

Modulation of Cytokine Production by Low and High Retinoid Diets in Ovalbumin-Sensitized Mice

Ralph Rühl¹, Ada Garcia¹, Florian J. Schweigert¹ and Margitta Worm²

¹ Institute of Nutritional Science, University of Potsdam, Potsdam-Rehbrücke, Germany

² Department of Dermatology and Allergy, Charité, Humboldt University Berlin, Germany

Received for publication: May 5, 2003; Accepted for publication: August 8, 2003

Abstract: Retinoids modulate many physiological processes such as the differentiation and growth of different cell types, including cells from the immune system. We have previously shown that retinoids modulate IgE production *in vitro* and *in vivo*. In the present study we investigated the effects of retinoids in non-sensitized and ovalbumin-sensitized mice that were fed for 11 weeks with three different vitamin A (VA) diets: a) VA-deficiency diet, b) base diet, and c) base diet supplemented with 0.5% all-trans-retinoic acid (ATRA). Phorbol-myristate-acetate (PMA)/ionomycin-stimulated SMC (splenic mononuclear cells) from mice fed with ATRA and the vitamin A-deficient diet group showed increased interleukin-4 (IL-4) responses in non-sensitized mice. After ovalbumin sensitization in the VA-deficient and the ATRA supplementation diet groups, no significant effects on IL-4 production were observed. By contrast, gamma interferon (IFN- γ) production from PMA/ionomycin-stimulated SMC was enhanced in the VA-deficient diet group in ovalbumin-sensitized mice, and also in non-sensitized mice compared to the base and the ATRA-supplemented diet group. The data indicate that VA and retinoid content in a diet influences the cytokine response in non-sensitized and also ovalbumin-sensitized mice. Therefore these molecules may serve as active modulators of cytokine production *in vivo* that are responsible for the induction and persistence of atopic diseases.

Key words: Allergy, retinoic acid, IL-4, IFN- γ , retinoid, vitamin A

Introduction

Allergic diseases are characterized by several alterations of the immune system, including a deviated T-cell cytokine response. An initial T-helper-2 (TH2) – dominated lymphocyte response with the production of interleukin-4 (IL-4) plays an important role in this context, since IL-4 induces the isotype switching to immunoglobulin E (IgE). By contrast, gamma interferon (IFN- γ) counteracts IL-4 and is produced by T-helper-1 (TH1) cells. However, IFN-

γ may also play an important role in the chronic state of an allergic inflammatory process [1, 2].

Ligands of the nuclear hormone receptor superfamily, such as 1,25-dihydroxy-vitamin D₃ [the active ligand of the vitamin D receptor (VDR)] and all-trans retinoic acid [ATRA, the active ligand of the retinoic acid receptor (RAR)], downregulate IgE production in *in vitro* as well as *in vivo* systems [3, 4, 5]. The retinoid-X receptor is the heterodimer-binding partner of several nuclear receptors like the VDR, RAR, and the peroxisome proliferator ac-

tivated receptors (PPARs) and has been attributed to enhanced TH2 development in an *in vitro* model for lymphocyte development [6].

Furthermore, retinoids like ATRA and 13-cis-retinoic acid have been shown to inhibit IgE production in several *in vitro* systems including anti-CD40 and IL-4 stimulated peripheral blood mononuclear cells (PBMCs) [3] but also in PBMCs from patients with atopic dermatitis [4]. However, *in vivo* experiments with and without ovalbumin (OVA) sensitization have not shown any significant IgE downregulation or even higher specific IgE responses after application of a synthetic RAR ligand (CD336/Am580) [4] or 13-cis-retinoic acid [7].

Studies by Buck *et al* [8] and Blomhoff *et al* [9] indicated that retinoids are key regulators of cell growth, cytokine production, and differentiation of normal B lymphocytes. A specific role of retinoic acids [10] and the retinoid receptors RAR and RXR (retinoid-X receptor) [11] indicate important roles of ligands and receptors for cell activation, normal growth, and apoptosis of B lymphocytes.

VA-deficient diets have been shown to ameliorate the profile of several cytokines influencing IgE secretion [12, 13]. In particular, vitamin A deficiency diminished the antibody-mediated response directed by TH2 cells, although some aspects of TH1-mediated immunity were also diminished. Carman *et al* [14] provided evidence for a TH2 cell dysfunction in vitamin A-deficient mice, with reduced IL-4 and IL-5 secretion by TH2 cells. However, the TH2 deficiency in vitamin A-deficient rodents has not been shown to be entirely due to excessive IFN- γ synthesis, since neutralizing IFN- γ did not restore TH2 cells [15, 16]. Data derived from human cell cultures, however, have shown inhibition of IgE production independently from IFN- γ influence [3], suggesting different pathways of IgE regulation by retinoids.

The aim of the present study was to examine whether VA-enriched or depleted diets may affect the T cell dependent cytokine production in an allergic mouse model. Therefore different VA diets were applied to OVA-sensitized mice in comparison to non-sensitized mice and IL-4 and IFN- γ production was measured consecutively in the different groups.

Materials and Methods

Animals

Female mice (NMRI: Tierzucht Schönwalde, Schönwalde, Germany) were kept under specific pathogen-free (spf) conditions at $21 \pm 1^\circ\text{C}$, $55 \pm 5\%$ relative humidity, and 12-hour light/dark cycle with light between 8:00 AM

and 8:00 PM. Food and tap water were provided *ad libitum*. Due to photosensitivity of the applied retinoic acid, all mice were kept under dim yellow light. The experiments were approved by the respective ethical authorities from Land Brandenburg, Germany.

Diets

Three different diets consisted of casein, 12%; sucrose, 10%; wheat starch, 64%; sunflower oil, 5%; mineral mix, 5%; cellulose, 2%; and vitamin mix 2%; with a similar, vitamin A-free mix for the vitamin A-deficient diet. The base diet contained 4.5 retinol equivalents/kg diet. The all-trans retinoic acid-supplemented diet was supplemented with 0.5% all-trans-retinoic acid (from BASF AG, Ludwigshafen, Germany) added to the base diet. Twelve mice per group were fed with the respective diets for 11 weeks.

OVA sensitization

Six mice of each group were intraperitoneally injected with 100 μl ovalbumin/aluminum hydroxide suspension in saline (10 μg ovalbumin adsorbed on 1.5 mg aluminum hydroxide), or with saline as control [4]. The OVA sensitizations were performed 8, 9, and 10 days after beginning of the supplementations. Eleven weeks after beginning of the treatment the mice were sacrificed, the spleen was collected and immediately (maximum of two hours after death) used for the extraction of SMC (spleen mononuclear cells).

Stimulation of lymphocytes

Lymphocytes were harvested from the spleen by gently pressing the tissue through a fine Falcon[®] nylon mesh sieve (100 μm) (BD, PharMingen, San Diego, CA, USA) and washing the cell suspension with phosphate-buffered saline (PBS) without Ca^{2+} / Mg^{2+} . The lymphocytes were counted and 1×10^6 to 5×10^6 cells were stained with fluorescein-labeled monoclonal antibodies (mAb). The cell suspensions were mixed with 100 μL labeled mAb and buffer (10% bovine serum albumin in PBS) in a 1:200 dilution. A double-color staining using fluorescein isothiocyanate (FITC) and phycoerythrin (PE) was performed using the following mAb combinations: FITC-conjugated rat IgG_{2a} with PE-conjugated rat IgG_{2a}; FITC-conjugated rat anti-mouse CD8a(Ly-2) with PE-conjugated rat anti-mouse CD4(L3T4); and FITC-conjugated rat anti-mouse CD3 molecular complex with PE-conjugated rat anti-mouse CD45R/B220. All mAbs were purchased from BD PharMingen (San Diego CA, USA). After incubation (20 minutes at 4°C in darkness), the cells were washed. The supernatant was removed and the pellet resuspended with

500 μ L 2% paraformaldehyde-solution in PBS purchased from Sigma, Munich, Germany. The fixed cells were kept at 4°C until cytometric analyses.

Flow cytometry

The cells were acquired and analyzed using a fluorescence-activated cell sorter with Cell Quest 3.3 software (FACSsort, Becton Dickinson, San Jose, CA, USA). A gate was set to record 50 000 events using side (SSC) and forward (FSC) light scatter parameters that allowed the identification of the morphological cell populations. The lymphocytes were gated again and analyzed using FL1/FSC and FL2/FSC parameters. The FL1/FSC dot plot expressed FITC and the FL2/FSC expressed PE. For each staining the appropriate isotype control was done to evaluate nonspecific staining.

Cytokine measurements

10^6 splenocytes were cultured unstimulated or stimulated [20 ng/mL PMA (phorbol myristate acetate)/ 10^{-7} M ionomycin] for 24 hours. Supernatants were collected and kept frozen (-80°C) until further analysis. For detection of the cytokines (IL-4/ IFN- γ) enzyme-linked immunosorbent assays (ELISA) from R&D (Wiesbaden, Germany) were used according to the manufacturer's guidelines.

Statistics

Standard deviations have been calculated with MS-Excel using Student's t-test; a value of $p < 0.05$ indicates statistical significance.

Results

ATRA supplementation results in decreased lymphocyte subpopulations

Initially we determined lymphocyte counts in the spleen from mice receiving the different diets. Stimulation with OVA had no effects on percentages of lymphocyte subpopulations (CD8+, CD4+, and CD45R/B220+) extracted from the spleen. By contrast, the different diets exhibited considerable effects on the numbers of lymphocyte subpopulations. Mainly the ATRA-supplemented diet group showed a decrease of CD8+ ($1.8 \pm 0.5\%$), CD4+ ($9.1 \pm 3.0\%$), and CD45R/B220+ ($11.9 \pm 2.8\%$) lymphocyte subpopulations in comparison to base diet ($3.2 \pm 0.9\%$ CD8+; $12.2 \pm 2.7\%$ CD4+; $14.6 \pm 4.2\%$ CD45R/B220+). By contrast, the VA-deficient diet had a lowering effect on lymphocyte counts with reduced amounts of CD8+ and

CD45R/B220+ lymphocytes and higher amounts of CD4+ lymphocytes in non-OVA-sensitized mice. No effects on the amounts of lymphocyte subpopulations after OVA stimulation ($3.1 \pm 0.9\%$ CD8+; $12.1 \pm 1.6\%$ CD4+; $13.5 \pm 2.0\%$ CD45R/B220+) were observed (Fig. 1).

A VA-deficient diet induced an enhanced IFN- γ response

To examine the IFN- γ responses in mice with different diets with and without OVA-sensitization, splenic mononu-

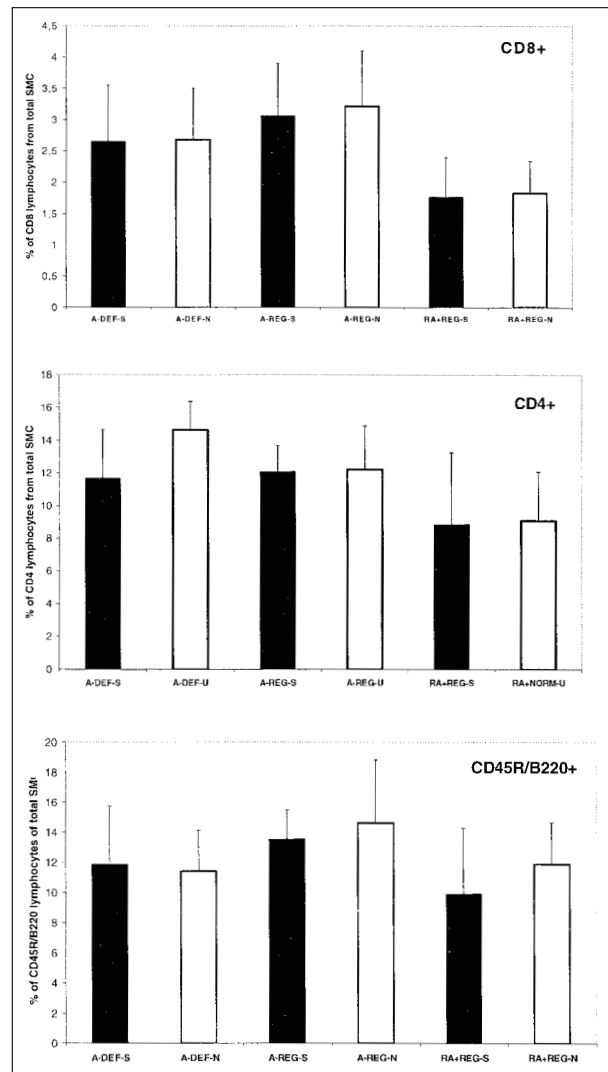


Figure 1: Lymphocyte numbers; CD8+ (top figure), CD4+ (middle figure) and CD45R/B220+ (bottom figure) lymphocytes from total extracted SMC in non- and OVA-sensitized mice after application of three different diets: Vitamin A-deficient diet (A-DEF), regular vitamin A diet (A-REG), and regular vitamin A diet with all-trans-retinoic acid supplementation (RA+REG). N – non-stimulated and S – stimulated with OVA. In each group the number of mice were $n = 6$.

clear cells (SMCs) were extracted and stimulated with PMA/ionomycin. The IFN- γ response of SMC significantly increased after OVA-sensitization in all treatment groups. The highest IFN- γ responses were determined in the vitamin A-deficient group (7.89 ± 3.09 ng/mL) and considerably lower concentrations in the base diet (4.45 ± 2.55 ng/mL) and ATRA-supplemented group (3.96 ± 3.34 ng/mL). Very low to undetectable IFN- γ concentrations were determined in non-sensitized mice (0 ng/mL base diet-fed group; 0.01 ± 0.03 ng/mL ATRA-supplemented group). Only the vitamin A-deficient mice showed low IFN- γ responses (1.26 ± 2.32 ng/mL), as shown in Figure 2.

Increased IL-4 response in VA-deficient mice

IL-4 was detectable after PMA/ionomycin stimulation in non-sensitized as well as in OVA-sensitized mice. The highest concentrations of IL-4 were determined in the VA-deficient non-OVA-sensitized mice (176 ± 92 pg/mL). In contrast to the data of IFN- γ , no further increase of IL-4 concentrations after OVA-stimulation was detected (102 ± 83 pg/mL). No significant differences of IL-4 production were determined in OVA-sensitized mice depending on the type of diet. However, a significant increase of IL-4 production was observed in non-sensitized VA-deficient (102 ± 83 pg/mL) or ATRA-supplemented mice (153 ± 93 pg/mL) in comparison to the base diet-fed mice (96 ± 52 pg/mL) (Fig. 2).

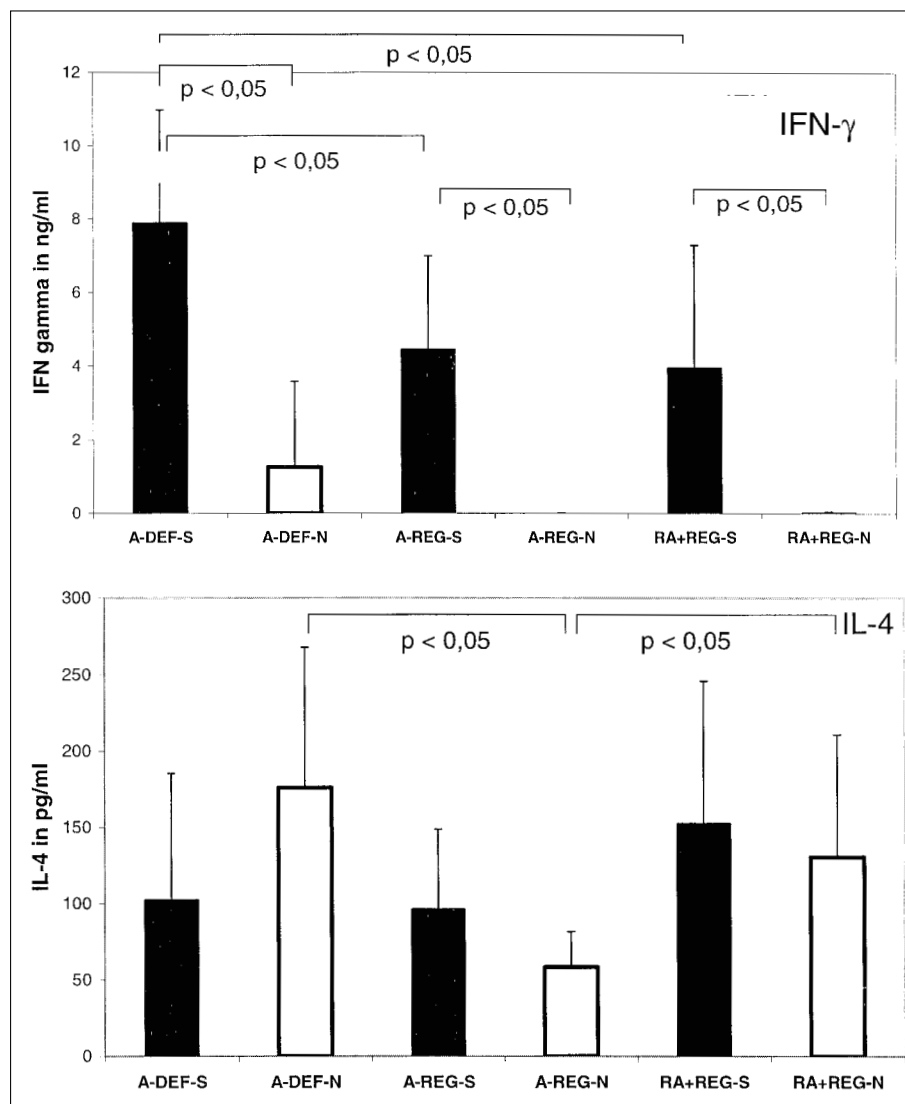


Figure 2: IFN- γ (top figure) and IL-4 (bottom figure) production after stimulation with PMA/ionomycin of SMC from non- and OVA-stimulated mice after application of three different diets: Vitamin A-deficient diet (A-DEF), regular vitamin A diet (A-REG) and regular vitamin A diet with all-trans-retinoic acid supplementation (RA+REG). N – non-stimulated and S – stimulated with OVA. Significances were determined using Student's t-test. In each group the number of mice were n = 6.

Discussion

In the present study we demonstrate a statistically significant modulation of the cytokine response (IL-4 and IFN- γ) using a murine *in vivo* model receiving VA-deficient and ATRA-supplemented diets. However, we do not find a statistically significant alteration of lymphocyte subpopulations after OVA-sensitization (CD4+, CD8+, and CD45R/B220+). These data are in correspondence with previously published studies where VA-deficient diets lead to an abnormal IFN- γ secretion and an imbalance of TH1/TH2 function [15, 16]. Novel is the finding of a high IL-4 response in vitamin A-deficient fed, non-OVA-sensitized mice and the increase of IFN- γ production by SMC in vitamin A-deficient fed, OVA-sensitized mice, clearly pointing out an immunomodulatory role of retinoids during an allergic immune response.

In previous studies a clear connection between IgE secretion and retinoid application has been shown [3, 4, 13]. In contrast to the *in vitro* experiments, during *in vivo* application of ATRA or a synthetic retinoid (CD336/Am580), the OVA-specific IgG but also total IgE were upregulated. In the present study we elucidate possible reasons for these previous findings since VA-deficient or ATRA-supplemented diets resulted in a modulation of the TH1-derived IFN- γ and the TH-2 derived IL-4 response *in vivo*. IFN- γ production was upregulated during OVA-sensitization and further enhanced by retinoids in the diet, whereas IL-4 production was modulated by a retinoid-enriched diet in non-sensitized mice only.

The cell-specific mechanisms that are involved for the IFN- γ or IL-4 response by retinoids were not determined in the present study. IFN- γ and IL-4 are not the only T cell-specific cytokines and the effects of retinoids through the diet may also be mediated by different pathways including other cell types like monocytes or B cells [3, 4]. Retinoids have additionally been shown to be isotype-specific modulators towards an IL-4 dependent immunoglobulin response, like IgG1 and IgE production [17]. These effects have been associated mainly with attenuation of IL-4 production [18] or an upregulation of IFN- γ secretion during vitamin A deficiency [6, 14, 16, 19].

Retinoids are also known to be involved in the regulation of differentiation pathways [20] and apoptotic processes [21] mediated by RARs. Inhibitory effects are mainly mediated via the RAR α [22] and stimulation of apoptosis via the RAR γ receptors [23]. A decrease of CD4+ and CD8+ cells after a supplementary diet with ATRA suggests an influence of retinoic acid on T cell development and/or survival. Pathways via retinoid receptors in T lymphocytes have been described by dietary vitamin A [24] and the presence of these receptors may be associated with the induction of apoptosis.

Increased IL-4 secretion in non-sensitized vitamin A-deficient fed mice is a novel and highly important finding of this study. The fact that retinoids inhibit IL-4-dependent IgE and IgG1 production in lipopolysaccharide (LPS)-stimulated murine splenic B cells has been reported by Tokuyama *et al* [18].

Taken together, our data show that retinoid supplementation or VA deficiency in the diet have the potential to modulate the cytokine responses *in vivo*, namely IL-4 and IFN- γ production by splenic lymphocytes. Since these cytokines are involved in the development and induction of an allergic immune response, we conclude that modulation of the amount of retinoids in the diet may play an important role in the induction and course of atopic diseases via alteration of TH1-TH2 cytokine production.

Acknowledgements

The authors would like to thank Dr. Klaus Krämer from BASF AG for donation of ATRA, and we are grateful to Solveig Stazewski and Katrin Anton for excellent technical assistance.

References

1. Worm, M. and Henz, B. M. (1997) Molecular regulation of human IgE synthesis. *J. Mol. Med.* 75, 440.
2. Bacharier, L. B. and Geha, R. S. (2000) Molecular mechanisms of IgE regulation. *J. Allergy Clin. Immunol.* 105, S547.
3. Worm, M., Krah, J. M., Manz, R. A. and Henz, B. M. (1998) Retinoic acid inhibits CD40+ Interleukin-4-mediated IgE production *in vitro*. *Blood* 92, 1713.
4. Worm, M., Herz, U., Krah, J. M., Renz, H. and Henz, B. M. (2001) Effects of retinoids on *in vitro* and *in vivo* IgE production. *Int. Arch. Allergy Immunol.* 124, 233.
5. Heine, G., Anton, K., Henz, B. M. and Worm, M. (2003) 1,25-Dihydroxyvitamin D3 inhibits anti-CD40 + IL-4 mediated IgE production *in vitro*. *Eur. J. Immunol.* 32, 3395.
6. Stephensen, C. B., Rasooly, R., Jiang, X., Ceddia, M. A., Weaver, C. T., Chandraratna, R. A. S. and Bucy, R. P. (2002) Vitamin A enhances *in vitro* Th2 development via Retinoid X receptor pathways. *J. Immunol.* 168, 4495.
7. Barnett, J. B. (2002) Immunopotential of the IgE antibody response by 13-*cis*-retinoic acid. *Int. Arch. Allergy. Appl. Immunol.* 67, 287.
8. Buck, J., Dannecker, L., Viswanathan, K., Cohen, S. L., Chait, B. T. and Hämmerling, U. (1990) Retinol is essential for growth of activated human B cells. *J. Exp. Med.* 171, 1613.
9. Blomhoff, H. K., Smeland, E. B., Erikstein, B., Rasmussen, A. M., Skrede, B., Skjonsberg, C. and Blomhoff, R. (1992) Vitamin A is a key regulator for cell growth, cytokine production, and differentiation in normal B cells. *J. Biol. Chem.* 267, 23988.

10. Fahlman, C., Jacobsen, S.E., Smeland, E.B., Lomo, J., Naess, C.E., Funderud, S. and Blomhoff, H.K. (1995) All-trans- and 9-cis-retinoic acid inhibit growth of normal human and murine B cell precursors. *J. Immunol.* 155, 58.
11. Lomo, J., Smeland, E.B., Ulven, S., Natarajan, V., Blomhoff, R., Gandhi, U., Dawson, M.I. and Blomhoff, H.K. (1998) RAR-, not RXR, ligands inhibit cell activation and prevent apoptosis in B-lymphocytes. *J. Cell. Physiol.* 175, 68.
12. Hayes, C.E., Nashold, F.E., Enrique Gomez, F. and Hoang, F.A. (1999) Retinoids and Immunity. In: *Retinoids: The biochemical and molecular basis of vitamin A and retinoid action*. (Nau, H. and Blaner, W.S., eds.) pp. 589–610, Springer-Verlag, Berlin.
13. Stephensen, C.B. (2001) Vitamin A, infection, and immune function. *Annu. Rev. Nutr.* 21, 167.
14. Carman, J.A., Smith, S.M. and Hayes, C.E. (1989) Characterization of a helper T lymphocyte defect in vitamin A-deficient mice. *J. Immunol.* 142, 388.
15. Cantora, M.T., Nashold, F.E. and Hayes, C.E. (1994) In vitamin A deficiency multiple mechanisms establish a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function. *J. Immunol.* 152, 1515.
16. Carman, J.A. and Hayes, C.E. (1991) Abnormal regulation of IFN-gamma secretion in vitamin A deficiency. *J. Immunol.* 147, 1247.
17. Tokuyama, Y. and Tokuyama, H. (1996) Retinoids as Ig isotype-switch modulators. The role of retinoids in directing isotype switching to IgA and IgG1 (IgE) in association with IL-4 and IL-5. *Cell. Immunol.* 170, 230.
18. Tokuyama, H., Tokuyama, Y. and Nakanishi, K. (1995) Retinoids inhibit IL-4-dependent IgE and IgG1 production by LPS-stimulated murine splenic B cells. *Cell. Immunol.* 162, 153.
19. Cantorna, M.T., Nashold, F.E. and Hayes, C.E. (1994) Vitamin A deficiency results in a priming environment conducive for Th1 cell development. *Eur. J. Immunol.* 25, 1673.
20. Yagi, J., Uchida, T., Kuroda, K. and Uchiyama, T. (1997) Influence of retinoic acid on the differentiation pathway of T cells in the thymus. *Cell. Immunol.* 181, 153.
21. Szondy, Z., Reichert, U. and Fesus, L. (1998) Retinoic acids regulate apoptosis of T lymphocytes through an interplay between RAR and RXR receptors. *Cell. Death. Differ.* 5, 4.
22. Szondy, Z., Reichert, U., Bernardon, J.M., Michel, S., Toth, R., Karaszi, E. and Fesus, L. (1998) Inhibition of activation-induced apoptosis of thymocytes by all-trans- and 9-cis-retinoic acid is mediated via retinoic acid receptor alpha. *Biochem. J.* 331, 767.
23. Szondy, Z., Reichert, U., Bernardon, J.M., Michel, S., Toth, R., Ancian, P., Ajzner, E. and Fesus, L. (1997) Induction of apoptosis by retinoids and retinoic acid receptor gamma-selective compounds in mouse thymocytes through a novel apoptosis pathway. *Mol. Pharmacol.* 51, 972.
24. Halevy, O., Arazi, Y., Melamed, D., Friedman, A. and Sklan, D. (1994) Retinoic acid receptor-alpha gene expression is modulated by dietary vitamin A and by retinoic acid in chicken T lymphocytes. *J. Nutr.* 124, 2139.

PD Dr. Margitta Worm

Charité Campus Mitte
Humboldt University Berlin
Schumannstr. 20-21
D-10117 Berlin
Tel. +49 30 4505 18105
Fax +49 30 4505 18958
E-mail: margitta.worm@charite.de