The effects of the omega-3 fatty acid eicosapentaenoic acid on dendritic cells and their ability to stimulate $CD4^+$ T cells *in vitro*

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HÁSKÓLI ÍSLANDS

Áhrif ómega-3 fitusýrunnar eikósapentaensýru á þroskun angafrumna og getu þeirra til að ræsa $CD4^+$ T frumur

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Ágrip

Áhrif ómega-3 fitusýrunnar eikósapentaensýru á þroskun angafrumna og getu þeirra til að ræsa $CD4^+$ T frumur

Angafrumur eru aðalsýnifrumur ónæmiskerfisins en hlutverk þeirra er að taka upp vaka og sýna þá óreyndum T frumum. Angafrumur gegna lykilhlutverki við stjórnun sérhæfingar T frumna. Ómega-3 fjölómettaðar fitusýrur hafa áhrif á ýmsar frumur ónæmiskerfisins, svo sem T frumur og makrófaga, og yfirleitt eru áhrif þeirra talin vera bælandi. Lítið er vitað um áhrif ómega-3 fitusýra á angafrumur.

Markmið verkefnisins var að athuga áhrif ómega-3 fitusýrunnar eikósapentaensýru (EPA) og ómega-6 fitusýrunnar arakídónsýru (AA) á angafrumur og getu angafrumna til að ræsa bæði samgena og ósamgena $CD4^+$ T frumur.

Óþroskaðar angafrumur voru ræktaðar með eða án EPA eða AA í sólarhring og fitusýrurnar síðan þvegnar af. Óþroskuðu angafrumurnar voru síðan þroskaðar áfram og þroskun þeirra metin með því að mæla tjáningu á yfirborðssameindum með frumuflæðisjá og seytun á boðefnum með ELISA aðferð. Einnig voru þroskaðar angafrumur sem höfðu verið meðhöndlaðar með eða án fitusýranna settar í rækt, annars vegar með ósamgena $CD4^+$ T frumum og hins vegar með samgena óreyndum ($CD45RA^+$) $CD4^+$ T frumum og virkjun T frumnanna skoðuð með mælingu á boðefnum í floti með ELISA aðferð og fjölgun þeirra með innlimun á geislavirku tímidíni.

Ræktun með EPA olli því að færri þroskaðar angafrumur tjáðu yfirborðssameindirnar HLA-DR, CD86, CD80, CD40, CCR7 og DC-SIGN miðað við angafrumur sem voru ræktaðar með AA eða án fitusýra, og var fækkunin styrkháð fyrir CD86 og HLA-DR. Þrátt fyrir að enginn munur hafi verið á meðaltjáningu ofangreindra sameinda hjá angafrumum sem voru ræktaðar með eða án fitusýra þegar skoðuð var tjáning allra frumna, þá reyndist meðaltjáning CD40 sameinda vera meiri hjá angafrumum ræktuðum með EPA miðað við angafrumur ræktuðum með AA eða án fitusýra þegar einungis voru skoðaðar CD40 jákvæðar frumur og einnig tilhneiging til þess sama fyrir meðaltjáningu á CD86 og HLA-DR. Angafrumur sem voru ræktaðar með EPA seyttu minna af IL-10 og höfðu tilhneigingu til minni seytunar á IL-12p40 en meiri seytunar á IL-6 en angafrumur sem voru ræktaðar með AA eða án fitusýra.

Ósamgena $CD4^+$ T frumur sem voru samræktaðar með EPA meðhöndluðum angafrumum höfðu tilhneigingu til meiri seytunar á IL-17 og IFN-γ en minni seytunar á IL-4 en ósamgena $CD4^+$ T frumur sem voru ræktaðar með AA meðhöndluðum angafrumum eða með ómeðhöndluðum angafrumum.

Enginn munur var hins vegar á fjölgun ósamgena $CD4^+$ T frumna eftir því hvaða meðferð angafrumurnar höfðu fengið. Samgena óreyndar $CD4^+$ T frumur sem voru ræktaðar með EPA eða AA meðhöndluðum angafrumum seyttu minna af IL-4 og höfðu tilhneigingu til minni seytunar á IL-10 en samgena óreyndar $CD4^+$ T frumur sem voru ræktaðar með ómeðhöndluðum angafrumum, en tilhneiging var til aukinnar seytunar á IL-17 og IFN-γ.

Þrátt fyrir að meðhöndlun óþroskaðra angafrumna með EPA fækkaði frumum sem þroskuðust yfir í þroskaðar angafrumur voru þær frumur sem þroskuðust og höfðu verið meðhöndlaðar með EPA jafn hæfar til að ræsa $CD4^+$ T frumur og hæfari í að stýra ónæmissvari $CD4^+$ T frumna í Th1 eða Th17 svar en frumur sem höfðu verið meðhöndlaðar með AA eða án fitusýra. Þetta getur verið vegna þess að angafrumur sem ræktaðar voru með EPA höfðu meira magn af sameindum sem taka þátt í virkjun T frumna á yfirborði sínu og $HLA\text{-}DR^+$ angafrumur sem voru ræstar í gegnum CD40 höfðu tilheygingu til að seyta meira af IL-12p40 og IL-6 en angafrumur sem voru meðhöndlaðar með AA eða án fitusýra, sem bendir til þess að EPA meðhöndlaðar angafrumur sem ná að þroskast séu hæfari til að virkja T frumur en samsvarandi angafrumur sem voru meðhöndlaðar með AA eða án fitusýra.

Abstract

The effects of the omega-3 fatty acid eicosapentaenoic acid on dendritic cells and their ability to activate $CD4^+$ T cells *in vitro*

Dendritic cells (DCs) are the main antigen presenting cells. Their role is to sample the environment and present antigens to naïve T cells. DCs have a key role in determining the differential pathway of T cells. Omega-3 polyunsaturated fatty acids affect many cells of the immune system, for example T cells and macrophages, and their effects are generally thought to be anti-inflammatory. However, little is known about the effects of omega-3 fatty acids on DCs.

The aim of this project was to evaluate the effects of the omega-3 fatty acid eicosapentaenoic acid (EPA) and the omega-6 fatty acid arachidonic acid (AA) on DC maturation and the ability of EPA or AA pre-treated DCs to activate both allogeneic and autologous $CD4^+$ T cells *in vitro*.

Human monocyte-derived immature DCs were cultured in the absence or presence of EPA or AA for 24 hours before being matured into mature DCs. DC maturation was assessed by measuring expression of surface molecules by flow cytometry and concentration of cytokines in the cell culture supernatants by ELISA. Mature DCs treated with or without fatty acids were co-cultured with allogeneic $CD4^+$ T or autologous naïve ($CD45RA^+$) $CD4^+$ T cells and T cell activation determined by measuring cytokines in the supernatants by ELISA and proliferation by incorporation of radioactive thymidine.

Fewer mDCs upregulated, upon maturation, expression of the surface molecules CD40, CD80, CD86, CCR7, DC-SIGN and HLA-DR after incubation with EPA compared with cells cultured with AA or without fatty acids, the reduction being dose dependent for CD86 and HLA-DR. Although there was no difference between treatments in the mean expression levels of the surface molecules when the whole cell population was examined, the mean expression levels of CD40, were higher in CD40 positive mDCs that had been treated with EPA than in CD40 positive mDCs that had been pre-treated with AA or not treated with fatty acids. In addition, there was a tendency towards higher mean expression levels of CD86 and HLA-DR by CD86 and HLA-DR positive mDCs that had been treated with EPA than by mDCs that had been pre-treated with AA or not treated with fatty acids. mDCs treated with EPA secreted less IL-10 and had a tendency towards less secretion of IL-12p40, but more secretion of IL-6 than mDCs treated with AA or not treated with fatty acids.

Allogeneic $CD4^+$ T cells cultured with mDCs treated with EPA had a tendency towards more IL-17 and INF-γ secretion but less IL-4 secretion than allogeneic $CD4^+$ T cells cultured with mDCs treated with

AA or without fatty acids. No difference was seen in T cell proliferation of allogeneic $CD4^+$ T cells between different treatment groups. Autologous naïve $CD4^+$ T cells cultured with mDCs treated with EPA secreted less IL-4 and had a tendency towards less secretion of IL-10 than autologous naïve $CD4^+$ T cell cultured with mDCs that were not treated with fatty acids, and there was a tendency towards increased secretion of IL-17 and INF-γ.

Although treatment of imDC with EPA decreased the number of cells that matured into mature DCs, the EPA-treated DCs were equally capable of activating $CD4^+$ T cells and more efficient in inducing Th1/Th17 phenotype than AA-treated DCs or DCs not treated with fatty acids. This may be linked to the increased levels of expression of molecules linked to T cell activation of those EPA treated cells that managed to mature and a tendency towards increased IL-12p40 and IL-6 secretion by $HLA\text{-}DR^+$ DCs stimulated with CD40, suggesting that EPA-treated DCs that mature are more effective in activating T cells and inducing a Th1/Th17 phenotype than the mature DCs obtained after AA-treatment or no treatment.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AA	Arachidonic acid
APC	Allophycocyanin
APCs	Antigen presenting cells
BCR	B cell receptor
BHT	Butylated hydroxytoluene
BF3	Boron trifluoride
BSA	Bovine serum albumin
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CMP	Common myeloid progenitor
CPM	Counts per minute
CTLs	Cytotoxic T lymphocytes
CTL	C-type lectin
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DTA	Docosatetraenoic acid
EDTA	Ethylenediamineteraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluoresence-activated cell sorter
FITC	Fluorescein isothicyanate
GEO mean	Geometrical mean
GM-CSF	Granulocyte macrophage – colony stimulating factor
GPI	Glycosylphosphatidylinositol
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICAM	Inter-cellular adhesion molecule
IFN	Interferon

IL	Interleukin
IRAK	The interleukin-1 receptor associated kinase
iTregs	Inducible Tregs
imDCs	Immature dendritic cells
JNK	c-Jun N-terminal kinase
LA	Linoleic acid
LFA	Leukocyte function associated antigen
LNA	α-Linolenic acid
LPS	Lipopolysaccharide
MACS	Magnetic antibody cell sorting
MAP kinases	Mitogen-activated protein kinases
mDCs	Mature dendritic cells
MeOH	Methanol
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MCs	Monocytes
MMR	Macrophage mannose receptor
NF-κB	Nuclear factor- κB
NLRs	NOD-like receptors
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
nTregs	Natural Tregs
OA	Octanoic acid
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PMA	phorbol 12-myristate 13-acetate
PPAR	Peroxisome-proliferator-activated receptor
PPRs	Pattern recognition receptors
PBS	Phosphate buffered saline
PE	Phycoerythrin
PUFAs	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
Rosi	Rosiglitazone
RT	Room temperature

SEM	Standard error of mean
SI	Secretion index
TCR	T cell receptor
TGF	Transforming growth factor
Tfh cells	Follicular helper T cells
Th cells	T helper cells
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-α
TLRs	Toll like receptors
Tregs	Regulatory T cells

1 INTRODUCTION

Dietary omega-3 polyunsaturated fatty acids (PUFAs) have beneficial effects in some inflammatory diseases and may affect different aspects of the immune system. They alter T cell activation and suppress the activity of monocytes, B cells and natural killer (NK) cells. Although dendritic cells (DCs) are key players in induction and differentiation of adaptive immune responses, little is known about the effects of omega-3 PUFAs on DC activation and subsequent ability of DCs to prime naïve T cells.

1.1 The immune system

The role of the immune system is to protect the body from invading pathogens. To fulfill that role the immune system is divided into the innate immune system and the adaptive immune system. The cells of the innate immune system are the first to arrive at the site of infection. They engulf the pathogens and secrete cytokines that attract the cells of the adaptive immune system. The cells of the adaptive immune system arrive later and together the innate and the adaptive immune systems can eliminate the infection (1).

1.2 The innate immune system

The cells of the innate immune system include monocytes, macrophages, neutrophils, eosinophils, basophils, NK cells and DCs. All of these cells, with the exception of NK cells, are derived from the common myeloid progenitor (CMP) in the bone marrow. These cells are the first to respond when pathogens invade the body.

Monocytes are the immature form of macrophages. Monocytes circulate in the blood before they enter tissues and mature into macrophages. Macrophages reside in almost every tissue of the body. Their role is mainly to phagocytose invading pathogens and present peptides from the pathogens to T cells by their major histocompatibility complex (MHC) class II molecules. Macrophages obtain help from T cells, in order to enhance their response against the pathogen so that they can kill the engulfed pathogen. Macrophages express receptors that participate in phagocytosis of pathogens, including Fc receptors, complement receptors and pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) on the pathogens. Amongst the PRRs are the scavenger receptors and macrophage mannose receptor (MMR). Upon binding to these receptors, the pathogen is engulfed into phagosomes and killed, most often after fusion of the phagosomes with lysosomes that contain enzymes, proteins and peptides that can help with killing of the pathogen. Some of the PRRs, e.g. the toll-like receptors (TLRs), do not participate in phagocytosis but activate the macrophages.

Neutrophils are another type of phagocytic cells, which are found in blood where they are short-lived. During infection they leave the blood and migrate into the infected tissue and help the macrophages to kill the pathogens. Eosinophils and basophils are thought to be important in protection against parasites. They have granules containing many different kinds of enzymes and toxic proteins which are secreted when the cells are activated.

NK cells are derived from the common lymphoid progenitor (CLP). Although they belong to the lymphoid lineage they do not express traditional antigen receptors. They are activated early during infections, where their role is to kill abnormal cells, mainly virus-infected cells and tumour cells.

DCs are part of the innate immune system but because of their link with the adaptive immune system they will be discussed in connection with the adaptive immune system.

If the innate immune system is not able to destroy the invading pathogens the adaptive immune system is activated and together these two systems eradicate the infection (2).

1.3 The adaptive immune system

The adaptive immune system developed with the purpose to make the defence more versatile and effective against infecting pathogens. The cells of the adaptive immune system are divided into T and B lymphocytes, which are derived from CLPs in the bone marrow. The T and B cells have a large repertoire of antigen receptors, which makes them able to recognise almost every pathogen molecule they may come across. The reason for the large variety of receptors on the lymphocytes is rearrangement of the genes that make up the antigen-binding site of the receptors during their development (2).

The B cells complete their maturation in the bone marrow. During that time they rearrange their genes for the B cell receptor (BCR) and undergo negative selection, where all B cells expressing BCR that recognise self antigens are deleted or made anergic. After development in the bone marrow naïve B cells leave the bone marrow and travel between the blood and lymphoid tissues. If the B cells encounter a pathogen they have a specific receptor for, they differentiate into plasma cells and start secreting antibodies. B cells need stimulation from helper T cells to be fully activated, except when responding to T cell independent antigens (2).

The developing T cells travel from the bone marrow to the thymus where they finish their development. As for the B cells, they rearrange their antigen receptor (T cell receptor, TCR). In the thymus, the developing T cells become MHC-restricted so that $CD4^+$ T cells will recognise antigens presented by MHC class II molecules and $CD8^+$ T cells will recognise antigens presented by MHC class I molecules. Thereafter the T cells undergo negative selection, where T cells that recognise self antigens undergo apoptosis. The T cells leave the thymus as naïve T cells and start to circulate

between the blood and lymphoid tissues where the T cells survey the peptides presented by the antigen presenting cells (APCs). When a TCR binds an MHC-peptide complex, presented by the APC, the T cell activates and starts to proliferate and differentiate into a specific effector T cell. When naïve T cells have fully matured into specialized effector T cells, they either leave the lymphoid tissue and migrate to the site of the infection or reside within the lymphoid tissues providing help for B cells (2).

1.4 T cell subsets

T cells are divided into $CD4^+$ T cells and $CD8^+$ T cells, depending on their expression of the CD4 or the CD8 co-receptor. When $CD8^+$ T cells recognise antigens presented to them in connection with MHC class I molecules they become cytotoxic T lymphocytes (CTLs). The role of CTLs is to kill virus-infected cells or tumour cells. They do so by connecting to the target cell and delivering signals to the cell that promote apoptosis (2).

The main role of effector $CD4^+$ T cells is to activate other cells of the immune system (T helper cells, Th cells), with a small part of them having a role in mediating regulation (T regulatory cells, Tregs). $CD4^+$ T cells can differentiate into four known subgroups of effector $CD4^+$ T cells, depending on the signals they receive from the APCs and their environment. These are Th1, Th2, Th17 and Tregs. These T cell subsets have different roles in the immune response (2).

Th1 cells are produced when effector T cells receive signals from interleukin (IL)-12 secreted by DCs and interferon (IFN)-γ secreted by NK cells. Their main role is to induce immune response to intracellular pathogens by activating infected macrophages to kill pathogens they have engulfed. They mediate this activation by secreting IFN-γ and expressing CD40L (3). Th1 cells also induce antibody production by B cells (2).

Naïve T cells become Th2 cells when IL-4, produced by mast cells and NKT cells, is present in their surroundings during activation. Th2 cells secrete a wide range of cytokines, such as IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. The main role of Th2 cells is to induce immune response against extracellular pathogens. They do so by helping B cells to undergo antibody switching and produce antibodies (3).

Th17 cells are produced in mice when IL-6 and transforming growth factor (TGF)-β are present; however, it is not yet clear which cytokine has to accompany IL-6 in humans for their production, but the most likely cytokine is IL-1β (4). IL-23 was initially thought to be a differentiation factor for Th17 cells, but now it is believed to be associated with the survival of the Th17 cells (5). Th17 cells produce IL-17a, IL-17f, IL-21 and IL-22. IL-17a and IL-17f recruit and activate neutrophils, whereas IL-21 is a stimulatory factor for Th17 differentiation (3, 6). Th17 cells enhance neutrophil response and play a role in extracellular defence, especially against extracellular bacteria and fungi (2).

Tregs play a role in maintaining tolerance, both against self and non-self antigens. Tregs are divided into subgroups, natural Tregs (nTregs) and adaptive/inducible Tregs (iTregs) (7). The nTregs develop in the thymus. They express high amounts of CD25, the α chain of the IL-2 receptor, and secrete IL-10 and TGF-β. These cells are important for maintaining tolerance to self antigens. The iTregs are derived from naïve circulating T cells, which in lymphoid tissues receive signals rendering them tolerogenic. The iTregs most likely comprise a heterogeneous group of regulatory cells. Th3 are iTregs which exist in mucosa and are activated when harmless environmental antigens are presented to them in mucosa. When activated they produce IL-10, IL-4 and TGF-β. Tr1, another type of iTregs, which are produced in the presence of IL-10, have only been produced *in vitro* but are believed to exist also *in vivo* (7, 8).

Two new subtypes of T helper cells have recently been discovered. Th9 cells are produced in the presence of TGF-β and IL-4. Th9 cells secrete IL-9 and IL-10 but in much more concentration than Th2 cells, and are therefore thought to be another subtype of T helper cells rather than a specialized form of Th2 cells. Follicular helper T cells (Tfh cells) secrete IL-10 and IL-21 but IL-21 is essential for their production. Tfh cells have a role in the germinal centre reaction in the lymph node (9, 10). Very little is known about these two new subtypes and these new subgroups are still under investigation.

1.5 Dendritic cells

DCs are the main APCs. They derive from the bone marrow progenitors, either through the myeloid or the lymphoid lineage and are accordingly divided into two subgroups, i.e. into myeloid (sometimes called conventional) and plasmacytoid DCs (11). Plasmacytoid DCs produce type I IFNs, mainly against viral infections (12). Myeloid DCs can be divided into subgroups based on their localization in tissues, their expression of surface markers or their maturation state. DCs are found in immature state in peripheral tissues, mainly in skin and mucosa (13, 14).

Two myeloid DCs subsets exist in the skin, epidermal Langerhans cells which reside in the epidermis and dermal interstitial DCs. These subgroups express different collections of molecules, e.g. different TLRs, adhesion molecules and C-type lectins (CTL). Langerhans cells express Langerin and E-cadherin but dermal interstitial DCs express dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN, CD209), CD11b and CD14. Myeloid DC subgroups are also found in the blood. They have a common expression of CD11c but can be further subdivided according to their expression of CD1a, CD16 or CD34 (13, 15).

DCs reside in an immature state in peripheral tissues where they sample the environment and act as sentinels. Immature DCs (imDCs) capture antigens, both self and foreign antigens, via phagocytosis or macropinocytosis. ImDCs capture and present antigens from many different types of pathogens, mostly bacteria and viruses but they are also believed to present antigens from fungi and parasites. In addition, they also present antigens from damaged tissue or dying cells in the periphery (2).

Microbes invading the body contain PAMPs, which are repeated molecular structures specific for microbes and found on many types of pathogens. Different molecules serve as PAMPs, e.g. components of bacterial cell walls, such as lipids, lipoproteins, proteins, lipopolysaccharides (LPS) and peptidoglycans. Fungal β-glucans and viral nucleic acids are also examples of PAMPs. DCs express many types of PRRs that recognise different PAMPs on microbes (16, 17). TLRs are the best described group of PRRs. There are 10 functional human TLRs, all of which have different roles in recognising distinct PAMPs from different types of microorganisms (17). TLRs are divided into groups depending on the types of PAMPs they recognise. TLR1, 2, 4 and 6 recognise lipids and TLR5 and 11 recognise protein ligands. TLR3, 7, 8 and 9 are localised intracellularly and detect nucleic acids derived from bacteria and viruses (17). Different DC subgroups express different types of TLRs but human blood plasmacytoid DCs express TLR1, 6, 7, 9 and 10, blood myeloid DCs express TLR1, 2, 3, 4, 5, 6, 7, 8 and 10 and epidermal DCs from the skin express TLR1, 2, 3, 6 and 10 (13).

C-type lectin receptors (CLR) are another large group of PRRs but they bind to carbohydrate structures on antigens. Examples of CLR are MMR (CD206) and DEC-205 (CD205), which have a role in phagocytosis, and DC-SIGN, which is an adhesion molecule that increases adhesion between DCs and naïve T cells, but DC-SIGN is expressed on both imDCs and mature DCs (mDCs). Dectin-1 is another CLR expressed on DCs. It binds to β-glucans and has a role in anti-fungal defence. Nucleotide-binding oligomerization domain (NOD) -like receptors (NLRs) are located intracellularly and bind to viral antigens and some bacteria. MDA5 and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) are also located intracellularly and bind to viral RNA (16, 18, 19).

ImDCs are poor stimulators of T cells and they express low levels of the molecules involved in activation of naïve T cells, such as MHC class II molecules and the B7 molecules (CD80 and CD86). When PRRs on imDCs recognise and bind to PAMPs on microbes, the imDCs are activated; they stop engulfing antigens, downregulate expression of chemokine receptors that maintain the DCs within the tissues, upregulate expression of the chemokine receptor CCR7 (CD197) and upregulate their expression of CD80/CD86 and MHC class II molecules. Following the upregulation of activation molecules and downregulation of molecules involved in phagocytosis, the DCs migrate to the nearest lymph nodes where they become potent APCs and can activate naïve T cells, which have specific receptors for the MHC-peptide complex on the DC surface. As well as activating naïve T cells, the mature DCs also secrete cytokines which determine the T cell differentiation pathway (20).

The naïve T cells must receive three signals from the mDCs to become activated. It is through this binding of the TCR with the MHC-peptide complex that the naïve T cells receive the first signal. The second signal comes through CD28 molecules when they bind to the co-stimulatory molecules CD80 and CD86. As a result of these two signals, the naïve T cells upregulate expression of CD40L, which

binds to CD40 on the activated DCs. This interaction has two roles; it both activates the T cells as well as activating the DCs to express more CD80 and CD86 (2).

The first two signals are sufficient for the activation and proliferation of the naïve T cells. The third signal determines their differentiation pathway. This signal is provided by the cytokine microenvironment of the naïve T cells and is mainly provided by the activated DCs (20). The signals that DCs receive from their microenvironment, as well as the type of antigen or microbe that activated the DCs, determine the array of cytokines the DCs secrete, and hence the type of T helper cell response (21, 22). Cytokines secreted by other neighbouring cells, such as macrophages, mast cells and epithelial cells also affect the outcome of the DCs response to the antigen and subsequently their cytokine secretion (21, 22). Tumour necrosis factor (TNF)-α and IL-1β are among the cytokines secreted early in inflammation by activated macrophages and these cytokines are able to activate the imDCs (2), a fact that has been used in the project presented here.

Mature DCs secrete a variety of cytokines with different functions and different roles. As IL-6, IL-10 and IL-12 play important roles in the differentiation of Th17, Tregs and Th1 cells, respectively, these will be described in some detail.

IL-12 and IL-23 belong to the IL-12 cytokine family, which also consists of IL-27 and IL-35. They are heterodimeric cytokines, composed of an α chain (p35 and p19, respectively) and they share the p40 β chain. IL-12 and IL-23 are both secreted by DCs as well as macrophages and B cells. They are pro-inflammatory cytokines but with different functions. IL-12 polarizes naïve T cells to become Th1 cells (23) but IL-23 is needed for the survival of Th17 cells. IL-23 has also been connected with chronic autoimmune diseases, e.g. psoriasis in humans (5, 23, 24) and in experimental animals, such as in collagen-induced arthritis and experimental autoimmune encephalitis (25, 26).

IL-6 is a pro-inflammatory cytokine secreted by DCs and is thought to participate, in conjunction with IL-1β, in the differentiation of naïve T cells into Th17 cells in humans, but in mice IL-6 in conjunction with TGF-β induces differentiation of naïve T cells into Th17 cells (4, 24). IL-10 is an anti-inflammatory cytokine secreted by DCs, macrophages, monocytes, B cells and Tregs. It inhibits production of pro-inflammatory cytokines and diminishes tissue damage. IL-10 also downregulates activation and co-stimulatory molecules on DCs and thereby decreases T cell activation and proliferation induced by DCs (27).

When DCs first encounter a pathogen, they often secrete both IL-1β and IL-6, which induces differentiation of Th17 cells. Later, other cytokines become more prominent and if the mDCs secrete IL-12, Th1 cells are induced. Less is known about the role of DCs in the differentiation of Th2 cells, as Th2 cells are mainly induced in the presence of IL-4, which is not secreted by DCs. It has been suggested that lack of IL-12 secretion induces differentiation of Th2 cells (28, 29).

As the role of imDCs is to take up and present antigens in the periphery, in the absence of infection they present self antigens. ImDCs, not activated through infection or by pro-inflammatory cytokines, can migrate to the draining lymph nodes. If self-reacting naïve T cells encounter such DCs they will be rendered anergic because they lack co-stimulation, a process called peripheral tolerance. In addition, these DCs can also activate nTregs, which then can suppress the potential activation of naïve T cells with self-specific receptors (2).

1.6 Polyunsaturated fatty acids

Fatty acids are hydrocarbon chains of varying length. They have a methyl group on one end (omega end) and a carboxyl group on the other end (alpha end). Fatty acids can be saturated or unsaturated. Saturated fatty acids contain no double bonds, monounsaturated fatty acids contain one double bond and PUFAs contain two or more double bonds. Omega-3 fatty acids have the first double bond on the third carbon from the methyl end but omega-6 fatty acids have the first double bond on the sixth carbon from the methyl end.

The omega-6 fatty acid linoleic acid (LA, 18:2 n-6) and the omega-3 fatty acid α-linolenic acid (LNA, 18:3 n-3) are both essential fatty acids. They are essential because humans do not have desaturases necessary for formation of double bonds between carbon atoms beyond carbon 9 and the methyl end of the fatty acids. LA is found in most vegetable oils, e.g. corn oil and safflower oil. LNA is scarce in food but it is present in some vegetable oils including flaxseed oil and soybean oil. Arachidonic acid (AA, 20:4 n-6) is produced from LA and eicosapentaenoic acid (EPA, 20:5 n-3) is produced from LNA. The same desaturases and elongases are used in the body to produce AA and EPA. LA is more abundant in the body than LNA so more AA than EPA is produced. It is debated whether production of EPA and docosahexaenoic acid (DHA) from LNA can fulfill the need for these fatty acids, but DHA is another omega-3 fatty acid derived from EPA (30). Therefore, it is advised that EPA and DHA are acquired from food, e.g. fish oil and fatty fish.

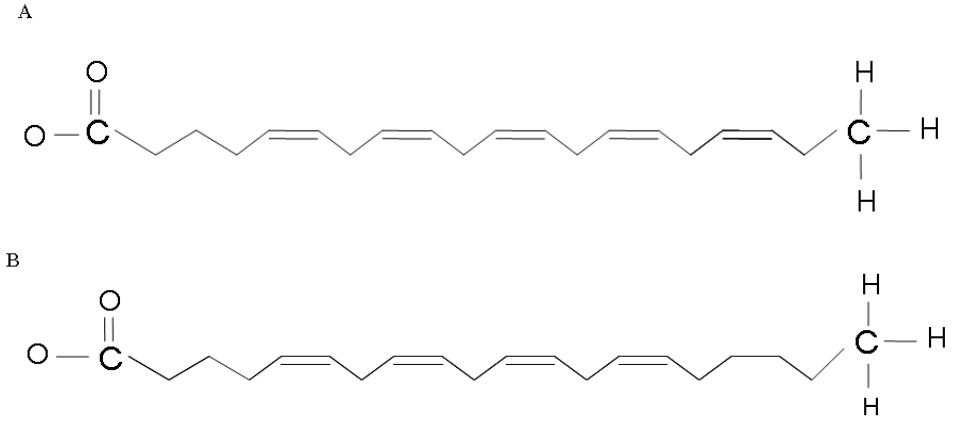

Figure 1. Structure of eicosapentaenoic acid, 20:5, n-3 (A) and arachidonic acid, 20:4, n-6 (B).

1.7 Fatty acids in cell membranes

PUFAs are absorbed in the intestines and transported in chylomicrons to virtually all cells of the body, where they are incorporated into cell membranes (31). Cell membranes are mostly composed of glycerophospolipids. The L-glycerol backbone of the phospholipid usually has a saturated fatty acid attached to carbon 1 and an unsaturated fatty acid on carbon 2. The composition of fatty acids linked to the L-glycerol backbone can vary but higher amount of unsaturated fatty acids increase the mobility and fluidity of the membrane.

Culturing cells *in vitro* with PUFAs alters the fatty acid composition of the cell membrane (32). It is also possible to alter the fatty acid composition of both human and animal cells *in vivo* by altering the dietary intake of specific PUFAs. Increasing the intake of omega-3 fatty acids, such as EPA and DHA, can increase the amount of these fatty acids in the cell membranes, at the expense of omega-6 fatty acids, especially AA. The higher the dose of the omega-3 PUFAs in the diet, the more the incorporation of omega-3 PUFAs into cell membranes, with the omega-3 fatty acid accumulation reaching a limit at 4% of the total fatty acids in the cell membranes (33, 34).

1.8 Mechanism by which PUFAs can affect immune cells

Fatty acids have many different roles within immune cells. They serve as fuel for the cell, they are a part of the cell membrane, they affect intracellular responses and hence gene expression, and they act as precursors for eicosanoids (35).

The best examined effects of omega-3 PUFAs on immune cell function is their effects on eicosanoid production. Eicosanoids are lipid mediators derived from 20 carbon PUFAs that have widespread effects on immune responses. Among eicosanoids are prostaglandins, leukotrienes, thromboxanes, lipoxins and resolvins. EPA and AA are substrates for the same enzymes for eicosanoid production. Because AA is in higher amount in cell membranes than EPA, AA is usually the major substrate for eicosanoid production. Eicosanoids produced from EPA are in general less potent than eicosanoids produced from AA, so incorporating more EPA into cell membranes instead of AA could dampen the inflammatory response (33, 36-40).

PUFAs in cell membranes may also modify the physical properties of lipid rafts. Lipid rafts contain high amounts of proteins necessary for signal transduction. They are rich in saturated fatty acids, sphingolipids, cholesterol and glycosylphosphatidilinositol (GPI)-anchored proteins. The sphingolipids and cholesterol in the outer leaflet of the membrane bilayer connect to phospholipids and cholesterol in the inner leaflet of the membrane bilayer. Because of the high amount of saturated fatty acids in the phospholipids and sphingolipids in the lipid rafts they are more tightly packed than the phospholipids

and sphingolipids in the surrounding membranes, which contain more of PUFAs. By increasing intake of PUFAs the amount of PUFAs in cell membranes increases. Substituting saturated fatty acids by PUFAs alters the structure and the microviscocity of the cell membranes and leads to changes in the lipid rafts with subsequent changes in the profile of signalling proteins (38, 41-43). Altered expression of proteins in lipid rafts can lead to changes in signalling pathways inside the cell and cause indirect effects of PUFAs on gene expression. PUFAs can also directly affect intracellular signalling pathways and gene expression (43, 44).

1.9 Polyunsaturated fatty acids and immune responses

The effects of omega-3 PUFAs on immune responses have generally been found to be anti-inflammatory. Omega-3 PUFAs have been shown to have beneficial effects in inflammatory diseases, particularly in rheumatoid arthritis (RA). Patients suffering from RA experience reduced morning stiffness and their requirement for non-steroid anti-inflammatory drugs declines when they receive omega-3 PUFAs (45-48). It is still controversial whether omega-3 fatty acids have beneficial effects in other inflammatory diseases but omega-3 PUFAs have been implied to have anti-inflammatory effects in asthma, psoriasis, inflammatory bowel disease and IgA nephropathy (37, 48, 49). How omega-3 PUFAs exert their effects on immune responses is not known in detail but a number of studies have examined their effects on immune cells *ex vivo* and *in vitro*.

1.10 The effects of culturing T cells with omega-3 PUFAs *in vitro* and *ex vivo*

The effects of PUFAs on T cell proliferation and cytokine secretion has been widely studied and is probably the most explored field of omega-3 PUFAs effects on immune cells. Culturing T cells with omega-3 PUFAs leads to diminished IL-2 secretion and decreased T cell proliferation of both rodent and human T cells, as well as immortalized Jurkat T cell lines (36, 50-52). Omega-3 PUFAs also affect lipid raft composition and expression of surface molecules essential for T cell activation and communication with other immune cells (53-55).

1.11 The effects of culturing monocytes or macrophages with PUFAs

A couple of studies have demonstrated that EPA and DHA can suppress the activity of human monocytes by decreasing the number of stimulated monocytes expressing human leukocyte antigen (HLA)-DR, HLA-DP, inter-cellular adhesion molecule (ICAM) and leukocyte function associated antigen (LFA)-3 as well as the intensity of the expression of these molecules (56, 57).

A couple of studies have also examined the effects of omega-3 PUFA on surface molecule expression by macrophages. In a dietary study, mice fed a fish oil diet had a higher proportion of splenic macrophages with high expression of CD11b than mice fed a corn oil diet but there was no effect on

the expression of CD80 or CD86 (58). In contrast, in an *in vitro* study, DHA reduced CD40 and CD86 expression of J774A.1 cells that had been infected with *Mycobacterium tuberculosis* (59).

Studies on the effects of omega-3 fatty acids on macrophages have focused more on the effects of the omega-3 PUFAs on their cytokine secretion than on expression of surface molecules. Most of the studies examining the effects of dietary omega-3 PUFAs on cytokine secretion by resident peritoneal macrophages show that they increase secretion of the pro-inflammatory cytokines TNF-α and IL-1β when compared with omega-6 PUFAs (reviewed in (60)). For example, in a study by Petursdottir et al. (61), murine resident peritoneal macrophages obtained from mice fed a fish oil diet and stimulated with LPS *ex vivo* secreted more TNF-α than murine resident peritoneal macrophages obtained from mice fed a corn oil diet. In addition, murine resident peritoneal macrophages cultured with EPA or DHA *in vitro* secreted more TNF-α than macrophages cultured without fatty acids or with AA or LA (62). Skuladottir et al. (62) also showed that thioglycolate-elicited peritoneal macrophages cultured with EPA or DHA secreted more TNF-α than macrophages cultured with LA or AA although Wallace et al. (63) showed that thioglycollate-elicited macrophages from mice fed fish oil secreted less TNF-α than thioglycollate-elicited macrophages from mice fed coconut oil, safflower oil, olive oil or low fat diet.

Most studies examining the effects of culturing immortalized macrophage cell lines with omega-3 PUFAs show that cells incubated with DHA and/or EPA or emulsions containing omega-3 PUFAs secreted less TNF-α than cells cultured without fatty acids or with an emulsion containing omega-6 PUFA (64-68), although Skuladottir et al. (62) found that EPA and DHA increase TNF-α secretion by RAW 264.7 macrophages *in vitro*.

1.12 The effects of PUFAs on DCs *ex vivo* and *in vitro*

Few studies have investigated the effects of omega-3 fatty acids on DCs. These studies have evaluated the effects of omega-3 PUFAs on murine and human monocyte-derived DCs *ex vivo* and *in vitro* and show that omega-3 fatty acids or PUFAs have a tendency to reduce expression of activation molecules and antigen presentation by DCs (69-76) .

When rats were given fish oil their DCs had lower expression of CD2, CD11a, CD18, MHC class II molecules and CD54 and less ability to present antigens than DCs from rats receiving a diet containing saffran oil (69), indicating that the omega-3 fatty acids in the fish oil reduced expression of co-stimulatory molecules and antigen presentation when compared with the omega-6 fatty acids in the saffran oil diet. When DCs derived from mouse bone marrow were incubated *in vitro* with DHA, DHA inhibited endotoxin induced increase in MHC class II expression as well as IL-12p70 and IL-6 secretion compared with that by DCs cultured with the saturated fatty acid lauric acid or without fatty acids (70). The DCs cultured with DHA also attenuated T cell proliferation, compared with that by DCs

cultured with lauric acid. EPA and AA also suppressed IL-12 production in addition to reducing expression of co-stimulatory molecules in mouse bone marrow derived DCs (71). Rat DCs exposed to AA and oleic acid had less expression of MHC class II molecules than DCs exposed to the medium chain fatty acid octanoic acid (OA) and DCs exposed to AA had less upregulation of co-stimulatory molecules than DCs exposed to OA (73).

There are only three studies that have evaluated the effects of omega-3 fatty acids on human DCs (74-76). These studies show that culturing human monocyte-derived DCs with PUFAs downregulates or fails to upregulate, upon stimulation, expression of many cell surface molecules as well as secretion of cytokines playing roles in DC maturation and T cell activation. When monocytes (MCs) were cultured with EPA or AA the imDCs derived from them failed to upregulate CD40, CD80, CD83 and CD86 after stimulation with LPS and their secretion of TNF-α and IL-12p40 was also diminished compared with that by imDCs derived from MCs that had not been cultured with EPA or AA (76). Similarly, when MCs were cultured with DHA in the study by Zapata-Gonzales et al. (75) the DCs failed to upregulate CD1a, CD80, CD83, CD86 and HLA-DR, and their secretion of IL12p70 and IL-10 was reduced compared with that by DCs derived from MCs cultured with oleic acid or linoleic acid. The same pattern was seen in a study by Wang et al. (74) where the expression of CD80, CD86 and HLA-DR was downregulated after incubation of imDCs with DHA or EPA, and the secretion of IL-12p70 and TNF-α was diminished compared with cells cultured without fatty acids or with stearic acid (74).

Attempting to elucidate the mechanism by which PUFAs affect DCs, Zeyda et al. (76) showed that the effects observed by the PUFAs were independent of the transcription factor nuclear factor (NF)-κB), as interleukin-1 receptor associated kinase (IRAK) degradation, phosphorylation of IκB and phosphorylation and degradation of p38 were not affected. Furthermore, phosphorylation of the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) were not affected by the PUFAs (76). In contrast to the results obtained by Zeyda et al. (76), Draper et al. (71) showed that EPA and DHA decreased NF-κBp65 expression by DCs and Wang et al. (74) showed that treatment with EPA and DHA reduced phosphorylation of p38. Furthermore, Zapata-Gonzales et al. (75) showed that DHA activated the transcription factor peroxisome-proliferator-activated receptor (PPAR)-γ in combination with Rosiglitazone (Rosi, a known PPAR-γ activator) more than when Rosi was added alone to the DC culture and Draper et al. (71) showed that EPA and DHA increased PPAR-γ expression in LPS stimulated DCs. However, DHA and EPA retained the ability to inhibit IL-12p70 production by DCs when the PPAR-γ antagonist GW9662 was present in the culture, indicating that the fatty acids influenced the DCs independent of PPAR-γ (75).

The results from the studies discussed above show that culturing murine derived DCs *ex vivo* or *in vitro* with omega-3 or omega-6 PUFAs results in less expression of surface molecules and cytokine secretion. In contrast, the results from most of the studies using human derived DCs indicate that only

omega-3 PUFAs but not omega-6 PUFAs inhibit DC expression of many activation and co-stimulatory molecules as well as affecting secretion of cytokines essential for T cell activation and stimulation *in vitro*.

DCs play a key role in controlling immune responses and determining the differential pathways of naïve T cells. Therefore the objective of this study was to further define the effects of the omega-3 PUFA, EPA, on DC maturation and activation and also on the ability of DCs cultured with EPA to activate and differentiation naïve T cells.

2 OBJECTIVES

The objectives of this study were to determine the effects of the omega-3 fatty acid EPA and the omega-6 fatty acid AA on maturation of monocyte-derived DCs *in vitro* and the effects of DCs cultured with EPA or AA on T cell activation and proliferation.

Specific objectives were to:

1) Determine the effects of EPA and AA on the phenotype of the DCs and on their secretion of cytokines.
2) Determine the effects of DCs treated with EPA or AA on cytokine secretion by autologous naïve T cells.
3) Determine the effects of DCs treated with EPA or AA on cytokine secretion by allogeneic T cells.

3 MATERIALS AND METHODS

3.1 Preparation of fatty acids

The fatty acids AA and EPA (Sigma Aldrich, St Louis, MO) were dissolved in 100% ethanol, flushed with nitrogen and then stored at -20°C. Low endotoxin bovine serum albumin (BSA) (Sigma Aldrich) was dissolved in phosphate buffered saline (PBS) (Sigma Aldrich). The fatty acids were incubated with the BSA at a ratio of 3:1 for 16 hours at 37°C prior to use.

3.2 Preparation of dendritic cells

3.2.1 Collection and isolation of monocytes

Peripheral blood was collected from healthy volunteers (ethical approval number 06-068). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Monocytes were isolated from the blood with magnetic antibody cell sorting (MACS) using CD14 microbeads (Miltenyi Biotech, Germany) and magnetic cell sorting columns (Miltenyi Biotech).

The purity of the $CD14^+$ monocytes was on average 96%, with average expression of 101 but CD14 is a surface molecule expressed on monocytes but not on other blood cells.

3.2.2 Differentiation of monocytes into dendritic cells and activation of dendritic cells

Monocytes were suspended at 0.5 x 10^6 cells/ml in 1640 RPMI culture medium (Invitrogen, Paisley, England) supplemented with 10% foetal calf serum (Invitrogen) and penicillin/streptomycin 10000 U/ml:10000 µg/ml (Invitrogen) (complete medium). The cells were cultured in 48 well plates (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 for 7 days. After 7 days of incubation the average expression of CD14 was 10 and 3% of the imDCs were positive for CD14. Granulocyte macrophage – colony stimulating factor (GM-CSF) at 25 ng/ml (R&D Systems, Abingdon, England) and IL-4 at 12.5 ng/ml (R&D Systems) were added to the culture on day 0 and day 3, inducing differentiation of the monocytes to imDCs. Different concentrations (50 µM, 25 µM or 12.5 µM) of the fatty acids, EPA or AA, were added to the cell culture at day 6. Untreated cells were cultured with BSA alone. At day 7 the imDCs were collected and washed thoroughly in order to remove the fatty acids. The cells were resuspended at 2.5 x 10^5 cells/ml in complete medium and cultured with TNF-α at 50 ng/ml (R&D Systems) and IL-1β at 10 ng/ml (R&D Systems), with or without extra stimulation with LPS at 0.5 µg/ml (Fluka, Sigma), for two days in 48 well plates. At day 9 the imDC had become mDCs. The cells were

collected and stained for flow cytometry to determine expression of surface molecules and the supernatant was collected and stored at -70°C until cytokines were measured by enzyme-linked immunosorbent assay (ELISA).

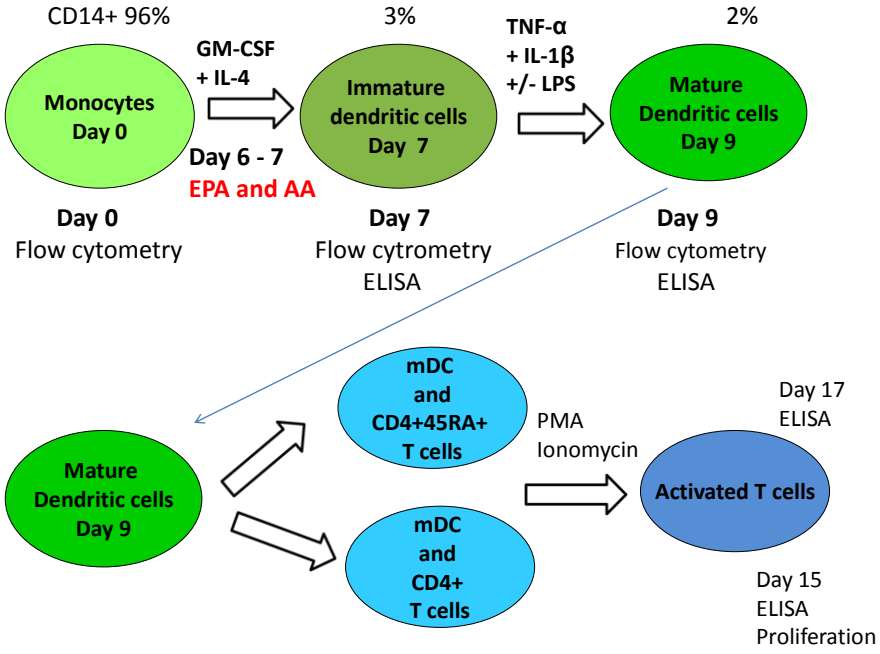

Figure 2. Schematic picture of the dendritic cell model

3.2.3 Flow cytometry analysis

Cells were collected on day 0 (purified $CD14^+$ cells), day 7 (imDCs) and day 9 (mDCs) and analysed for expression of various molecules by flow cytometry. Cells were suspended at 0.5 x 10^5 cells in staining buffer (PBS with 0.5% BSA (MP Biomedicals, Irvine, CA), 2mM ethylenediamineteraacetic acid (EDTA) (Sigma-Aldrich), and 0.1% NaN_3 (Sigma-Aldrich)) and stained with mouse monoclonal antibodies conjugated with fluorescein isothicyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) (see details in table 1). The cells were incubated for twenty minutes at 4°C, then washed with 2 ml staining buffer and fixed with 0.5% paraformaldehyde in PBS. Cells were stained with isotype-matched antibodies as negative control. Ten thousand cells were collected using FACScalibur (BD Biosciences, CA) and analysed using the Cellquest programme (BD Biosciences). DCs were located on a dot plot showing size and granularity and the results expressed as percentage positive cells (with isotype control antibodies set at 2%) and mean fluorescence intensity (MFI).

Granulation was determined as geometrical mean (Geo mean) which is the calculated mean of the side scattered light.

Table 1. List of antibodies used in this study.

Antigen	Fluorochrome	Isotype	Clone	Dilution	Company*
CD14	APC	IgG2a	UCHM1	1/10	AbD-Serotec
CD40	PE	IgG2a	LOB7/6	1/5	AbD-Serotec
CD80	FITC	IgG1	MEM-233	1/10	AbD-Serotec
CD86	FITC	IgG1	BU63	1/10	AbD-Serotec
CD197	PE	IgG2a	150503	1/1	R&D Systems
CD209	FITC	IgG1	MR-1	1/10	AbD-Serotec
HLA-DR	PE	IgG2a	G46-6 (243)	1/10	BD Biosciences
Isotype control	APC	IgG2a		1/5	AbD-Serotec
Isotype control	FITC	IgG1	MOPC-21	1/5	BD Biosciences
Isotype control	PE	IgG2a	OX-34	1/10	AbD-Serotec

* AbD-Serotec, Abingdon, England; R&D Systems, Abingdon, England; BD Biosciences, CA

3.2.4 Intracellular staining

Cells were suspended at 5 x 10^5 cells/ml and incubated for 10 minutes with 500 µl of cold 4% paraformaldehyde in PBS. Cells were then washed with staining buffer and then with saponin buffer (staining buffer with 0.1% saponin). Cells were incubated with mouse monoclonal antibodies against HLA-DR for twenty minutes on ice. The cells were then washed with saponin buffer, followed by staining buffer, and then fixed with 1% paraformaldehyde in PBS.

3.3 Membrane fatty acid composition

Fatty acid analysis was performed on DCs collected before incubation with EPA or AA at day 6, on imDCs after removal of the fatty acids on day 7 and on mDCs on day 9. Cells were suspended in 1.0 ml 0.9 % NaCl and the antioxidant butylated hydroxytoluene (BHT) dissolved in methanol (MeOH; 500 mg/l) was added to the cell samples at a final concentration of 20 mg/l, before they were stored at -70°C.

Total DCs lipids were extracted with MeOH/chloroform ($CHCl_3$) (2:1, v/v) as described by Bligh & Dyer (77). Briefly, cells from different experiments were pooled together, spun down and re-suspended in 0.8 ml 0.9% NaCl at 1 x 10^7 cells/ml and mixed with 2 ml MeOH-BHT and 1 ml $CHCl_3$-BHT. The antioxidant BHT was added to the MeOH and the $CHCl_3$ at a final concentration of 50 mg/L. The

samples were mixed before the addition of one ml NaCl (0.9%) and 1 ml $CHCl_3$ to the sample. After centrifugation (1700 rpm, 15 min) two layers had formed. The lower layer containing total lipids was collected and dried down under a stream of nitrogen. The lipid sample was dissolved in $CHCl_3$ and then added to a thin layer chromatography (TLC) plate (Adsorbosil H, Alltech, Deerfield, IL) and put into a jar containing 100 ml $CHCl_3$:MeOH (8:1). The phospholipid band was collected from the plate. Heneicosanoic acid (C21:0) methyl ester standard (Sigma-Aldrich) and 2 ml boron trifluoride (BF3) (Sigma-Aldrich) were added to the sample and it flushed with nitrogen. The fatty acids were methylated at 110°C for 45 minutes and then 1.5 ml distilled water and 1.5 ml hexane added to the sample, mixed well and centrifuged (1700 rpm, 10 min). After centrifugation (1700 rpm, 10 min) two layers had formed. The hexane layer containing the fatty acid methyl esters (the upper phase) was removed and flushed with nitrogen. The fatty acid methyl esters were dissolved in 125 µl isooctane and stored at -30°C before analysis using a gas-chromatohraph (Hewlet Packard 7673, HP/Agilent, Palo Alto, CA) containing a CP-WAX 52CB capillary column (25m*0.25mm i.d.*0.2µm film thickness) and a flame ionization detector with hydrogen as the carrier gas. The peaks of the fatty acid methyl esters were detected by comparing their retention times with that of commercial standards (Sigma-Aldrich, Nu-Chek-Prep, Elysian, MN).

3.4 Co-culturing of dendritic cells and T cells

3.4.1 Collection and isolation of T cells

PBMCs were isolated as described above. PBMCs for autologous co-culture were isolated from the same volunteer as the PBMCs used for the isolation of monocytes, or from another volunteer for allogeneic co-culture. $CD4^+$ T cells were isolated using CD4 microbeads (Miltenyi Biotech). For allogeneic co-culture experiments the $CD4^+$ T cells were used after isolation, whereas for autologous co-culture the $CD4^+$ microbeads were enzymatically removed and $CD45RA^+$ naïve T cells isolated using CD45RA microbeads (Miltenyi Biotech).

3.4.2 Co-culturing of dendritic cells and autologous T cells

Mature DCs, previously cultured with or without EPA or AA and activated with TNF-α and IL-1β, at 5 x 10^4 cells/well in 96 well plates were co-cultured with autologous naïve T cells ($CD4^+CD45^+$) at 1 x 10^4 cells/well for 8 days (days 9 to 17). IL-2 at 30 units/ml (R&D Systems) was added to the culture at day 9 and again at day 13. T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) at 1 ng/ml (Sigma-Aldrich) and ionomycin at 1 nmol/ml (Sigma-Aldrich) at day 15. At day 17 the cells and supernatants were harvested and the supernatants stored at -70°C.

3.4.3 Co-culturing of dendritic cells and allogeneic T cells

Mature DCs, previously cultured with or without EPA or AA and activated with TNF-α and IL-1β, at 2 x 10^4 cells/well in 96 well plates were co-cultured with allogeneic $CD4^+$ T cells at 5 x 10^4 cells/well for 6 days (days 9 to 15). T cells were stimulated with PMA at 1 ng/ml (Sigma) and ionomycin at 1 nmol/ml (Sigma) at day 14. At day 15 the cells and supernatants were harvested and the supernatants stored at -70°C.

3.4.4 T cell proliferation

Mature DCs, previously cultured with or without EPA or AA and activated with TNF-α and IL-1β, were resuspended at 2 x 10^5 cells /ml and titrated in 3-fold dilutions. $CD4^+$ T cells from different volunteer were suspended at 0.5 x 10^5 cells/well. Mature DCs and $CD4^+$ T cells were co-cultured in DC:T cell ratio 1:2.5, 1:7.5, 1:22.5 and 1:67.5 for 5 days (days 9 to 14) in 96 well plates. T cells were pulsed on day 13 with 1 μC ^{3}H-thymidine (Amersham, GE Healthcare) for 16 hours and T cell proliferation measured with Packard Topcount NXT scintillation counter (Packard BioScience, Meriden, CT).

3.5 Cytokine determination

IL-6, IL-10 and IL-12p40 in supernatants from cells collected on day 7 and 9 were measured by ELISA. IL-2, IL-4, IL-10, IL-17 and IFN-γ were measured by ELISA in supernatants from co-cultures collected on day 17. All ELISA reagents were purchased from R&D Systems, except where indicated, and used according to the manufacturer's instructions. Maxisorp ELISA plates (Nunc) were incubated with capture antibodies specific for IL-6, IL-10, IL-12p40, IL-2, IL-4, IL-17 or IFN-γ, at room temperature (RT) overnight. After removing the capture antibodies, but without washing, the plates were blocked with 1% BSA, 5% sucrose (Sigma Aldrich) and 0.05 % NaN_3 in PBS for one hour at RT. After a wash with PBS with 0.5% tween-20, the samples and standards (at seven concentrations) were added to the plates and incubated overnight at 4°C. Plates were then washed and detection antibodies specific for IL-6, IL-10, IL-12p40, IL-2, IL-4, IL-17 or IFN-γ added to the plates and incubated for two hours at RT. Plates were washed again and horseradish peroxidase (HRP)-labelled streptavidin added to the plates and incubated for twenty minutes at RT. After washing the plates again, substrate solution (TMB substrate) (KEM-EN-TEC Diagnostics, Taastrup, Denmark) was added to the plates and incubated until blue colour had developed. Stop solution (0.18 M H_2SO_4) was added to the plates and the absorbance measured immediately at 450 nm in Titertek Multiscan (Biological Instrumentation Services, England). The concentration in ng/ml of each sample was read from a seven point standard curve constructed using absorbance on y-axis and concentrations on x-axis. In order to reduce variation caused by using blood from various individuals, the cytokine secretion by DCs treated with EPA or AA was calculated as a ratio of the cytokine secretion by DCs treated with BSA only and expressed as secretion index (SI).

3.6 Statistical analysis

Results were expressed as the mean ± standard error of mean (SEM) for each treatment group. Analysis of variance (ANOVA), followed by Bonferroni post hoc test was used to compare the groups using SigmaStat, with $P \leq 0.05$ being considered significant.

4 Results

4.1 Fatty acid composition of dendritic cells cultured with AA or EPA

Preliminary results indicate that DCs that had been incubated with EPA (50 µM) at day 6 for 24 h contained EPA and an elongation product of EPA, i.e. docosapentaenoic acid (DPA) both at day 7 as well as on day 9. Cells incubated with AA for 24 h from day 6 to 7 contained both AA and the elongation product docosatetraenoic acid (DTA) on day 7 and 9 (data not shown). Due to the large number of cells needed for the fatty acid analysis, cells from ten experiments were pooled to extract and analyse the fatty acid composition. However, the fatty acids were in limited amounts and the results are only preliminary.

4.2 Viability of dendritic cells cultured with AA or EPA

Twenty four hour incubation with AA or EPA at concentrations of 12.5 µM, 25 µM or 50 µM did not affect cell viability as there was no difference in the number of cells between different treatment groups after incubation with AA or EPA or without fatty acids, neither on day 7 (imDCs) nor after stimulation on day 9 (mDCs) (table 2).

Table 2. The effects of AA and EPA on cell viability of imDCs and mDCs

	imDC x 10^5 cells/ml			mDC x10^5 cells/ml		
Exp.	**wo FA**	**AA**	**EPA**	**wo FA**	**AA**	**EPA**
1	1.15	1.15	1.25	1.35	1.15	1.90
2	1.10	1.10	0.85	1.80	1.75	2.20
3	0.75	1.30	0.95	2.4	1.5	1.3
4	1.25	1.30	1.25	1.75	1.70	1.40
5	0.80	1.10	0.80	2.75	1.10	2.50
Average	1.01	1.19	1.02	2.01	1.44	1.86
Std	0.22	0.10	0.22	0.56	0.30	0.51

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA (50 µM) were added to the cells at day 6. The cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β. Cells were counted in trypan blue and results expressed as number of cells per ml. wo FA: without fatty acids, EPA: eicosapentaenoic acid, AA: arachidonic acid, imDC: immature dendritic cells, mDC: mature dendritic cells, n = 5.

4.3 The effects of AA and EPA on dendritic cell phenotype

When imDCs were matured with TNF-α and IL-1β there was an increase in the percentage of cells expressing HLA-DR, CD86, CD80 and CD40 when cells had been incubated without FA or with 50 µM AA (fig. 3). There was no further increase in the percentage of cells expressing these surface molecules upon stimulation with LPS. The percentage of mDCs expressing HLA-DR, CD86, CD80 and

CD40 was statistically lower for mDCs that had been incubated with EPA compared with mDCs that had been incubated without FA or with AA (fig. 3) and there was no increase in the percentage of cells expressing HLA-DR or CD80 upon maturation of the DCs. When imDCs that were incubated with EPA were matured there was an increase in the proportion of cells expressing CD86 but this increase was less than the increase in the proportion of cells expressing CD86 after maturation of the imDCs without FA or with AA. Incubation with EPA caused less proportion of mDCs than imDCs to express CD40. The percentage of cells expressing CCR7 increased upon maturation when the cells were incubated without FA but not when cells were incubated with AA. Similar proportion of mDCs expressed DC-SIGN as imDCs when the cells were incubated without FA or with AA, but when cells were incubated with EPA less proportion of mDCs were positive for DC-SIGN than observed for imDCs. Very low percentage of imDCs and mDCs expressed CD14, regardless of whether they had been incubated with or without FA (fig. 3).

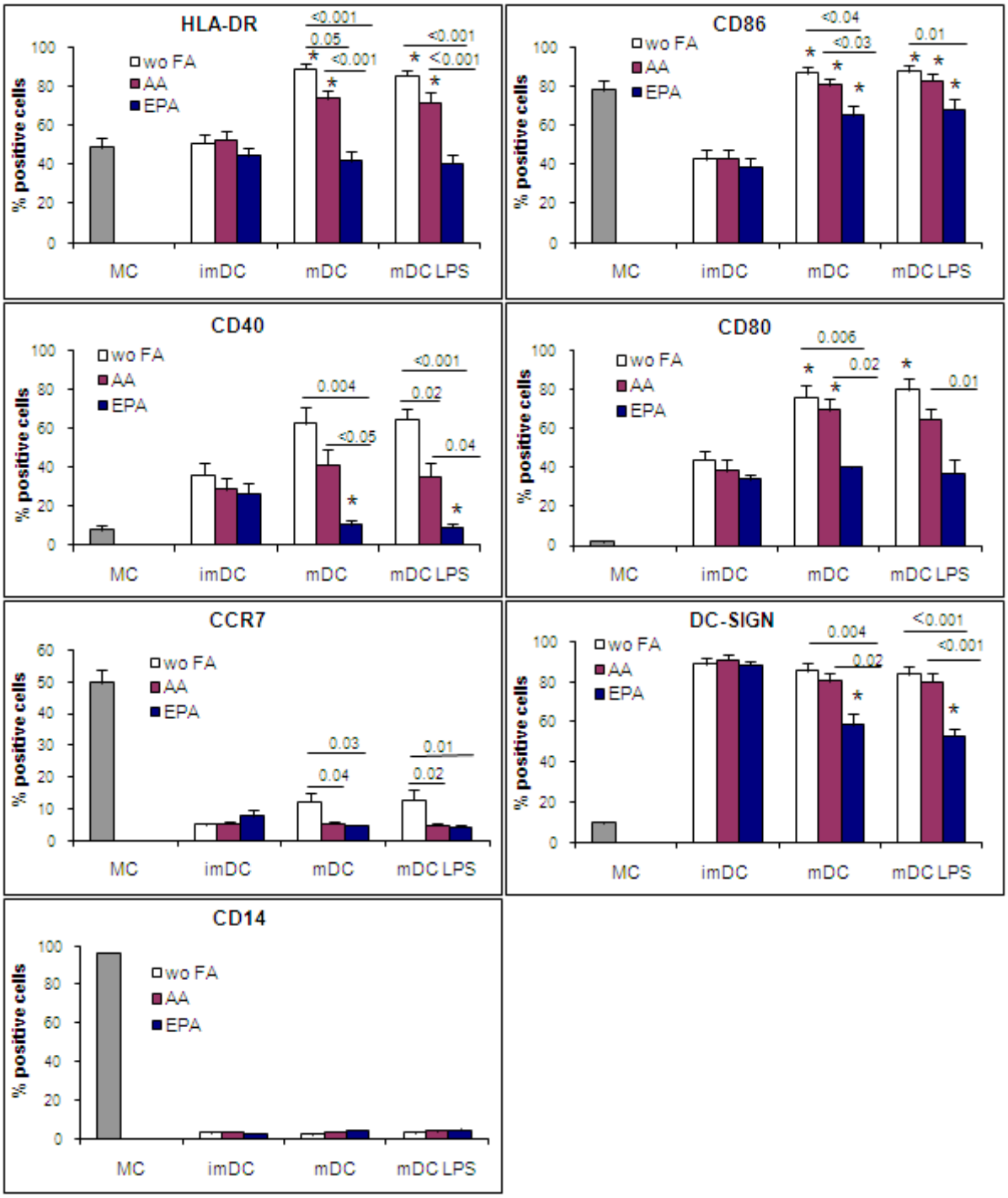

Figure 3. The effects of AA and EPA on the percentage of monocytes and monocyte-derived dendritic cells expressing the surface molecules indicated.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA (50 μM) were added to the cells at day 6. The cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β, with or without LPS. Percentage of positive cells was determined by flow cytometry. wo FA: without fatty acids, EPA: eicosapentaenoic acid, AA: arachidonic acid, MC: monocytes, imDC: immature dendritic cells, mDC: mature dendritic cells, LPS: lipopolysaccharide. Values are mean + SEM, n = 3-9. * different from imDC previously incubated without or with AA or EPA ($P<0.05$). P values are shown for differences between imDCs, mDCs and mDCs stimulated with LPS and incubated with AA or EPA compared with cells at the same maturation stage but not incubated with fatty acids.

The effects of AA and EPA on the percentage of mDCs expressing HLA-DR or CD86 were dose dependent (fig. 4).

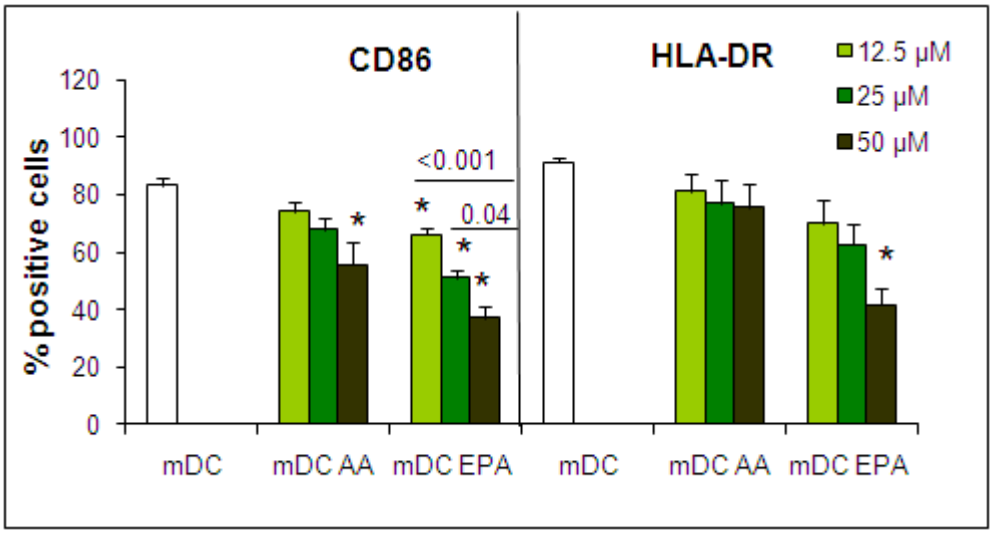

Figure 4. The effects of different concentrations of AA and EPA on the percentage of mature dendritic cells expressing CD86 and HLA-DR.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA at concentrations of 12.5, 25 or 50 µM were added to the culture at day 6. Cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β. Percentage of positive cells was determined by flow cytometry. Abbreviations are the same as in Figure 2. Values are mean + SEM, n = 3, * different from mDCs previously incubated without FA ($P<0.05$). P values are shown for differences between mDCs incubated with AA or EPA compared with mDCs not incubated with fatty acids.

The average expression levels (mean expression of the whole cell population) of HLA-DR, CD86, CD80, CD40 and CD14 increased when the cells were maturated to mDCs from imDCs (fig. 5). On the contrary, the average expression level of DC-SIGN decreased upon maturation to mDCs (fig. 5). There was no difference in the average expression levels of these molecules between cells incubated without FA or with AA or EPA (fig. 5). EPA only affected average expression of CCR7 but mDCs that had been incubated with EPA and stimulated with LPS had higher expression of CCR7 compared with mDCs incubated without FA or with AA (fig. 5).

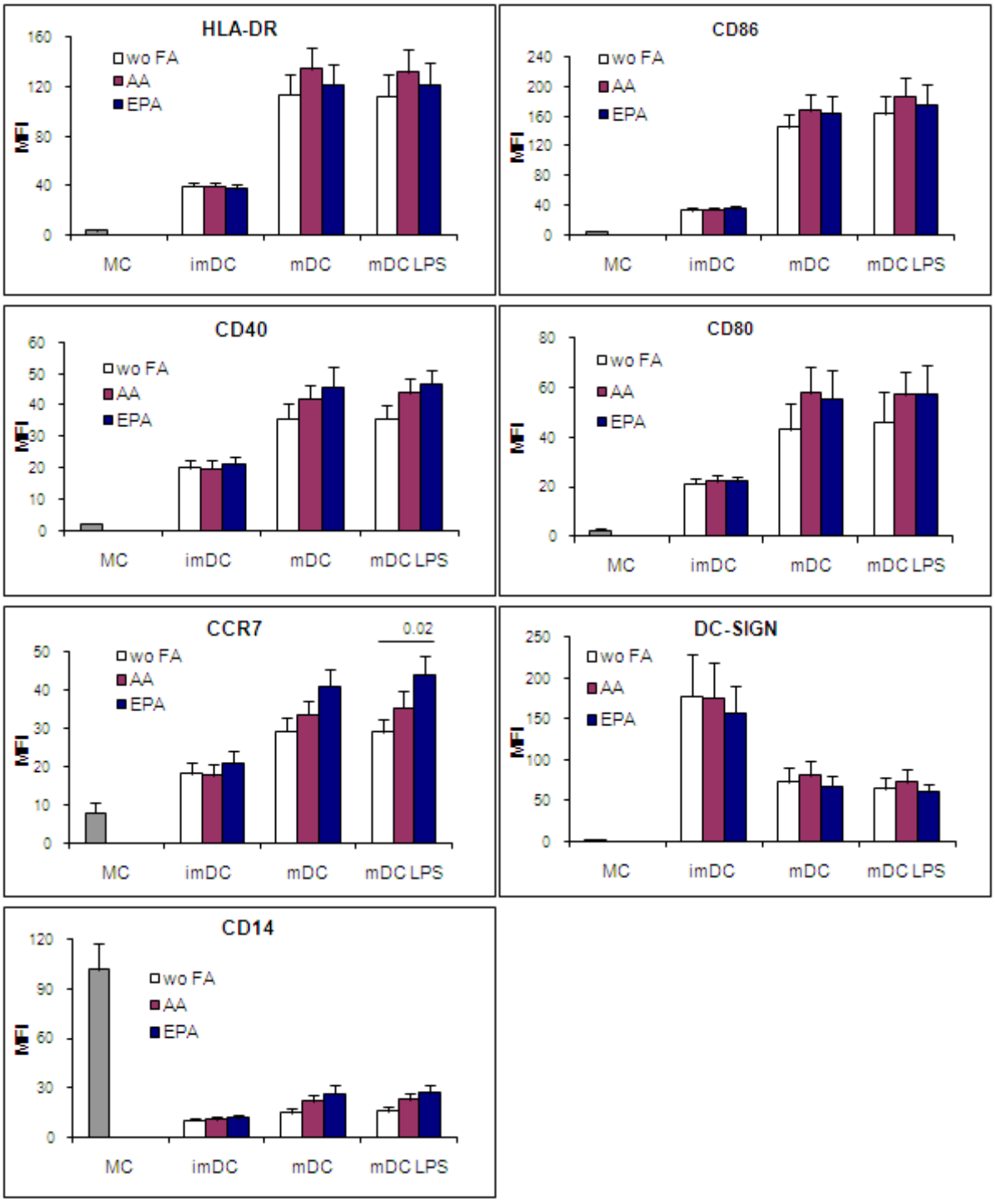

Figure 5. The effects of AA and EPA on the average expression of surface molecules on monocytes and monocyte-derived dendritic cells.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA (50 μM) were added to the cells at day 6. The cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β, with or without LPS. The average expression of surface molecules on each cell, or the MFI (mean fluorescence intensity), was determined by flow cytometry. Abbreviations are the same as in Figure 2. Values are mean + SEM, n = 3-9. P value is shown for difference between mDCs stimulated with LPS and incubated with EPA compared with mDCs stimulated with LPS not incubated with fatty acids.

When the average expression levels were examined for the positive population only, mDCs expressing CD40 that had been incubated with EPA had significantly higher average expression of CD40 than mDCs that had been incubated with AA or without FA (fig. 6). There was a tendency towards the same effect of EPA on average expression of CD86 and HLA-DR for the CD86 and the HLA-DR positive populations (fig. 6).

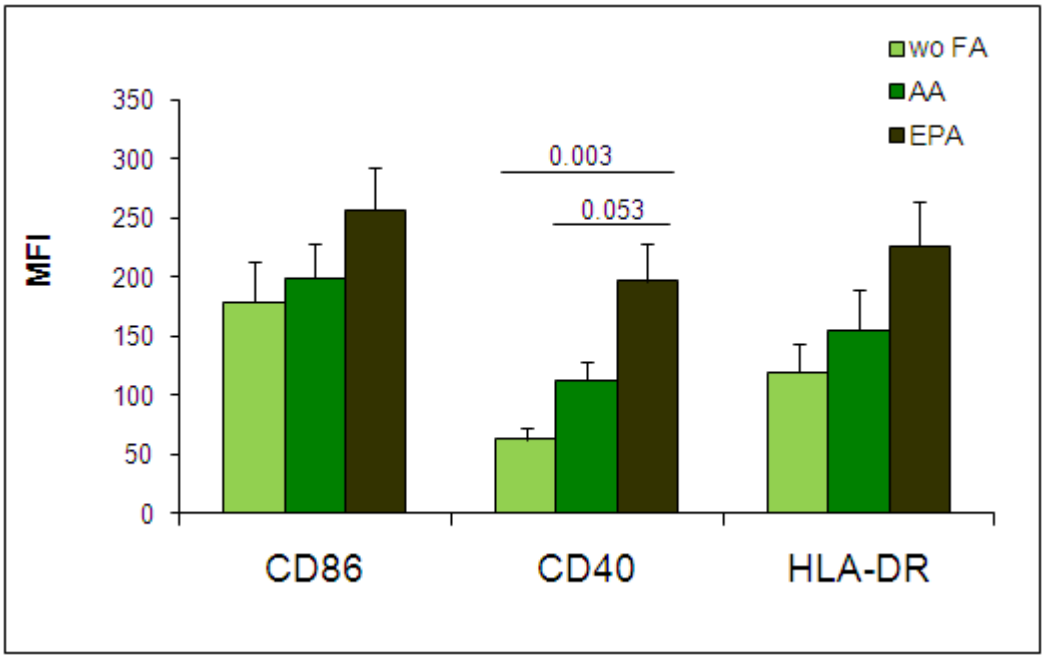

Figure 6. The effects of AA and EPA on the average expression of CD86, CD40 and HLA-DR on mature dendritic cells positive for the surface molecules.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA 50 μM were added to the cells at day 6. Cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β. The average expression of surface molecules on each cell, or the MFI (mean fluorescence intensity), was determined by flow cytometry. Abbreviations are the same as in Figure 2. Values are mean + SEM, n = 5. P values are shown for differences between mDCs incubated with AA or EPA compared with mDCs not incubated with fatty acids.

4.4 The effects of AA and EPA on dendritic cell granulation

ImDCs and mDCs that were incubated with EPA or AA were more granulated than imDCs and mDCs incubated without FA (fig. 7). There was no difference in the granulation of cells incubated with EPA or AA. The effects of EPA and AA on the granulation were dose-dependent (fig. 7).

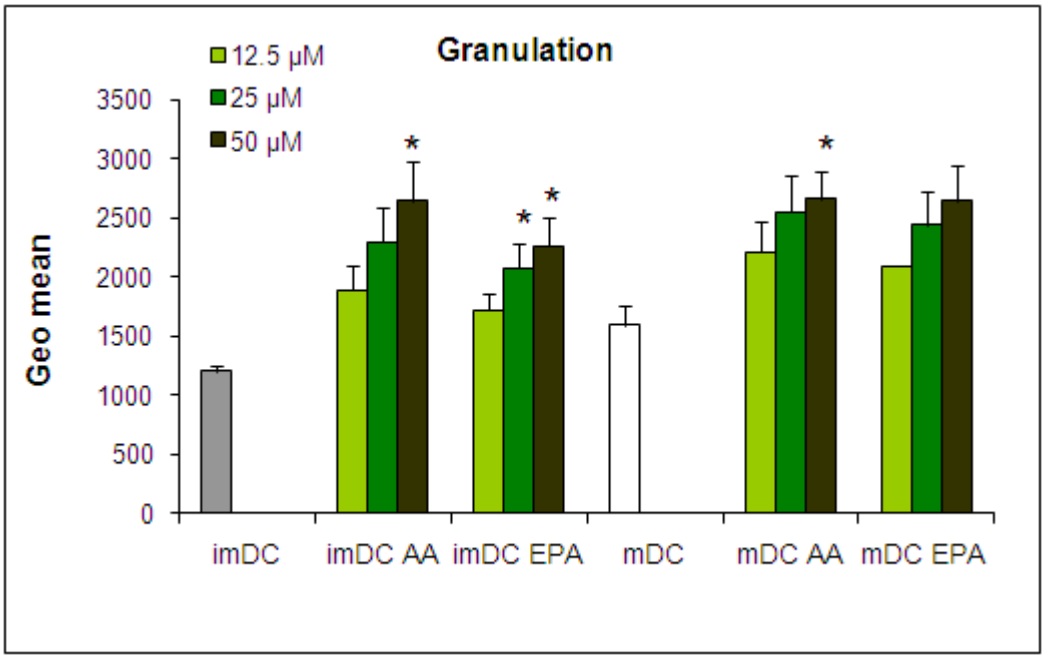

Figure 7. The effects of AA and EPA on the granulation of monocyte-derived dendritic cells

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA at concentrations of 12.5 µM, 25 µM or 50 µM were added to the cultures at day 6. Cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β. Percentage of positive cells was determined by flow cytometry. Abbreviations are the same as in Figure 2. Values are mean + SEM, n = 3.* different from imDCs or mDCs previously incubated without FA ($P<0.05$).

4.5 The effects of AA and EPA on intracellular expression of HLA-DR

To determine if surface molecules accumulated inside the DCs, intracellular staining, which detects simultaneously molecules expressed both internally and on the surface of the cells, was performed. There was no difference in the percentage of imDCs or mDCs expressing HLA-DR for cells incubated without or with AA or EPA (fig. 8). There was no difference in the average expression of HLA-DR for cells incubated with or without FA (data not shown).

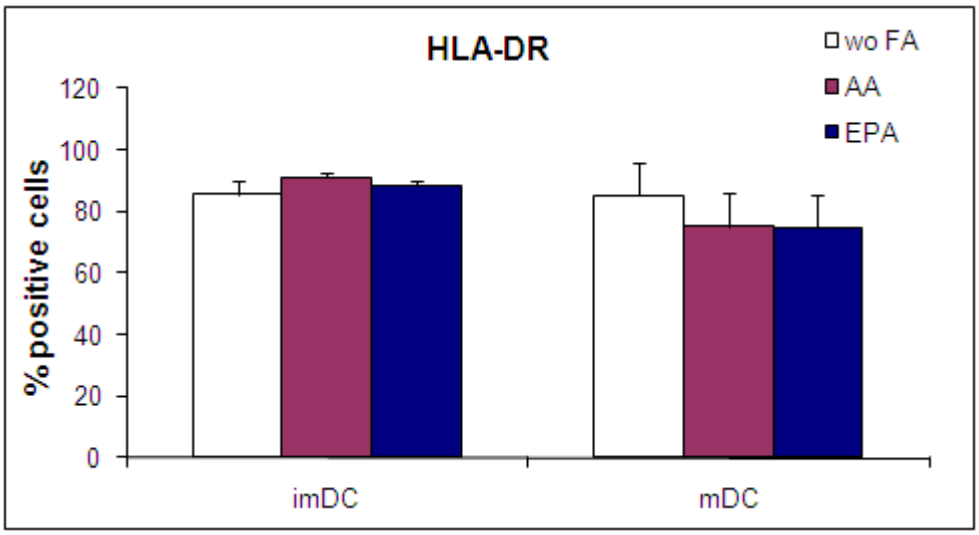

Figure 8. The effects of AA and EPA on the percentage of monocyte-derived dendritic cells expressing HLA-DR intracellularly and on the surface.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA (50 μM) were added to the cultured cells at day 6. Cells were washed thoroughly on day 7 and cultured without fatty acids for two more days with TNF-α and IL-1β. Percentage of positive cells was determined by flow cytometry. Abbreviations are the same as in figure 3. Values are mean + SEM, n=3.

4.6 The effects of AA and EPA on cytokine secretion by dendritic cells

ImDCs secreted very little IL-10, IL-12p40 or IL-6, regardless of whether they were incubated without FA or with AA or EPA (data not shown). IL-10 and IL-12p40 secretion increased upon maturation of the DCs (IL-10 from 0.08 to 0.50 ng/ml and IL-12p40 from 0.12 to 12.91 ng/ml). ImDCs and mDCs secreted similar amounts of IL-6 (1.01 and 0.93 ng/ml, respectively). Stimulation with LPS increased cytokine secretion by the mDCs (1.22 ng/ml for IL-10; 27.86 ng/ml for IL-12p40; and 3.75 ng/ml for IL-6).

Mature DCs previously incubated with AA or EPA secreted less IL-10 than mDCs incubated without FA and there was a tendency towards a decrease in the secretion of IL-12p40 by mDCs when they had previously been cultured with AA or EPA (fig. 9). On the other hand, there was a tendency towards an increase in secretion of IL-6 by mDCs stimulated with LPS when they had previously been incubated with EPA, compared with that when they were incubated without fatty acids.

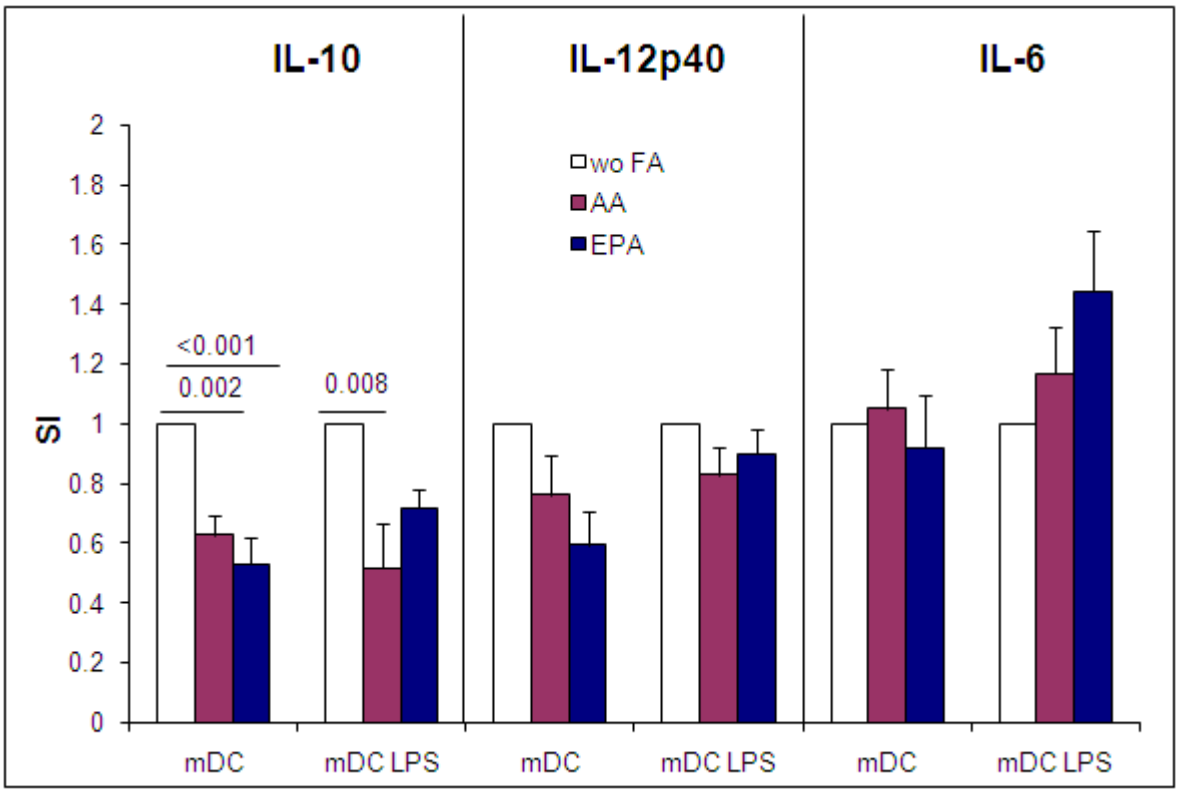

Figure 9. The effects of AA and EPA on cytokine secretion by monocyte-derived dendritic cells.

Monocytes were cultured with GM-CSF and IL-4 for 7 days. AA or EPA (50 μM) were added to the culture at day 6. Cells were washed thoroughly on day 7 and incubated without fatty acids for two more days with TNF-α and IL-1β, with or without LPS. Concentration of IL-10, IL-12p40 and IL-6 in the medium was measured by ELISA. Abbreviations are the same as in figure 2. Results are shown as a ratio of cytokine concentration in cultures of mDC incubated with and without fatty acids. n = 4-8. P values are shown for differences between mDCs or mDCs stimulated with LPS incubated with AA or EPA compared with mDCs or mDCs stimulated with LPS not incubated with fatty acids.

4.7 The effects of AA- and EPA-treated dendritic cells on allogeneic $CD4^+$ T cell proliferation

There was no difference in proliferation of T cells when they were co-cultured with mDCs that had been incubated without FA or with AA or EPA (fig. 10). $CD4^+$ T cell proliferation increased with increasing number of DCs in the DC:T cell co-culture. When $CD4^+$ T cells or mDCs were cultured alone very little proliferation occurred.

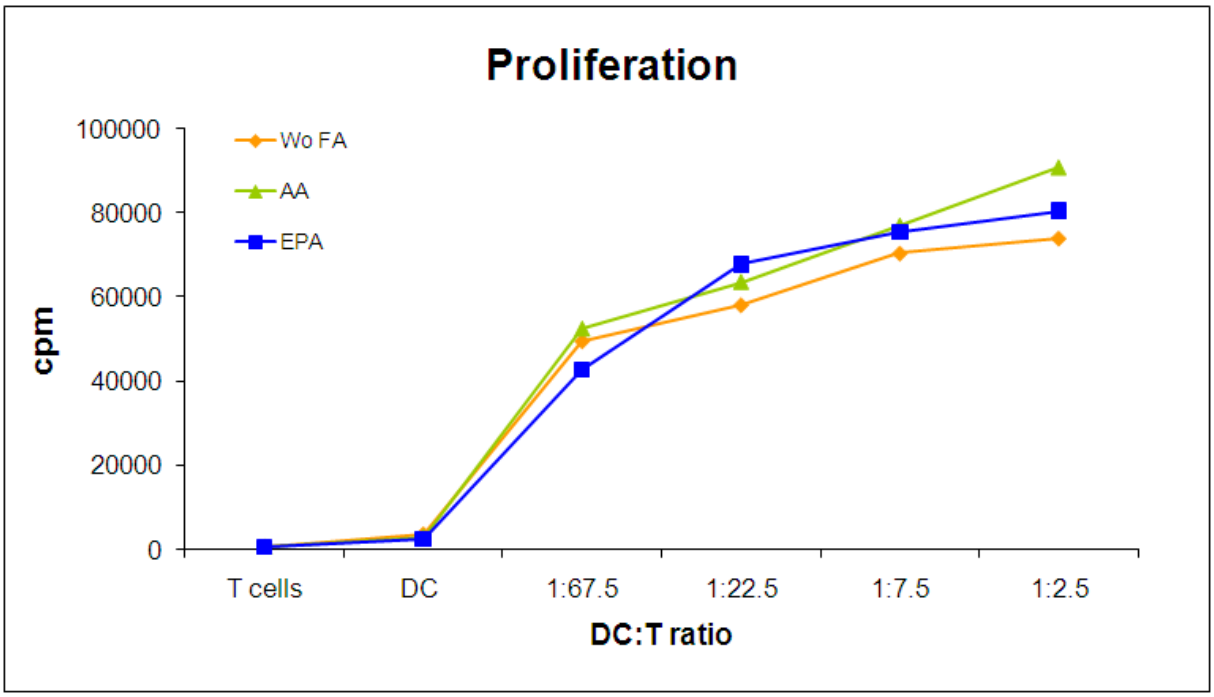

Figure 10. The effects of AA- and EPA-treated dendritic cells on T cell proliferation.

Mature dendritic cells incubated with 50 µM EPA or AA or without FA were collected on day 9. Peripheral blood from another donor was also collected on day 9 and mDCs and $CD4^+$ cells co-cultured in DC:T cell ratios of 1:67.5, 1:22.5, 1:7.5 and 1:2.5 for 5 days. T cells were pulsed with 1 µCi ^{3}H-thymidine for 16 hours and T cell proliferation measured as counts per minute (cpm). n=3.

4.8 The effects of AA- and EPA-treated dendritic cells on cytokine secretion by allogeneic $CD4^+$ T cells

When mDCs that had previously been incubated with EPA were co-cultured with allogeneic $CD4^+$ T cells, there was a tendency towards less secretion of IL-4 compared with that by allogeneic $CD4^+$ T cells cultured with AA-treated DCs or DCs incubated without FA (fig. 11). Allogeneic $CD4^+$ T cells, co-cultured with AA-treated DCs had a tendency towards more IL-4 secretion than $CD4^+$ T cells cultured with DCs incubated without FA. When DCs were incubated with EPA, the allogeneic $CD4^+$ T cells had a tendency towards more IL-17 and IFN-γ secretion than allogeneic $CD4^+$ T cells co-cultured with DCs incubated without FA or with AA. Allogeneic $CD4^+$ T cells, co-cultured with AA-treated DCs, also had a tendency towards more IL-17 and IFN-γ secretion than allogeneic $CD4^+$ T cells co-cultured with DCs incubated without FA, but less than allogeneic $CD4^+$ T cell co-cultured with EPA-treated DCs.

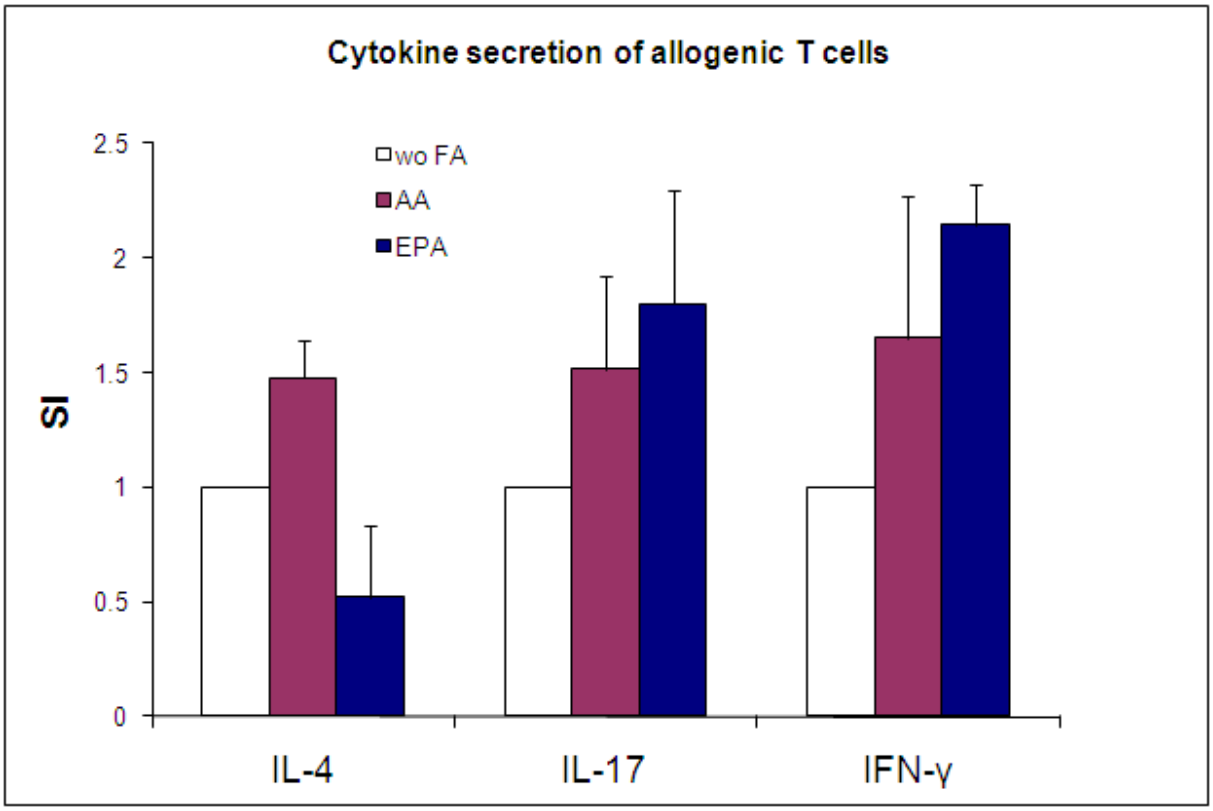

Figure 11. The effects of AA and EPA-treated dendritic cells on cytokine secretion by allogeneic $CD4^+$ T cells.

Mature dendritic cells treated without or with 50 μM AA or EPA were collected on day 9. Peripheral blood from another donor was also collected on day 9 and mDCs and $CD4^+$ cells co-cultured at DC:T cell ratio of 1:2.5 for 6 days. At day 14 PMA and ionomycin were added to the culture for T cell stimulation for 24 hours. At day 15, the supernatants were collected and IL-4, IL-17 and IFN-γ were measured by ELISA. Results are shown as a ratio of cytokine concentration in culture of mDC incubated with and without fatty acids/allogeneic T cell co-culture. n=2. Cytokine concentration in co-cultures of allogeneic $CD4^+$ T cells with mDCs (wo FA) were 0.17 ng/ml of IL-4, 1.20 ng/ml of IL-17 and 0.10 ng/ml of IFN-γ.

4.9 The effects of AA- and EPA-treated dendritic cells on cytokine secretion by autologous naïve $CD4^+$ T cells

Autologous $CD4^+$ T cells co-cultured with AA-treated mDCs secreted less IL-4 than $CD4^+$ T cells co-cultured with DCs incubated without FA and autologous $CD4^+$ T cells co-cultured with EPA-treated mDCs had a lower secretion of IL-4 than $CD4^+$ T cells co-cultured with DCs incubated without FA (fig. 12). There was a tendency towards less secretion of IL-10 by $CD4^+$ T cells co-cultured with mDCs that had been incubated with AA or EPA, but a tendency towards more IL-17 and IFN-γ secretion by autologous $CD4^+$ T cells cultured with mDCs that had been incubated with AA or EPA compared with autologous $CD4^+$ T cells cultured with mDCs that had been incubated without fatty acids (fig. 12).

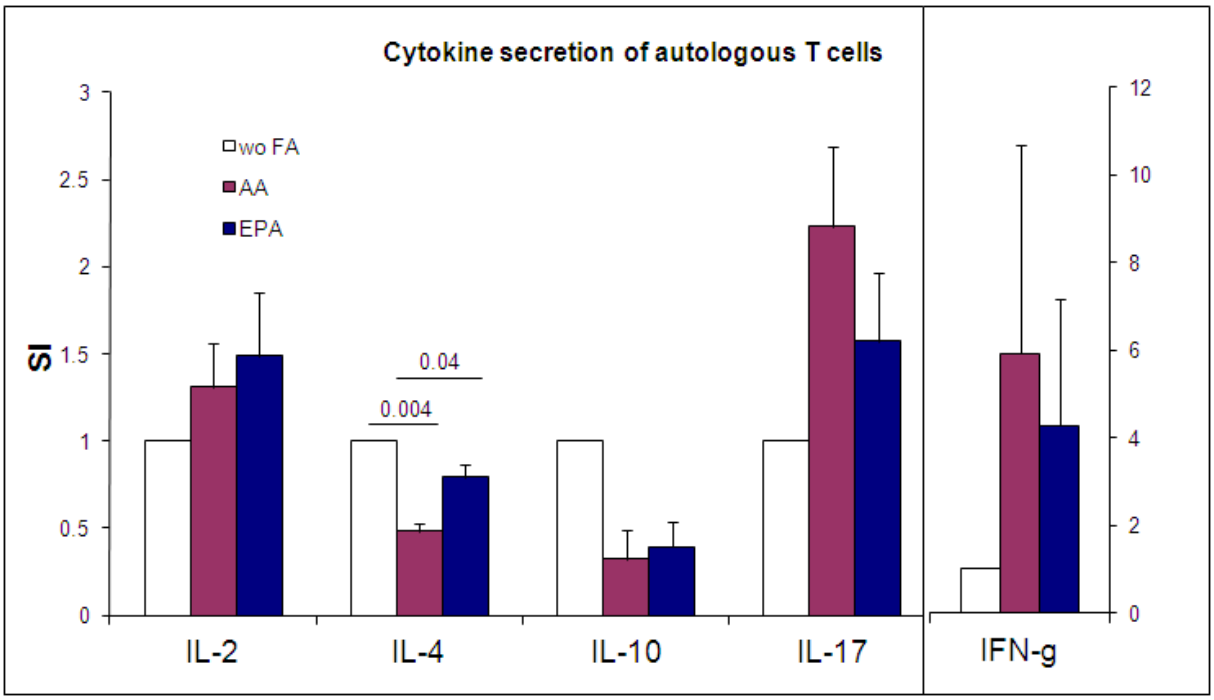

Figure 12. The effects of AA- and EPA-treated dendritic cells on cytokine secretion by autologous naïve T cells.

Mature dendritic cells treated without or with 50 μM AA or EPA were collected on day 9. Peripheral blood from the same donor was also collected on day 9 and mDCs and naïve $CD4^+$ cells co-cultured for 8 days. IL-2 was added to the culture at day 9 and 13. T cells were stimulated with PMA and ionomycin at day 15. The supernatants were collected at day 17 and IL-2, IL-4, IL-10, IL-17 and IFN-γ measured by ELISA. Results are shown as a ratio of cytokine concentration in cultures of mDC incubated with and without fatty acids/autologous T cell co-culture. n = 3. Cytokine concentration in co-cultures of autologous T cells with mDCs (wo FAs) were 75.4 ng/ml, 0.88 ng/ml for IL-4, 0.80 ng/ml for IL-10, 5.64 ng/ml for IL-17 and 45.45 ng/ml for IFN-γ.

5 Discussion

In this study we investigated the effects of the omega-3 fatty acid, EPA, on DC differentiation and maturation and compared it with the effects of the omega-6 fatty acid, AA. The results show that EPA affects the phenotype of the mDCs as well as their cytokine secretion. Fewer of the mDCs that had been cultured with EPA expressed several surface molecules known to be important for T cell activation and stimulation, than of the mDCs cultured without fatty acids or with AA. However, DCs that had been incubated with EPA and were expressing the surface molecules associated with T cell activation and stimulation, expressed higher average levels of some of the molecules than DCs that had been incubated with AA or without fatty acids. Mature DCs that had been incubated with EPA secreted less IL-10 and IL-12p40 than mDCs that had been incubated with AA or without fatty acids. When naïve T cells were co-cultured with mDCs, previously incubated with EPA, they proliferated as well as naïve T cells co-cultured with mDCs previously incubated without fatty acids or with AA. However, mDCs that had been incubated with EPA activated allogeneic and autologous naïve T cells to secrete more IFN-γ and IL-17 than mDCs that had been incubated without fatty acids or with AA.

When imDCs that had been cultured without fatty acids were matured with TNF-α and IL-1β, there was an increase in the proportion of cells expressing HLA-DR, CD86, CD80, CD40, CCR7 and DC-SIGN. These results are in accordance with results from other studies showing that upon maturation DCs upregulate these and other molecules (78-81). However, when in the present study, imDCs were cultured with EPA prior to maturation, a significant proportion of the DCs failed completely or partly to upregulate these surface molecules. These results are partly in accordance with results from two other studies that investigated the effects of omega-3 fatty acids on DC differentiation and maturation (74-76). These studies examined the effects of EPA (74) or DHA (75) on the proportion of mDCs expressing surface molecules associated with T cell stimulation and activation and showed that incubation with EPA reduced the proportion of mDCs expressing CD80 and HLA-DR and incubation with DHA decreased the proportion of mDCs expressing CD80. However, in contrast to the results from the present study, incubation of the cells with EPA did not affect the proportion of cells expressing CD40 and DHA had no effect on the proportion of mDCs expressing HLA-DR or CD86. The reason for the discrepancy between the results from these studies and the present one may have to do with differences in the experimental setup as will be discussed below.

Interestingly, in the present study, despite the reduction in the proportion of cells upregulating the surface molecules associated with T cell activation, incubation with EPA caused a higher average expression of HLA-DR, CD86 and CD40 on the DCs that did express these molecules, compared with DCs previously incubated without fatty acids or with AA. These results indicate that EPA can impede upregulation of molecules involved in T cell activation and stimulation but also enhance upregulation of these molecules by the mDCs that do express them. Results from other studies that have examined the effects of EPA and/or DHA on the average expression levels of surface molecules on mDCs are not in accordance with the results from the present study as they show a decrease in the average

expression levels of HLA-DR, CD86, CD80 (74, 75) and CD40 (76) on mDCs when the cells had been cultured with EPA and/or DHA, compared with that when the cells were incubated without fatty acids.

Only two of the studies mentioned above reported results concerning the effects of EPA or DHA on surface molecule expression by imDCs. In contrast to the results from the present study showing no effect of EPA on the proportion or average expression levels of surface molecules by imDCs, these studies showed that EPA and DHA decreased the average expression levels of CD80 and CD40 and the proportion of imDCs expressing CD80 but increased the average expression levels and the proportion of cells expressing CD86 (75, 76).

The discrepancy in the results from the present study and the other three studies may be due to differences in the experimental setup between this study and the other studies that have examined the effects of omega-3 PUFA on DC maturation (74-76). One of the major differences between the setup of the present study and the others is that the fatty acids in the present study were removed from the culture before maturation of the imDCs, whereas in the other studies the fatty acids were present in the cultures from the beginning of the differentiation into imDCs (75, 76) or from day 5 (74) and were kept in the cultures for the remainder of the studies. PUFAs are susceptible to oxidation and prolonged presence of added PUFAs in the cell culture could lead to their oxidation. Oxidation of PUFAs in the cell membrane could lead to damages in the structure of the cell membrane (82), which could affect transportation of surface molecules to the surface of the cell membrane. However, Zeyda et al. (76) showed that adding the anti-oxidant BHT to the culture did not affect surface molecule expression of the DCs indicating that oxidation of PUFAs is not a likely reason for fewer positive cells expressing the surface molecules after incubation with PUFAs or the discrepancy between the results from the present study and the others. Furthermore, Zeyda et al. (76), in addition to using the experimental setup described above, also added the fatty acids post-differentiation and removed them before stimulating the mDCs and showed that it had similar effects as adding the fatty acids earlier, in that they decreased average expression levels of CD40 and increased (had a tendency towards increase when added earlier) average expression levels of CD86. Thus, the addition of the fatty acids earlier and keeping them in the cultures longer than in the present study does not seem to explain the different results obtained.

The presence or absence of foetal calf serum in the cell culture medium is another factor that may affect the results from the present study and the previous ones and perhaps explain some of the differences between the results. In the present study, 10% foetal calf serum was added to the cell culture medium, as it may provide more physiological conditions than using serum free medium. Wang et al. (74) used 5% foetal calf serum, Zeyda et al. (76) incubated the monocytes under serum free conditions and Zapata-Gonzales et al. (75) did not mention the use of foetal calf serum. Foetal calf serum contains fatty acids and these fatty acids may become incorporated into the cell membranes in exchange for the fatty acids present there. Thus, serum free conditions are usually chosen to see the

effects of the fatty acids without the interruption of fatty acids from the serum. Yaqoob et al. (83) has shown that when foetal calf serum is used in the medium of cultured cells retrieved from animals that have been fed dietary fatty acids, the effects of the dietary fatty acids disappear. Thus, in the present study, fatty acids from the foetal calf serum may have been incorporated into the cell membranes at the expense of the fatty acids incorporated there from the medium containing AA or EPA. However, in the present study, EPA affected the stimulation and maturation of the DCs, despite the fact that foetal calf serum was present in the culture medium. It might even be considered that the effects could have been even greater if foetal calf serum would not have been used.

The effects of EPA on DCs seen in the present study are likely due to the fatty acid being incorporated into the cell membranes, as our preliminary results indicate that EPA and AA were indeed incorporated into the cell membranes on day 7 and were still present in the membranes of the cells on day 9 (data not shown). Only one of the studies discussed above (76) reported fatty acid composition of the cells and showed, similar to our results, that cells incubated with EPA had incorporated EPA into the membranes and elongated some of it to DPA. Zeyda et al. (76) used the fatty acids at 20 µM concentration, compared with most of the experiments in the present study, as well as the other published studies, being conducted with the fatty acids at 50 µM concentration (74, 75).

In the present study, the fatty acids were allowed to bind to BSA before they were added to the culture medium. This was also the case in the study by Zeyda et al. (76) but not the other studies investigating the effects of PUFAs on DC maturation. Long chain fatty acids are hydrophobic and are bound to serum albumin in blood. Allowing the fatty acids to bind to BSA before adding them to the culture medium may prevent their potential detergent effects and facilitate efficient uptake into the cell membranes (84).

In the present study, incubation with both EPA and AA led to increased granulation of both imDCs and mDCs as indicated by an increase in side scatter in the flow cytometer analysis. Increased granulation of DCs following incubation with PUFAs has not been reported previously. As there was no difference between the granulation of imDCs and mDCs it may be concluded that the fatty acids exerted their effects on the granulation from day 6 and that the effect remained, although the fatty acids were removed from the culture medium. Increased granulation may indicate accumulation of molecules in the cytosol. When both surface and internal expression of HLA-DR molecules was examined simultaneously, it revealed that treatment with AA or EPA did not affect the proportion of cells expressing this molecule. As fewer EPA-treated cells expressed HLA-DR only on the surface, these results indicate that in EPA-treated cells the HLA-DR molecules may not be transported to the surface. Whether there was accumulation in the cytosol of other molecules is not known. As both EPA and AA led to an increase in granulation of mDCs but AA had little or no effect on the expression of surface molecules of mDCs, accumulation of surface molecules in the cytosol may not be the only explanation of the effect of EPA on expression of molecules on DCs.

Other possible targets for the fatty acids to affect the DCs are the cell membranes but because of the bend in their shape, PUFAs may modify the structure and the microviscosity of the cell membranes. This change in the structure and fluidity of the cell membranes might affect the transfer of the molecules to the surface. The fatty acids may also affect production of transcription factors or mediators in intracellular pathways of the DCs, preventing signals to reach the nucleus and consequently preventing production of the surface molecules.

In the present study stimulation with LPS, in addition to TNF-α and IL-1β, did not affect the proportion of cells expressing the surface molecules or the average expression of the surface molecules, with the exception of CCR7 which was more expressed when LPS was added to the culture. The limited effect of LPS stimulation on the expression of surface molecules may be in agreement with the speculations that LPS does not affect DCs because they do not express the CD14 receptor, which is necessary for LPS to bind to TLR4 (85). However, cytokine secretion was greatly enhanced by mDCs upon stimulation with LPS as compared with mDCs that had been stimulated with TNF-α and IL-1β alone, suggesting that the LPS used may contain substances such as lipoproteins that can connect to toll like receptors other than the CD14/TLR4 complex. In the studies by Zeyda et al. (76) and Wang et al. (74), LPS was the only stimulation factor used and since monocyte-derived DCs do not express CD14 this may cause inadequate stimulation that might affect their results.

As mentioned earlier, stimulation of mDCs with LPS, along with TNF-α and IL-1β, in the present study increased secretion of the cytokines, compared with that by mDCs stimulated with TNF-α and IL-1β alone. Cells that were cultured with EPA or AA previous to stimulation secreted less IL-10 and had a tendency towards less secretion of IL-12p40 than cells cultured without fatty acids. Other studies that have investigated the effects of omega-3 fatty acids on DC maturation have also shown that mDCs cultured with EPA secreted less IL-10, IL-12p40 and IL-12p70 than DCs cultured without fatty acids (74-76). Mature DCs cultured with EPA in the present study secreted more IL-6 than mDCs cultured with AA or without fatty acids, which is opposite to the results by Zapata-Gonzales et al. (75) who showed that culturing cells with DHA inhibited IL-6 expression.

In the present study, EPA caused fewer mDCs to express surface molecules necessary for T cell activation and mDCs that had been incubated with EPA secreted less IL-10 and had a tendency towards less secretion of IL-12p40 than DCs incubated without fatty acids, implying that their potential to act as antigen presenting cells is diminished. But as shown by their capability to activate both allogeneic and autologous T cells, that did not prove to be the case. An explanation for this may be the high levels of costimulatory molecules expressed on the positive mDCs that had been incubated with EPA as compared with those that had been incubated with AA or without fatty acids. In addition, Magnusdottir et al. (unpublished observations) showed that HLA-DR^+ mDCs that had been incubated with EPA had a tendency towards increased secretion of IL-12p40 and IL-6 after stimulation via CD40,

suggesting that the HLA-DR$^+$ mDCs that had been incubated with EPA were not only expressing higher levels of surface molecules but also secreting more cytokines than HLA-DR$^+$ mDCs that had been incubated with AA or without fatty acids. This may indicate that the few DCs that reached maturation after incubation with EPA were capable of inducing T cell activation.

The cytokine secretion of T cells co-cultured with mDCs that had been incubated with EPA was different from the cytokine secretion of T cells co-cultured with mDCs that had been incubated with AA or without fatty acids. Autologous naïve CD4$^+$ T cells co-cultured with mDCs that had been incubated with EPA secreted less IL-4 and slightly less IL-10 than autologous naïve CD4$^+$ T cells co-cultured with mDCs that been incubated with AA or without fatty acids. This tendency was also observed for allogeneic CD4$^+$ T cells co-cultured with mDCs that been incubated with EPA. In contrast, co-culturing both autologous naïve CD4$^+$ T and allogeneic CD4$^+$ T cells with mDCs that had been incubated with EPA resulted in a tendency towards increased secretion of IFN-γ and IL-17, which suggests that the T cells developed a Th1 and/or a Th17 phenotype. The results obtained from the co-culture studies indicate that although culturing imDCs with EPA results in fewer cells expressing surface molecules necessary for T cell activation, they are fully capable of activating T cells for proliferation and inducing them to secrete cytokines linked with Th1 and/or Th17 phenotype. That is in contrast with previous results showing that DCs cultured with omega-3 fatty acids induced less T cell proliferation than DCs cultured without omega-3 fatty acids (74-76) and caused a reduction in the secretion of IL-2 and IFN-γ (76), suggestive of inhibition of Th1 polarization.

PMA and ionomycin were used to activate the T cells for cytokine production in the co-culture experiments. It might skew the results because PMA and ionomycin cause unspecific activation of the T cells. However, the difference found between T cells co-cultured with mDC that had been incubated with EPA and T cells co-cultured with mDCs that had been incubated without fatty acids was only due to differences in the mDCs as in both instances the T cells received the same stimulation by the PMA and ionomycin.

As IL-12p40 is a shared chain between IL-12 and IL-23, the tendency towards a decrease in IL-12p40 secretion by mDCs that had been incubated with EPA could indicate a reduction in either or both cytokines. As IL-12 is strongly linked to Th1 cytokine polarization (23) and IL-23 has been linked to Th17 survival (5), the reduction in IL-12p40 could predict a reduction in Th1 and/or Th17 cytokine secretion by the T cells in the co-culture experiments. However, the results obtained were opposite to that and the data suggestive of increased Th1 and Th17 cytokine secretion by the T cells following co-culture with mDCs that had been incubated with EPA. The tendency towards increased IL-17 secretion by the activated T cells when co-cultured with DCs treated with EPA may be linked to increased IL-6 secretion by the DCs, as IL-6 induces differentiation of naïve T cells into Th17 cells (4, 24). As discussed earlier, although there were fewer mDCs expressing molecules associated with T cell activation and stimulation, following EPA-treatment, those that are expressing these molecules

may be more potent than the mDCs incubated without fatty acids and therefore more capable of inducing Th1 and Th17 cytokine secretion.

6 Summary and conclusion

Incubation of imDCs with EPA resulted in fewer mDCs expressing HLA-DR, CD86, CD80, CD40, CCR7 and DC-SIGN than among mDCs cultured without fatty acids or with AA. However, the cells expressing these molecules expressed higher average levels of them. Incubation with EPA also affected cytokine secretion by the mDCs with reduced IL-10 secretion, a tendency towards reduced IL-12p40 secretion but increased secretion of IL-6. Despite this immature phenotype of EPA-treated DCs they were just as capable of activating allogeneic and autologous $CD4^+$ T cells as DCs cultured without EPA. T cells co-cultured with EPA-treated mDCs had a tendency towards increased IFN-γ and IL-17 secretion indicating that the EPA-treated mDCs seemed to polarize the cells towards a Th1 or Th17 phenotype. Therefore, although culturing imDCs with EPA lead to fewer mDCs with a mature phenotype, the DCs seemed to be fully capable of activating and differentiating T cells.

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