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Inflammation Research

Retinoic acid inhibits CD40 plus IL-4 mediated IgE production through alterations of sCD23, sCD54 and IL-6 production

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Abstract. *Background:* All-trans retinoic acid (ATRA) inhibits IgE synthesis from anti-CD40 plus IL-4 stimulated human B lymphocytes.
Objective: To study the underlying mechanisms, we examined here molecules which are known to have an impact on IgE production, namely CD23, CD54 and IL-6.
Methods: Human anti-CD40 plus IL-4 stimulated B cells were cultured in the absence and presence of ATRA (10^{-6}–10^{-10} M). ELISAs were performed to determine soluble (s) CD23 and sCD54, IL-6 and IgE-levels. CD23 and CD54 surface expression were determined by flow cytometric analysis. Semiquantitative-RT-PCR was employed to analyse IL-6, CD23 and CD54 mRNA expression.
Results: ATRA induced a dose-dependent increase of percent CD23 (3.4 fold) or CD54 (1.6 fold) positive B cells. At the mRNA level, this was reflected by a modest increase of CD54 mRNA (46.5 ± 15.8%) only. By contrast, levels of sCD54 were decreased dose-dependently in the presence of ATRA (56.6 ± 7.6%). Cytokine analysis showed that IL-6 secretion was significantly inhibited by ATRA (53.6 ± 0.6%) and also IL-6 mRNA synthesis was reduced (66.3 ± 11.6%). The observed inhibition of IgE production mediated by ATRA was significantly reversed to 90.5 ± 12% by the addition of 100 pg/mL recombinant IL-6.
Conclusions: ATRA interferes through several pathways with the anti-CD40 plus IL-4 mediated B cell activation, namely IL-6, CD23 and CD54.

Key words: B cells – Retinoic acid – IgE – IL-6 – CD23

Introduction

Vitamin A and its derivates (retinoids) exert a broad range of biological activities, including modulatory effects on the proliferation and differentiation of several cell types of the immune system such as T- and B-lymphocytes [1, 2]. Previously, we could show that all-trans retinoic acid (ATRA) and its isomer 13-cis ATRA inhibit IgE production in anti-CD40 plus IL-4 stimulated PBMC and B cells in vitro in a dose-dependent manner [3]. This effect was isotype selective, since the production of other immunoglobulins (Ig) including IgA, IgG and IgM was not significantly affected. Because these findings may offer new therapeutic approaches for the treatment of IgE related diseases, including allergic rhinitis or atopic eczema, it is important to further clarify the mechanisms involved in these ATRA mediated effects by dissecting the various aspects of anti-CD40 plus IL-4 induced activation and IgE production by B cells.

The CD23 antigen, also known as the low affinity receptor for IgE, is expressed on B cells and has been shown to be upregulated during IL-4-dependent stimulation [4]. By proteolysis, a soluble form (sCD23) is released by shedding [5], which has been shown to enhance IgE production by itself [4]. On the other hand, membrane-bound CD23 on B cells inhibits IgE production via a negative feedback loop [6–8]. This indicates that CD23 plays a major role in the regulation of IgE synthesis. Other surface molecules like CD54 or CD86 have been described to influence IgE production as well, although their exact contribution to the regulation of IgE production is not clear [9, 10].

Besides surface molecules, many cytokines also modulate IgE synthesis in vitro. Among these, IL-6 is a particulary important autocrine growth factor for B cells, but IL-8, IL-10 and IFNγ must also be regarded as possible inhibitory molecules of IgE production and are therefore potentially important modulators of anti-CD40 plus IL-4 stimulated B cells in the presence of ATRA [11–14].

In search of mechanisms which may account for the inhibition of IgE production in anti-CD40 plus IL-4 stimulated cells by RA, we have studied different cell surface molecules and cytokines which might be potential targets of ATRA-induced inhibition of IgE production by B cells. These molecules which are currently viewed to significantly modulate the regulation of human IgE synthesis, include the surface markers CD23, CD54 and CD86 as well as the cytokines IL-6, IL-8, IL-10, TGFβ and IFNγ.

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Materials and methods

Cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of clinically non-allergic, healthy donors by Ficoll Hypaque (Biochrom KG, Berlin, Germany; D = 1.077) separation (1500 rpm, 30 min, room temperature). A basal IgE production (< 1 ng/mL IgE) was an inclusion criterium for the non-atopic group. B cells were purified using anti-CD19-coupled magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) by magnetic cell sorting (MACS). Briefly, 4×10^8 PBMC were resuspended in 800 µl PBS containing 10 mM EDTA and 0.2% BSA incubated for 10 min on ice with 2,4 µg of human immunoglobulin (15 µl Beriglobin®, Aventis Behring, Marburg, Germany) to block unspecific binding. Thereafter, 200 µl of conjugated beads (1:5 ratio) were added, and incubation was allowed to proceed for 10 min at 4 °C. Finally, CD19-positive B cells were separated by magnetic positive selection with LS^+ columns (Miltenyi Biotec). The cells were then resuspended in medium and counted. The purified cell population contained ≥ 95% B cells, as assessed by immunofluorescence with anti-CD20-FITC conjugated antibody. In previous studies, this procedure has been shown not to activate B cells [15].

Cell culture

Cells (10^6/mL) were cultured for 1 day for cytokine-mRNA production, 2 days for flow cytometric analysis, 3 days for cytokine ELISA and for 10 days for Ig-assays. The culture medium RPMI 1640 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.

Reagents

IL-4 (5 ng/mL) was purchased from Pharmingen, Hamburg, Germany. Purified anti-CD40 mAb (1 µg/mL; clone 626.1) was a kind gift of R. Geha (Children's Hospital, Boston, MA, USA), and ATRA (10^{-6}–10^{-10} M) was from Sigma (Dreieich, Germany).

Immunoglobulin production

3×10^5 cells were cultured for 10 days, and Ig's were measured in the supernatants of stimulated cells by ELISA. The mAb´s for IgE detection (clones HP6061 and HP6029) were kindly provided by Robert Hamilton, Asthma and Allergy Center, Johns Hopkins University, Baltimore, USA.

For Ig analysis, immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with anti-human Ig-Fc antibodies diluted in 0.1 M bicarbonate buffer (pH = 8.2). The wells were then blocked for one hour with 2% BSA-TBS. After several washings, supernatants and standards were incubated in duplicates for 2 h, and after further washings, the second biotinylated anti-IgE mAb was added, followed by another 1 h incubation with alkaline phosphatase conjugated streptavidin. After the final reaction with p-nitrophenylphosphate (Sigma, Dreieich, Germany), plates were read in a microplate ELISA reader at 405 nm, and the amount of Ig was calculated according to the standard curve. For the standard curve served diluted human serum of an atopic individual, with a total IgE level of 2300 ng/mL as determined by Pharmacia CAP-System (Freiburg, Germany). The sensitivity of the ELISA was 100 pg/mL. The variations of readings in duplicate cultures never exceeded 15%.

sCD23-, sCD54- and IL-6 ELISAs

The sCD23 and sCD54-ELISAs were from Endogen, Woburn, MA, USA and IL-10-, TGFβ, and IFNγ-ELISAs from Genzyme, Cambridge, MA, USA. Assays were performed according to the manufacturers' instructions. The IL-6-ELISA was established by using capture and detector antibodies (at 1 µg/mL final concentration) purchased from BD-Biosciences (Heidelberg, Germany). The sensitivity of the IL-6 ELISA was 30 pg/mL and was performed according to the manufacturers' instructions.

Flow cytometry

For fluorescent staining, 2×10^5 cells were suspended in 90 µl staining buffer (2% BSA in PBS, pH 7.4, 0.1% sodium azide) with 10 µl of the appropiate antibody and incubated for 30 min on ice. After several washings, cells were fixed in 2% paraformaldehyde. The antibodies used were CD23-PE and CD19-FITC (Biosource, Ratingen, Germany), CD54-PE (Immunotech, Hamburg, Germany) and CD86-PE (BD Biosciences, Heidelberg, Germany).

PCR

Total cellular RNA was extracted from purified B cells with an RNA preparation kit (Quiagen, Hilden, Germany) including a DNAse-treatment. The mRNA was reversely transcribed into cDNA by reverse transcriptase (InVitroGen, Carlsbad, USA). PCR amplification was performed with CD23-, CD54- and IL-6 specific primers [16]. CD23-specific primers were sense (ACG CAG ATT TCA CAG GAA CTG) and antisense (TTC CAT GTC GTC ACA GGC ATA) resulting in a 350 bp fragment. CD54-specific primers were sense (TAG CAG CCG CAG TCA TAA TG) and antisense (AGT CTT GCT CCT TCC TCT TGG) resulting in a 383 bp fragment.

For control, a 438 bp band corresponding to GAPDH was amplified using (GGG AAG GTG AAG GTC GGA GTC) as sense and (CTG ATG ATC TTG AGG CTG TTG) as antisense. PCR was performed with a Perkin Elmer thermocycler using the following program: 33 cycles (1 min at each 94 °C, 62 °C and 72 °C) for CD23, 38 cycles (1 min at each 94 °C, 58 °C and 72 °C) for CD54, and 27 cycles (1 min at each 94 °C, 60 °C and 72 °C) for GAPDH control. Semiquantitative RT-PCR was used for detection of CD23 and CD54. Briefly, differences in gene expression were determined by comparing the density of bands obtained from different samples against normalized GAPDH expression. Each sample was electrophoresed in 2% agarose gels. Quantification of ethidium bromide-stained gels was performed using Gelworks 1D. Detection and quantification of IL-6 mRNA was done by using TaqMan™ PCR analysis (K. Asadullah and A. Häusler, Schering AG, Berlin, Germany), as described previously [16].

Statistical analysis

The Wilcoxon test for non-parametric, paired data using SPSS software 10.0 was carried out to analyse the statistical significance of within-group changes. Between-group changes were analysed by the Mann–Whitney U-test for non-parametric, unpaired data. Statistically significant differences were considered at $p \leq 0.05$.

Results

Increased CD23 and CD54 expression on B cells in the presence of ATRA

Initially, the time course of surface expression of the low affinity IgE receptor CD23 and of the costimulatory molecules CD54 and CD86 were determined by flowcytometric analysis. Purified B cells were stimulated with ATRA alone and in combination with anti-CD40 plus IL-4, and the sur-

face molecules were analysed thereafter. Maximal expression of the analysed molecules was observed after 48 h of incubation (data not shown). This timepoint was taken for the studies on cell surface protein expression.

As evident from Figure 1, ATRA (10^{-6}–10^{-10} M) induced in anti-CD40 plus IL-4 stimulated B cells an increase of CD23 and CD54 positive cells and of the mean fluorescence intensity (MFI, revealed comparable data, not shown). This effect was statistically significant ($p < 0.05$) for ATRA ≥ 10^{-8} M for CD23 expression (max. 3.4 fold) and ATRA ≥ 10^{-10} for CD54 (max. 1.6 fold). In unstimulated B cells, CD23 and CD54 were dose-dependently and significantly upregulated by ATRA in terms of both percentage of positive cells and MFI (data not shown). CD86 surface expression remained unaffected by ATRA (data not shown).

Analysis of CD23 mRNA and CD54 mRNA on exposure to ATRA

Next, we examined whether the increased surface expression of CD23 and CD54 by ATRA was related to an increased mRNA expression, using semiquantitative RT-PCR. Time course analysis revealed maximal CD23 and CD54 mRNA expression after a 24 h incubation (data not shown). As is evident from Table 1, stimulation of B cells with anti-CD40 plus IL-4 induces mRNA of both molecules, as described previously [4, 9, 17], but on addition of ATRA (10^{-6} M), no effects were seen on CD23 mRNA expression, and only a modest increase was noted for CD54 mRNA (46.5 ± 15.8%). These data suggest that the ATRA-induced increase of CD23 and CD54 surface expression is governed by divergent mechanisms.

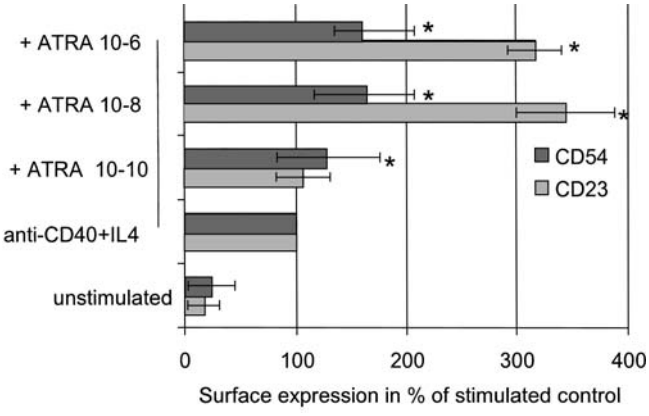

Fig. 1. CD23 and CD54 surface expression of B cells stimulated with anti-CD40 plus IL-4 for 48 h alone or in the presence of ATRA. Surface protein expression (% pos cells) was determined by flow cytometry. Mean values of 3 experiments with B cells from different donors and standard error of mean (SEM) are shown, the asterisk indicates $p < 0.05$ compared to anti-CD40 plus IL-4 stimulated control.

Decreased sCD23 and sCD54 release in ATRA stimulated B cells

Another possibility whereby the molecules CD23 and CD54 might affect B cell-dependent IgE synthesis relates to the fact that both surface proteins are cleaved by proteolysis from the cell membrane and are detectable in the supernatants of stimulated cells as soluble proteins. We have therefore examined whether the production of the soluble proteins (sCD23 and sCD54) is affected by treatment of anti-CD40 plus IL-4 stimulated cells with ATRA. An initial time course analysis revealed 72 h incubation as the optimal time point (data not shown). As depicted in Figure 2a and 2b, the amounts of sCD23 and sCD54 in the supernatants were decreased dose-dependently by ATRA (10^{-6}–10^{-10} M) to a max. inhibition of 24.3 ± 4.5% of sCD23 and 56.6 ± 7.6% of sCD54 release. This indicates that a reduced release of sCD23 and sCD54 occurs concomitantly with an increase in CD23 and CD54 surface expression, suggesting that ATRA inhibits the cleavage rate of CD23 and CD54 surface molecules.

sCD23 and sCD54 partially reverse ATRA mediated inhibition of IgE production

In order to analyse the effect of sCD23 and sCD54 on ATRA-modulated anti-CD40 plus IL-4 mediated IgE synthesis, sCD23 or sCD54 was added at the beginning of the 10 day incubation to stimulated B cells in the presence and absence of ATRA. As shown in Table 2 in the absence of ATRA, only minor or no effects were seen with sCD23 and sCD54, respectively. In the presence of ATRA, there was on the other hand a marked and highly significant inhibition of IgE synthesis which was reversed to a significant but small extent on addition of sCD23 (30 U/mL) and sCD54 (10 pg/mL) (from 72.7 ± 3% inhibition to 46.6 ± 3% and to 52.2 ± 3%). This suggests that inhibition of IgE production by ATRA depends at least in part on decreased sCD23 and sCD54 release.

Table 1. Expression of mRNA of CD23, CD54 and IL-6 in B cells in the presence of ATRA. B cells were incubated for 24 h in the presence and absence of anti-CD40 plus IL-4 (1 µg/mL and 5 ng/mL) in combination with ATRA (10^{-6} M). The table displays the mRNA expression of CD23, CD54, and IL-6 respectively normalized to GAPDH measurements. The expression of CD23, CD54, and IL-6 mRNA of anti-CD40 plus IL-4 in stimulated B cells is set at 100% (n = 4). The arithmetic mean values and the SEM are shown. The asterisk indicates $p < 0.05$.

Conditions	mRNA in % of anti-CD40 plus IL-4		
	CD23	CD54	IL-6
unstimulated	32.5 ± 12.0	27.1 ± 8.3	17.7 ± 6.1
unstimulated + ATRA	44.1 ± 10.6	31.7 ± 1.4	18.7 ± 8.8
anti-CD40 plus IL-4	100.0 ± 0	100.0 ± 0	100.0 ± 0
anti-CD40 plus IL-4 + ATRA	108.2 ± 2.2	146.5 ± 15.8*	66.3 ± 11.6

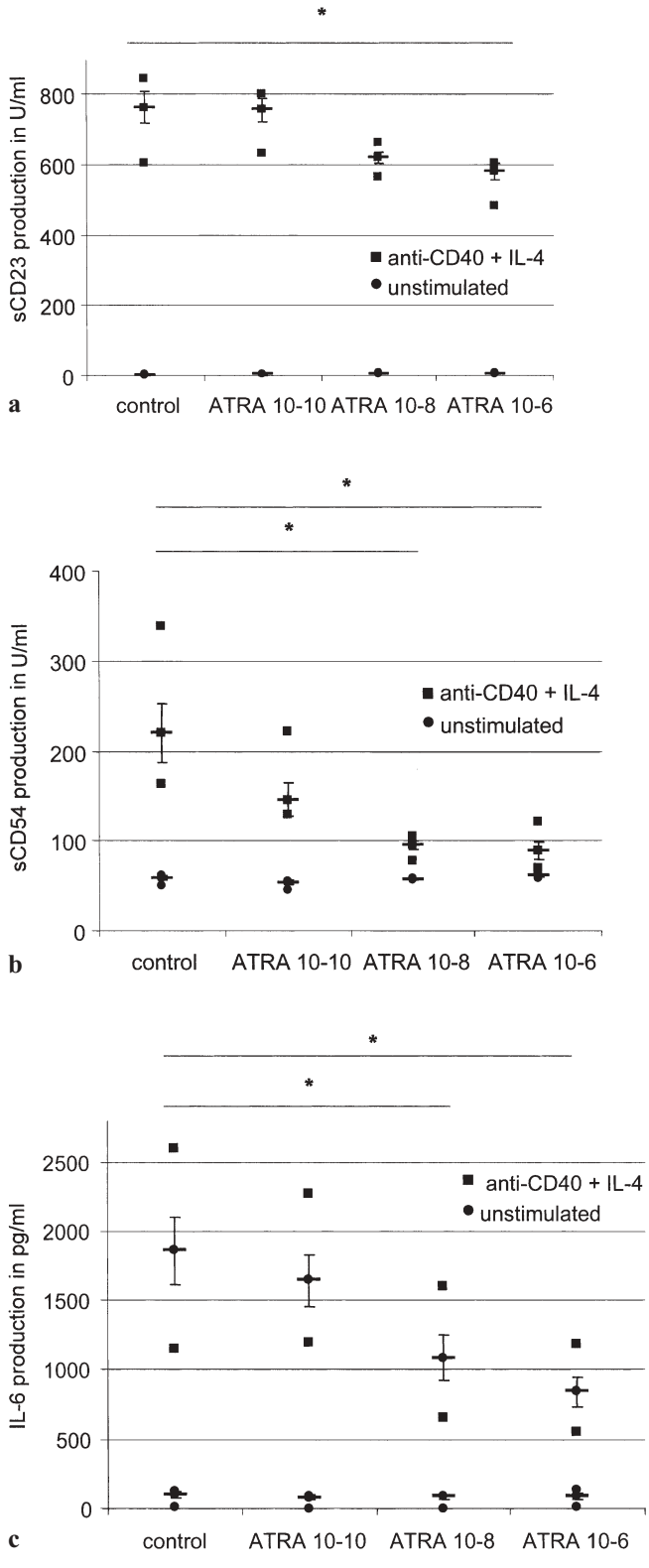

Fig. 2. Inhibitory effects of ATRA on sCD23 (a), CD54 (b) and IL-6 (c) protein release from B cells were incubated for 72 h under the conditions listed, and sCD23, sCD54 and IL-6 protein release was determined by ELISA in duplicates. The results of 3 different experiments and the means ± SEM were shown. The asterisk indicates $p < 0.05$ compared to anti-CD40 plus IL-4 stimulated control.

Down-regulation of IL-6 production by ATRA in anti-CD40 plus IL-4 mediated B cell activation

Since sCD23 and sCD54 did not reverse ATRA mediated inhibition of IgE synthesis completely (see Table 2), we searched for further molecules that might explain the effects of ATRA on this process by analysing the production of cytokines like IL-6, IL-8, IL-10, TGFβ and IFNγ which are all known to modulate IgE production in B cells [11, 12, 14, 18]. Under these conditions, production of IL-8 and of IFNγ remained unchanged in the presence of ATRA in unstimulated and anti-CD40 plus IL-4 stimulated B cells (data not shown). TGFβ and IL-10, in the presence of ATRA, were below detection levels in the supernatants of stimulated B cells, indicating that stimulated B cells fail to produce these cytokines under the given circumstances (not shown).

IL-6 is an important autocrine factor for B cell activation including IgE production [11], and IL-6 secretion was examined next in the presence of ATRA. Peripheral B cells were incubated for 72 h in the absence and presence of ATRA. As shown in Figure 2c, spontaneous secretion of IL-6 protein in unstimulated peripheral B cells is not significantly affected by ATRA. After anti-CD40 plus IL-4 stimulation, B cells produce increased amounts of IL-6 (23.7 fold induction) which are clearly inhibited by ATRA in a dose-dependent manner (Figure 2c, max. inhibition of 53.6 ± 0.6%). Besides the dose-dependent decrease in IL-6 protein secretion, a markedly reduced IL-6 mRNA expression was detected after 24 h (66.3 ± 11.6%, see Table 1) in the presence of ATRA (10^{-6} M).

In order to confirm the role of IL-6 in ATRA-mediated inhibition of IgE synthesis in B cells, addition of recombinant IL-6 at the beginning of the culture to anti-CD40 plus IL-4 stimulated B cells was examined next. While exogenously added IL-6 (100 pg/mL) to anti-CD40 plus IL-4 stimulated B cells enhanced IgE production (112.1 ± 20% of stimulated control, see Table 2), addition of IL-6 together with ATRA nearly reversed the ATRA-induced inhibition (90.5 ± 12% of stimulated control, $p < 0.05$, see Table 2).

Discussion

In the present study, we have demonstrated inhibitory effects of ATRA on anti-CD40 plus IL-4 mediated IgE production by at least two different mechanisms. For one, IL-6 production which plays an essential role in IgE production [11], is inhibited by ATRA at the mRNA and protein level and the inhibitory effects of ATRA were reversed to a major extent by addition of IL-6. Furthermore, a decreased proteolysis of the surface molecules sCD23 and sCD54 occurs in the presence of ATRA which is in part responsible for inhibition of IgE production, because addition of these molecules to anti-CD40 plus IL-4 stimulated cells in the presence of ATRA counteracted in part ATRA mediated inhibition of IgE production.

CD23 is a type II integral membrane glycoprotein which is continuously cleaved by proteolysis into soluble fragments [19]. Membrane CD23 is a multifunctional molecule and exerts different effects, dependent on the cell type on which it is expressed. On B cells, CD23 focusses IgE dependent

Table 2. Effects of sCD23, sCD54 and IL-6 on IgE production in anti-CD40 plus IL-4 stimulated peripheral B cells in the presence or absence of ATRA. B cells (10^6/mL) were incubated for 10 days in the presence of anti-CD40 plus IL-4 (1 µg/mL and 5 ng/mL) alone, in combination with ATRA (10^{-8} M) and with addition of recombinant sCD23 (30 U/mL), sCD54 (10 pg/mL), or IL-6 (100 pg/mL) at the beginning of cell culture. IgE was detected in the supernatants by ELISA and is expressed as pg/mL. Unstimulated cells produced no IgE (<100 pg/mL). IgE level in medium control were <100 pg/mL. Values are shown as % of anti-CD40 plus IL-4 stimulated control (n = 4). The arithmetic mean values and the SEM are shown. The asterisk indicates $p < 0.05$.

Conditions	Additions			
medium	control	sCD23	sCD54	IL-6
anti-CD40 plus IL-4	100.0	111.7* ± 4	92.7 ± 3	112.1 ± 20
anti-CD40 plus IL-4 + ATRA	27.3* ± 3	53.4* ± 3	47.8* ± 3	90.5* ± 12

presentation of antigen to T cells [20]. Similarly, soluble fragments of CD23 also exert a variety of functions, including the regulation of IgE synthesis [21].

The expression of CD23 depends strictly upon the rate of CD23 mRNA and CD23 surface synthesis and finally on the cleavage rate of surface CD23 to sCD23. Since it has been reported that under certain circumstances, stabilization of membrane CD23 may induce an upregulation of CD23 expression with a concomitant decrease in sCD23, without affecting the synthesis of the CD23 protein [4], a possible dissociation between the expression of CD23 and the release of sCD23 seems feasible. Such a mechanism may be effective in the presence of ATRA, suggesting that ATRA inhibits the proteolysis of CD23 into its soluble form. The inhibition of CD23 cleavage by metalloproteinase inhibitors results indeed in an inhibition of IgE production, as reported recently [22]. On the other hand, ATRA has been shown to inhibit metalloproteinase synthesis [23]. The metalloproteinases ADAM8, ADAM15 and ADAM28 have recently been identified as responsible proteins for CD23 cleavage [5]. Further investigations are necessary to clarifiy the exact role of ATRA as a modulator of these metalloproteinases.

The activation of human B cells by anti-CD40 plus IL-4 is accompanied by homotypic aggregation which is mediated by an interaction of the adhesion molecules LFA-1 and CD54. Homotypic aggregation results in physical contact which regulates IgE production since anti-CD54 has been shown to augment IgE production [9]. In the present study, we observed an induction of CD54 mRNA and surface protein expression by ATRA, whereas the amount of soluble CD54 was decreased. In agreement with these findings, it has been shown previously that the treatment of breast or cervix cancer cells with ATRA results in an upregulation of CD54 expression [24]. This indicates that interference by ATRA with regulatory elements of CD54 gene expression in different cell types may be a general mechanism. In fact, it has been demonstrated earlier that an upregulation of CD54 expression occurs through an ATRA responsive element in the NF-κB binding site which is found in the upstream promotor region of CD54 [25]. Similiar mechanisms may occur in B cells in the presence of ATRA. Since ATRA leads to an inhibition of IgE synthesis, it must be postulated that enhancement of CD54 protein expression is not as important as inhibition of sCD54 shedding from ATRA-treated B cells.

IL-6 is expressed by a number of cell types and plays a major role in the differentiation of B cells [26]. IL-4 and anti-CD40 synergize to induce IL-6 production, and it has been shown that autocrine IL-6 production is obligatory for IgE production [11]. In the present study, we observed an inhibition of IL-6 mRNA and protein production by ATRA which indicates that the inhibition of IgE by ATRA depends on the inhibition of IL-6 production. This conclusion is strengthened by the finding that addition of IL-6 to anti-CD40 plus IL-4 stimulated cells in the presence of ATRA reverses the inhibitory effects of ATRA on IgE production to a major extent (90.5 ± 12%). The inhibition of IL-6 production by ATRA seems however not to be B cell specific since several groups have reported similar effects in different normal and malignant human cells [27, 28]. Studies on mechanisms by which this inhibition of IL-6 production by ATRA occurs suggest that ATRA inhibits IL-6 promoter action by antagonizing the enhancer action of nuclear factor-IL-6 [29].

Taken together, we show here that ATRA interferes with anti-CD40 plus IL-4 mediated IgE production by B cells via several mechanisms. ATRA is a pan ligand, binding to all three currently known retinoic acid receptors (RARs) and may therefore be expected to modulate gene expression through several pathways. IL-6 plays a central role during B cell differentiation and amplification of IgE synthesis [11]. Also in view of the marked effects of this molecule and its modulation in the present study, compared to the sCD23 and sCD54 effects, IL-6 inhibition through ATRA may be of major importance for the control of anti-CD40 plus IL-4 mediated IgE production. It is a worthwhile target for further investigations aimed at controlling IgE production.

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