Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 269–275
Journal of Nutritional Biochemistry

Inhibition of IgE production by docosahexaenoic acid is mediated by direct interference with STAT6 and NFκB pathway in human B cells

Christin Weise[a], Kerstin Hilt[a,1], Milena Milovanovic[a], Dennis Ernst[a], Ralph Rühl[b], Margitta Worm[a,*]

[a]Allergy-Center-Charité, Department of Dermatology and Allergy, Charité-Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany
[b]Laboratory of Nutritional Bioactivation and Bioanalysis and Apoptosis and Genomics, Research Center of the Hungarian Academy of Sciences, Department of Biochemistry and Molecular Biology, University of Debrecen, Hungary

Received 8 October 2009; received in revised form 1 February 2010; accepted 8 February 2010

Abstract

Nutrition can modify the onset or severity of diseases and recent changes in eating habits are supposed to promote immunoglobulin (Ig) E-dependent disorders. The n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) possesses immunomodulatory properties and has been shown to influence chronic and allergic inflammatory disorders in vivo. Here, we examined the impact of DHA on primary human B cells to elucidate its potential role in direct regulation of IgE production and the underlying mechanisms of action. Therefore, cells were stimulated with anti-CD40/interleukin (IL)-4 in the presence of DHA. Subsequently, Ig production, generation of antibody secreting cells, epsilon-germline transcript (εGLT) and activation induced desaminase (AID) expression as well as IgE relevant signaling pathways were analyzed. Our results reveal that DHA inhibits IgE production (75±14%) and, depending on concentration, the differentiation of IgE secreting cells (59±27%). The reduction of IgE is accompanied by a direct inhibition of the switching process indicated by decreased εGLT and AID transcription. DHA causes both a reduced CD40 dependent nuclear factor κB-p50 translocation into the nucleus and a decreased IL-4 receptor expression which was associated with a reduction of IL-4 driven signal transducer and activator of transcription 6 phosphorylation. Taken together, DHA inhibits IgE production of human B cells by direct interference with both the CD40 and the IL-4 signaling pathway. The data provide one explanation for the anti-allergic role of DHA at the molecular level.

Keywords: Docosahexaenoic acid; n-3 PUFA; IgE; B cells; STAT6; NFκB

1. Introduction

The incidence of common allergic disorders has increased quite dramatically during the last decades and in many countries the prevalence has reached almost epidemic proportions [1]. At this moment, a cure for these diseases does not really exist, although a wide range of treatments is used to control the symptoms. Thus, allergies have become a major medical and social issue of the Western world [2]. Since there is an indication that the nutritional factors, like fatty acids, may contribute to this alarming development, many investigations have been performed in this field [1,3,4].

IgE is the dominant immunoglobulin (Ig) isotype of type I allergy. Amongst others, IgE production requires class switch recombination (CSR) and plasma cell differentiation. In general, isotype switching is regulated at the level of germline transcription of constant heavy chain genes and the induction of AID expression. Two signals induce the class switch to IgE: antigen specific T cell-B cell interaction via CD40L/CD40 and IL-4 secreted by activated T cells [5]. CD40, a member of the tumor necrosis factor (TNF) receptor family, transmits its signaling via TNF-receptor-associated factors followed by activation of a mitogen-activated protein kinase which phosphorylates and thus activates the inhibitor of nuclear factor κB (IκB) kinase leading to phosphorylation of IκB proteins [6,7]. Subsequent to IκB degradation, liberated nuclear factor κB (NFκB) translocates into the nucleus where it regulates the expression of a wide spectrum of genes [7–9]. IL-4 binding to its receptor complex mediates activation of cytoplasmic protein tyrosine kinases JAK1/JAK3 [10,11] and the following signal transducer and activator of transcription 6 (STAT6) phosphorylation. Afterwards, dimerized STAT6 modulates the transcription of IL-4 responsive genes [11]. NFκB and STAT6 synergize for IgE transcription and optimal IgE production. Both factors bind to the ε-germline gene promoter initiating the transcription of the sterile εGLT [1,12]. Association of εGLT with the ε-switch region leads to RNA-DNA hybrids with single stranded DNA, which is targeted by AID. After deamination within the switch region, DNA repair mechanisms lead to CSR [1,12]. In addition, STAT6 and NFκB also bind to an up-stream region of the AID gene and increase the availability of AID enzyme during the DNA rearrangement process [12].

A growing body of evidence shows the beneficial effects of the n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) (22:6n-3) on

* Corresponding author. Tel.: +49 30 450 518 105; fax: +49 30 450 518 919.
E-mail address: margitta.worm@charite.de (M. Worm).
[1] KH was supported by the Deutsche Forschungsgemeinschaft (DFG – SFB650 TP5).

0955-2863/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.02.004

inflammatory symptoms in diseases such as rheumatoid arthritis, autoimmune nephritis, inflammatory bowel disease, lupus erythematosus, psoriasis and asthma [13–16] which have led to more intensive research on different cell types of the immune system. Thereby, it has been observed that DHA and eicosapentaenoic acid alter lymphocyte, monocyte, macrophage, dendritic cell, and natural killer phenotype and function [17–25]. Although all of these immunomodulatory effects have been well documented, the underlying mechanism remains unclear in many cases. However, DHA can act via the membrane, influencing signaling in lipid rafts, by direct interference with signal transduction, as substrate for phospholipase A and by alteration of lipid mediators [24,26–30].

In a recent clinical trial, we showed that high dose DHA supplementation improves the clinical outcome of atopic eczema and causes a prominent inhibition of anti-CD40/IL-4 stimulated IgE production in PBMC ex vivo [31]. These data prompted us to investigate the underlying molecular mechanisms of DHA action on B cells.

2. Methods

2.1. Cell preparation and cell culture

Following the approval by the local ethics committee, human peripheral mononuclear blood cells (PBMC) were isolated by ficoll hypaque-isolation (d=1.077 g/ml; Biochrom KG, Berlin, Germany) from heparinized whole blood from non-allergic donors. Healthy subjects were defined as individuals who have not experienced allergic symptoms by history and had total IgE concentrations below 100 ng/ml.

B cells were purified by magnetic cell sorting using anti-CD19-coupled magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). B cell populations were more than 99% pure. This procedure has been shown not to activate B cells [32].

The culture medium RPMI1640 was supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat inactivated fetal calf serum (all from Biochrom KG). All cell cultures were carried out at 37°C in humidified air and 5% CO_2 atmosphere.

If not mentioned otherwise cells were stimulated with anti-CD40 (1 μg/ml; 82111; R&D, Wiesbaden, Germany) and IL-4 (5 ng/ml; ImmunoTools, Friesoythe, Germany) to induce IgE synthesis [33]. The DHA concentrations (Cayman Chemicals, Ann Arbor, USA) used were 0.1, 1 and 10 μM. DHA was dissolved in 100% ethanol, aliquotted, and stored at −20°C for a maximum of 6 months. The vehicle control was performed in culture media containing the same volume of ethanol (0.001%).

2.2. CFSE labeling

B cells were diluted in phosphate-buffered saline (PBS) containing 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) for 5 min at room temperature. After washing, cells were stimulated with anti-CD40/IL-4 for 4 and 7 days. Analysis was performed by means of flow cytometry (FACS Calibur; Becton Dickinson, Heidelberg, Germany).

Toxic effects of DHA were excluded by propidium iodide and trypan blue (both Sigma, Dreieich, Germany) staining of anti-CD40/IL-4 stimulated cells after 4, 7 and 10 days.

2.3. Immunoglobulin production

For Ig assays cells (10^6/ml) were stimulated with anti-CD40/IL-4. After 10 days Igs were measured in cell free supernatants by enzyme-linked immunosorbent assay (ELISA).

Clones HP6061 and HP6029 from Southern Biotech (Birmingham, AL, USA) were used for the IgE ELISA. For IgA, IgG and IgM analysis, matched antibody pairs (anti-IgA, anti-IgG and anti-IgM) were purchased from Dianova (Hamburg, Germany). Immuno plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with anti-human Ig-Fc antibody HP6061, anti-IgA, anti-IgG, or anti-IgM diluted in 0.1 M bicarbonate buffer. Blocking was performed by adding 2% bovine serum albumin (BSA)/tris-buffered saline (TBS) for 1 h, followed by several washings. Next, supernatants and standards were incubated for 2 h. After several washing steps, the second alkaline phosphatase conjugated anti-Immunoglobulin (A, G, M) was added. Since the secondary anti-IgE HP6029 was biotinylated an additional incubation with alkaline phosphatase conjugated streptavidin was performed. Following the final reaction with phosphatase substrate (Sigma), plates were read in a micro plate ELISA reader at 405 nm. The amount of Ig was calculated according to a standard curve.

2.4. ELISpot assay

The number of antibody secreting cells was determined by ELISpot technique. To this end, B cells were stimulated with anti-CD40/IL-4 for 7 days. 10^6 B cells were cultured for IgA and IgG, whereas 3×10^6 cells were used for IgE ELISpot. MultiScreen-High Protein Binding Immibilion-P Membrane plates (Millipore Schwallbach, Germany) were coated overnight with HP6061 (Southern Biotech) for IgE and with goat anti-Ig (Dianova) for IgA and IgG in 0.1 M bicarbonate buffer. Unspecific binding was blocked by adding 2% BSA/TBS for 1 h. After several washings, serial dilutions of B cells were incubated at 37°C for adequate period of time. After excessive washing, detection was performed with biotin-conjugated antibodies: HP6029 for IgE (Southern Biotech), G20-359 for IgA (BD PharMingen, San Diego, USA) and G18-145 for IgG (BD). The reaction was developed with streptavidin-horse radish peroxidase (HRP) and peroxidase substrate 33-Amino-9-ethyl-carbazole/*n*,*n*-dimethylormamide (Sigma). Antibody secreting cells appeared as red spots and were counted with CTL Immuno-Spot S4 (Cellular technology, Cleveland, OH, USA).

2.5. Real time reverse transcriptase-quantitative polymerase chain reaction

For the analysis of εGLT and AID mRNA expression, 10^6 B cells were stimulated with anti-CD40 and/or IL-4 for 4 days. Total cellular RNA was extracted using RNA preparation kit from Macherey-Nagel (Düren, Germany) and was reversely transcribed into cDNA (Applied Biosystem, Darmstadt, Germany) according to the manufacturer's guidelines.

Polymerase chain reaction (PCR) amplification was performed with hypoxanthine phophoribosyltransferase (HPRT), AID and εGLT specific primers. εGLT specific primers were sense gACgggCCACACCATCCACAggCACCAAATggACgAC and anti-sense CAggAC-gACTgTAAgATCTTCACg resulting in a 409 bp fragment. AID specific primers were sense AgAggCgTgACAgTgCTACA and anti-sense ATgTAgCggAggAAgAgCAA resulting in a 93-bp fragment. The expression levels of target gene mRNA were relatively quantified to the expression of the reference gene HPRT with the following primer pair TggCTTATATCCAACACTTCgTg (sense) and ATCAgACTgAAgAgCTATTgTAATgACCA (anti-sense) by real-time reverse transcriptase-quantitative PCR (RT-qPCR) using LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany). As negative control the template was replaced by sterile water. Amplification products were purified by electrophoresis in a 2.5% agarose gel stained with ethidium bromide. The melting curve analysis was used to control the melting point specificity. Efficiency corrected relative quantification was performed by 2-ΔΔCT method [34].

2.6. Protein isolation and Western blot of NFκB-p50

Cells were preincubated with DHA for 2 h, stimulated with 1 μg/ml anti-CD40 (G28.5; DRFZ, Berlin, Germany) for 1 h and finally lysed at 5×10^6 cells/100 μl with the NE-PER extraction reagent (Pierce, Rockford, USA) containing a protease inhibitor cocktail (Roche), followed by the extraction of nuclear proteins (30 μl). Protein concentration was quantified by standard Coomassie plus protein assay (Pierce). Nuclear extracts (4 μg) were separated in a 12% Tris glycine sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to Tris-buffered saline (PVDF) membrane (Amersham Biosciences, Freiburg, Germany) and blocked for 1 h with 5% milk powder/PBS. The membrane was incubated overnight at 4°C with 0.2 μg/ml anti-human p50 (H119, Santa Cruz, Heidelberg, Germany) in 1% milk powder/PBS. After washing the membrane with PBS-Tween, HRP conjugated goat anti-rabbit IgG (0.1 μg/ml, Santa Cruz) was added for 1 h at room temperature. The antigen detection was performed with the chemiluminescent detection system ECL Plus (Amersham Biosciences). The results were densitometrically analyzed by using freeware ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.7. Flow cytometric analysis of STAT6 phosphorylation and IL-4R expression

Purified B cells were preincubated with DHA overnight, stimulated with 10 ng/ml IL-4 for 1 h and harvested into Phosflow Fix Buffer I (BD) according to the manufacturer's recommendation. Unstimulated cells served as control. Cells were stained with anti-pSTAT6 (pY641; clone 18; BD), anti-STAT6 (clone 23, BD), anti-CD27 (LG.7F9; eBioscience, San Diego, CA, USA) and anti-CD38 (HIT2; eBioscience) for 1 h at room temperature. For IL-4 receptor (IL-4R) expression analysis, B cells were harvested directly into 4% paraformaldehyde without IL-4 stimulation and subsequently stained with anti-CD124 (clone hIL4R-M57, BD), anti-CD27 and anti-CD38 for 15 min on ice. Cells were immediately analyzed by flow cytometry. At least 30,000 gated B cells were collected for each sample and the geometric mean fluorescence was analyzed.

2.8. Flow cytometric analysis of IκBα degradation

B cells were treated as described for NFκB-p50 analysis. After fixation with 4% paraformaldehyde for 10 min at 37°C, B cells were incubated with anti-CD27 (LG.7F9) and anti-CD38 (HIT2, all eBioscience) for 15 min on ice. After an additional washing step, cells were stained with anti-IκBα (L35A5, Cell Signaling Technology, Boston, MA, USA) in 1% saponin/1% BSA/PBS for 30 min at 4°C. Cells were immediately measured as above.

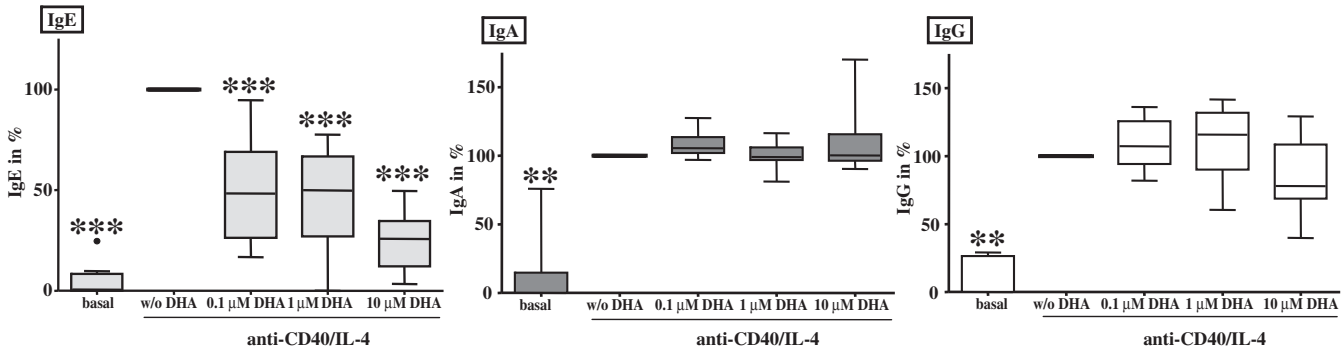

Fig. 1. DHA and its role on IgE, IgA and IgG production in anti-CD40/IL-4 stimulated B cells. Data are shown relative to DHA untreated stimulated control (100 %), as box plot (25. and 75. percentile, median); $n=16$. $^{**}P<.01$; $^{***}P<.001$, statistically significant difference compared to anti-CD40/IL-4 stimulated, DHA untreated sample.

2.9. Statistical analysis

To analyze the statistical significance Wilcoxon test for non-parametrical, paired data using SPSS software package for Windows version 12.0 (Chicago, IL, USA) was carried out. $P<.05$ was considered statistically significant.

3. Results

3.1. DHA and its role on anti-CD40/IL-4 mediated IgE production in B cells

To investigate the impact of DHA on IgE production, PBMC were incubated with or without DHA for 10 days.

IgE production from PBMC of non-allergic donors was negligible (<20 pg/ml). After anti-CD40/IL-4 stimulation, IgE increased in supernatants up to 7378±4475 pg/ml (data not shown). DHA treatment led to a dose-dependent reduction of IgE production. 10 µM DHA inhibited most efficiently down to 714±382 pg/ml ($P=.028$).

Next, we investigated whether the inhibition of IgE was due to direct action of DHA on B cells. To this end, B cells' IgE production and number of IgE secreting cells were analyzed using ELISA and ELISpot, respectively. For better illustration, the DHA untreated stimulated sample was set as 100%. After anti-CD40/IL-4 stimulation, IgE production increased up to 25-fold (2270±1610 pg/ml) and was dose-dependently reduced by DHA treatment (Fig. 1). The highest DHA concentration caused the strongest inhibition down to 485±719 pg/ml ($P<.001$, Fig. 1).

Furthermore, IgA and IgG production were examined to delineate whether DHA acts in an isotype specific manner. Since DHA treatment did not alter IgA and only slightly decreased IgG production (in a statistically insignificant manner), we concluded that DHA had a selective effect on IgE production (Fig. 1).

3.2. Effect of DHA on the number of IgE secreting cells

To prove that the reduction of IgE in supernatants was due to a reduced number of antibody secreting cells, ELISpot analysis was performed after 7 days of culture.

As depicted in Fig. 2, DHA treatment of B cell cultures caused a concentration-dependent effect on IgE secreting cells. 10 µM DHA significantly reduced the number of IgE secreting cells (51±34%, $P=.043$). Interestingly, 0.1 µM DHA did not decrease but rather increased IgE secreting cell numbers (139±30%, $P=.043$). Here, 100% correspond to 75±79 IgE-secreting cells/10^6 B cells.

In contrast, DHA had no impact on the number of IgA secreting cells (Fig. 2). However, 10 µM DHA slightly reduced the number of IgG secreting cells to 91±25% of DHA untreated sample ($P=.043$). Thereby, 237±180 IgA secreting cells/10^6 B cells and 343±284 IgG secreting cells/10^6 B cells represent 100%.

Next, anti-proliferative or toxic effects were ruled out. Toxicity by DHA was excluded by propidium-iodide (Fig. 3A) and trypan-blue staining (data not shown). Furthermore, DHA exerted no inhibition of B cell proliferation as measured by CFSE labeling (Fig. 3B).

3.3. DHA and AID mRNA expression in B cells

AID is essentially involved in the isotype switch process. Therefore, we investigated whether AID expression is altered by DHA.

AID mRNA expression was induced by anti-CD40/IL-4 stimulation up to fivefold ($P=.043$) and decreased in DHA treated B cells. As

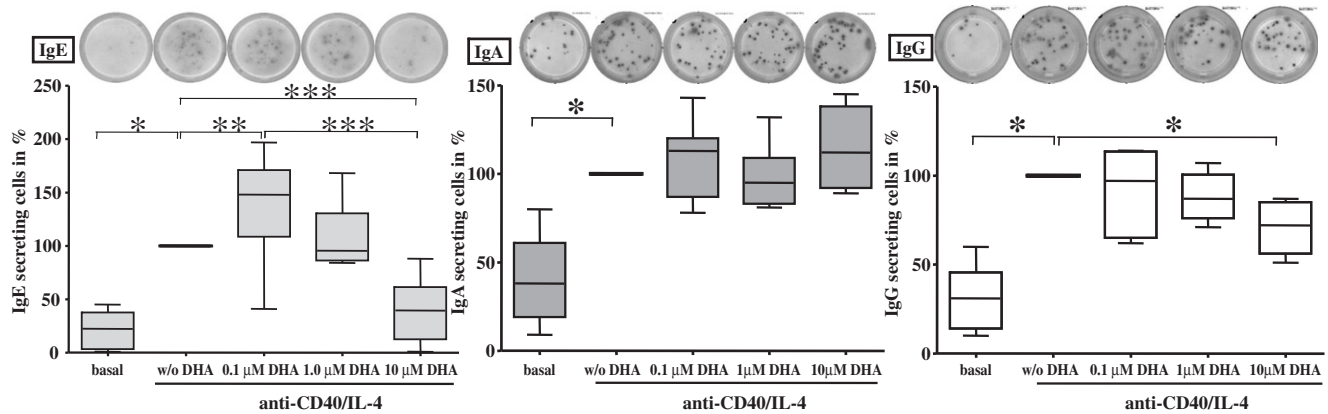

Fig. 2. Effect of DHA on the generation of IgE, IgA and IgG secreting cells in anti-CD40/IL-4 stimulated B cells. Data are shown relative to DHA untreated stimulated control (100 %), as box plot (25. and 75. percentile, median); $n\geq5$. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$, statistically significant difference compared to anti-CD40/IL-4 stimulated, DHA untreated sample.

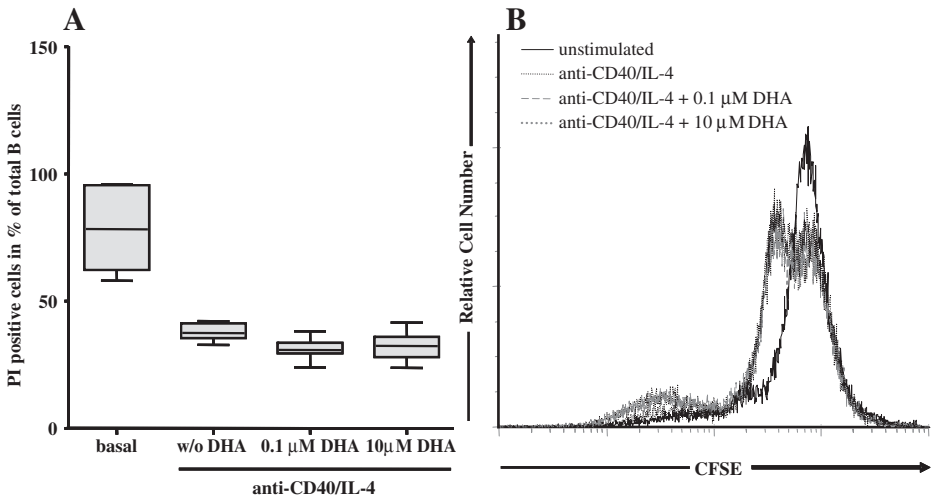

Fig. 3. Viability (A) or proliferation (B) of B cells in the presence of DHA. (A) Propidium iodide (PI) positive cells after 10 days; data are shown as box plot (25. and 75. percentile, median); n=5. (B) CFSE dilution on day 4; histogram is a representative of three experiments.

depicted in Fig. 4, the strongest inhibition was detected with the highest DHA concentration (44±32%, P=.018).

To delineate whether the IL-4 and/or the CD40 pathway were disrupted by DHA, B cells were stimulated with anti-CD40 or IL-4 alone (Supplemental Figure 1). 10 µM DHA inhibited AID expression by cells stimulated with IL-4 alone (32±22%, P=.006). Additionally, in anti-CD40 stimulated B cells 10 µM DHA tended to decrease the AID mRNA expression (39±28%) but failed to reach statistical significance (P=.0625).

3.4. Impact of DHA on IgE switch recombination

Ig heavy chain CSR correlates with the induction of specific germline transcript. The expression of εGLT indicates the switching process towards IgE.

In B cells εGLT expression was induced 5-fold by anti-CD40/IL-4 stimulation (P=.043, Fig. 4). The presence of DHA led to a reduction of εGLT expression. The maximal inhibition was observed with 10 µM DHA reaching 87±13% (P=.028). However, the overall inhibition was more prominent than the DHA mediated AID inhibition. Moreover, 10 µM DHA inhibited εGLT expression induced by CD40 ligation (54±19%, P=.036) as well as by IL-4 (73±26%, P=.016) (Supplemental Figure 1). Incidentally, the DHA mediated inhibition of both AID and εGLT was more pronounced when anti-CD40 and IL-4 were used in combination suggesting that DHA acts on both signaling pathways synergistically.

3.5. Modulation of NFκB-p50 translocation by DHA

To detect whether the impairment of IgE production was due to a modulation of p50 translocation, we analyzed the nuclear NFκB in DHA treated B cells by Western blot.

As shown in Fig. 5, p50 protein was detectable in nuclear extracts of unstimulated B cells. Stimulation with anti-CD40 caused a translocation of p50 into the nucleus from 129±20 to 177±29 densitometric arbitrary units (P=.018). However, concomitant treatment with 10 µM DHA decreased the anti-CD40 driven nuclear p50 translocation down to control levels (125±41 densitometric arbitrary units; P=.028).

Next, we determined whether IκBα degradation was affected by DHA and responsible for the modulation of p50 translocation. The

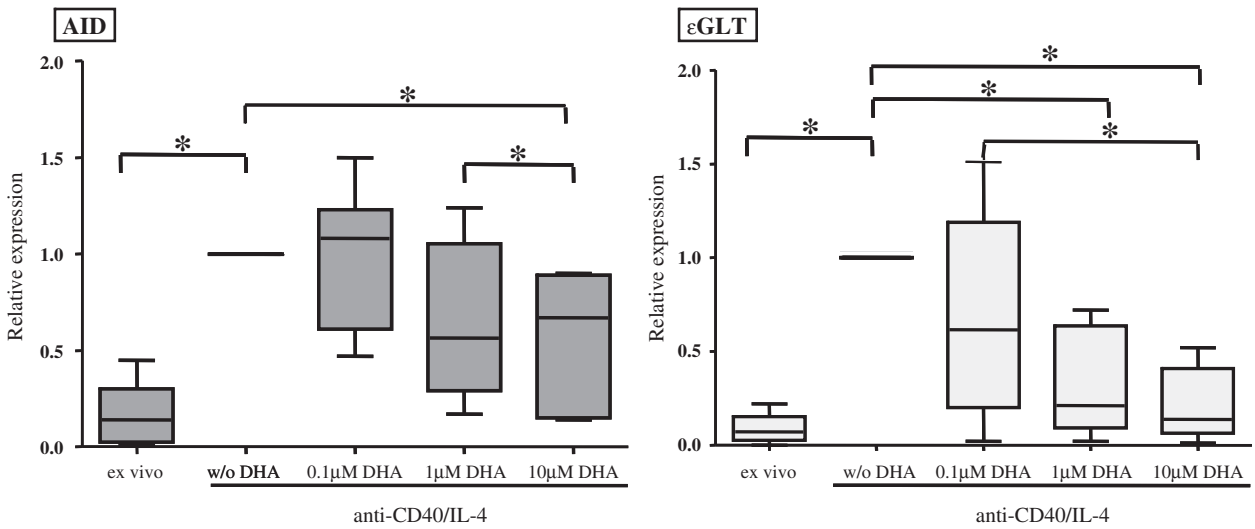

Fig. 4. Impact of DHA on AID and εGLT expression in anti-CD40 and/or IL-4 stimulated B cells. Data are shown as fold expression of DHA untreated stimulated sample; as box plot (25. and 75. percentile, median); n≥6. *P<.05, statistically significant difference compared to anti-CD40/IL-4-stimulated, DHA-untreated sample.

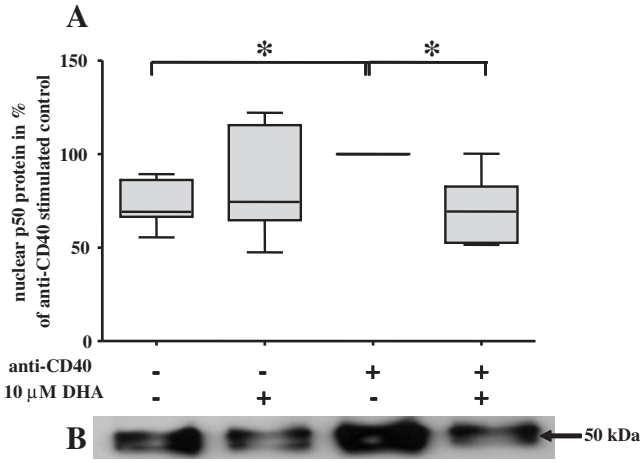

Fig. 5. Modulation of anti-CD40 induced NFκB-p50 translocation into the nucleus by DHA. (A) Data are shown as box plot (25. and 75. percentile, median). (B) Blot is a representative of seven similar experiments (n=7). *P<.05, statistically significant difference compared to anti-CD40 stimulated, DHA untreated sample.

intracellular IκBα content decreased from a basal geometric mean fluorescence intensity (MFI) of 40.2±5.0 to a MFI of 33.5±4.3 upon anti-CD40 stimulation and remained stable under DHA treatment (10 μM DHA: MFI 35.0±8.2). The data suggest that DHA did not operate by influencing the anti-CD40 driven degradation of IκBα (Supplemental Figure 2).

3.6. DHA and STAT6 phosphorylation in B cells

IL-4 signaling regulates transcription of IL-4 responsible genes by binding to its receptor followed by STAT6 phosphorylation and dimerization. Therefore, we determined the phosphorylation of STAT6 (pSTAT6) in the presence of DHA.

The basal phosphorylation level of STAT6 was low (MFI 3.0±0.6, Fig. 6A). IL-4 stimulation increased the number of pSTAT6 positive cells by 50-fold and the MFI threefold (11.0±3.7, P=.008). In the presence of DHA, a reduction of STAT6 phosphorylation was observed for 1 μM (MFI 9.3±4.4, P=.022) and 10 μM DHA (MFI 8.1 ±3.6, P=.008). Furthermore, 10 μM DHA reduced the number of pSTAT6 positive cells to 79.7±13.6% of DHA untreated stimulated sample (P=.008; data not shown), resulting in a decreased ratio between STAT6 and pSTAT6 (P=.018, Supplemental Figure 3). DHA 0.1 μM did not affect the number of pSTAT6 positive cells nor STAT6 phosphorylation.

3.7. IL-4Rα chain (CD124) expression upon DHA treatment

To analyze whether the DHA mediated reduction of STAT6 phosphorylation was due to a diminished IL-4R expression we investigated IL-4Rα chain (CD124) expression in B cells upon DHA incubation.

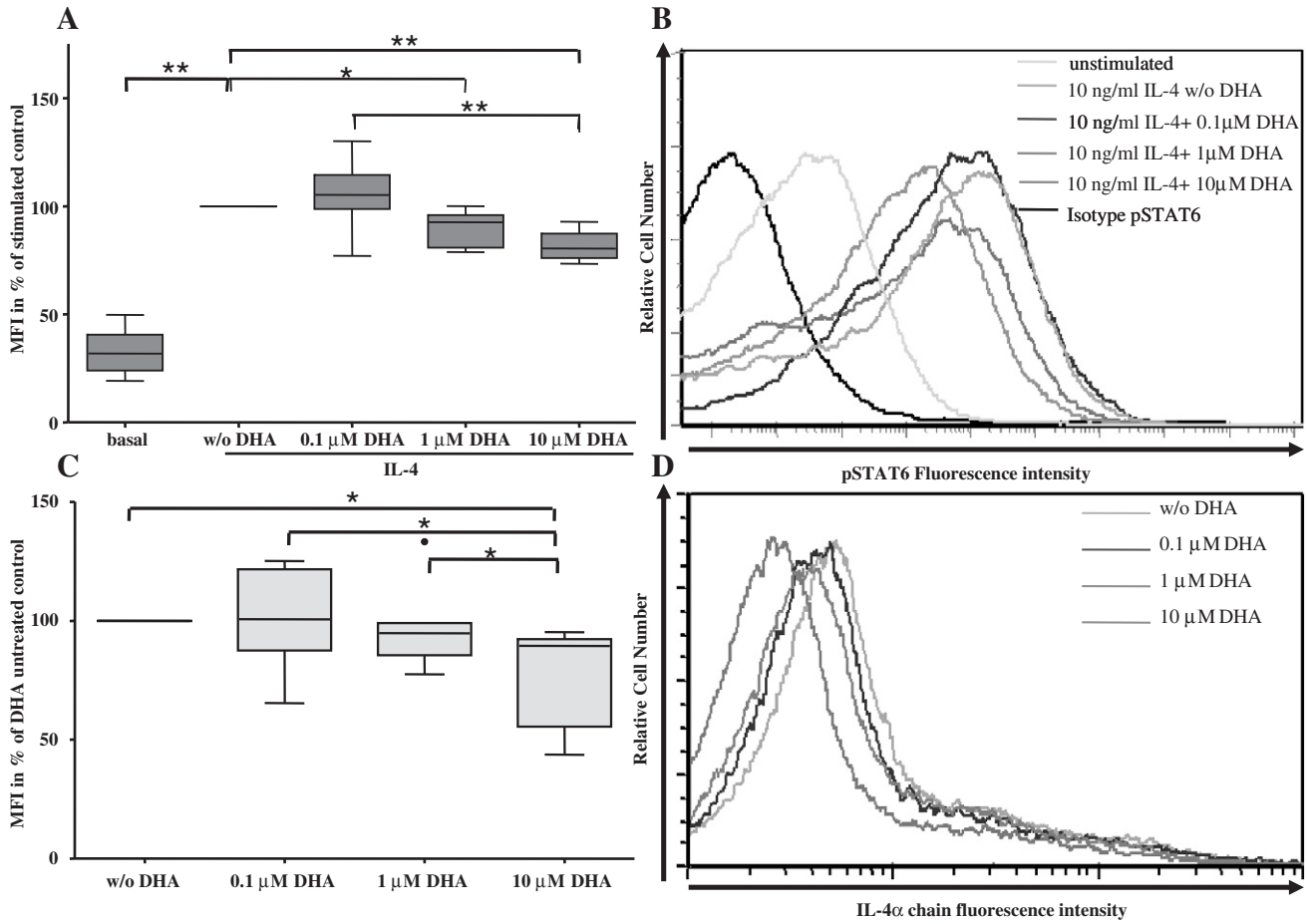

Fig. 6. (A) STAT6 phosphorylation in IL-4-stimulated B cells and (C) CD124 expression in unstimulated B cells upon DHA treatment. Data are shown relative to DHA untreated control (100 %) and as box plot (25. and 75. percentile, median); representative histogram for pSTAT6 measurement (B) (n=8) and CD124 expression analysis (D) (n=7). *P<.05; **P<.01, statistically significant difference compared to DHA untreated sample.

As shown in Fig. 6C, 10 μM DHA significantly reduced CD124 expression to 79±21% of DHA untreated B cells as determined by MFI (P=.037). Furthermore, in samples treated with 10 μM DHA the number of CD124 positive cells were reduced to 77±15 % of the DHA untreated control (P=.016, data not shown).

4. Discussion

Previously we have shown that dietary derived factors, e.g. retinoic acid and vitamin D3, modulate IgE production [35–37]. In this study, we show for the first time that DHA inhibits the IgE switching process and subsequent IgE production by down-regulating CD40- and IL-4-dependent signaling pathways.

Incubation with DHA resulted in a dose-dependent inhibition of IgE production in both PBMC and highly purified B cells. This inhibition was not due to decreased proliferation or increased cell death as demonstrated by CFSE proliferation assay and trypan blue as well as propidium iodide staining.

DHA inhibited the anti-CD40/IL-4 driven differentiation of IgE secreting cells. However, the numbers of IgA and IgG secreting cells were not or only slightly altered by DHA, excluding the possibility of a general inhibition of Ig production. This suggested a highly IgE specific inhibition of plasma cell differentiation.

In line with this, we observed that DHA specifically interfered with the IgE switching process by reducing AID and εGLT transcription using real time RT-qPCR.

The importance of AID in CSR is emphasized by its absence in AID $^{-/-}$ mice [38] as well as in patients with autosomal recessive hyper-IgM syndrome carrying mutations in the AID gene [39]. Although AID is required for CSR to all isotypes, IgA and IgG remained almost stable upon DHA treatment. This observation indicates that DHA mediated AID inhibition was not sufficient to affect the CSR in general. Indeed, we observed a more pronounced inhibition of εGLT rather than AID.

CSR towards IgE in B cells involves a number of molecular events [40]. The transcription of εGLT precedes B cell IgE synthesis and the connection between its expression and following switching to IgE is well appreciated [5,41]. The strong inhibition of εGLT under DHA treatment emphasizes its IgE specific action.

B cell-derived IgE production is regulated by direct contact between T and B cells [42], in particular CD40L/CD40 interaction [33] as well as by soluble cytokines like IL-4 [43]. Importantly, both AID [44] and εGLT [45] are dependent on CD40 and IL-4 down-stream events, which include NFκB and STAT6 signaling. DHA down-regulated AID and εGLT expression in B cells stimulated with anti-CD40 or IL-4 alone. This indicated that DHA was able to interfere with both signaling pathways. Therefore, we analyzed the impact of DHA on NFκB-p50 translocation into the nucleus and STAT6 phosphorylation.

CD40 ligation, along with other B cell stimuli, results in activation of transcription factors belonging to the Rel/NFκB family [7,9]. Genes involved in isotype switching like AID are regulated by type 1 but not type 2 NFκB activity [46]. Transcriptional regulation by type 1 NFκB involves activation of the IκB kinase complex resulting in the degradation of IκBα and IκBβ, which release NFκB p50:p65 and p50:cRel dimers [46]. NFκB-p50 is the most relevant down-stream event of CD40 involved in isotype switching [8] as mice lacking p50 show a marked reduction of IgE [47]. Therefore, the impact of DHA on the degradation of IκBα and subsequent nuclear translocation of p50 was investigated in detail. We found that DHA reduced the anti-CD40 driven translocation of p50 into the nucleus. These data are in line with previous reports showing that DHA can counteract NFκB activation [48,49]. Interestingly, DHA did not affect IκBα degradation, an up-stream molecule for p50 release. However, NFκB can dissociate from phosphorylated IκBα and translocate into the nucleus without IκBα degradation, indicating that a reduced p50 translocation is not necessarily related to a reduced IκBα degradation [50]. Another potential mechanism of NFκB-p50 translocation is based on direct ubiquitinylation and proteasomal degradation of p105 precursor without involvement of IκBα [8,51].

CD40 ligation plays a key role in the production of IgE [52] and triggers B cell εGLT, but by itself is insufficient to induce IgE production. Rather, in addition to CD40 signaling, IL-4 is indispensable for the induction of the mature IgE transcripts and consequent IgE synthesis [33,53,54]. We therefore tested whether DHA could interfere with IL-4 mediated STAT6 activation. Indeed, DHA caused an inhibition of STAT6 phosphorylation, thereby affecting the second essential signal for the IgE switch, IgE plasma cell development and IgE production. DHA had no impact on total STAT6 protein expression, but a decreased pSTAT6/STAT6 ratio points towards direct interference with the IL-4 driven signal transduction. In search of the mechanism we found that DHA decreased the expression of the IL-4Rα chain, thereby explaining at least in part the reduced signaling capacity via the IL-4R mediated by DHA. This reduction of IL-4R expression fits with previous evidence showing decreased IL-2 mediated STAT5 phosphorylation by DHA as a result of reduced surface expression of the IL-2 receptor [55]. This reduced surface expression was the result of an altered lipid raft organization. Other studies confirmed DHA as a molecule with lipid raft properties [27,56]. Stillwell et al. [57] recently described that dietary DHA consumption creates DHA-rich membrane domains which lead to a dislocation of sterol into sphingomyelin-rich lipid raft domains. In these two environments, the acyl chain order is extremely opposed. The protein movement between such domains is associated with a change in conformation and has the potential to mediate cellular signaling. Along the same line, it has been reported that DHA causes in human PBMC a partial disorganization of membrane microdomains disrupting signal transduction events [58]. Such mechanisms may well apply to DHA action on IL-4R expression and STAT6 phosphorylation.

CD40 ligation and IL-4 induce both, IgE and IgG_4 [59,60]. Therefore, an inhibited switch towards IgG_4 may potentially lead to the slight reduction of IgG secreting cells after treatment with 10 μM DHA.

Application of 0.1 μM DHA resulted in a significant reduction of IgE in supernatants, whereas the numbers of IgE secreting cells were not inhibited. This data indicates an additional impact on IgE gene expression and/or protein secretion, rather than an exclusive inhibition of the IgE switching process by DHA.

Taken together, DHA leads to a profound repression of the IgE switching process, IgE plasma cell development and IgE production in human B cells through early inhibition of CD40 and IL-4 signaling pathways. These results comprise a great biological relevance regarding the development of allergies. In fact, in our recently published clinical trial oral DHA supplementation led to a significant amelioration of atopic eczema which was accompanied by a strong inhibition of the anti-CD40/IL-4 driven IgE production of PBMC [31]. Thus, our data presented here provide one explanation for this observed IgE inhibition after DHA supplementation, offer the molecular basis for a well documented anti-allergic effect of DHA and are therefore important for dietary management. Although it will need further investigation to reveal the best time-point and optimal dose of DHA application, it is clear that DHA offers an excellent opportunity for an effective prevention or treatment of allergic diseases.

Acknowledgments

The authors thank Dr. Magda Babina for scientific discussions.

Conflict of interest: all authors declare that there is no conflict of interest regarding the study described in the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.02.004.

References

[1] Poulsen LK, Hummelshoj L. Triggers of IgE class switching and allergy development. Ann Med 2007;39(6):440–56.
[2] Mancini AJ, Kaulback K, Chamlin SL. The socioeconomic impact of atopic dermatitis in the United States: a systematic review. Pediatr Dermatol 2008;25 (1):1–6.
[3] Devereux G, Seaton A. Diet as a risk factor for atopy and asthma. J Allergy Clin Immunol 2005;115(6):1109–17 quiz 1118.
[4] Prescott SL, Calder PC. N-3 polyunsaturated fatty acids and allergic disease. Curr Opin Clin Nutr Metab Care 2004;7(2):123–9.
[5] Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. Nat Rev Immunol 2003;3(9):721–32.
[6] Lougaris V, et al. Hyper immunoglobulin M syndrome due to CD40 deficiency: clinical, molecular, and immunological features. Immunol Rev 2005;203:48–66.
[7] Bishop GA. The multifaceted roles of TRAFs in the regulation of B-cell function. Nat Rev Immunol 2004;4(10):775–86.
[8] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621–63.
[9] Bishop GA, Hostager BS. The CD40-CD154 interaction in B cell-T cell liaisons. Cytokine Growth Factor Rev 2003;14(3-4):297–309.
[10] Chatila TA. Interleukin-4 receptor signaling pathways in asthma pathogenesis. Trends Mol Med 2004;10(10):493–9.
[11] Hebenstreit D, et al. Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev 2006;17(3):173–88.
[12] Hellman L. Regulation of IgE homeostasis, and the identification of potential targets for therapeutic intervention. Biomed Pharmacother 2007;61(1):34–49.
[13] Horrocks LA, Yeo YK. Health benefits of docosahexaenoic acid (DHA). Pharmacol Res 1999;40(3):211–25.
[14] Simopoulos A. Omega-3 fatty acids in inflammation and autoimmune diseases. Am Coll Nutr 2002;21(6):495–505.
[15] Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83(6 Suppl):1505S–19S.
[16] Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc 2007;66(2):237–59.
[17] Calder P. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. Lipids 2003;38(4):343–52.
[18] Kelley DS, et al. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. Lipids 1999;34 (4):317–24.
[19] Calder PC, et al. Fatty acids and lymphocyte functions. Br J Nutr 2002;87(Suppl 1): S31–S48.
[20] Hwang D. Fatty acids and immune responses–a new perspective in searching for clues to mechanism. Annu Rev Nutr 2000;20:431–56.
[21] Zapata-Gonzalez F, et al. Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly through PPAR(gamma):RXR heterodimers: comparison with other polyunsaturated fatty acids. J Leukoc Biol 2008;84(4):1172–82.
[22] Weldon SM, et al. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. J Nutr Biochem 2007;18(4):250–8.
[23] Kew S, et al. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am J Clin Nutr 2004;79 (4):674–81.
[24] Arrington JL, et al. Docosahexaenoic acid suppresses function of the CD28 costimulatory membrane receptor in primary murine and Jurkat T cells. J Nutr 2001;131(4):1147–53.
[25] Wang H, et al. Omega-3 polyunsaturated fatty acids affect lipopolysaccharide-induced maturation of dendritic cells through mitogen-activated protein kinases p38. Nutrition 2007;23(6):474–82.
[26] Arrington JL, et al. Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. Clin Exp Immunol 2001;125(3):499–507.
[27] Stillwell W, Wassall SR. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 2003;126(1):1–27.
[28] Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res 2008;47(2):147–55.
[29] Seo J, et al. Docosahexaenoic acid selectively inhibits plasma membrane targeting of lipidated proteins. Faseb J 2006;20(6):770–2.
[30] McMurray DN, Jolly CA, Chapkin RS. Effects of dietary n-3 fatty acids on T cell activation and T cell receptor-mediated signaling in a murine model. J Infect Dis 2000;182(Suppl 1):S103–7.
[31] Koch C, et al. Docosahexaenoic acid (DHA) supplementation in atopic eczema: a randomized, double-blind, controlled trial. Br J Dermatol 2008;158(4):786–92.
[32] Funderud S, et al. Functional properties of CD19+ B lymphocytes positively selected from buffy coats by immunomagnetic separation. Eur J Immunol 1990;20 (1):201–6.
[33] Jabara HH, et al. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. J Exp Med 1990;172(6):1861–4.
[34] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4): 402–8.
[35] Worm M, et al. Retinoic acid inhibits CD40 + interleukin-4-mediated IgE production in vitro. Blood 1998;92(5):1713–20.
[36] Heine G, et al. 1alpha,25-dihydroxyvitamin D3 inhibits anti-CD40 plus IL-4-mediated IgE production in vitro. Eur J Immunol 2002;32(12):3395–404.
[37] Ruhl R, et al. Inhibition of IgE-production by peroxisome proliferator-activated receptor ligands. J Invest Dermatol 2003;121(4):757–64.
[38] Muramatsu M, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000;102(5):553–63.
[39] Okazaki IM, et al. The AID enzyme induces class switch recombination in fibroblasts. Nature 2002;416(6878):340–5.
[40] Manis JP, Tian M, Alt FW. Mechanism and control of class-switch recombination. Trends Immunol 2002;23(1):31–9.
[41] Shapira SK, et al. Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. J Exp Med 1992;175(1):289–92.
[42] Vercelli D, et al. Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. J Exp Med 1989;169(4):1295–307.
[43] Claassen JL, Levine AD, Buckley RH. Recombinant human IL-4 induces IgE and IgG synthesis by normal and atopic donor mononuclear cells. Similar dose response, time course, requirement for T cells, and effect of pokeweed mitogen. J Immunol 1990;144(6):2123–30.
[44] Dedeoglu F, et al. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkappaB. Int Immunol 2004;16(3):395–404.
[45] Iciek LA, Delphin SA, Stavnezer J. CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-kappaB: synergy with IL-4 induction. J Immunol 1997;158(10):4769–79.
[46] Zarnegar B, et al. Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappa B activation pathways. Proc Natl Acad Sci U S A 2004;101(21):8108–13.
[47] Snapper CM, et al. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. J Immunol 1996;156(1):183–91.
[48] Li H, et al. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. Kidney Int 2005;67 (3):867–74.
[49] Denys A, Hichami A, Khan NA. n-3 PUFAs modulate T-cell activation via protein kinase C-alpha and -epsilon and the NF-kappaB signaling pathway. J Lipid Res 2005;46(4):752–8.
[50] Imbert V, et al. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. Cell 1996;86(5):787–98.
[51] Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol 2008;8(11):837–48.
[52] Snapper CM, Finkelman FD, Paul WE. Regulation of Igg1 and Ige Production by Interleukin-4. Immunol Rev 1988;102:51–75.
[53] Gascan H, et al. Anti-Cd40 monoclonal-antibodies or Cd4+ T-cell clones and Il-4 induce Igg4 and Ige switching in purified human B-cells via different signaling pathways. J Immunol 1991;147(1):8–13.
[54] Kawabe T, et al. The immune-responses in Cd40-deficient mice — impaired immunoglobulin class switching and germinal center formation. Immunity 1994;1(3):167–78.
[55] Gorjao R, et al. Regulation of interleukin-2 signaling by fatty acids in human lymphocytes. J Lipid Res 2007;48(9):2009–19.
[56] Fan YY, et al. Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production. J Immunol 2004;173(10): 6151–60.
[57] Wassall SR, Stillwell W. Docosahexaenoic acid domains: the ultimate non-raft membrane domain. Chem Phys Lipids 2008;153(1):57–63.
[58] Diaz O, et al. The mechanism of docosahexaenoic acid-induced phospholipase D activation in human lymphocytes involves exclusion of the enzyme from lipid rafts. J Biol Chem 2002;277(42):39368–78.
[59] Armitage RJ, et al. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J Immunol 1993;150(9):3671–80.
[60] Wood N, et al. IL-21 effects on human IgE production in response to IL-4 or IL-13. Cell Immunol 2004;231(1-2):133–45.