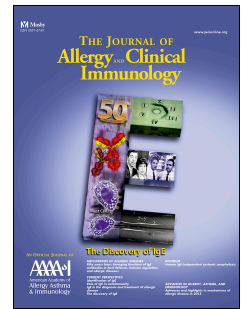


Accepted Manuscript

9-cis retinoic acid modulates the type I allergic immune response

Guido Heine, MD, Tim Hollstein, MD, Sandra Treptow, Dipl. ing., Andreas Radbruch, PhD, Margitta Worm, MD



PII: S0091-6749(17)30762-5

DOI: [10.1016/j.jaci.2017.03.046](https://doi.org/10.1016/j.jaci.2017.03.046)

Reference: YMAI 12809

To appear in: *Journal of Allergy and Clinical Immunology*

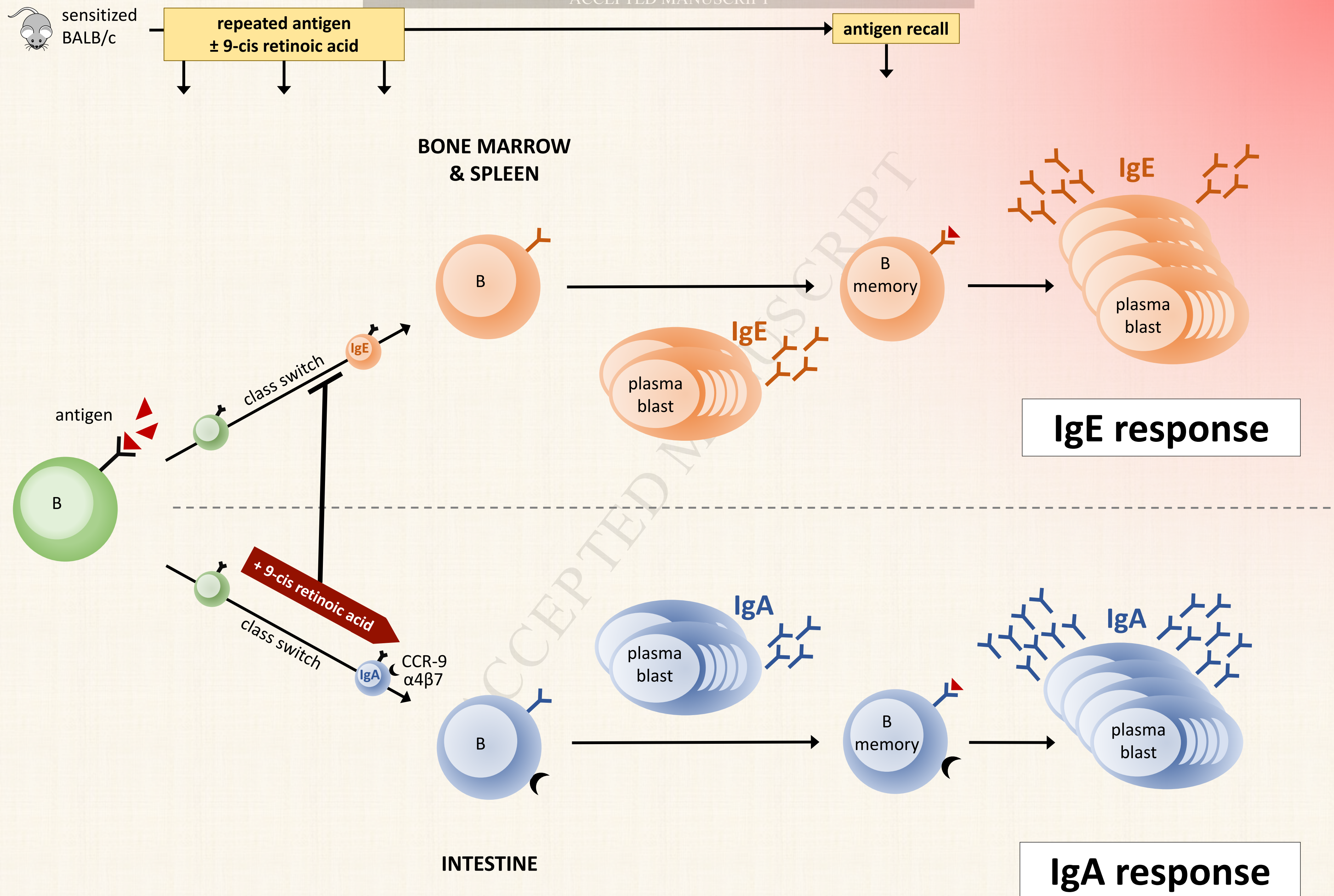
Received Date: 13 July 2016

Revised Date: 6 March 2017

Accepted Date: 16 March 2017

Please cite this article as: Heine G, Hollstein T, Treptow S, Radbruch A, Worm M, 9-cis retinoic acid modulates the type I allergic immune response, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.03.046.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



9-cis retinoic acid modulates the type I allergic immune response

Guido Heine, MD^{*,†,1}, Tim Hollstein, MD^{*,1}, Sandra Treptow, Dipl. ing.^{*}, Andreas Radbruch, PhD[†],
Margitta Worm, MD^{*,†}

^{*} Charité-Universitätsmedizin Berlin, Klinik für Dermatologie und Allergologie, Allergie-Centrum-Charité, Charité Platz 1, D-10117 Berlin, Germany

[†] Deutsches Rheuma-Forschungszentrum Berlin, A Leibniz Institute, Charitéplatz 1, D-10117 Berlin, Germany

¹ these authors contributed equally to this manuscript

Running Title: 9cRA and IgE

Word count abstract: 240 words. Manuscript: 3639 words. 41 references. 5 Figures. 5

Supplementary Figures

Corresponding author: Prof. Dr. Margitta Worm and PD Dr. med. Guido Heine; Klinik für Dermatologie, Venerologie und Allergologie; Allergie-Centrum-Charité, CCM; Charité - Universitätsmedizin Berlin; Charitéplatz 1; D-10117 Berlin; Tel. +49.30.450.518.105; Fax +49.30.450.518.931; email: margitta.worm@charite.de

Abstract

Background: Vitamin A is a potent regulator of adaptive immunity. The impact of the endogenous metabolite 9-cis retinoic acid (9cRA) on allergic sensitization is unknown.

Objective: To investigate whether and to what extent 9cRA modulates the humoral immune response.

Methods: BALB/c mice were sensitized and challenged with ovalbumin (OVA). 9cRA was applied repeatedly together with the antigen. Immunoglobulin production and cellular analysis were performed by ELISA, ELISPOTs and flow-cytometry. Human CD19⁺ B cells were activated *in vitro* in the presence or absence of 9cRA and activation markers, proliferation and secreted Ig were analyzed by flow-cytometry and ELISA.

Results: 9cRA applied together with repeated OVA challenges transiently increased specific serum IgA, IgE and IgG1 serum levels (2.0-8.9-fold). After OVA recall, specific IgE concentrations were reduced by a mean of 57% after adding 9cRA, while IgA was strongly induced (20-fold) and IgG1 remained unchanged. Correspondingly, less specific IgE- and more IgA secreting cells resided in the spleen in the 9cRA groups. Additionally, 9cRA promoted the migration of specific B cells to the mesenteric, but not the draining lymph nodes. In purified stimulated human B cells, 9cRA markedly reduced IgE production and enhanced IgA production. B cell activation was modulated by 9cRA reducing the expression of CD86 and promoting IL-10.

Conclusions: Our data indicate that 9cRA modulates the allergic immune response by reducing the IgE response but promoting the IgA response. Thus, 9cRA can modulate the allergic immune response towards a non-IgE condition.

240/250 words

Key Messages.

- Repeated antigen challenge in the presence of 9cRA induces IgA instead of IgE in mice
- Induction of IgE-producing human B cells by Th2-signals is hampered with 9cRA
- 9cRA contributes to the control of type I allergy

Capsule summary.

The physiological vitamin A receptor agonist 9-cis retinoic acid contributes to the control of type I allergic immune reactions in mice and humans by reducing IgE and inducing IgA.

Key Words: allergy, B cell, IgE, 9-cis retinoic acid, vitamin A

Abbreviations: 7-AAD, 7-aminoactinomycin; 9cRA, 9-cis retinoic acid; $\alpha 4\beta 7$, integrin- $\alpha 4\beta 7$; ASC, antibody secreting cell; ATRA, all-trans retinoic acid; BCR, B cell receptor; CCR, chemokine receptor; CD, cluster of differentiation; CFSE, carboxyfluorescein diacetat succinimidyl ester; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneal; NP-KLH, nitrophenyl sensitized mice; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid-X-receptor; SEM, standard error mean; TGF, transforming growth factor; TLR, toll-like receptor.

63 Introduction

64 The adaptive immune response is shaped by environmental factors including vitamin A.^{1, 2} Their
65 effective impact on the protective immune response prompted the WHO to recommend vitamin A
66 treatment in conjunction with vaccination against measles, tetanus, or polio in developing
67 countries.^{3, 4} Analysis of vitamin A deficient mice confirms an abrogated specific IgG1/3-humoral
68 immune response after vaccination or infection, which is effectively restored by vitamin A repletion
69 or the active metabolite retinoic acid.⁴⁻⁶ The underlying mechanisms include a defective T cell
70 function, reduced B cell proliferation, and differentiation during primary and memory antibody
71 response.^{4, 7} Moreover, vitamin A was shown to facilitate the physiologic IgA response under
72 homeostatic conditions,⁸ but also during infection,⁹ and immunization.¹⁰ Human B cells can be
73 directly targeted by the active vitamin A metabolite all-trans retinoic acid (ATRA), which enhances
74 toll-like receptor-9-(TLR9)-induced proliferation and differentiation into IgG secreting cells.¹¹
75 However, the function of vitamin A in allergy is not clear. We previously demonstrated in human B
76 cells that IgE production is inhibited by impaired IgE-germline transcript expression and blocked
77 interleukin-(IL)-6 production when activated in the presence of ATRA.^{12, 13}
78 At the molecular level, vitamin A operates in its active forms by activation of retinoic acid- (RAR α -
79 γ) and retinoic-X-receptors (RXR α - γ) and transcriptional regulation of target genes.¹⁴ The diversity
80 of vitamin A functions results from cell-specific expression of vitamin A receptors, its activating
81 metabolite, and also transcriptional cofactors.¹⁴ Human B cells express RAR α , RAR β , and RXR α .^{12,}
82 ¹⁵ 9-cis retinoic acid (9cRA) is a naturally occurring bioactive vitamin A metabolite, which is
83 formed by spontaneous isomerization of ATRA.¹⁴ In addition to targeting RAR α - γ like ATRA,
84 9cRA also activates RXR α - γ .¹⁴ Currently, 9cRA is used for the treatment of chronic therapy-
85 resistant hand eczema (alitretinoin). Our previous data show that different RXR α partner receptors
86 modulate B cell activation, including vitamin D receptor,¹⁶⁻¹⁹ RAR α ,^{12, 13} liver-X-receptor,²⁰ and

87 Peroxisome proliferator-activated receptor (PPAR γ).²¹ However, the impact of 9cRA on B cells and
88 the allergic immune response is not known.

89

90

Methods

Animals and treatments

All animal procedures complied with the German institutional, state and federal guidelines (LaGeSo Berlin). Female BALB/c mice (strain cByJRj, Elevage Janvier, Le Genest St. Isle, France) were kept specific-pathogen free and treated as described previously.²² Briefly, the mice were sensitized intraperitoneally (i.p.) with 10 µg Ovalbumin (OVA) absorbed to alum (Figure 1A). To target specific lymphocytes with 9cRA, starting on day 56 when the sensitization-induced plasmablast response declined,²³ 100 µg OVA s.c. was applied in weekly intervals with or without 9cRA (Basilea, Switzerland, dissolved in 10% DMSO, 20% Tween80 in 0.9% NaCl). Control mice received PBS/solvents accordingly. The doses of 0.43 and 2.43 mg/kg 9cRA in mice correspond to the human treatment daily dose of 3 and 30 mg considering the body surface area.²⁴ Finally, the specific memory B cells were challenged by i.p. injection with 100 µg OVA after allergen-free intervals of 4-5 weeks on day 109.

Cell culture

All procedures were approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. After a written informed consent was obtained, CD19⁺ B cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured as previously described.^{18, 25} Consecutive separation of CD27⁻ naïve and CD27⁺ memory B cells in some experiments was performed magnetically according to the manufacturer's instructions (Miltenyi Biotec). *In vitro*-induced IgE expression was analyzed from CD19⁺ human B cells stimulated by anti-CD40 (clone 626.1, Santa Cruz Biotechnology, USA or clone 83111 R&D system, Wiesbaden, Germany, both 1 µg/mL) and IL-4 (Miltenyi Biotec, 5 ng/mL) after 10 days. To analyze the impact on IgA-secretion, CD27⁻ naïve B cells were activated 4 days with anti-IgM-F(ab2)'-fragments (Jackson ImmunoResearch, Dianova, Hamburg, Germany, 2 µg/ml) to crosslink the antigen receptor

(BCR), CD40L (Miltenyi Biotec, 1 µg/mL), IL-4 (Miltenyi Biotec, 5 ng/mL) and IL-21 (Miltenyi Biotec, 50 ng/mL) followed by IL-10 and IL-21 (Miltenyi Biotec, 50 ng/mL) stimulation for 72 hrs in IMDM (supplemented with 4 mM L-glutamine non essential amino acids, 5 µg/mL transferrin and 5 µg/mL insulin (Sigma Aldrich), respectively. 9cRA was added at the beginning of cell culturing in concentrations ranging from 10 pmol to 1 µmol (used in 100 nmol/mL if not stated otherwise; kindly provided by Basilea or Sigma Aldrich).

Flow cytometry

For the flow-cytometric analysis, murine B cells were stained after Fcγ receptor blockade (clone 2.4G2), using fluorescent labeled antibodies against B220 (clone RA3.6B2), CD38 (clone 90, BioLegend), CD138 (clone 281-2, BD Pharmingen), 7-Aminoactinomycin (7-AAD, eBioscience), CCR9-PE (R&D Systems), anti-α4β7 (clone DATK32, DREZ). For plasma cell analysis, the cells were additionally fixed in 1 % paraformaldehyde, permeabilized in 0.5 % saponin/PBS and stained intracellular with OVA-FITC (Sigma) and IgG1-APC (clone X56, BD Pharmingen). The human B cells were stained by fluorescence conjugated antibodies against IgD, CD23, CD54, CD86, CD38, IgD and CCR-9 (BD Pharmingen), CD27, (Miltenyi Biotec), 7-aminoactinomycin D (7-AAD, eBioScience, San Diego, USA). B cell proliferation was analyzed after labeling with carboxyfluorescein diacetat succinimidyl ester (CFSE, Sigma) before activation, as previously described.^{18, 20} Cells were cultured 3-10 days as indicated, including a medium change for the Ig-cultures on day 3 (measurement intervals were determined previously by optimal expression levels, data not shown).^{13, 16} All flow cytometric analyses were performed using a MACSQuant® Analyzer (Miltenyi Biotec) and Flowjo software (Tree Star, Inc., Ashland, OR, USA).

Detection of IL-10 expression in human B cells

Human viable B cells were stained for IL-10 secretion by the IL-10 capture assay (Miltenyi Biotec) as previously described.²⁵ Briefly, activated B cells were activated for 4 days at 1.5×10^6 cells/mL as indicated following restimulation with PMA (phorbol myristate acetate, 10 ng/mL) and

ionomycin (1 µg/ml, both Sigma Aldrich) for 2.5 h. Harvested CD19⁺ B cells were incubated with an anti-CD45-IL-10 bivalent antibody matrix which relocates secreted IL-10 on the B cell surface and 45 min. Positive cells are subsequently labeled with a second fluorescent IL-10-specific antibody.

Enzyme-linked Immunosorbent Assay (ELISA) and ELISPOT

Murine Ig serum concentrations and ELISPOT assays were determined as described in detail previously.^{20, 26} The OVA-specific Ig levels were obtained by serial dilution of a highly positive serum pool and 4-point analysis calculating relative laboratory units (RU/L). Human IgM, IgA, IgG and IgE ELISA were analyzed from cell free supernatants after 10 day of cell culture as described previously.¹⁶ Human IL-10 protein were determined from cell free supernatants according to the manufacturer's instructions (Duoset, R&D Systems).

Statistical analysis

Statistical analyses and graphical presentation were performed using GraphPad Prism software (GraphPad Software, La Jolla, USA). The values are depicted as mean ± standard error mean (SEM). Comparisons between two groups were determined using Mann-Whitney-U-Test or Students T-Test. Statistical significance was considered at p-values ≤ 0.05.

Results

9-cis retinoic acid (9cRA) promotes specific IgA and reduces the IgE allergic immune response

To investigate the impact of 9cRA on the allergic immune response, OVA-sensitized BALB/c mice were challenged three times with OVA in weekly intervals as described²² in the presence or absence of increasing doses of 9cRA. The reactivation of OVA-specific memory cells (OVA-recall) was performed 6 weeks after challenge and 1 week before organ analysis of the mice by administering OVA i.p. (Figure 1A). Before sensitization, OVA-specific antibodies were below the detection threshold. The sensitization induced mainly OVA-specific IgE and IgG1 in all groups (Figure 1B-D). By repetitive OVA-challenges starting at day 56, the addition of 9cRA increased all specific isotypes in the 30 mg 9cRA group compared to control group (Figure 1B-D; OVA-IgA $p<0.0001$, OVA-IgE $p=0.06$, OVA-IgG1 $p=0.008$) as well as total IgA with $p<0.01$ and IgE with $p<0.05$ (Supplementary Figure 1 in this article's Online repository at www.jacionline.org). Interestingly, 6 weeks after challenge, OVA-specific IgA remained higher in the 30 mg 9cRA group (Figure 1B, $p<0.001$) and OVA-specific IgE declined to values lower than control (Figure 1C, $p<0.01$), respectively. Challenging the specific memory immune response at day 109 (OVA-recall) showed within 1 week an induction of OVA-specific IgA by 9cRA resulting in 20-fold higher concentrations (Figure 1B, $p<0.001$, 1600-fold intergroup difference) and OVA-specific IgE remained stable and still lower than in control mice (Figure 1C, $p<0.01$), but total IgE was induced compared to control (Supplementary Fig. 1B in this article's Online repository at www.jacionline.org). OVA-IgG1, total-IgA and total-IgG1 memory response were not significantly affected by 9cRA after recall. (Figure 1 and Supplementary Fig. 1 in this article's Online repository at www.jacionline.org).

Increased numbers of specific IgA secreting cells by 9cRA

To further elucidate whether the observed changes in specific Ig serum concentrations by 9cRA

were induced by altered differentiation of Ig secreting cells and/or homing, ELISPOT analyses were performed on day 115 from spleen and bone marrow, the two major accumulation sites of antibody secreting cells in this model (Figure 2). The total number of splenocytes and bone marrow cells did not differ between the groups ($p=0.73 - 0.34$, data not shown). However, in the spleen, the frequencies of OVA-IgA secreting cells were increased in the presence of 9cRA in a dose dependent manner compared to control (Figure 2, $p=0.015$), and robustly decreased regarding OVA-IgE ($p=0.052$) and OVA-IgG1 ($p<0.01$) at the 30 mg dose. In the bone marrow, the presence of 9cRA reduced the numbers of OVA-IgG1 secreting cells marginally and the other isotypes were comparable among the groups. Accordingly, the absolute numbers of OVA-IgA secreting cells were increased in the presence of 30 mg 9cRA compared to the control group in the spleen and bone marrow ($p=0.015$ and 0.013), while those regarding OVA-IgG1 were reduced in the spleen ($p=0.015$, both data not shown). Finally, OVA-IgG1 and OVA-IgE cell numbers in the bone marrow were reduced although statistical significance was not reached ($p>0.05$, data not shown).

OVA-specific B cells and plasmablasts migrate towards the intestine by 9cRA

As 9cRA modulated the presence of specific IgA and IgE secreting cell numbers in the spleen, we addressed the question, whether specific B cells are altered in their homing to lymphoid tissue, too. Therefore, OVA-specific memory B cells ($B220^+CD138^-$ small lymphocytes), and plasmablasts ($CD138^+B220^-$ lymphoblasts) were analyzed by flow cytometry from the bone marrow, spleen and lymph nodes (Figure 3, gating strategy and OVA-specificity shown in Supplementary Figure 2 in this article's Online repository at www.jacionline.org). Due to the limitations in IgE^+ cell numbers we focused our analysis on $IgG1^+$ cells, which are also induced by IL-4. On day 115, the frequencies of OVA-IgG1 B memory cells and plasmablasts were reduced by 9cRA in the spleen in the 30 mg 9cRA group ($p=0.051$, $p>0.05$, respectively, data not shown). Moreover, the data show a reduced presence within the axillar and inguinal, but increased presence in the mesenteric lymph

nodes (Figure 3B), whereby statistical calculation was limited due to only 2 samples. We detected, in line with OVA-IgA induction in the blood, IgG1⁻ OVA-specific B cells in the mesenteric LN, which are most likely of IgA isotype, as OVA IgE cells are very rare and are not induced by OVA challenge (Figure 1C).

To further analyze whether 9cRA induces gut homing of B cells during an allergic immune response, B cells were stained for the retinoic acid-inducible gut-homing molecules CC-chemokine receptor 9 (CCR9) and integrin- α 4 β 7 (α 4 β 7). CCR9 was expressed on mostly IgG1⁻ B cells, but also few IgG1⁺ B cells (Supplementary Figure 3A in this article's Online repository at www.jacionline.org), but not on plasmablasts. In mice with 9cRA-treatment, more CCR9⁺ mesenteric IgG1⁻ B cells were detectable. The cell numbers of α 4 β 7⁺ B cells were comparable in the spleen in all 3 study groups (data not shown). However, in the mesenteric lymph nodes, higher frequencies of all 3 B cell subsets were observed with an increasing dose of 9cRA (Supplementary Figure 3B). This data indicates that 9cRA mediated the expression of gut-homing surface molecules in a Th2 mediated immune response, namely CCR9 on B cells and α 4 β 7 on plasmablasts.

9cRA inhibits anti-CD40 and IL-4 induced IgE production in human B cells

As our data show an impaired IgE response by 9cRA in a murine type I sensitization model and in 9cRA treated patients with increased serum IgE,²⁷ we investigated its impact on IgE production by human B cells. Following anti-CD40 + IL-4 stimulation of B cells, IgE concentrations were strongly induced (24.4-fold), but not IgM, IgG, IgA (Figure 4A). The addition of 9cRA inhibited IgE production significantly ($-67\% \pm 8.5$, $p < 0.01$), while secreted IgM was moderately increased ($+26\% \pm 52$, $p < 0.05$) and IgG/IgA secretion remained stable ($p > 0.05$; Figure 4A). The comparison of CD27⁻ naïve and CD27⁺ memory B cells sorted before cell culture showed no significant differences between both subsets regarding survival, proliferation and Ig-secretion (data not shown). Of note, IgA was neither induced by the culture conditions, nor by 9cRA. To investigate

whether IgA-inducing conditions are required to facilitate the enhancing action of 9cRA observed in mice (Figure 1B), we activated naïve human B cells in the presence of IL-21 and BCR-crosslinking F(ab2)'-fragments in addition to CD40 and IL-4 receptor stimulation to mimic physiological B cell activation in the lymph node with T follicular helper cells. Upon these conditions, secreted IgA was significantly induced by the stimulation (mean 60 ng/ml, in unstimulated cells not detectable, not shown). Additional 9cRA further increased IgA production up to mean 7.4-fold (Figure 4B). Secretion of IgM and IgG were also induced by the stimulation containing IL-21 to mean 110 ng/ml and 206 ng/ml, which was further increased by 9cRA (mean 9-fold and 3-fold), respectively, whereas IgE was not significantly affected (mean 1.3 ng/ml, $p=0.14$). To exclude a bias by potential reduced proliferation or viability by 9cRA, flow cytometric analysis of 7AAD-stained cells and CFSE dilution was analyzed in cultured B lymphocytes. The data showed that the viability remained unchanged by 9cRA following stimulations ($p>0.05$, data not shown). The analysis of CFSE-labeled B cells showed induced proliferation by anti-CD40 + IL-4 (12-fold compared to unstimulated cells), which was reduced by trend in the presence of 9cRA by $49\% \pm 10$ ($p=0.09$, data not shown).

9-cis retinoic acid (9cRA) targets human B cells directly and modulates its activation

To assess which and how human B cell populations are targeted by 9cRA, we determined the surface expression of the vitamin A-inducible protein CD38 on B cells as surrogate marker for retinoid signaling¹⁵ (Supplementary Figure 4 in this article's Online repository at www.jacionline.org) and the production of cytokines which are crucial for immunoglobulin production in an autocrine manner.¹⁷ Kinetic studies identified day 4 as an optimal timepoint (data not shown). The data show an increased surface CD38 expression on anti-CD40 + IL-4 as well as anti-CD40 + IL-4 + anti-BCR + IL-21 activated B cells (Supplementary Figure 4A in this article's Online repository at www.jacionline.org).

To assess how 9cRA impacts B cell activation, we first determined the expression of the IgE receptor-II (CD23) and the costimulatory molecules CD54 (ICAM-I) and CD86 on B cells. Kinetic studies identified day 3 as an optimal timepoint (data not shown) for further analysis. In anti-CD40 + IL-4 stimulated cells, additional 9cRA did not alter CD23 and CD54, but CD86 expression on B cells was significantly reduced by 9cRA (mean 2-fold, Figure 5A). However, unlike in murine B cells, chemokine receptor 9 (CCR-9) surface expression was not altered in the presence of 9cRA on human B cells (data not shown). Secondly, we examined whether 9cRA controls B cell cytokines, involved in differentiation to IgE-secreting cells, namely IL-10 promoting plasmablast differentiation.²⁵ We determined the secretion of IL-10, considering the kinetics of cytokine expression as reported previously.^{17, 25} After 3 days IL-10 was induced in B cells by stimulation with anti-CD40 + IL-4. We detected more IL-10 in the supernatants of anti-CD40 + IL-4-stimulated B cells in the presence of 9cRA, as validated also in conditions including IL-21 and anti-BCR. To clarify, whether this results from a higher frequency of IL-10 secreting cells, or more secretion per cell, we performed flow cytometric analysis. We analyzed B cells activated according the established conditions to induce IgA *in vitro*, including IL-21 + anti-BCR, as the frequencies of IL-10⁺ B cells and amounts of secreted IL-10 were low in response to anti-CD40 + IL-4 stimulation.²⁵ The data show significant induction of IL-10 secreting cells by the stimulation (mean 2.8% ± 0.6), which is increased by 9cRA (mean 3.3% ± 1.1, data not shown and Supplementary Figure 5 in this article's Online repository at www.jacionline.org).

Discussion

We have provided evidence that exogenous 9-cis retinoic acid (9cRA) in the context of an allergic sensitization profoundly modulates an established humoral IgE response, resulting in reduced specific IgE responses and increased specific IgA responses in mice. Data from human *in vitro* studies underline that 9cRA directly targets activated human B cells and decreases IgE production. Our data suggest a novel mechanism in type 1 allergy to control IgE by a strong modulatory action of 9cRA on allergen-specific IgA. The generation of an IgA⁺ allergen-specific memory B cell pool irreversibly limits the regeneration of IgE memory B cells, as the gene encoding for IgE and IgG subclasses is excised during the process of isotype class switch recombination to IgA.²⁸ We demonstrated a 9cRA-driven plasmablast generation (early 9cRA function) and polarization of specific memory B cells (late 9cRA function) towards IgA instead of IgE/IgG1. Accordingly, serum OVA-IgA rapidly increased in the 9cRA groups on day 115 after OVA recall (day 109) while OVA-IgE concentrations and numbers of specific Ig-secreting cells were unaffected. However, data from human studies indicate that IgA remained stable under 9cRA treatment in hand eczema²⁷ but also in CD40 + IL-4 activated B cells *in vitro* as shown in this study. By simulating the B cell activation in the lymph node with additional BCR-activation and IL-21, IgA production by a factor of up to 7.4 due to 9cRA. These findings suggest that a temporo-spatial presence of antigens with 9cRA in a specific microenvironment, e.g. in the gut,⁸ is required to achieve IgA polarization. Our data from the murine allergic sensitization model using 9cRA confirms a gut-tropism of B cells activated in the presence of vitamin A. This was reflected by the increased presence of CCR9⁺ B cells and integrin- $\alpha 4\beta 7$ ⁺ plasmablasts in mesenteric lymph nodes rather than the draining lymph nodes. In this respect, our study extends the findings of previous reports.²⁹ We also detected a large proportion of IgG1⁺ B cells and plasmablasts in the mesenteric lymph nodes in the 9cRA groups. These were most likely IgA isotype cells and their number was dependent on the dose of 9cRA administered.

Whether the induction of gut-homing molecules by 9cRA directly occurs in B cells or indirectly via specialized gut-derived dendritic cells that were in contact with vitamin A² is as yet unknown. However, the latter option is supported by our finding that these homing receptors were not induced by 9cRA in purified human B cells.

It is still not clear how 9cRA induced IgA *in vivo*. TGF- β is essential for the induction of IgA²⁸ as IgA is absent in mice with a B cell-specific deletion of the TGF- β receptor.³⁰ There is still no conclusive evidence whether retinoic acid can substitute for TGF- β in the induction of IgA.³¹ But autocrine TGF- β induces IgA-switching³² and is known to be induced by retinoic acid.³³ Therefore, an indirect mechanism which causes 9cRA to induce IgA in B cells seems probable. Alternatively, retinoic acid-induced TGF- β from other cell types like intestinal dendritic cells³⁴ or epithelial cells²⁸ and contact to IL-21-secreting T follicular helper cells³⁵ may serve as indirect mechanisms causing IgA to be produced in B cells. The latter hypothesis is supported by our finding that *in vitro* anti-CD40 + IL-4 activated human B cells fail to secrete IgA in the presence of 9cRA, but not upon BCR- and IL-21-induced IgA secretion.

In addition to its impact on specific IgA induction, our data are of great importance for the understanding how established IgE responses are regulated by endogenous and exogenous cofactors, as 9cRA is synthesized from vitamin A enzymatically¹⁴ but is also used systemically for chronic inflammatory dermatological conditions (Alitretinoin). Our observation that 9cRA inhibits IgE production in isolated human B cells is in line with reduced serum IgE concentrations in 9cRA treated patients,²⁷ suggesting that B cells may be targeted directly. Significantly, the concentrations of 9cRA used in this study were adjusted to levels used during systemic treatment. In addition, our data improve the understanding of 9cRA, building on the insights previously gained from the isomer all-trans retinoic acid (ATRA), which inhibits IgE production in purified human or murine B cells and sensitized mice.^{13, 36, 37} In human B cells, 9cRA promoted IL-10 as previously demonstrated for ATRA.¹¹⁻¹³ Flow-cytometric analysis showed that 9cRA increases IL-10

expression by increasing the frequencies of producing cells similar to vitamin D.¹⁷ Surface CD86 expression decreased as a result of 9cRA as well as of ATRA,^{38, 39} suggesting a common mode of action, namely via RAR and RXR receptors.

However, the fact that B cell viability remained unaffected and the beneficial safety and tolerability profile of 9cRA⁴⁰ suggest superiority over other retinoids such as ATRA. B cell-derived IL-10 negatively controls IgE expression,⁴¹ which supports the findings of a recent study that 9cRA-induced IL-10 from B cells contributes to the control of serum IgE.²⁷ However, further detailed studies are required to validate this hypothesis. It would be particularly relevant to analyze the lamina propria, the peyers patches, and the B cell phenotypes in the mesenteric lymph nodes to further understand the mechanism of action of 9cRA.

To sum up, 9cRA targets the humoral immune response in an allergic sensitization model by promoting a specific IgA response and decreasing the IgE response. Our findings indicate that RXR activating retinoids play a major role in the physiologic regulation of IgE due to the endogenous synthesis of 9cRA. Further research is necessary to clarify whether a specific allergic response can be biased in a long-term manner towards IgA using 9cRA.

Author contributions: G.H. planned experiments, analyzed the data and wrote the paper, T.H. performed the experiments, analyzed the data and wrote the paper, S.T. performed experiments and analyzed the data, A.R. and M.W. performed conceptual work and wrote the paper.

Acknowledgements

Thanks go to Björn Hartmann, Ph.D. (now Faculty of Medicine, Stanford University, USA) and to Kerstin Geldmeyer-Hilt, Ph.D. for helping with the cell culturing setup and biochemical analysis, Rene Riedel (Deutsches Rheuma-Forschungszentrum Berlin, A Leibniz Institute), and Dennis Ernst for excellent technical assistance.

Conflict of interest: The authors declare no conflict of interest.

Funding sources: This work was supported by grants awarded by grants to MW, AR and GH of the Deutsche Forschungsgemeinschaft (DFG–SFB650-TP5 and DFG-TRR130-P19) and by Charité – Universitätsmedizin Berlin. T.H. was supported by a student grant awarded by Deutsches Rheuma-Forschungszentrum Berlin. S. T. is currently financed by the DFG (TRR130-TP19).

359 References

- 360 1. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJ, Furman D, et al. Variation in the
361 human immune system is largely driven by non-heritable influences. *Cell* 2015; 160:37-47.
- 362 2. Hall JA, Grainger JR, Spencer SP, Belkaid Y. The role of retinoic acid in tolerance and
363 immunity. *Immunity* 2011; 35:13-22.
- 364 3. Sudfeld CR, Navar AM, Halsey NA. Effectiveness of measles vaccination and vitamin A
365 treatment. *Int J Epidemiol* 2010; 39 Suppl 1:i48-55.
- 366 4. Ross AC. Vitamin A supplementation and retinoic acid treatment in the regulation of
367 antibody responses in vivo. *Vitam Horm* 2007; 75:197-222.
- 368 5. Smith SM, Hayes CE. Contrasting impairments in IgM and IgG responses of vitamin A-
369 deficient mice. *Proc Natl Acad Sci U S A* 1987; 84:5878-82.
- 370 6. Chun TY, Carman JA, Hayes CE. Retinoid repletion of vitamin A-deficient mice restores
371 IgG responses. *J Nutr* 1992; 122:1062-9.
- 372 7. Chen Q, Ross AC. Retinoic acid promotes mouse splenic B cell surface IgG expression and
373 maturation stimulated by CD40 and IL-4. *Cell Immunol* 2007; 249:37-45.
- 374 8. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing
375 IgA-secreting B cells by intestinal dendritic cells. *Science* 2006; 314:1157-60.
- 376 9. Surman SL, Rudraraju R, Sealy R, Jones B, Hurwitz JL. Vitamin A deficiency disrupts
377 vaccine-induced antibody-forming cells and the balance of IgA/IgG isotypes in the upper
378 and lower respiratory tract. *Viral Immunol* 2012; 25:341-4.
- 379 10. Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O, et al.
380 Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous
381 immunization in mice. *J Clin Invest* 2011; 121:3051-61.
- 382 11. Ertesvag A, Aasheim HC, Naderi S, Blomhoff HK. Vitamin A potentiates CpG-mediated
383 memory B-cell proliferation and differentiation: involvement of early activation of
384 p38MAPK. *Blood* 2007; 109:3865-72.
- 385 12. Worm M, Krah JM, Manz RA, Henz BM. Retinoic acid inhibits CD40 + interleukin-4-
386 mediated IgE production in vitro. *Blood* 1998; 92:1713-20.
- 387 13. Scheffel F, Heine G, Henz BM, Worm M. Retinoic acid inhibits CD40 plus IL-4 mediated
388 IgE production through alterations of sCD23, sCD54 and IL-6 production. *Inflamm Res*
389 2005; 54:113-8.
- 390 14. Chambon P. A decade of molecular biology of retinoic acid receptors. *Faseb J* 1996; 10:940-
391 54.
- 392 15. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, Collins SJ, et al. Retinoic acid-
393 induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid
394 receptor-alpha. *Cancer Res* 1994; 54:1746-52.
- 395 16. Heine G, Anton K, Henz BM, Worm M. 1alpha,25-dihydroxyvitamin D3 inhibits anti-CD40
396 plus IL-4-mediated IgE production in vitro. *Eur J Immunol* 2002; 32:3395-404.
- 397 17. Heine G, Niesner U, Chang HD, Steinmeyer A, Zugel U, Zuberbier T, et al. 1,25-
398 dihydroxyvitamin D(3) promotes IL-10 production in human B cells. *Eur J Immunol* 2008;
399 38:2210-8.
- 400 18. Geldmeyer-Hilt K, Heine G, Hartmann B, Baumgrass R, Radbruch A, Worm M. 1,25-
401 dihydroxyvitamin D impairs NF-kappa B in human naive B cells. *Biochem Biophys Res*
402 *Commun* 2011; 407:669-702.
- 403 19. Milovanovic M, Heine G, Hallatschek W, Opitz B, Radbruch A, Worm M. Vitamin D
404 receptor binds to the epsilon germline gene promoter and exhibits transrepressive activity. *J*
405 *Allergy Clin Immunol* 2010; 126:1016-23, 23 e1-4.
- 406 20. Heine G, Dahten A, Hilt K, Ernst D, Milovanovic M, Hartmann B, et al. Liver X receptors
407 control IgE expression in B cells. *J Immunol* 2009; 182:5276-82.

- 408 21. Ruhl R, Dahten A, Schweigert FJ, Herz U, Worm M. Inhibition of IgE-production by
409 peroxisome proliferator-activated receptor ligands. *J Invest Dermatol* 2003; 121:757-64.
- 410 22. Heine G, Tabeling C, Hartmann B, Gonzalez Calera CR, Kuhl AA, Lindner J, et al. 25-
411 hydroxvitamin D3 promotes the long-term effect of specific immunotherapy in a murine
412 allergy model. *J Immunol* 2014; 193:1017-23.
- 413 23. Luger EO, Fokuhl V, Wegmann M, Abram M, Tillack K, Achatz G, et al. Induction of long-
414 lived allergen-specific plasma cells by mucosal allergen challenge. *J Allergy Clin Immunol*
415 2009; 124:819-26 e4.
- 416 24. Reichling J. *Heilpflanzenkunde für die Veterinärpraxis*. Springer 2008.
- 417 25. Heine G, Drozdenko G, Grun JR, Chang HD, Radbruch A, Worm M. Autocrine IL-10
418 promotes human B-cell differentiation into IgM- or IgG-secreting plasmablasts. *Eur J*
419 *Immunol* 2014; 44:1615-21.
- 420 26. Weise C, Zhu Y, Ernst D, Kuhl AA, Worm M. Oral administration of *Escherichia coli*
421 Nissle 1917 prevents allergen-induced dermatitis in mice. *Exp Dermatol* 2011; 20:805-9.
- 422 27. Schindler M, Drozdenko G, Kuhl AA, Worm M. Immunomodulation in patients with
423 chronic hand eczema treated with oral alitretinoin. *Int Arch Allergy Immunol* 2014; 165:18-
424 26.
- 425 28. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol* 2008; 8:421-34.
- 426 29. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and
427 D take centre stage. *Nat Rev Immunol* 2008; 8:685-98.
- 428 30. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in
429 vivo. *Immunity* 2000; 13:443-51.
- 430 31. Tokuyama H, Tokuyama Y. Retinoic acid induces the expression of germ-line C alpha
431 transcript mainly by a TGF-beta-independent mechanism. *Cell Immunol* 1997; 176:14-21.
- 432 32. Zan H, Cerutti A, Dramitinos P, Schaffer A, Casali P. CD40 engagement triggers switching
433 to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence
434 for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S
435 gamma, S gamma-->S alpha DNA recombination. *J Immunol* 1998; 161:5217-25.
- 436 33. Tokuyama H, Tokuyama Y. Endogenous cytokine expression profiles in retinoic acid-
437 induced IgA production by LPS-stimulated murine splenocytes. *Cell Immunol* 1995;
438 166:247-53.
- 439 34. Suzuki K, Maruya M, Kawamoto S, Sitnik K, Kitamura H, Agace WW, et al. The sensing of
440 environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation
441 in the gut. *Immunity* 2010; 33:71-83.
- 442 35. Dullaers M, Li D, Xue Y, Ni L, Gayet I, Morita R, et al. A T cell-dependent mechanism for
443 the induction of human mucosal homing immunoglobulin A-secreting plasmablasts.
444 *Immunity* 2009; 30:120-9.
- 445 36. Worm M, Herz U, Krah JM, Renz H, Henz BM. Effects of retinoids on in vitro and in vivo
446 IgE production. *Int Arch Allergy Immunol* 2001; 124:233-6.
- 447 37. Tokuyama H, Tokuyama Y, Nakanishi K. Retinoids inhibit IL-4-dependent IgE and IgG1
448 production by LPS-stimulated murine splenic B cells. *Cell Immunol* 1995; 162:153-8.
- 449 38. Zhan XX, Liu Y, Yang JF, Wang GY, Mu L, Zhang TS, et al. All-trans-retinoic acid
450 ameliorates experimental allergic encephalomyelitis by affecting dendritic cell and
451 monocyte development. *Immunology* 2013; 138:333-45.
- 452 39. den Hartog G, van Altena C, Savelkoul HF, van Neerven RJ. The mucosal factors retinoic
453 acid and TGF-beta1 induce phenotypically and functionally distinct dendritic cell types. *Int*
454 *Arch Allergy Immunol* 2013; 162:225-36.
- 455 40. Diepgen TL, Pfarr E, Zimmermann T. Efficacy and tolerability of alitretinoin for chronic
456 hand eczema under daily practice conditions: results of the TOCCATA open study
457 comprising 680 patients. *Acta Derm Venereol* 2012; 92:251-5.

- 458 41. Milovanovic M, Heine G, Zuberbier T, Worm M. Allergen extract-induced interleukin-10 in
459 human memory B cells inhibits immunoglobulin E production. Clin Exp Allergy 2009;
460 39:671-8.

461

462

Figure 1. Repetitive antigen promotes the IgA response in the presence of 9cRA. (A)

Experimental setup. Arrows indicate OVA-sensitization, OVA-challenges ($\pm$ 9cRA at the same time) and OVA-recall. OVA-specific serum concentrations of (B) IgA, (C) IgE and (D) IgG1 were determined after sensitization on day 51, after challenges on day 77 (open bars=without 9cRA; orange=3 mg/kg 9cRA; red=30 mg 9cRA), pre and post OVA recall on day 109 and day 116, respectively. Data are shown as mean + SEM, n=6. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2. Increased numbers of IgA- and diminished IgE- and IgG1-secreting cells by 9cRA in the spleen.

Mice were treated as described in Figure 1 legend and Ig-secreting cells were analyzed on day 115 by OVA-specific ELISPOT regarding (A) IgA, (B) IgE and (C) IgG1 isotype. The left column depicts the data from spleen, the right from the bone marrow. The line indicates the mean value, n=5-6, * $p \leq 0.05$, ** $p \leq 0.01$, Mann-Whitney-U-Test compared to control.

Figure 3. 9cRA promotes the intestinal homing of OVA-specific B cells and plasmablasts.

Mice were treated as described in Figure 1A. OVA-specific B cells and plasmablasts were analyzed on day 115 by flow cytometry. The dot plots were gated on live single cells (Supplementary figure 2). The data are shown (A) as dot plots from representative 1 experiment, (B) as mean + SEM from different lymphatic tissues, nd=not detectable, n=5-6 mice; cells from lymph nodes were pooled from 3 individual mice, Mann-Whitney-U-Test compared to control.

Figure 4. Effect of 9cRA on human immunoglobulin production.

CD19⁺ B cells were stimulated with (A) anti-CD40 + IL-4 for 10 days or (B) anti-CD40 + IL-4 + anti-BCR + IL-21 for 7 days in the absence or presence of increasing doses of 9cRA and the secreted Ig concentrations were detected by ELISA. The mean concentration after anti-CD40 + IL-4 stimulation was 1.9 ng/ml for IgE, 29 ng/ml for IgM, 96 ng/ml for IgG and 710 ng/ml for IgA. The mean concentration after anti-

489 CD40 + IL-4 + anti-BCR + IL-21 stimulation was 1.3 ng/ml for IgE, 110 ng/ml for IgM, 206 ng/ml
490 for IgG and 60 ng/ml for IgA. Values are normalized to stimulated control shown as mean + SEM
491 (n=4, *=p<0.05, **=p<0.01).

492

493 **Figure 5:** Impact of 9cRA on B cell activation. (A, B) Human CD19⁺ B cells were activated by
494 antiCD40 + IL-4 or (C) anti-CD40 + IL-4 + anti-BCR + IL-21 ± 9cRA for 4 days. CD23, CD54 and
495 CD86 MFI was analyzed by flow cytometry. IL-10 concentrations were detected by ELISA. Mean
496 IL-10 concentrations in the stimulated control group were (B) 16 pg/ml and (C) 1367 pg/ml. (A)
497 Exemplary histograms are gated on live B cells (filled grey=stimulated, black line=stimulated +
498 9cRA). The bars are normalized to stimulated control and represent mean + SEM (n=4, *=p<0.05).

499

Supplementary Figure 1. Stable total Ig-serum concentrations upon treatment with 9cRA together with the antigen. Experimental setup as described in Figure 1 legend. Total serum-Ig concentrations were determined regarding (A) IgA, (B) IgE and (C) IgG1 at day 51 (post-sensitization), day 77 (post OVA challenges without (white), with 3 mg/kg 9cRA (orange) or 30 mg 9cRA (red), day 109 and day 116 (pre and post OVA recall). Data are shown as mean + SEM, n=6. ** $p \leq 0.01$.

Supplementary Figure 2. Detection of OVA-specific B cells and plasmablasts. A) Gating strategy to detect OVA-specific IgG1⁺ plasmablasts (left panel) and memory B cells (right panel) and backgating to the scatter characteristics (size=FSC, granularity=SSC). B) Specificity of OVA⁺ plasmablasts compared by cells from naive mice, nitrophenyl sensitized mice (NP-KLH) and OVA sensitized mice following preincubation with unlabeled OVA in excess (OVA-block).

Supplementary Figure 3. Detection of homing parameters of OVA-specific B cells. The mice were treated as described in Figure 1A and analyzed on day 115 by flow cytometry. (A) Gating strategy to detect homing parameters on B cell subsets. (B) Expression of CCR9 and $\alpha 4\beta 7$ on mesenteric lymph node B cells. nd=not detectable; n=5-6 mice; cells from lymph nodes were pooled from 3 individual mice.

Supplementary Figure 4. 9cRA enhances surface CD38 expression in activated B cells. CD19⁺ B cells were activated by (A) anti-CD40 + IL-4 for 4 days or (B) anti-CD40 + IL-4 + anti-BCR + IL-21 for 7 days $\pm$ 9cRA and analyzed by flow cytometry. A representative histogram of each stimulation (right to the diagram) is gated on live B cells (filled grey=stimulated, black

line=stimulated + 9cRA). The bars are normalized to stimulated control and represent mean $\pm$ SEM (n=4, *=p<0.05, ***=p<0.001).

Supplementary Figure 5: 9cRA upregulates IL-10 resulting in a higher frequency of IL-10 secreting cells. Naive human B cells (CD19⁺/CD27⁻) were stimulated for 4 days by CD40L + IL-4 + IL-21 + anti-BCR in the absence or presence of increasing concentrations of 9cRA. The data show dot plots of intracellular IL-10 stained cells from one representative donor.

