assay system (Yamashita, Maruyama, Yamazaki, Hamazaki, & Yano, 1991). However, an emulsion of omega-3 fatty acids and anti-oxidants along with resolvin D1 or curcuminoids enhanced the cytocidal activity of NK cells in a co-culture with pancreatic cancer cells (Halder et al., 2015).

The effects of different dietary PUFAs, including ALA, GLA, EPA and DHA, on NK cell activity in humans has also been studied (Thies et al., 2001). In the study the participants received different encapsulated oil blends daily for 12 weeks. Blood was collected and the cytotoxic activity of NK cells, in preparations of PBMCs, measured against the K562 target cell line. No significant effects on NK cell activity were observed in groups receiving ALA, GLA, AA or DHA alone while the group supplemented with fish oil, providing both EPA and DHA, showed significantly decreased NK cell activity. Prior to this study, dietary studies on rats had shown that a diet rich in omega-3 ALA and a diet containing fish oil rich in EPA and DHA lowered spleen NK cell activity when compared with a diet rich in omega-6 LA and low-fat diet, respectively (Jeffery, Sanderson, Sherrington, Newsholme, & Calder, 1996; Yaqoob, Newsholme, & Calder, 1994).

1.7 The connection between omega-3 PUFAs, resolution of inflammation and NK cells

A prior study by the research group, using methylated BSA (mBSA)-induced peritonitis in mice, sought to determine the effects of dietary fish oil on the induction and resolution of inflammation (Tomasdottir et al., 2013). The results of the study indicate that dietary fish oil, rich in the omega-3 PUFAs EPA and DHA, enhances resolution of inflammation. In this study there was also an increase in NK cell count in the peritoneum early in the inflammation in mice fed the fish oil, but not in mice fed the control diet. Following this, Anuforo studied the role of NK cells in the resolution of antigen-induced inflammation in mice and demonstrated that NK cells were indeed necessary for the resolution of the inflammation (Anuforo, 2015). Prior to these studies it had been shown that human NK cells induce apoptosis of neutrophils in co-culture of NK cells and

neutrophils, indicating their possible role in resolution of an acute inflammation as neutrophil apoptosis is an important factor in that process (Thoren et al., 2012).

These studies indicate that omega-3 PUFAs in the diet of mice increase NK cell count early in inflammation and increase resolution of inflammation, and that NK cells have a role in resolution of inflammation, as resolution is hampered by NK cell depletion. However, it is not known whether omega-3 fatty acids affect resolution through the increase in NK cell count and/or by affecting NK cell activity. Therefore, the aim of this study was to determine whether the omega-3 fatty acids EPA and DHA affect the activity of human NK cells, namely their secretion of cytokines and chemokines *in vitro*.

2 AIM

The aim of this study was to determine whether the omega-3 fatty acids EPA and DHA affect the activity of human NK cells, namely their secretion of cytokines and chemokines *in vitro.*

3 MATERIALS AND METHODS

The study was performed at the Department of Immunology and Centre for Rheumatology Research at Landspitali – The National University Hospital of Iceland (Dept. of Immunology).

3.1 Materials

Buffy coats, prepared from peripheral blood of healthy donors upon informed consent (ethical approval number 06-068 SI), were supplied by The Icelandic Blood Bank. Other materials used in the study are listed in table 1. Recipes for buffers and solutions prepared at the Dept. of Immunology can be seen in table 2.

Table 1: List of materials used in the study

Material	Batch number	Manufacturer
Antibodies for flow cytometry		
- Anti-Human CD56- phycoerythrin (PE) Clone: CMSSB	E12343-1636	eBioscience
- Mouse IgG_1 Isotype - (PE) Clone: X40	33023	BD Biosciences
Arachidonic acid (AA)	0470116	Cayman Chemical Company
Blocking solution	-	Dept. of Immunology
Bovine serum albumin (BSA)	778	Millipore
Cytokines used in cell culture		
- Recombinant human IL-2	AE5014121	R&D Systems
- Recombinant human IL-12	PY1815081	R&D Systems
- Recombinant human IL-15	TLM0915094	R&D Systems
Dimethyl sulfoxide (DMSO)	SZBE1610V	Sigma-Aldrich
Distilled water (dH_2O)	-	Dept. of Immunology
Docosahexaenoic acid (DHA)	0470118	Cayman Chemical Company
Eicosapentaenoic acid (EPA)	0470117	Cayman Chemical Company
Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate	K17596618	Merck
Enzyme-linked immunosorbent assay (ELISA) buffer	-	Dept. of Immunology

ELISA DuoSet: capture antibodies, detection antibodies, standards and Streptavidin-HRP		
- CCL3	328926	R&D Systems
- GM-CSF	1367359	R&D Systems
- IFN-γ	-	R&D Systems
- IL-8	-	R&D Systems
- IL-10	-329864	R&D Systems
- IL-13	-	R&D Systems
- IL-17	324878	R&D Systems
- TNF-α	1276651	R&D Systems
Fetal bovine serum (FBS)	41F9011K	Gibco, Invitrogen
Formalin solution 10% v/v	0614206001	J.T. Baker
Histopaque-1077	RNBD6320	Sigma-Aldrich
Magnetic activated cell sorting (MACS) buffer	-	Dept. of Immunology
Nitrogen gas (N_2)	-	ÍSAGA
NK cell isolation kit **- NK cell Biotin antibody cocktail** **- NK cell Microbead cocktail**	5151210353	Miltenyi Biotec
Normal goat serum	-	AbD Serotec
Normal human serum:Normal mouse serum (NHS:NMS) 1:1	-	AbD Serotec
Penicillin:Streptomycin (Pen:Strep) 10.000 U/ml:10.000 µg/ml	1377475	Gibco, Life Technologies
Phosphate buffered saline (PBS)	-	Dept. of Immunology
Potassium chloride (KCl)	80520	Sigma-Aldrich
Potassium dihydrogen phosphate (KH_2PO_4)	304 A707573	Merck
RPMI culture medium	-	Dept. of Immunology
RPMI 1640 [+]L-Glutamine	1667127	Gibco, Life Technologies
Sodium azide (NaN_3)	BCBJ4642V	Sigma-Aldrich
Sodium chloride (NaCl)	SZBF0919V	Sigma-Aldrich

Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \bullet 2H_2O$)	SZBA3290V	Sigma-Aldrich
Substrate solution; 3,3´,5,5´-Tetramethylbenzidine (TMB)	120611	Kem-En-Tec Diagnostics
Sucrose	011M00421V	Sigma-Aldrich
Sulfuric acid 0,5 M (H_2SO_4)	0063	EliA, Thermo Fisher Scientific
Staining buffer	-	Dept. of Immunology
Stop solution	-	Dept. of Immunology
Tris-buffered saline (TBS)	-	Dept. of Immunology
Tris ELISA buffer	-	Dept. of Immunology
Trizma base	088K5413	Sigma-Aldrich
Trypan blue 0.4%	RNBC1670	Sigma-Aldrich
Trypan blue 0.4% (for use in Countess)	1700609	Life technologies
Tween 20	SZBA3190V	Sigma-Aldrich
Wash buffer	-	Dept. of Immunology
1% Paraformaldehyde in PBS	-	Dept. of Immunology
20 mM EDTA	-	Dept. of Immunology
20% BSA in PBS	-	Dept. of Immunology

3.1.1 Recipes for solutions and buffers

Recipes for solutions and buffers prepared at the Dept. of Immunology are shown in table 2.

Table 2: Recipes for solutions prepared at the Dept. of immunology

Solution	Formulation
Blocking solution (1% BSA, 5% sucrose, 0.05% NaN_3 in PBS)	5 g BSA 25 g sucrose 0.25 g NaN_3 500 ml PBS keep refrigerated
Enzyme-linked immunosorbent assay (ELISA) buffer (1% BSA in PBS)	2 g BSA 200 ml PBS keep refrigerated

Magnetic activated cell sorting (MACS) buffer (PBS with 0.5% BSA, 2 mM EDTA)	440 ml PBS, sterile 12.5 ml 20% BSA in PBS, filtered 50 ml 20 mM EDTA, sterile keep refrigerated
Phosphate-buffered saline (PBS) pH 7.4	40 g NaCl 5.75 g Na_2HPO_4 (or 7.2 g Na_2HPO_4 x $2H_2O$) 1.0 g KH_2PO_4 1.0 g KCl dH_2O ad 5 l autoclave if to be used for cell culture
RPMI medium	RPMI 1640 [+]L-Glutamine (500 ml flask) add 50 ml of FBS (10%) and 5 ml of Pen Strep keep refrigerated
Staining buffer (PBS with 0.5% BSA, 2mM EDTA, 0.1% NaN_3)	440 ml PBS 12.5 ml 20% BSA in PBS, filtered 50 ml 20 mM EDTA, sterile 0.5 g NaN_3 keep refrigerated
Stop solution (0.18 M Sulfuric acid)	20 ml Sulfuric acid 0,5 M 35.5 ml dH_2O
Tris-buffered saline (TBS) pH 7.3	2.4 g Trizma base (20 mM) 8.7 g NaCl (150 mM) dH_2O ad 1 l adjust pH to 7.3 with HCl
TRIS ELISA buffer (0.1% BSA, 0.05% Tween 20 in TBS)	0.2 g BSA 0.1 ml Tween 20 200 ml TBS pH 7.3 keep refrigerated
Wash buffer (PBS with 0.05% Tween-20)	40 g NaCl 5.75 g Na_2HPO_4 (or 7.2 g Na_2HPO_4 x $2H_2O$) 1.0 g KH_2PO_4

	1.0 g KCl dH_2O ad 5 l 2.5 ml Tween-20
1% Paraformaldehyde in PBS	20 ml Formalin solution 10% (v/v) 180 ml PBS
20 mM EDTA	3.72 g EDTA 500 ml PBS autoclaved keep at room temperature
20% BSA in PBS	40 g BSA fill up to 180 ml with PBS let stand overnight before filling up to 200 ml mark with PBS filter the solution with a 0.22 µm filter into four sterile 50 ml tubes keep refrigerated

3.2 Equipments

Following is a list of the principal equipments used.

Table 3: List of equipments used in the study

Equipment	Type	Manufacturer
Autoclave	AAC046	Astell Scientific
Automated cell counter	Countess	Invitrogen
Biological Safety Cabinet	NU-425 Class II	NuAire
Cell counting chamber	Neubauer Improved	Assistent
Cell counting chamber slide	Countess	Invitrogen
Cell culture plate; **96 well, U-bottom**	Nuclon Delta Surface (sterile)	Thermo Scientific
Centrifuge	Heraeus Multifuge 3SR+	Thermo Scientific
ELISA plate; 96 well, flat-bottom	Nunc, MaxiSorp	Thermo Scientific

ELISA plate sealing tape	Nunc, BarSeal	Thermo Scientific
Eppendorf tubes	0.5 ml; 1.5 ml	Sarstedt
Flow cytometer	Navios	Beckman Coulter
Flow cytometry tubes	5 ml	Sarstedt
Freezer; -80°C	Glacier NU-9668E	NuAire
Fume hood	KP-12	KEBO Healthcare Systems
MACS kit	MACS MultiStand; MidiMACS separator; 25 LS column	Miltenyi Biotec
Microplate photometer	Original multiskan EX	Thermo Electron Corp.
Microplate washer	Elx405	BioTek
Microplate stacker	BioStack 2WR	BioTek
Microscopes	Leitz Fluov.; DMLS; DMI3000B	Leica
Microscope camera	DFC450 C	Leica
Micro tube	2 ml	Sarstedt
Micro tube with cap	2 ml (sterile)	Sarstedt
Milligram balance	430-21	KERN
Mixer	Vortex-Genie 2	Scientific Industries
Multichannel pipette	Finnpipette: 5-50 µl; 50-300 µl	Labsystems
Nitrogen evaporator	Reacti-Vap III	Pierce
Pipet aid	Pipetboy acu	Integra Biosciences
Pipettes	Finnpipette: 0.5-10 µl; 5-40 µl; 40-200 µl; 200-1000 µl	Labsystems
Pipettes	Finnpipette: 5-50 µl; 10-100 µl; 20-200 µl; 100-1000 µl	Thermo Electron Corp.
Pipette tips	200 µl; 1000 µl	Sarstedt
Serological pipettes	10 ml; 25 ml (sterile)	Sarstedt
Steri-Cult CO_2 Incubator	HEPA Class 100	Thermo Electron Corp.
Transfer pipette	3.5 ml (sterile)	Sarstedt
Tube	15 ml (sterile)	BD Falcon
Tube	50 ml (sterile)	Sarstedt
Universal tube	25 ml (sterile)	Thermo Scientific
Water distiller	Distinction	Bibby Scientific

3.3 Methods

3.3.1 Isolation of NK cells

The isolation of NK cells from blood was performed in two steps. First, peripheral blood mononuclear cells (PBMCs) were isolated from supplied buffy coats by centrifugation on histopaque. Then, NK cells were isolated from the PBMCs by negative selection on magnetic activated cell sorting (MACS) columns. The isolation was performed under sterile conditions in a biological safety cabinet.

3.3.1.1 Isolation of PBMCs

PBMCs were isolated from buffy coats by centrifugation on histopaque, a ficoll density gradient. Briefly, a buffy coat was diluted 1:2 in phosphate buffered saline (PBS). Then, 11 ml of the diluted buffy coat were carefully placed with a serological pipette onto 10 ml of histopaque liquid in four universal tubes. The tubes were then centrifuged for 30 minutes at 400 g and 22°C, without brake. As red blood cells and granulocytes are denser than the histopaque, they travel to the bottom of the tube through the histopaque gradient while the PBMCs form a layer on top of it. The centrifugation thus resulted in the formation of four layers as shown in figure 4. The layer of PBMCs at the interface was collected with a transfer pipette and placed in a 50 ml tube.

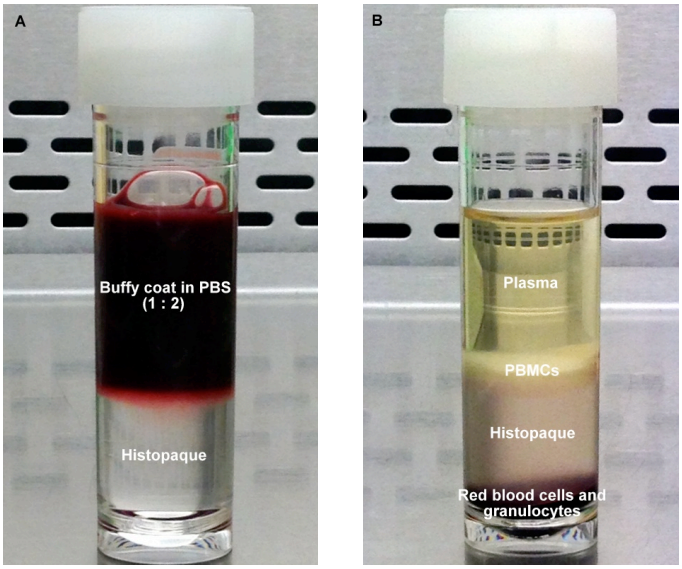

Figure 4: Isolation of PBMCs with centrifugation on histopaque, a ficoll density gradient

A buffy coat diluted in PBS was layered on a histopaque density gradient in a universal tube **(A)**. After centrifugation for 30 minutes at 400 g and 22°C without brake, four layers formed: Plasma at top, then PBMCs, histopaque and red blood cells and granulocytes at the bottom **(B).** The layer of PBMCs was collected with a transfer pipette.

The PBMCs were washed three times by filling to the mark of the 50 ml tube with MACS buffer, centrifuging at 300 g and 4°C for 10 minutes and discarding the supernatant. Before centrifuging for the third and last time, a sample of cell suspension was taken for cell counting. The sample was diluted 1:9 in 0.4% trypan blue and the cells counted in an Improved Neubauer cell counting chamber. After centrifuging and discarding the supernatant in the third wash, the PBMCs were diluted in MACS buffer to a desired concentration for the isolation of NK cells on MACS column.

3.3.1.2 Isolation of NK cells from PBMCs

NK cells were isolated from the PBMCs by negative selection on MACS columns where non-NK cells are indirectly magnetically labeled, i.e. labeled with a cocktail of biotin-conjugated antibodies against antigens not expressed by NK cells (NK cell Biotin-Antibody Cocktail) followed by a cocktail of MicroBeads (NK cell MicroBead Cocktail), and depleted by retaining them within the MACS column when placed in a strong magnetic field. NK cell isolation kit from Miltenyi Biotec was used according to the manufacturer's instructions. In short, the PBMCs were diluted in MACS buffer to a concentration of 25 x 10^7 cells/ml, equivalent to 1 x 10^7 cells per 40 µl. For every 40 µl of cell suspension, 10 µl of NK cell Biotin-Antibody Cocktail were added, mixed well and incubated on ice for 5 minutes. Then, 20 µl of NK cell MicroBead Cocktail were added for every 40 µl of cell suspension, mixed well and incubated for another 10 minutes on ice. The volume was then adjusted to 500 µl with MACS buffer, before the magnetic separation on the MACS column.

An LS MACS column was placed in a magnetic field and washed with 3 ml of MACS buffer. The PBMC suspension was placed on the column and the flow-through, containing the NK cells, collected in a 15 ml tube. Remaining NK cells were flushed trough the column by washing it two times with 3 ml of MACS buffer. The collected NK cells were then counted as described before, only now the cell suspension was diluted 1:1 before counting. Then, the cell suspension was centrifuged at 300 g and 4°C for 10 minutes and the supernatant discarded before diluting the NK cells to the desired concentration of 1 x 10^6 cells/ml for the cell culture. At this point two 100 µl samples were taken for flow cytometry purity check, described in chapter *3.3.5.2.*

3.3.2 Preparation of PUFAs

AA, EPA and DHA were supplied in ampules containing 500 µg of the given fatty acid dissolved in 1 ml ethanol. The contents of one ampule were transferred to a 1.5 ml eppendorf tube and the ethanol evaporated under nitrogen gas. Then, 20 µl of dimethyl sulfoxide (DMSO) were added to the tube and mixed well before pipetting 5 µl into each of four sterile 0.5 ml eppendorf tubes. Nitrogen gas was then blown into the tubes before they were closed and stored at -80°C

until used. Five µl aliquots of DMSO solvent alone were also prepared in 0.5 ml eppendorf tubes to be used as a control.

Fatty acids and DMSO were diluted in RPMI culture medium before use in cell culture and let stand for an hour, giving the fatty acids a chance to bind to BSA in the culture medium. The concentration of fatty acids after dilution in RPMI medium was 2000 µM. Five µl were added to appropriate wells in culture plates, into a final volume of 200 µl, for a final concentration of 50 µM of the fatty acids.

3.3.3 Optimization of NK cell culture conditions

Before studying the effects of EPA and DHA on NK cells *in vitro*, a suitable NK cell model was developed. After isolation, described in 3.3.1, NK cells were cultured at 37°C, 5.0% CO_2 and 95% humidity. The NK cells were cultured in RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum (FBS) and antibiotics. This medium has been used in prior studies with NK cells isolated from blood (Chiossone et al., 2007; Girart, Fuertes, Domaica, Rossi, & Zwirner, 2007; Tanaka, Porter, Horvath-Arcidiacono, & Bloom, 2007).

Different densities of NK cells in 96-well U-bottomed plates were tested. Cells were cultured with various concentrations of IL-2, IL-15 or IL-12, alone or in combinations, as these cytokines have been used for stimulating NK cell cytokine production in prior studies (Cooper, Fehniger, Turner, et al., 2001; Fehniger et al., 1999). Different duration of stimulation ranging from 24-96 h was also tested. Results from this screening for optimal NK cell stimulation can be seen in appendix A.

3.3.4 Culturing of NK cells with fatty acids

NK cells were seeded in a 96 well, U-bottomed culture plate, at 1 x 10^5 cells/well in RPMI culture medium. NK cells were cultured with or without 50 µM of AA, EPA or DHA, which had been dissolved in DMSO and diluted in culture medium. DMSO alone was used as control, clarification of control selection can be seen in appendix B. After 18 h of incubation with or without fatty acids, NK cells were stimulated with IL-15 (10 ng/ml) and IL-12 (2 ng/ml) for another 48 h before harvesting supernatants for measurements of cytokine and chemokine concentration by enzyme-linked immunosorbent assay (ELISA) (*3.3.5.3*). The supernatants were frozen at -80°C until assayed. The cells were also harvested as they were used in another project where their expression of receptors was studied.

3.3.5 Assays

Viability of NK cells cultured with or without fatty acids was assessed. Flow cytometry analysis was performed to evaluate the purity of the NK cell population isolated from buffy coats. ELISA was used to determine the concentration of cytokines and chemokines in supernatants.

3.3.5.1 Assessment of cell viability

Viability of NK cells, cultured with or without fatty acids, was assessed when cells and supernatants were harvested at the end of culture. Sample was taken and diluted 1:1 in 0.4% trypan blue stain and the cells counted in Countess®, an automated cell counter. As the trypan blue stains dead cells blue, the number of both dead and live cells could be counted.

3.3.5.2 Flow cytometry analysis: evaluation of NK cell purity

To evaluate the purity of the NK cell population after isolation, described in 3.3.1, cells were stained with phycoerythrin (PE) conjugated anti-CD56 antibodies and CD56 expression of the cells analysed using the Navios flow cytometer. Briefly, 100 µl of NK cell suspension from isolation (1 x 10^6 cells/ml) were put in two flow cytometry tubes; one for anti-CD56 PE staining and the other for staining with a PE conjugated mouse IgG_1 isotype control, used to account for non-specific Fc receptor binding. To block non-specific binding of antibodies to cell surfaces, 2% normal human serum:normal mouse serum (NHS:NMS) was added to the tubes and incubated on ice for 10 minutes. The appropriate volume of the antibodies was then added to the tubes, in accordance with the manufacturer's instructions, and incubated on ice for 20 minutes. Cells were then washed by adding approximately 2 ml of staining buffer to the tubes and centrifuging for 5 minutes at 1200 revolutions per minute (rpm) and 4°C. The supernatant was discarded and cells resuspended and fixed by adding 300 µl of 1% paraformaldehyde. The cells were then stored in dark at 4°C and data on forward scatter, side scatter and fluorescence emission collected on the Navios flow cytometer within a week. Twenty thousand events were collected per tube and Kaluza® 1.3 software used for data analysis. The data was presented as percentage of positive cells, as compared with cells stained with isotype control antibodies.

3.3.5.3 ELISA: determination of cytokine and chemokine concentration in supernatants

Sandwich ELISA can be used to measure analytes such as cytokines and chemokines in biological fluids. In this study, DuoSet® ELISA kits from R&D Systems were used according to the manufacturer's instructions, to measure the concentration of the cytokines and chemokines of interest. The principle of the assay can be seen in figure 5.

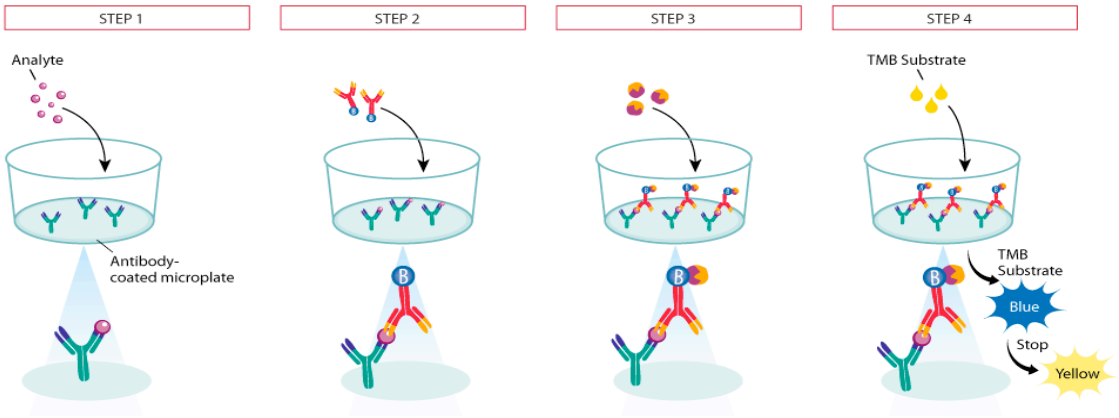

Figure 5: Principle of a DuoSet ® ELISA

In step 1, a microplate is coated with a capture antibody specific for the analyte to be measured. Unbound antibody is washed away and a protein containing blocking solution added to bind to uncovered area of the microplate wells before washing again. Standards of the analyte of interest and the samples to be assayed are added to the microplate and the analyte present allowed to bind to the capture antibody. In step 2, unbound material is washed away before adding a biotinylated detection antibody specific for the analyte. Again, unbound material is washed away before adding Streptavidin-Horseradish Peroxidase (HRP) in step 3, which binds to the biotin on the detection antibody. Unbound Streptavidin-HRP is washed away and 3,3´,5,5´-Tetramethylbenzidine (TMB) substrate solution added in step 4, developing a blue color in proportion to the amount of analyte originally present in each sample. Stop solution is added when considerable blue color has developed, which turns it to yellow. The optical density (OD) of the yellow color is measured in a microplate photoreader at 450 nm. Figure adapted from the R&D Systems website (R&D, 2016).

The concentration in pg/ml of TNF-α, GM-CSF, IFN-γ, IL-17, IL-13, IL-10, CCL3 and IL-8 in supernatants harvested from NK cells after 18 h incubation with or without 50 µM AA, EPA or DHA followed by stimulation with IL-15 (10 ng/ml) and IL-12 (2 ng/ml) was determined.

Corresponding to step one (figure 5), a 96 well flat bottomed ELISA microplate was coated with capture antibody diluted in PBS. 100 µl were put in each well and the plate incubated at room temperature overnight. The following day, the solution was discarded from the plate and 300 µl of blocking solution applied to each well. After one hour of incubation at room temperature the plate was washed four times with wash buffer in a microplate washer. A standard of the cytokine to be assayed was diluted in the appropriate buffer and applied to the plate in two-fold dilutions to make a standard curve. 100 µl of supernatant samples, undiluted or diluted in buffer, were added to the plate in duplicate according to scheme (see appendix E). The dilution of samples differed according to which cytokine or chemokine was being measured, see table 4. The blank for each plate was buffer without a sample. When measuring TNF-α, GM-CSF, IL-13, IL-17, IL-10 and CCL3 an ELISA buffer was used but a Tris-ELISA buffer in the case of IFN-γ and IL-8.

After incubation at room temperature for two hours, the plate was washed four times as before (step 2, figure 5). Then, detection antibodies were diluted in buffer and 100 µl added to each well. Again, the plate was incubated at room temperature for two hours and then washed four times. Then, 100 µl of Streptavidin-Horseradish Peroxidase (HRP) diluted in buffer were added to each well in step 3 (figure 5) and the plate incubated at room temperature for 20 minutes away from light before washing four times. In step 4, 100 µl of substrate solution were added to each well and the plate kept away from light as blue color developed. When considerable blue color had developed, 50 µl of sulfuric acid stop solution were applied to each well; turning the color to yellow before reading the optical density (OD) at 450 nm in an Original multiskan EX microplate photometer. Ascent Software Version 2.6 was used when operating the photometer and analysing the data.

Table 4: Dilution of supernatant samples for different ELISAs

Cytokines	Dilution
IFN-γ; TNF-α	1:1
CCL3; GM-CSF; IL-8;IL-10; IL-13; IL-17	Undiluted

3.3.6 Statistical analysis

The results for the cytokine and chemokine secretion by NK cells are presented as secretion index (SI), i.e. the concentration of cytokine or chemokine in supernatants from NK cells cultured with fatty acids divided by the concentration of cytokine or chemokine in supernatants from NK cells cultured without fatty acids, to take into account the variability in the basal cytokine secretion between donors. Results represent the mean ± standard error of the mean (SEM) of 5 or more donors for each cytokine or chemokine, calculated in Microsoft Excel. SigmaStat 3.1 and GraphPad Prism softwares were used to analyse differences between treatments using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Differences were considered statistically significant if $p < 0.05$.

4 RESULTS

4.1 The effects of fatty acids on cell viability

Viability of NK cells, cultured with fatty acids dissolved in DMSO or DMSO alone in the same concentration as the fatty acids were dissolved in (control), was assessed using 0.4% trypan blue stain and Countess® automated cell counter. Culturing NK cells with 50 µM of the fatty acids, AA, EPA or DHA, did not affect the viability of the cells (figure 6). Raw data is shown in appendix C.

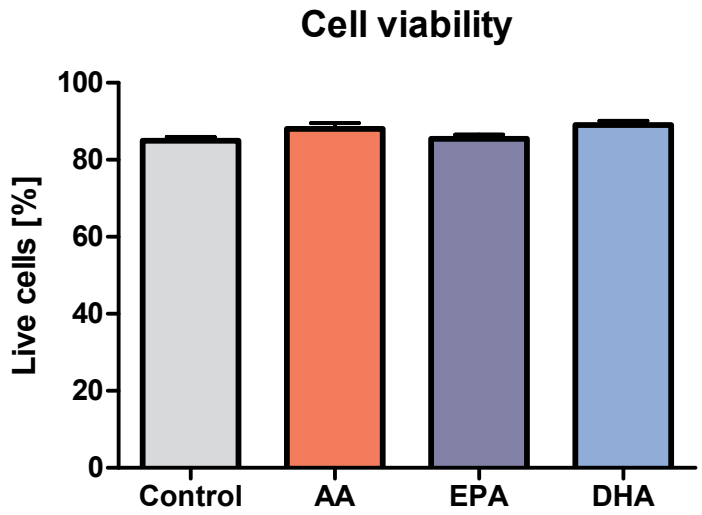

Figure 6: Cell viability

NK cells were cultured without fatty acid (control) or with 50 µM of AA, EPA or DHA for 18 h and then stimulated with IL-15 (10 ng/ml) and IL-12 (2 ng/ml) for another 48 h. Viability of the cells at the end of culture was assessed using 0.4% trypan blue stain and Countess® automated cell counter. Results are presented as the mean percentage of live cells ± SEM, n = 8. Statistical significance was determined using one-way ANOVA with Bonferroni post-hoc test. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

4.2 Purity of NK cell population after isolation

The purity of the NK cell population after isolation (at 0 h) was determined by staining the cells with fluorescent antibody against CD56 and determining the proportion of the cells that expressed this NK cell specific surface marker using flow cytometric analysis. The proportion of cells expressing CD56 after isolation is shown for each donor in figure 7. On average 84% of the cells expressed CD56. Raw data is shown in appendix D.

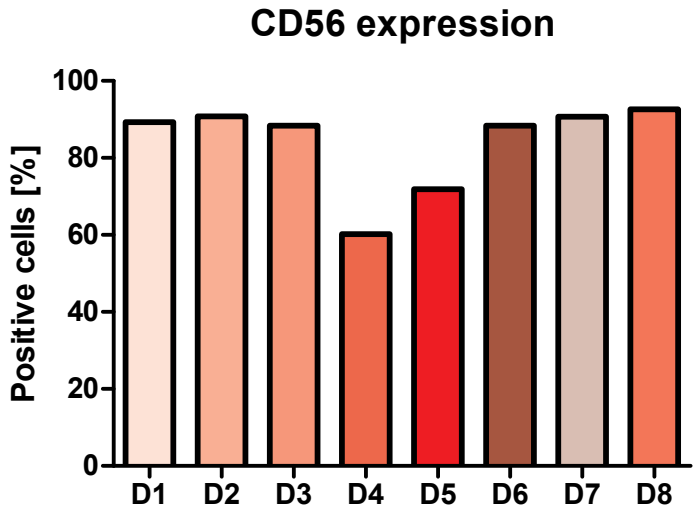

Figure 7: Proportion of cells expressing CD56 after isolation

Cell population obtained by negative selection for NK cells on MACS columns was stained with PE conjugated anti-CD56 antibodies at 0 h and the proportion of the cells expressing this NK cell specific surface marker determined. Analysis was performed on Navios flow cytometer. The proportion of cells expressing CD56 after isolation is shown for each donor. D, donor.

4.3 The effects of AA, EPA and DHA on cytokine secretion by NK cells

The concentration of cytokines and chemokines in NK cell culture supernatants, harvested after 18 h incubation with or without 50 µM AA, EPA or DHA followed by stimulation with IL-15 (10 ng/ml) and IL-12 (2 ng/ml) for another 48 h, was determined using sandwich ELISA. In some cases the OD values were below or above the lowest or highest standard in the standard curve, respectively, and were omitted. However, some exceptions were made in the case of determination of IL-13 concentration, see appendix E.

The results are expressed as SI, which is the concentration of cytokine or chemokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of the cytokine or chemokine secreted by NK cells cultured without fatty acid (control).

4.3.1 The effects of AA, EPA and DHA on secretion of type 1 and type 17 cytokines

Figures 8 – 10 show the effects of the fatty acids, AA, EPA and DHA, on NK cell secretion of the type 1 cytokines TNF-α, GM-CSF and IFN-γ. The type 17 cytokine IL-17 was not detectable by ELISA. Raw data is shown in appendix E.

NK cells cultured with 50 µM EPA or DHA secreted less TNF-α than NK cells cultured without fatty acids (figure 8). The concentration of TNF-α in the control was 110 ± 14 pg/ml. Culturing the NK cells with AA did not affect their secretion of TNF-α.

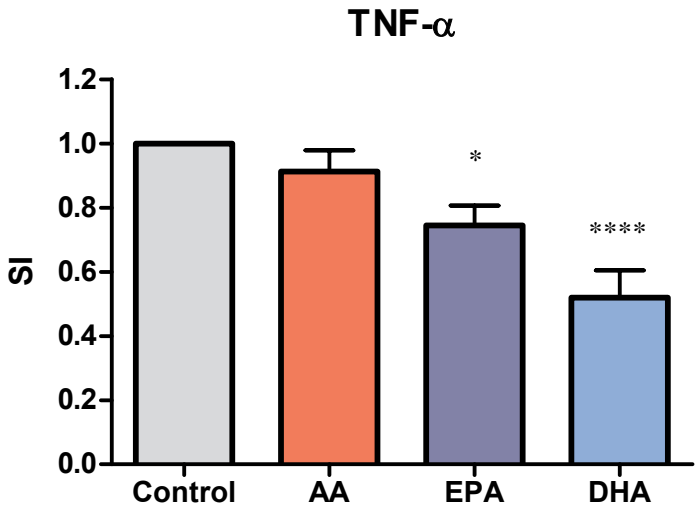

Figure 8: The effects of AA, EPA and DHA on TNF-α secretion by NK cells

TNF-α secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 7 for control and EPA, n = 5 for AA and n = 6 for DHA. Difference between TNF-α secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test; *, $p<0.05$; ****, $p<0.0001$. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SI, secretion index; TNF-α, tumor necrosis factor-α.

NK cells cultured with 50 µM DHA secreted less GM-CSF than NK cells cultured without fatty acids (figure 9). The concentration of GM-CSF in the control was 134 ± 41 pg/ml. AA and EPA did not affect the GM-CSF secretion by NK cells.

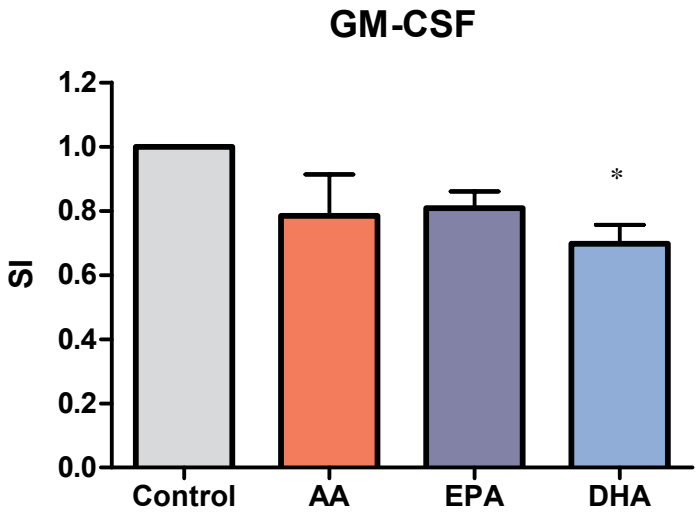

Figure 9: The effects of AA, EPA and DHA on GM-CSF secretion by NK cells

GM-CSF secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 9 for control and EPA, n = 8 for AA and DHA. Difference between GM-CSF secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test; ∗, $p<0.05$. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; SI, secretion index.

There was a tendency towards less IFN-γ secretion by NK cells cultured with 50 µM AA compared with NK cells cultured without fatty acids, but the difference was not statistically significant (figure 10). Culturing NK cells with 50 µM EPA or DHA did not affect their secretion of IFN-γ. The concentration of IFN-γ in the control was 1014 ± 230 pg/ml.

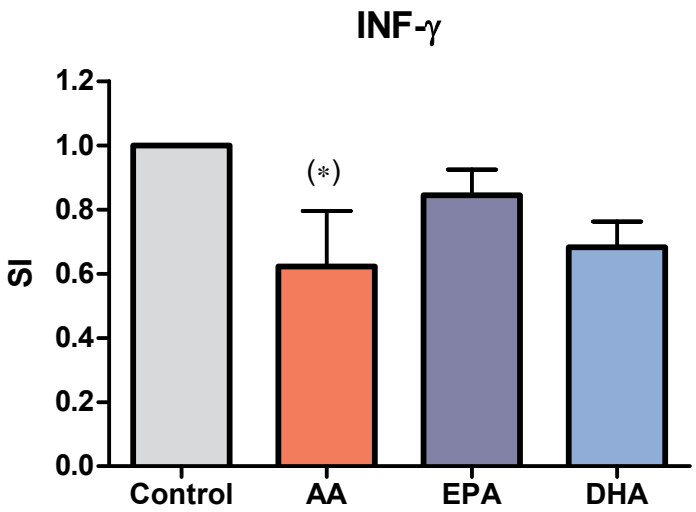

Figure 10: The effects of AA, EPA and DHA on IFN-γ secretion by NK cells

IFN-γ secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 7 for control, EPA and DHA, n = 6 for AA. Difference between IFN-γ secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test; (*), $p<0.1$. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IFN-γ, interferon-γ; SI, secretion index.

4.3.2 The effects of AA, EPA and DHA on secretion of type 2 cytokines

Figures 11 and 12 show the effects of the fatty acids, AA, EPA and DHA, on NK cell secretion of the type 2 cytokines IL-13 and IL-10. Raw data can be seen in appendix E.

NK cells cultured with 50 µM EPA or DHA secreted less IL-13 than NK cells cultured without fatty acids (figure 11). The concentration of IL-13 in the control was 234 ± 41 pg/ml. AA did not affect the IL-13 secretion by NK cells.

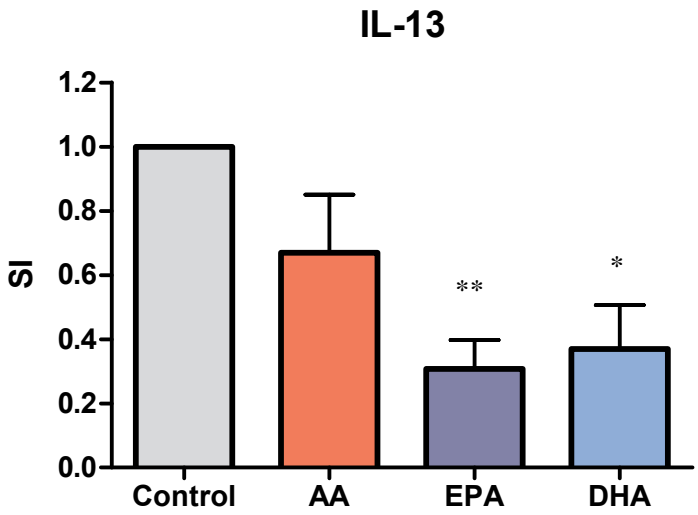

Figure 11: The effects of AA, EPA and DHA on IL-13 secretion by NK cells

IL-13 secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 5 for all treatments. Difference between IL-13 secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test; ∗, $p<0.05$; ∗∗, $p<0.01$. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-13, interleukin 13; SI, secretion index.

There was no difference in secretion of IL-10 between NK cells cultured without fatty acids (control) or with 50 µM AA, EPA or DHA (figure 12). The concentration of IL-10 in the control was 74 ± 4 pg/ml.

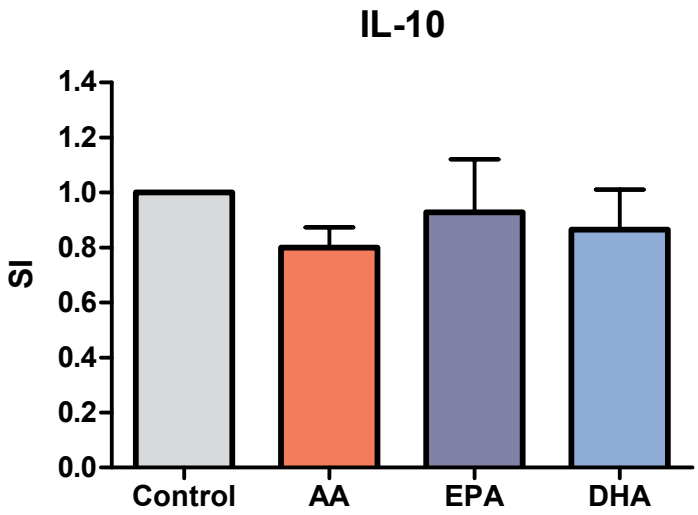

Figure 12: The effects of AA, EPA and DHA on IL-10 secretion by NK cells

IL-10 secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 5 for all treatments. Difference between IL-10 secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-1, interleukin 10; SI, secretion index.

4.3.3 The effects of AA, EPA and DHA on secretion of chemokines

The effects of AA, EPA and DHA, on NK cell secretion of two chemokines was examined. The effects of the fatty acids on CCL3 secretion can be seen in figure 13. The concentration of IL-8, however, was not detectable by ELISA in the NK cell supernatants; see appendix E for raw data.

There was no difference in CCL3 secretion by NK cells cultured without fatty acids (control) or with 50 µM AA, EPA or DHA (figure 13). The concentration of CCL3 in the control was 63 ± 13 pg/ml.

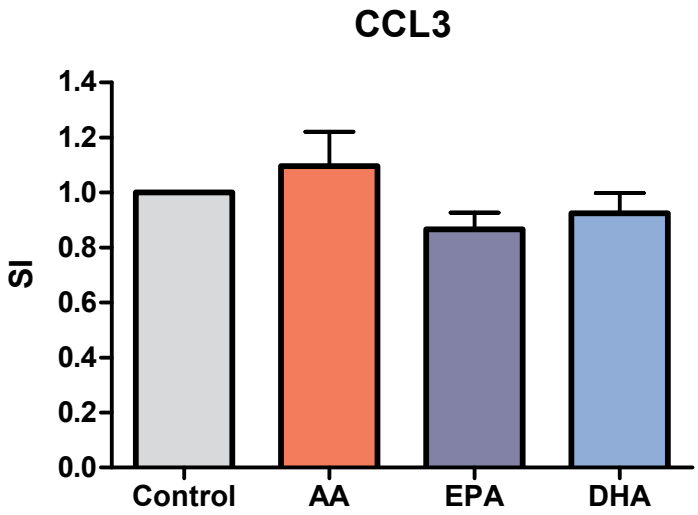

Figure 13: The effects of AA, EPA and DHA on CCL3 secretion by NK cells

CCL3 secretion by NK cells cultured without fatty acids (control) or with AA, EPA or DHA was measured by ELISA. The results are expressed as SI, i.e. the concentration of cytokine secreted by NK cells cultured with 50 µM of AA, EPA or DHA divided by the concentration of cytokine secreted by NK cells cultured without fatty acids (control). Results are presented as mean ± SEM, n = 7 for control and EPA, n = 6 for AA, and DHA. Difference between CCL3 secretion by NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; CCL3, CC chemokine ligand 3; SI, secretion index.

5 DISCUSSION

The results from this study show that the omega-3 fatty acids EPA and DHA decreased secretion of the type 1 cytokine TNF-α and the type 2 cytokine IL-13 when included in *in vitro* culture of NK cells, compared with secretion of these cytokines by NK cells cultured without fatty acids. DHA additionally decreased secretion of the cytokine and growth factor GM-CSF. EPA and DHA did not affect secretion of the type 1 cytokine IFN-γ, the type 2 cytokine IL-10 or the chemokine CCL3. This indicates that the effects by these fatty acids on the other cytokines were not caused merely by some unspecific or general decrease in cytokine secretion by the cells. Nor was the decrease in cytokine secretion restricted to either type 1 or type 2 cytokines. Culturing NK cells with the omega-6 fatty acid AA did not affect their secretion of the cytokines tested for. No conclusions can be draw from the present study on possible effects of fatty acids on IL-8 and IL-17 secretion by NK cells as those cytokines were undetectable in the NK cell supernatants.

In the resolution of inflammation some of the key steps include sequestration of pro-inflammatory cytokines, apoptosis of neutrophils and their efferocytosis by macrophages, in which lipid mediators including omega-3 derived SPMs are important signaling molecules (Buckley, Gilroy, & Serhan, 2014; Serhan, 2014). Various inflammatory cytokines, including TNF-α, IFN-γ, GM-CSF and IL-8, have been shown to enhance neutrophil survival and an *in vitro* study showed that TNF-α, IFN-γ and GM-CSF produced by cytokine-stimulated NK cells play a key role in delay of neutrophil apoptosis and retention of their function (Bhatnagar et al., 2010). Decrease of TNF-α secretion by NK cells cultured with EPA or DHA in the present study, and an additional decrease of GM-CSF secretion by cell cultured with DHA, thus might play a role in increasing resolution of inflammation by enhancing neutrophil apoptosis. The omega-6 fatty acid AA did not affect the cytokine secretion by NK cells but there was a tendency towards less IFN-γ secretion by NK cells cultured with AA. That AA may be able to decrease IFN-γ is not surprising as AA is a substrate for PGE_2 production and PGE_2 is known to inhibit IFN-γ secretion from Th1 cells (Betz & Fox, 1991).

To my knowledge, there have been no *in vitro* studies determining the effects of omega-3 fatty acids alone on the secretion of cytokines by NK cells. However, a previous study showed that an emulsion of omega-3 fatty acids and anti-oxidants did not affect IFN-γ production by NK cells in a co-culture with cancer cells (Halder et al., 2015). This is in accordance with the results of the present study where the omega-3 fatty acids EPA and DHA did not affect IFN-γ production by NK cells in culture. In contrast, culturing human blood lymphocytes with up to 20 µg/ml (equal to around 60 µM) of DHA increased IFN-γ production by the lymphocytes while EPA or the omega-6 fatty acid GLA had no effects (Purasiri et al., 1997). In the same study there was a decrease in TNF-α concentration in lymphocyte supernatants following exposure to EPA, DHA or GLA, which is in accordance with the results of the present study where EPA and

DHA decreased TNF-α secretion by NK cells. However, in the study by Purasiri et al. (1997) the fatty acids did not affect production of the type 2 cytokine IL-4 by the lymphocytes which is in contrast to EPA and DHA decreasing secretion of the type 2 cytokine IL-13 in the present study. In this context it should be kept in mind that NK cells comprise just about 10% of human peripheral blood lymphocytes (Mandal & Viswanathan, 2015).

The NK cells were stimulated in the culture in order to induce cytokine secretion as occurs in inflammation. $CD56^{bright}$ NK cells readily produce cytokines when stimulated with IL-12, IL-15 and IL-18 (Cooper et al., 2001). However, the optimal combination of these cytokines seems to be different for the production of different cytokines. For example, stimulation by IL-12 and IL-18 induces high levels of IFN-γ while stimulation by combinations of cytokines including IL-15 are appropriate for IL-13 production and stimulation by IL-12 with IL-15 or IL-2 can induce IL-10 secretion (Cooper et al., 2001; Fehniger et al, 1999; Wolk et al, 2002). Then again, IL-15 has been shown to inhibit production of IL-17 by NK cells in toxoplasmosis while e.g. IL-6 is important for the production (Passos et al., 2010). In the present study, stimulation of the NK cells with IL-12 and IL-15 led to considerably lower average concentration of IFN-γ (1 ng/ml), IL-10 (74 pg/ml), GM-CSF (134 pg/ml) and TNF-α (110 pg/ml) in control supernatants compared with the approximate concentration of IFN-γ (100 ng/ml), IL-10 (1600 pg/ml), GM-CSF (800 pg/ml) and TNF-α (less than 300 pg/ml) in supernatants of $CD56^{bright}$ NK cells stimulated with the same cytokines in a study by Cooper et al. (2001). In that same study, $CD56^{dim}$ NK cells produced considerably lower levels of these cytokines. $CD56^{bright}$ NK cells are thought to be the primary source of NK cell derived cytokines in response to stimulation by cytokines but $CD56^{dim}$ NK cells have been shown to produce cytokines and chemokines upon target cell recognition (Fauriat, Long, Ljunggren, & Bryceson, 2010). Relatively low concentration of the cytokines mentioned in the present study might be due to low percentage of $CD56^{bright}$ NK cells of the total NK cells isolated from peripheral blood.

It is known that high concentrations of fatty acids may affect the viability of cells due to detergent effects (Purasiri et al., 1997). Therefore, the viability of the NK cells was assessed at the end of the culture to make sure that the fatty acids were not killing the cells and thereby decreasing the amount of cytokines secreted. The results showed that the fatty acids did not affect the viability of the NK cells, compared with that when they were cultured with the vehicle in which the fatty acids were solubilized, i.e. DMSO. This demonstrates that the effect of the fatty acids on the cytokine secretion by the NK cells was not due to effects on cell viability.

In some previous studies the fatty acids were complexed with BSA prior to adding them to the cell culture to avoid possible toxic effects of the free fatty acids and decrease the risk of them affecting the cell viability. This was done for example in a study on macrophages *in vitro* (Calder, Bond, Harvey, Gordon, & Newsholme, 1990). On the other hand, in studies on human T cell lines, dendritic cells and human monocytes, EPA and DHA were added to cultures in an ethanol vehicle (Hughes & Pinder, 1997; Stulnig et al., 2001; Wang et al., 2007) and in a study

on THP-1 macrophages the cells were treated with EPA and DHA delivered in a DMSO vehicle (Weldon et al., 2007). In the present study, the fatty acids were dissolved in DMSO but prior to adding them to the cell culture they were diluted in culture medium containing serum, which again contains albumin. As they did not affect the viability of the cells it can be assumed that there were no toxic effects of the fatty acids on the cells.

The final concentration of the fatty acids was 50 μM. Similar concentration was used in a study of the effects of omega-3 and omega-6 PUFAs on LPS-induced TNF-α and IL-10 secretion by murine peritoneal macrophages, i.e. cells were cultured in the presence or absence of 10-50 μM of LA, AA, EPA or DHA for 4-24 h (Skuladottir, Petursdottir, & Hardardottir, 2007). Wang et al. (2007) similarly added either 50 μM of EPA or DHA to cell cultures with dendritic cells and incubated for 24 h before adding LPS to induce cell maturation. In Calder et al. (1990) fatty acids were kept in culture for 48 h with the final concentration of each fatty acid being 33 μM. That was stated to be a physiological concentration as the total plasma free fatty acid concentration in the fed state is approximately 0.3 mM and the individual concentrations of major fatty acids therefore thought to be in the range of 30-130 μM. The concentration of fatty acids and the incubation time in the present study thus seem to have been appropriate and within a physiological range.

It must be kept in mind that *in vitro* studies do not reflect what happens *in vivo*, where interplay between various cells and mediators occurs. In the present study NK cell were isolated from PBMCs and cultured in wells, which are considerably different from the natural environment of the cells. As can be seen by the percentage of cells expressing CD56 after isolation, there were cells other than NK cells in the culture which may have contributed to or affected the cytokine concentration in the culture. It is, however, hard to pinpoint the exact type of these contaminating cells as the NK cell population was isolated by negative selection. Another point to consider regarding the cell culture is the stimulation of the cells to produce cytokines but NK cells can secrete different cytokines and chemokines upon stimulation with different cytokines. Here IL-12 and IL-15, cytokines which activated macrophages are known to produce upon infection, were added to the culture for stimulation. It is questionable how this stimulation resembles the complex inflammatory milieu *in vivo*. Despite shortcomings like these, *in vitro* experiments have their advantages. For example, they are relatively inexpensive compared with *in vivo* studies, conditions can be carefully controlled, repeatability is good and they may give some prediction of how a specific cell type may be affected by a particular treatment *in vivo*.

In this study the aim was to investigate the effects of fatty acids on NK cells in connection with resolution of inflammation. In a previous study by the research group, dietary omega-3 PUFAs led to an increase in NK cell count in the peritoneum early in the inflammation as well as an early peak in concentration of the immunosuppressive mediator transforming growth factor-β (TGF-β) (Tomasdottir et al., 2013). Increased TGF-β in the resolution phase of murine peritonitis

suggest that it plays a role in resolution of acute inflammation (Bannenberg et al., 2005). In the light of this it might have been interesting to determine NK cell secretion of TGF-β. However, this was not feasible as serum was included in the culture medium and serum is abundant in TGF-β. The present study was, however, successful in elucidating the effects of fatty acids on the secretion of various cytokines by NK cells *in vitro.* Various inflammatory cytokines, including TNF-α and GM-CSF, have been shown to enhance neutrophil survival (Bhatnagar et al., 2010). It would thus have been interesting to co-culture the NK cells with neutrophils to see if the fatty acids affected their apoptosis but human NK cells have previously been shown to induce neutrophil apoptosis *in vitro* by mechanisms dependent on cell-cell contact (Thoren et al., 2012).

6 CONCLUSIONS

The results of the study indicate that the omega-3 fatty acids EPA and DHA affect the activity of human NK cells, namely their secretion of cytokines *in vitro*. EPA and DHA decreased secretion of the pro-inflammatory cytokine TNF-α by NK cells and DHA additionally decreased secretion of GM-CSF, compared with NK cells cultured without fatty acids. This might be contributive to the effects that omega-3 fatty acids have been shown to have on the resolution of inflammation, as these cytokines are known to delay neutrophil apoptosis.

The next step would be to investigate whether the decrease in secretion of these cytokines by NK cells can indeed affect neutrophil apoptosis, by co-culturing NK cells with neutrophils or alternatively, culturing neutrophils with supernatants from activated NK cells.

Studies aiming at further elucidation and understanding of the effects and mode of action of omega-3 fatty acids on inflammation resolution might include determination of resolving lipid mediators and TGF-β production by NK cells *in vitro*.

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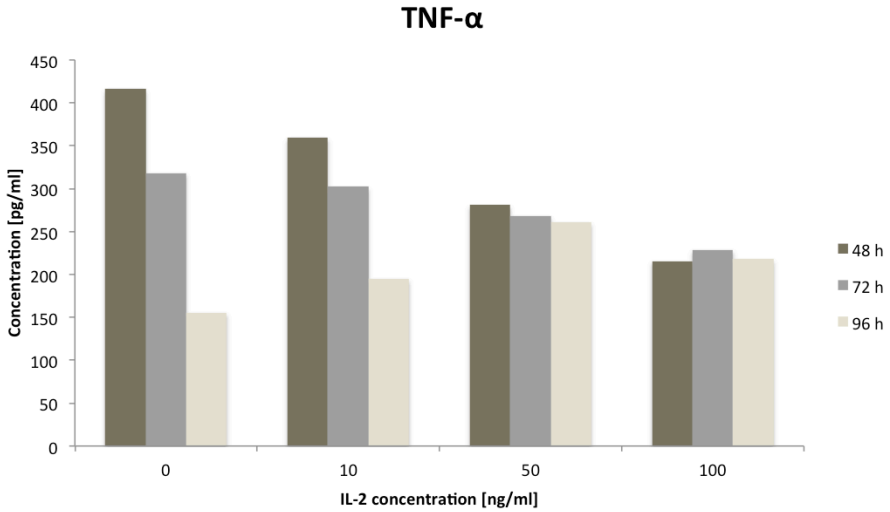
9 APPENDIXES

APPENDIX A

Optimization of NK cell culture conditions

Before studying the effects of AA, EPA and DHA on primary NK cells *in vitro*, a suitable NK cell model was developed. Production of IFN-γ and TNF-α cytokines, measured by ELISA on NK cell supernatants, was used as an indicator of suitable culture conditions.

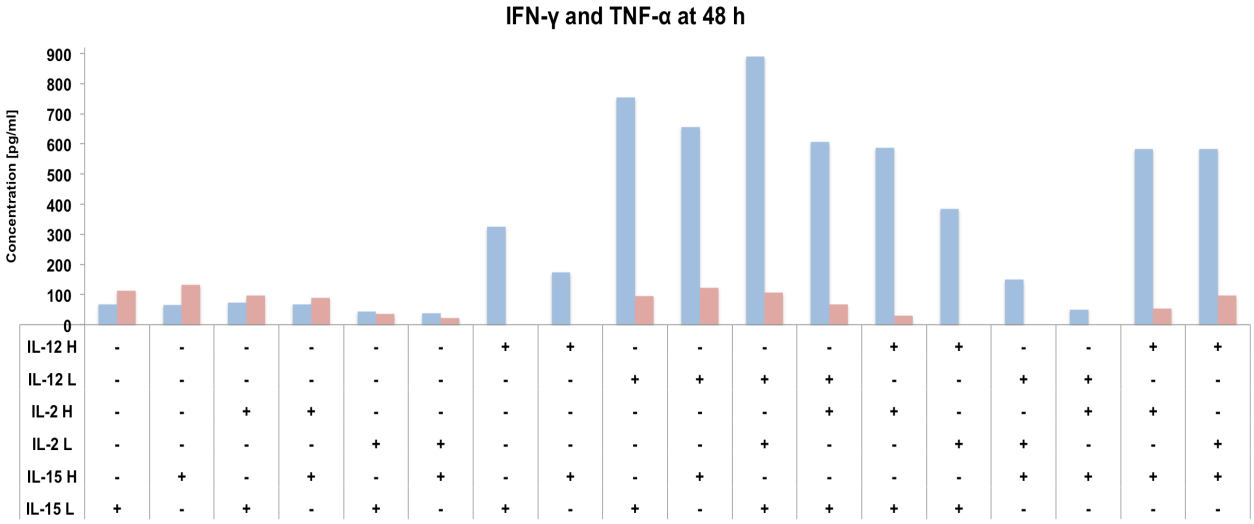
Three different experiments were performed, starting with two donors each time, where NK cells were seeded in a 96 well culture plate at 5 x 10^4 cells/well. In the first experiment, different duration of culture (48 h; 72 h; 96 h) with different concentrations of IL-2 for stimulation (0 ng/ml; 10 ng/ml; 50 ng/ml; 100 ng/ml) were tested. In the second experiment, different duration of culture (24 h; 48 h; 72 h) with different concentrations and combinations of IL-2 (10 ng/ml; 50 ng/ml) and IL-12 (2 ng/ml; 10 ng/ml) for stimulation were tested. The results of the first experiment hinted that 100 ng/ml of IL-2 did not induce greater TNF-α production than 10 ng/ml or 50 ng/ml. IFN-γ was unmeasurable. In addition, the two experiments hinted that a stimulation duration of 48 h or 72 h was more suitable than 24 h or 96 h. Data from the second experiment is not shown.



TNF-α secretion by NK cells stimulated with different concentrations of IL-2 for 48, 72 and 96 h

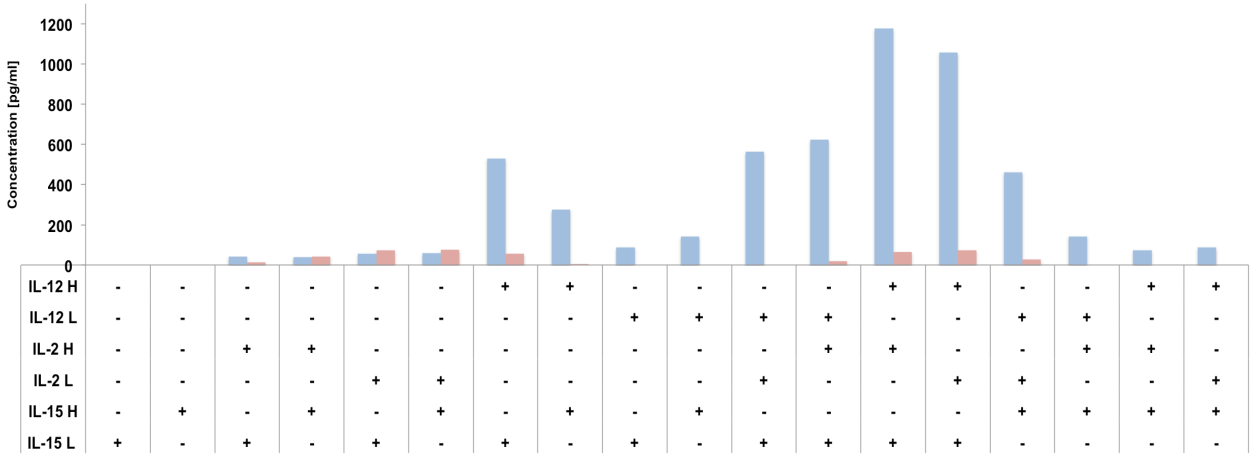
NK cells were seeded in 96 well culture plates at 5 x 10^4 cells/well. The cells were stimulated with different concentrations of IL-2 (0 ng/ml; 10 ng/ml; 50 ng/ml; 100 ng/ml) for 48 h (dark grey), 72 h (grey) or 96 h (beige) and TNF-α in the supernatants determined by ELISA, n = 2. IL-2, interleukin 2; TNF-α, tumor necrosis factor α.

In the third experiment, different duration of culture (48 h; 72 h) with different concentrations and combinations of IL-2 (10 ng/ml; 50 ng/ml), IL-12 (2 ng/ml; 10 ng/ml) and IL-15 (10 ng/ml; 50 ng/ml) for stimulation were tested.



IFN-γ and TNF-α secretion by NK cells stimulated with different combinations of IL-2, IL-12 and IL-15 for 48 h

NK cells were seeded in 96 well culture plates at 5 x 10^4 cells/well. The cells were stimulated for 48 h with different combinations of IL-2, IL-12 and IL-15 and the concentration of IFN-γ (blue) and TNF-α (red) in the supernatants determined by ELISA, n = 2 for IFN-γ and n = 1 for TNF-α. IFN-γ, interferon γ; IL-12 H, interleukin 12 higher concentration (10 ng/ml); IL-12 L, interleukin 12 lower concentration (2 ng/ml); IL-2 H, interleukin 2 higher concentration (50 ng/ml); IL-2 L, interleukin 2 lower concentration (10 ng/ml); IL-15 H, interleukin 15 higher concentration (50 ng/ml); IL-15 L, interleukin 15 lower concentration (10 ng/ml); TNF-α, tumor necrosis factor α.



IFN-γ and TNF-α secretion by NK cells stimulated with different combinations of IL-2, IL-12 and IL-15 for 72 h

NK cells were seeded in 96 well culture plates at 5 x 10^4 cells/well. The cells were stimulated for 72 h with different combinations of IL-2, IL-12 and IL-15 and the concentration of IFN-γ (blue) and TNF-α (red) in the supernatants determined by ELISA, n = 2 for IFN-γ and n = 1 for TNF-α. IFN-γ, interferon γ; IL-12 H, interleukin 12 higher concentration (10 ng/ml); IL-12 L, interleukin 12 lower concentration (2 ng/ml); IL-2 H, interleukin 2 higher concentration (50 ng/ml); IL-2 L, interleukin 2 lower concentration (10 ng/ml); IL-15 H, interleukin 15 higher concentration (50 ng/ml); IL-15 L, interleukin 15 lower concentration (10 ng/ml); TNF-α, tumor necrosis factor α.

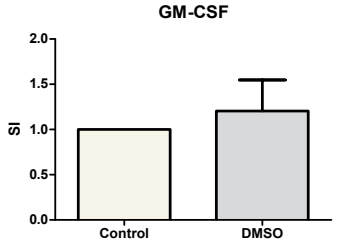
Concentrations of GM-CSF, IL-8 and IL-10 were measured in supernatants from NK cells stimulated with combinations of IL-2, IL-15 and IL-12 that resulted in the highest production of both IFN-γ and TNF-α. Taking the secretion of those cytokines into account, 48 h stimulation with the combination of IL-15 (10 ng/ml) plus IL-12 (2 ng/ml) seemed most suitable of the available alternatives.

After starting to test the appropriate control for studying the effects of the fatty acids on cytokine secretion by the NK cells (see appendix B) the initial seeding density of cells was incereased from 5 x 10^4 cells/well to 1 x 10^5 cells/well to increase the concentration of cytokines in supernatants.

APPENDIX B

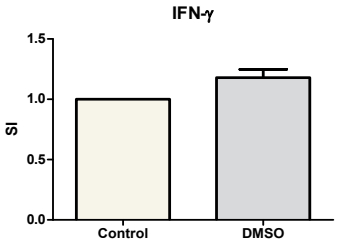
Selection of the appropriate control to use

The fatty acids used, AA, EPA and DHA, were dissolved in DMSO. Thus, DMSO alone was to be used as control. To ascertain that the DMSO was not affecting the cells in the culture, NK cells from three donors were additionally cultured in culture medium alone. NK cell production of three cytokines was used to evaluate if DMSO affected their cytokine secretion. Results are shown below with the cytokine production presented as SI. Differences between treatments were analysed using two-tailed, paired t-test and judged statistically significant if $p<0.05$. The difference in the production of these three cytokines by NK cells when cultured with or without DMSO was not statistically significant. DMSO was thus chosen as control.



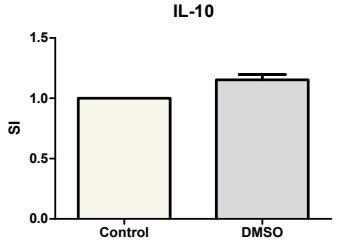
GM-CSF production by NK cells in culture with or without DMSO

DMSO, dimethyl sulfoxide; GM-CSF, granulocyte-macrophage colony-stimulating factor; SI, secretion index.



IFN-γ production by NK cells in culture with or without DMSO

DMSO, dimethyl sulfoxide; IFN-γ, interferon-γ; SI, secretion index.



IL-10 production by NK cells in culture with or without DMSO

DMSO, dimethyl sulfoxide; IL-10, interleukin-10; SI, secretion index.

APPENDIX C

Cell viability and number of cells at the end of culture

Countess® automated cell counter was used to assess viability of NK cells cultured with DMSO (control) or 50 μM of AA, EPA or DHA when cells and supernatants were harvested. The results are the mean of eight donors, n = 8, for all treatments and are shown below as percentage of live cells. Culturing NK cells with the fatty acids did not affect their viability. Statistical significance was determined by one-way ANOVA and judged statistically significant if $p<0.05$.

Percentage of live cells in NK cell cultures with or without fatty acids

Treatment	D1 [%]	D2 [%]	D3 [%]	D4 [%]	D5 [%]	D6 [%]	D7 [%]	D8 [%]	Mean [%]	SD [%]	SEM [%]
DMSO	84.5	82.5	85.0	87.0	86.5	79.0	87.0	88.0	84.9	3.0	1.0
AA	88.0	81.0	93.5	91.0	90.0	85.0	91.0	85.0	88.1	4.1	1.5
EPA	85.5	79.5	87.0	86.5	83.5	85.0	86.5	90.0	85.4	3.0	1.1
DHA	94.0	91.5	89.0	90.5	85.5	86.0	89.0	87.0	89.1	2.9	1.0

AA, arachidonic acid; D, donor; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; SD, standard deviation; SEM, standard error of the mean.

Similarly, the number of NK cells at the end of culture with DMSO (control) or 50 μM of AA, EPA or DHA was assessed. The concentration of cells was acquired by the Countess cell counter and the value used to calculate the number of NK cells per well. Culturing NK cells with the fatty acids did not affect the final number of cells. Statistical significance was determined by one-way ANOVA and judged statistically significant if $p<0.05$. These values for the number of cells per well at the end of culture (shown below) should not be compared with the number of cells seeded, i.e. 1×10^5 cells/well, as those values rely upon manual counting using a cell counting chamber and a microscope and these two methods give unequal results, unfortunately.

Number of cells x 10^4 per well in NK cell cultures with or without fatty acids

Treatment	D1	D2	D3	D4	D5	D6	D7	D8	Mean	SD	SEM
DMSO	5.0	2.4	6.0	4.8	6.7	4.8	5.1	6.8	5.2	1.4	0.5
AA	5.0	5.2	6.3	5.7	6.3	5.5	5.4	10.2	6.2	1.7	0.6
EPA	6.0	5.0	6.7	5.5	5.9	14.6	5.0	5.5	6.8	3.2	1.1
DHA	6.4	4.3	6.7	6.1	6.7	6.9	6.1	9.3	6.6	1.4	0.5

AA, arachidonic acid; D, donor; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; SD, standard deviation; SEM, standard error of the mean.

APPENDIX D

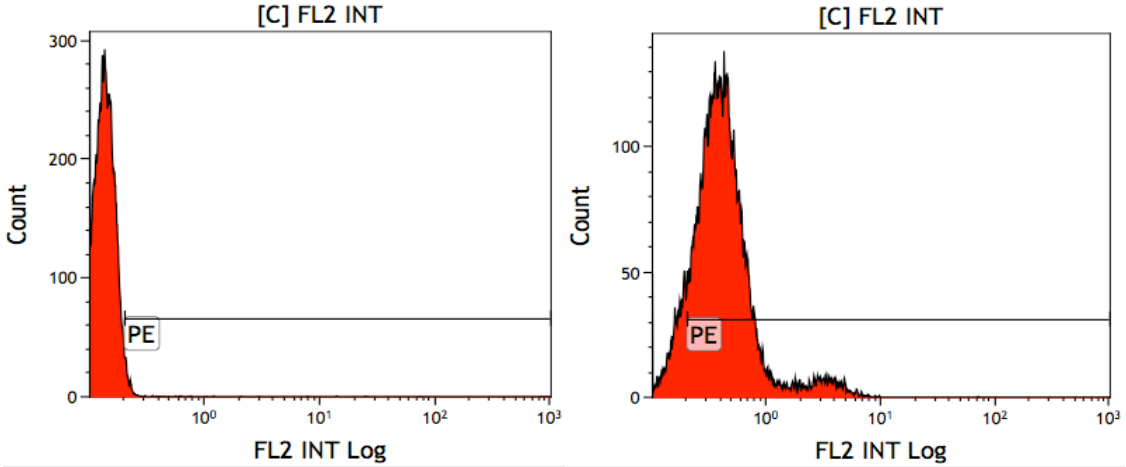
Purity of the NK cell population after isolation

To evaluate the purity of the NK cell population after isolation (at 0 h), cells were stained with PE conjugated anti-CD56 antibodies and cytrometric analysis performed on the Navios flow cytometer. Data analysis was performed in Kaluza® 1.3 software (example of analysis for donor 1 below). NK cell populations isolated from PBMCs of eight different donors were analysed. On average 84% of the cells expressed CD56. Mean fluorescence intensity (MFI) or geometric mean of CD56 was 0.46 ± 0.03.

Proportion of cells expressing CD56 and MFI after isolation

	D1	D2	D3	D4	D5	D6	D7	D8	Mean	SD	SEM
CD56 expression [%]	89.3	90.8	88.4	60.2	71.8	88.4	90.7	92.6	84.0	11.6	4.1
CD56 MFI	0.5	0.5	0.4	0.3	0.4	0.5	0.6	0.5	0.5	0.1	0.0

D, donor; MFI, mean fluorescence intensity; SEM, standard error of the mean; SD, standard deviation.



Gate	Number	%Total	%Gated	X-Med	X-GMean
All	16.991	96,90	100,00	0,14	0,14
PE	316	1,80	1,86	0,22	0,28

Gate	Number	%Total	%Gated	X-Med	X-GMean
All	16.554	96,51	100,00	0,39	0,42
PE	14.782	86,18	89,30	0,42	0,47

Example of histograms obtained by data analysis in Kaluza® 1.3 software

To account for non-specific Fc receptor binding of antibodies in the test (right), a gate was set by analysing cells stained with PE conjugated mouse IgG_1 isotype control (left) represented by a PE marked horizontal line. The histograms represent the data analysis of donor 1 (D1), percentage of CD56 positive cells and MFI are underlined with red. FL2, fluorescence 2 channel (on the Navios flow cytometer); GMean, geometric mean; PE, phycoerythrin.

APPENDIX E

Determination of cytokine and chemokine concentration by ELISA

Sandwich ELISA was used to determine the concentration of cytokines and chemokines in supernatants harvested from NK cells, after 18 h incubation without (control) or with 50 μM AA, EPA or DHA followed by stimulation with IL-15 (10 ng/ml) and IL-12 (2 ng/ml) for another 48 h. Supernatants were frozen at -80°C until assayed in duplicate for TNF-α, GM-CSF, IFN-γ, IL-17, IL-13, IL-10, CCL3, and IL-8. DuoSet® kits from R&D Systems were used according the manufacturer's instructions.

Examples of OD measurements are shown below. In some cases the OD values were below or above the lowest or highest standard in the standard curve, respectively. Those values were omitted in SI calculations, though some exceptions were made in determination of IL-13 concentration. Due to this, the number of donors behind different treatments and different cytokine measurements vary. Statistically significant difference between NK cells cultured with or without fatty acids was determined using one-way ANOVA with Bonferroni post-hoc test where $p<0.05$ was considered significant.

Determination of TNF-α concentration by ELISA

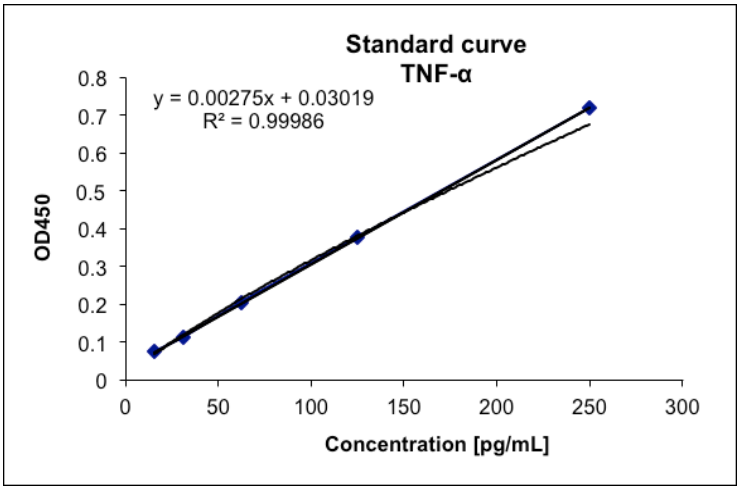
TNF-α ELISA on NK cell culture supernatants, donors 1-8 (D1-D8). Sample dilution 1:1.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	Blank
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	Blank
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	Blank
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	Blank
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	Blank
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	Blank
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	Blank
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	Blank

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.21	2.204	0.335	0.339	0.21	0.196	0.333	0.36	0.392	0.378	0.135	0.144
B	1.463	1.443	0.331	0.339	0.22	0.212	0.312	0.303	0.395	0.381	0.145	0.135
C	0.884	0.897	0.3	0.307	0.211	0.187	0.277	0.303	0.388	0.359	0.158	0.162
D	0.557	0.54	0.263	0.239	0.203	0.196	0.259	0.301	0.347	0.35	0.148	0.174
E	0.359	0.396	0.307	0.287	0.216	0.186	0.353	0.359	0.193	0.185	0.162	0.166
F	0.263	0.305	0.282	0.271	0.195	0.186	0.339	0.389	0.176	0.184	0.153	0.146
G	0.245	0.252	0.277	0.267	0.201	0.186	0.328	0.381	0.21	0.202	0.157	0.169
H	0.155	0.191	0.244	0.246	0.203	0.205	0.29	0.317	0.212	0.151	0.125	0.184



Standard curve for TNF-α and the equation of the best fit line

Standards

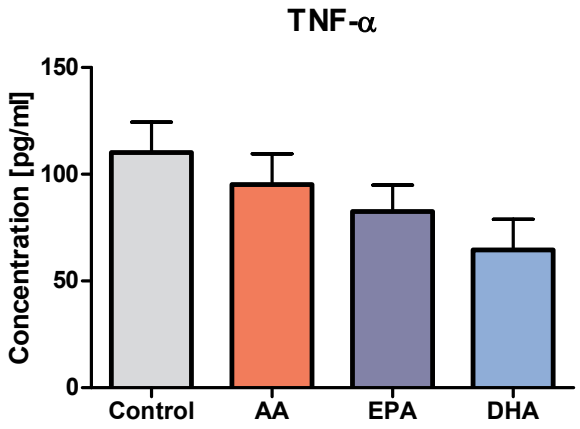
Concentration [pg/ml]	OD-blank
1000	2.034
500	1.28
250	0.7175
125	0.3755
62.5	0.2045
31.25	0.111
15.625	0.0755

The concentration of TNF-α in supernatant samples was calculated using the equation of the best fit line. The calculated values for TNF-α in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown.

TNF-α concentration

	Control [pg/ml]	AA [pg/ml]	EPA [pg/ml]	DHA [pg/ml]
D1	97	96	73	35
D2	68	53	50	NA
D3	NA	NA	NA	NA
D4	NA	NA	NA	NA
D5	104	76	63	56
D6	111	117	110	73
D7	132	134	124	106
D8	NA	NA	NA	NA
D9	78	NA	44	17
D10	NA	NA	NA	NA
D11	181	NA	114	100
Mean	110	95	83	64
SD	38	32	33	35
SEM	14	14	12	14
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on TNF-α secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

TNF-α secretion index

	Control	AA	EPA	DHA
D1	1	0.9851	0.7496	0.3573
D2	1	0.7815	0.7335	NA
D3	NA	NA	NA	NA
D4	NA	NA	NA	NA
D5	1	0.7279	0.6057	0.5360
D6	1	1.0524	0.9902	0.6564
D7	1	1.0165	0.9367	0.7992
D8	NA	NA	NA	NA
D9	1	NA	0.5641	0.2179
D10	NA	NA	NA	NA
D11	1	NA	0.6298	0.5525
Mean	1	0.9127	0.7442	0.5199
SD	0	0.1474	0.1645	0.2077
SEM	0	0.0659	0.0622	0.0848
ANOVA	-	-	0.025	<0.001

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of GM-CSF concentration by ELISA

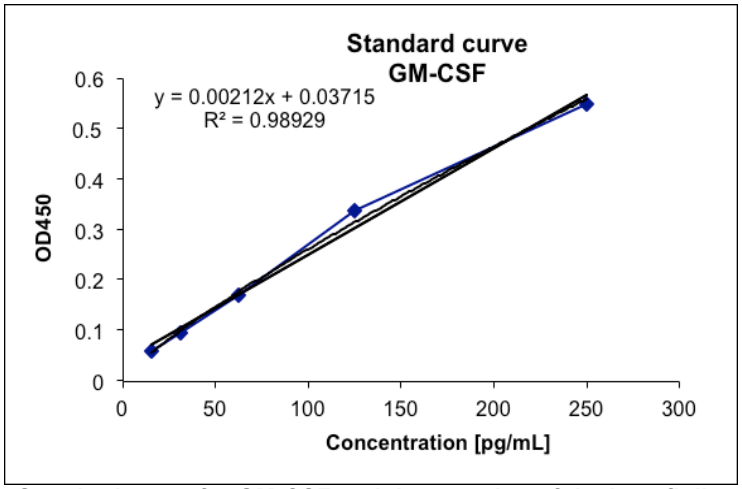
GM-CSF ELISA on NK cell culture supernatants, donors 1-8 (D1-D8). Samples undiluted.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	Blank
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	Blank
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	Blank
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	Blank
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	Blank
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	Blank
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	Blank
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	Blank

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.506	1.463	0.496	0.446	0.144	0.146	0.39	0.405	0.653	0.666	0.064	0.068
B	0.982	1	0.609	0.585	0.149	0.156	0.371	0.36	0.624	0.572	0.063	0.074
C	0.614	0.621	0.506	0.446	0.139	0.15	0.348	0.318	0.624	0.583	0.076	0.064
D	0.375	0.435	0.456	0.431	0.133	0.134	0.311	0.315	0.637	0.622	0.063	0.067
E	0.238	0.238	0.275	0.238	0.136	0.166	0.342	0.323	0.112	0.109	0.066	0.069
F	0.162	0.162	0.197	0.199	0.145	0.13	0.304	0.304	0.112	0.103	0.071	0.068
G	0.129	0.123	0.198	0.19	0.124	0.149	0.274	0.278	0.101	0.095	0.069	0.078
H	0.13	0.146	0.244	0.165	0.104	0.11	0.273	0.276	0.094	0.09	0.065	0.064



Standard curve for GM-CSF and the equation of the best fit line

Standards

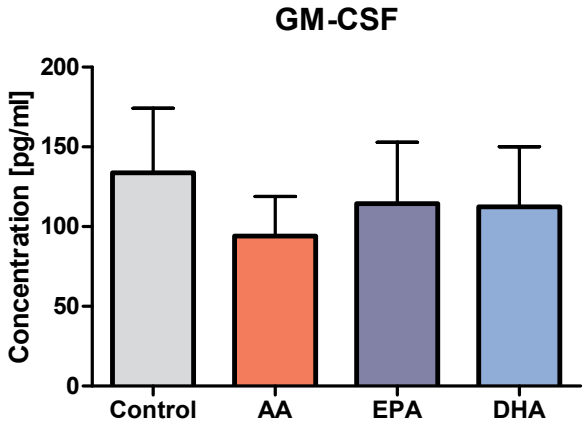
Concentration [pg/ml]	OD-blank
1000	1.417
500	0.9235
250	0.55
125	0.3375
62.5	0.1705
31.25	0.0945
15.625	0.0585

The concentration of GM-CSF in supernatant samples was calculated using the equation of the best fit line. The calculated values for GM-CSF in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown.

GM-CSF concentration

	Control [pg/ml]	AA [pg/ml]	EPA [pg/ml]	DHA [pg/ml]
D1	173	232	175	160
D2	80	44	42	47
D3	19	23	19	13
D4	22	15	15	NA
D5	138	123	108	98
D6	107	94	81	80
D7	NA	233	235	NA
D8	NA	NA	NA	NA
D9	272	129	224	158
D10	21	NA	16	9
D11	371	92	350	334
Mean	134	94	114	112
SD	122	71	115	107
SEM	41	25	38	38
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on GM-CSF secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

GM-CSF secretion index

	Control	AA	EPA	DHA
D1	1	1.3439	1.0136	0.9249
D2	1	0.5480	0.5245	0.5861
D3	1	1.1859	0.9876	0.7026
D4	1	0.7087	0.6872	NA
D5	1	0.8907	0.7798	0.7115
D6	1	0.8749	0.7520	0.7454
D7	NA	NA	NA	NA
D8	NA	NA	NA	NA
D9	1	0.4743	0.8235	0.5809
D10	1	NA	0.7619	0.4286
D11	1	0.2480	0.9434	0.9003
Mean	1	0.7843	0.8082	0.6975
SD	0	0.3663	0.1558	0.1663
SEM	0	0.1295	0.0519	0.0588
ANOVA	-	-	-	0.036

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of IFN-γ concentration by ELISA

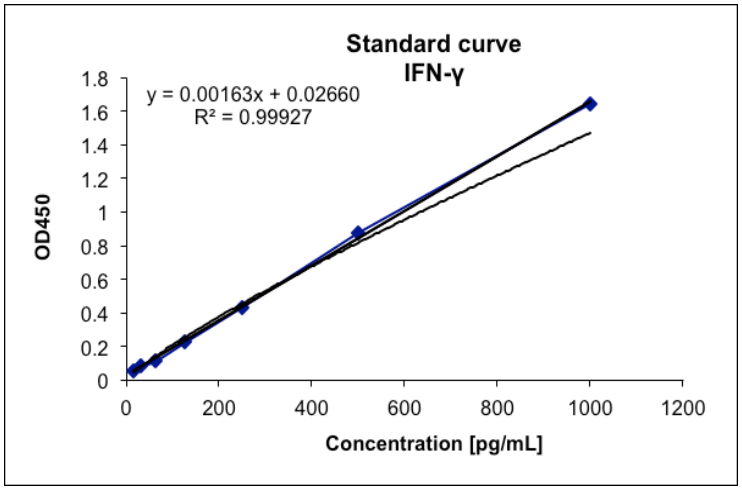
IFN-γ ELISA on NK cell culture supernatants from donors 1-8 (D1-D8). Sample dilution 1:1.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	Blank
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	Blank
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	Blank
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	Blank
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	Blank
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	Blank
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	Blank
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	Blank

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.801	1.618	0.878	0.77	1.06	1.045	1.484	1.55	1.96	1.922	0.068	0.08
B	0.933	0.947	0.67	0.566	1.098	0.987	1.684	1.891	1.961	1.962	0.064	0.08
C	0.508	0.491	0.68	0.657	0.858	0.95	1.8	1.673	1.941	1.982	0.064	0.064
D	0.29	0.292	0.548	0.492	0.821	0.768	1.475	1.378	2.146	2.049	0.065	0.064
E	0.183	0.176	1.942	1.962	0.188	0.172	2.135	2.125	0.643	0.657	0.06	0.07
F	0.165	0.131	1.917	1.871	0.189	0.169	2.316	2.256	0.555	0.587	0.068	0.077
G	0.137	0.102	1.912	1.95	0.172	0.171	2.31	2.176	0.493	0.536	0.065	0.08
H	0.104	0.097	1.552	1.53	0.132	0.126	2.268	2.309	0.46	0.46	0.064	0.069



Standard curve for IFN-γ and the equation of the best fit line

Standards

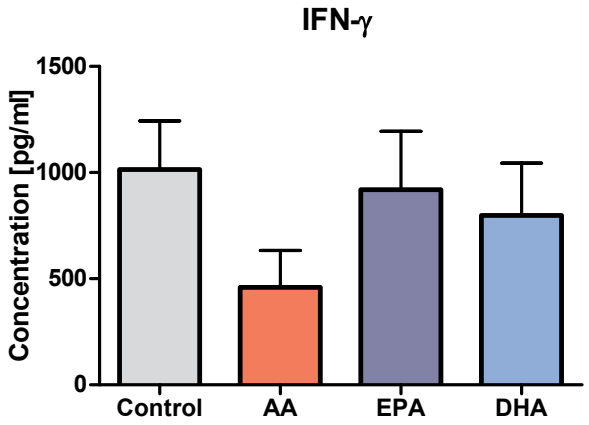
Concentration [pg/ml]	OD-blank
1000	1.643
500	0.8735
250	0.433
125	0.2245
62.5	0.113
31.25	0.0815
15.625	0.053

The concentration of IFN-γ in supernatant samples was calculated using the equation of the best fit line. The calculated values for IFN-γ in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown.

IFN-γ concentration

	Control [pg/ml]	AA [pg/ml]	EPA [pg/ml]	DHA [pg/ml]
D1	897	644	706	524
D2	NA	NA	NA	NA
D3	1177	1165	995	861
D4	107	105	96	44
D5	1747	NA	2016	1636
D6	NA	NA	NA	NA
D7	NA	NA	NA	NA
D8	683	586	517	450
D9	NA	NA	NA	NA
D10	693	43	331	339
D11	1793	210	1777	1733
Mean	1014	459	920	798
SD	608	426	728	653
SEM	230	174	275	247
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on IFN- secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

IFN-γ secretion index

	Control	AA	EPA	DHA
D1	1	0.7182	0.7872	0.5841
D2	NA	NA	NA	NA
D3	1	0.9896	0.8452	0.7311
D4	1	0.9885	0.9022	0.4131
D5	1	NA	1.1542	0.9364
D6	NA	NA	NA	NA
D7	NA	NA	NA	NA
D8	1	0.8581	0.7567	0.6588
D9	NA	NA	NA	NA
D10	1	0.0620	0.4776	0.4892
D11	1	0.1171	0.9911	0.9665
Mean	1	0.6223	0.8449	0.6827
SD	0	0.4250	0.2107	0.2112
SEM	0	0.1735	0.0796	0.0798
ANOVA	-	(0.074)	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of IL-13 concentration by ELISA

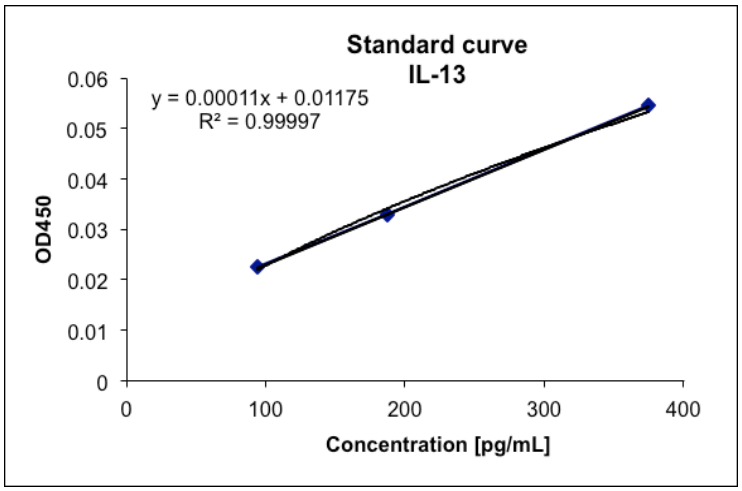
IL-13 ELISA on NK cell culture supernatants from donors 1-10 (D1-D10). Samples undiluted.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	D9 Control
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	D9 AA
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	D9 EPA
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	D9 DHA
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	D10 Control
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	D10 AA
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	D10 EPA
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	D10 DHA

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.163	1.958	0.076	0.127	0.103	0.082	0.117	0.101	0.092	0.098	0.092	0.072
B	1.022	1.015	0.068	0.115	0.112	0.064	0.116	0.123	0.084	0.071	0.084	0.061
C	0.45	0.455	0.064	0.066	0.105	0.059	0.09	0.087	0.059	0.062	0.057	0.071
D	0.2	0.208	0.064	0.102	0.086	0.052	0.106	0.102	0.06	0.067	0.058	0.062
E	0.113	0.111	0.064	0.098	0.076	0.059	0.068	0.09	0.06	0.068	0.048	0.057
F	0.091	0.09	0.07	0.08	0.11	0.058	0.06	0.109	0.052	0.056	0.054	0.058
G	0.082	0.078	0.062	0.059	0.076	0.054	0.054	0.064	0.059	0.052	0.059	0.054
H	0.06	0.055	0.05	0.049	0.084	0.048	0.049	0.074	0.047	0.048	0.061	0.054



Standard curve for IL-13 and the equation of the best fit line

Standards

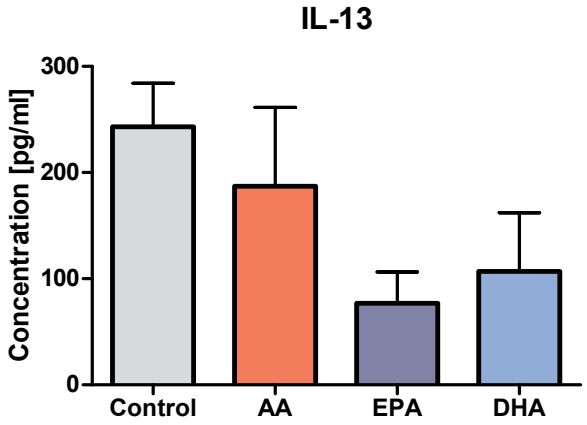
Concentration [pg/ml]	OD-blank
6000	2.003
3000	0.961
1500	0.395
750	0.1465
375	0.0545
187.5	0.033
93.75	0.0225

The concentration of IL-13 in supernatant samples was calculated using the equation of the best fit line. The calculated values for IL-13 in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown. The OD values that were below the lowest standard on the standard curve were given a substitute concentration value, equal to one third of the concentration of the lowest standard.

IL-13 concentration

	Control [pg/ml]	**AA [pg/ml]**	**EPA [pg/ml]**	**DHA [pg/ml]**
D1	293	202	**31**	125
D2	NA	NA	NA	NA
D3	211	170	116	**31**
D4	NA	NA	NA	NA
D5	361	457	175	316
D6	NA	NA	NA	NA
D7	234	75	**31**	**31**
D8	NA	NA	NA	NA
D9	116	**31**	**31**	**31**
Mean	243	187	77	107
SD	92	166	66	124
SEM	41	74	30	55
ANOVA	-	-	-	-

Substitute values are printed in bold. AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on IL-13 secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

IL-13 secretion index

	Control	AA	EPA	DHA
D1	1	0.6899	**0.1057**	0.4264
D2	NA	NA	NA	NA
D3	1	0.8065	0.5484	**0.1467**
D4	NA	NA	NA	NA
D5	1	1.2642	0.4843	0.8742
D6	NA	NA	NA	NA
D7	1	0.3204	**0.1324**	**0.1324**
D8	NA	NA	NA	NA
D9	1	**0.2675**	**0.2675**	**0.2675**
Mean	1	0.6697	0.3077	0,3694
SD	0	0.4051	0.2014	0,3059
SEM	0	0.1812	0.0901	0,1368
ANOVA	-	-	0.006	0.013

Values calculated using a substitute concentration value are printed in bold. AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of IL-10 concentration by ELISA

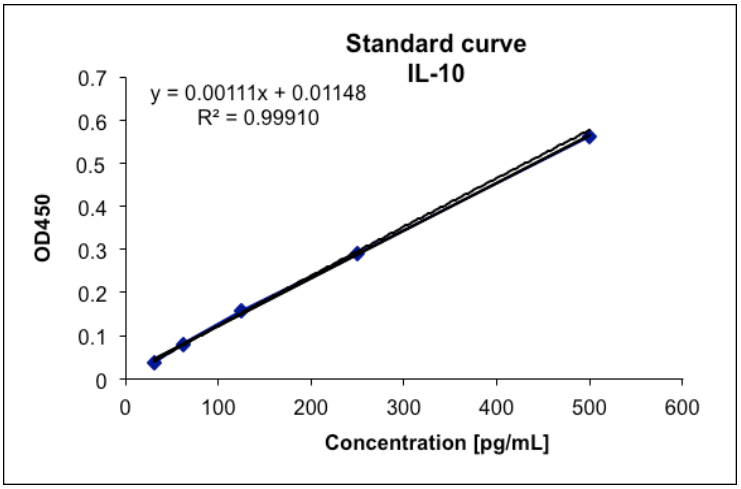
IL-10 ELISA on NK cell culture supernatants from donors 1-6 and donor 8 (D1-D6 and D8).

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D8 Control	Blank
B	Standard 2	D1 AA	D3 AA	D5 AA	D8 AA	Blank
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D8 EPA	Blank
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D8 DHA	Blank
E	Standard 5	D2 Control	D4 Control	D6 Control	Blank	Blank
F	Standard 6	D2 AA	D4 AA	D6 AA	Blank	Blank
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	Blank	Blank
H	Blank	D2 DHA	D4 DHA	D6 DHA	Blank	Blank

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.883	1.776	0.19	0.195	0.099	0.098	0.175	0.161	0.122	0.111	0.098	0.102
B	1.101	1.082	0.159	0.18	0.097	0.097	0.15	0.147	0.095	0.091	0.087	0.086
C	0.66	0.633	0.153	0.151	0.099	0.087	0.135	0.154	0.093	0.092	0.101	0.092
D	0.382	0.37	0.159	0.145	0.09	0.09	0.128	0.134	0.087	0.085	0.092	0.086
E	0.252	0.234	0.134	0.176	0.099	0.081	0.172	0.176	0.092	0.081	0.081	0.089
F	0.164	0.168	0.122	0.119	0.085	0.084	0.16	0.16	0.082	0.083	0.081	0.086
G	0.123	0.122	0.11	0.107	0.08	0.075	0.146	0.147	0.082	0.077	0.078	0.081
H	0.091	0.116	0.119	0.106	0.079	0.094	0.142	0.193	0.081	0.081	0.078	0.084



Standard curve for IL-10 and the equation of the best fit line

Standards

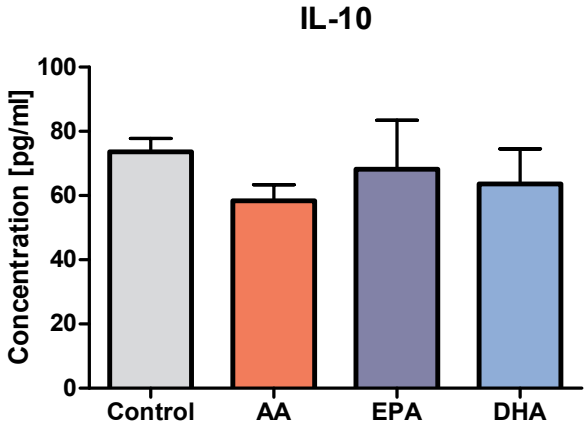
Concentration [pg/ml]	OD-blank
2000	1.7445
1000	1.0065
500	0.5615
250	0.291
125	0.158
62.5	0.081
31.25	0.0375

The concentration of IL-10 in supernatant samples was calculated using the equation of the best fit line. The calculated values for IL-10 in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown.

IL-10 concentration

	Control [pg/ml]	AA [pg/ml]	EPA [pg/ml]	DHA [pg/ml]
D1	87	66	50	50
D2	NA	NA	NA	NA
D3	NA	NA	NA	NA
D4	NA	NA	NA	NA
D5	64	47	43	31
D6	70	57	45	64
D7	NA	NA	NA	NA
D8	NA	NA	NA	NA
D9	68	73	80	79
D10	NA	NA	NA	NA
D11	79	49	123	94
Mean	74	58	68	64
SD	9	11	34	25
SEM	4	5	15	11
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on IL-10 secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid..

IL-10 secretion index

	Control	AA	EPA	DHA
D1	1	0.7605	0.5782	0.5782
D2	NA	NA	NA	NA
D3	NA	NA	NA	NA
D4	NA	NA	NA	NA
D5	1	0.7273	0.6714	0.4827
D6	1	0.8194	0.6453	0.9162
D7	NA	NA	NA	NA
D8	NA	NA	NA	NA
D9	1	1.0665	1.1729	1.1507
D10	NA	NA	NA	NA
D11	1	0.6224	1.5702	1.2004
Mean	1	0.7992	0.9276	0.8656
SD	0	0.1657	0.4303	0.3260
SEM	0	0.0741	0.1924	0.1458
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of IL-17 concentration by ELISA

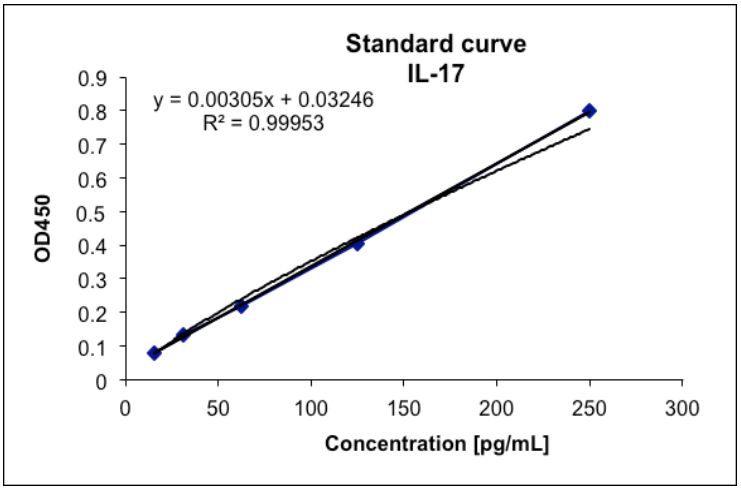
IL-17 ELISA on NK cell culture supernatants from donors 1-10 (D1-D10). Samples undiluted.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	D9 Control
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	D9 AA
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	D9 EPA
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	D9 DHA
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	D10 Control
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	D10 AA
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	D10 EPA
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	D10 DHA

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.516	2.419	0.093	0.092	0.15	0.137	0.096	0.088	0.11	0.126	0.131	0.107
B	1.483	1.527	0.086	0.086	0.107	0.09	0.117	0.129	0.136	0.073	0.119	0.083
C	0.88	0.886	0.085	0.083	0.08	0.085	0.078	0.089	0.096	0.07	0.075	0.075
D	0.492	0.484	0.079	0.081	0.078	0.075	0.076	0.095	0.091	0.069	0.071	0.068
E	0.32	0.285	0.081	0.077	0.073	0.066	0.068	0.074	0.108	0.106	0.068	0.061
F	0.218	0.217	0.086	0.083	0.078	0.073	0.072	0.081	0.068	0.068	0.068	0.066
G	0.166	0.161	0.078	0.08	0.077	0.074	0.075	0.077	0.107	0.106	0.067	0.068
H	0.086	0.079	0.077	0.073	0.071	0.07	0.066	0.066	0.065	0.065	0.062	0.057



Standard curve for IL-17 and the equation of the best fit line

Standards

Concentration [pg/ml]	OD-blank
1000	2.385
500	1.4225
250	0.8005
125	0.4055
62.5	0.22
31.25	0.135
15.625	0.081

IL-17 concentration in supernatants of DMSO, AA, EPA or DHA treated cells from D1-D8 (see above) and D9-D10 (not shown) was calculated from the equation of the best fit line of the standard curve. All samples were below the lowest standard of the standard curve. Thus, concentration or SI values are not available.

Determination of CCL3 concentration by ELISA

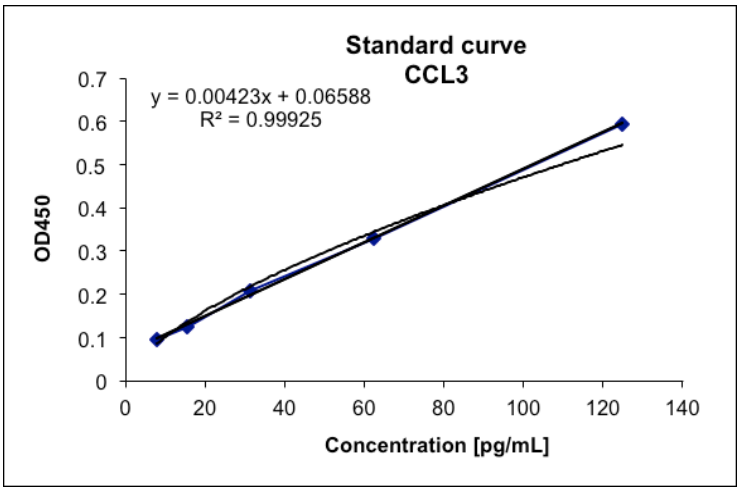
CCL3 ELISA on NK cell culture supernatants from donors 1-10 (D1-D10). Samples undiluted.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	D9 Control
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	D9 AA
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	D9 EPA
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	D9 DHA
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	D10 Control
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	D10 AA
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	D10 EPA
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	D10 DHA

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.814	0.753	0.916	0.717	0.682	0.718	0.594	0.43	0.387	0.352	0.292	0.261
B	0.744	0.629	0.842	0.733	0.809	0.611	0.522	0.602	0.319	0.313	0.244	0.324
C	0.795	0.755	0.831	0.679	0.612	0.614	0588	0.465	0.29	0.4	0.288	0.255
D	0.534	0.488	1.137	0.66	0.618	0.622	0.629	0.524	0.298	0.386	0.285	0.289
E	0.395	0.382	1.144	0.885	0.588	0.552	0.726	0.667	0.387	0.399	0.354	0.336
F	0.321	0.294	1.327	0.902	0.766	0.605	0.735	0.686	0.46	0.447	0.152	0.146
G	0.328	0.228	1.032	1.209	0.633	0.494	0.567	0.558	0.39	0.41	0.3	0.325
H	0.219	0.143	1.187	0.812	0.571	0.469	0.945	0.816	0.391	0.405	0.353	0.303



Standard curve for CCL3 and the equation of the best fit line

Standards

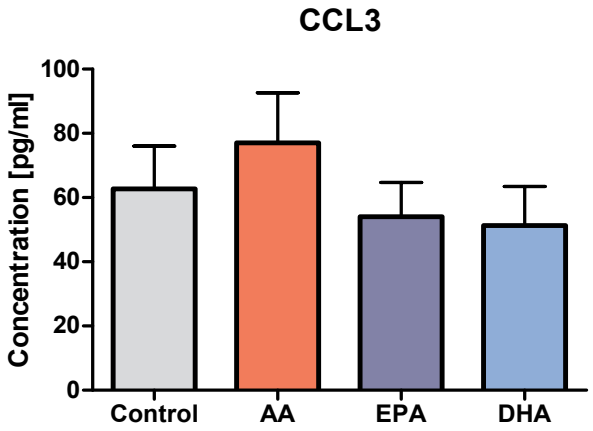
Concentration [pg/ml]	OD-blank
500	0.6025
250	0.5055
125	0.594
62,5	0.33
31.25	0.2075
15.625	0.1265
7.8125	0.097

The concentration of CCL3 in supernatant samples was calculated using the equation of the best fit line. The calculated values for CCL3 in the supernatants of NK cells from different donors treated by DMSO (control), AA, EPA or DHA are shown and depicted below. Corresponding SI values are also shown.

CCL3 concentration

	Control [pg/ml]	AA [pg/ml]	EPA [pg/ml]	DHA [pg/ml]
D1	NA	NA	NA	NA
D2	NA	NA	NA	NA
D3	107	109	87	88
D4	76	104	75	65
D5	63	74	66	78
D6	106	110	75	NA
D7	29	16	23	22
D8	35	49	36	36
D9	NA	NA	NA	NA
D10	23	NA	16	19
Mean	63	77	54	51
SD	35	38	28	30
SEM	13	16	11	12
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.



The effects of AA, EPA and DHA on CCL3 secretion by NK cells

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

CCL3 secretion index

	Control	AA	EPA	DHA
D1	NA	NA	NA	NA
D2	NA	NA	NA	NA
D3	1	1.0221	0.8080	0.8234
D4	1	1.3575	0.9799	0.8453
D5	1	1.1886	1.0547	1.2433
D6	1	1.0311	0.7020	NA
D7	1	0.5637	0.8002	0.7757
D8	1	1.4140	1.0479	1.0342
D9	NA	NA	NA	NA
D10	1	NA	0.6688	0.8267
Mean	1	1.0962	0.8659	0.9248
SD	0	0.3070	0.1608	0.1799
SEM	0	0.1253	0.0608	0.0735
ANOVA	-	-	-	-

AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, not available; SD, standard deviation; SEM, standard error of the mean.

Determination of IL-8 concentration by ELISA

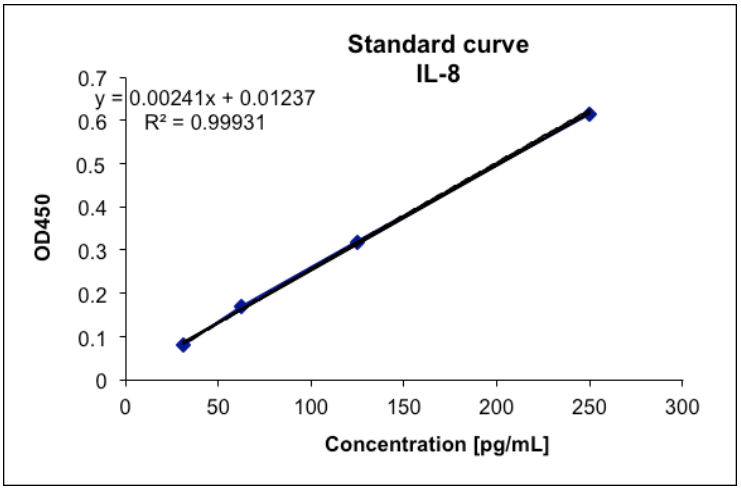
IL-8 ELISA on NK cell culture supernatants from donors 1-8 (D1-D8). Samples undiluted.

ELISA plate scheme

	Column 1-2	Column 3-4	Column 5-6	Column 7-8	Column 9-10	Column 11-12
A	Standard 1	D1 Control	D3 Control	D5 Control	D7 Control	Blank
B	Standard 2	D1 AA	D3 AA	D5 AA	D7 AA	Blank
C	Standard 3	D1 EPA	D3 EPA	D5 EPA	D7 EPA	Blank
D	Standard 4	D1 DHA	D3 DHA	D5 DHA	D7 DHA	Blank
E	Standard 5	D2 Control	D4 Control	D6 Control	D8 Control	Blank
F	Standard 6	D2 AA	D4 AA	D6 AA	D8 AA	Blank
G	Standard 7	D2 EPA	D4 EPA	D6 EPA	D8 EPA	Blank
H	Blank	D2 DHA	D4 DHA	D6 DHA	D8 DHA	Blank

Optical density (OD) measurement of ELISA plate read at 450 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.515	2.553	0.093	0.087	0.09	0.083	0.076	0.086	0.113	0.136	0.059	0.061
B	1.812	1.868	0.091	0.086	0.091	0.079	0.073	0.073	0.127	0.121	0.054	0.059
C	1.114	1.184	0.088	0.1	0.081	0.076	0.081	0.086	0.127	0.125	0.057	0.056
D	0.67	0.706	0.081	0.081	0.092	0.082	0.079	0.086	0.131	0.122	0.06	0.057
E	0.389	0.399	0.093	0.114	0.081	0.07	0.091	0.094	0.065	0.069	0.067	0.062
F	0.24	0.247	0.098	0.111	0.092	0.081	0.115	0.116	0.074	0.084	0.082	0.071
G	0.152	0.159	0.092	0.108	0.081	0.079	0.124	0.123	0.097	0.075	0.082	0.092
H	0.075	0.075	0.091	0.129	0.08	0.083	0.097	0.12	0.109	0.077	0.096	0.058



Standard curve for IL-8 and the equation of the best fit line

Standards

Concentration [pg/ml]	OD-blank
2000	2.459
1000	1.765
500	1.074
250	0.613
125	0.319
62.5	0.1685
31.25	0.0805

IL-8 concentration in supernatants of DMSO, AA, EPA or DHA treated cells from D1-D8 (see above) was calculated using the equation of the best fit line of the standard curve. All samples were below the lowest standard of the standard curve. Thus, concentration or SI values are not available.