



**University of  
Zurich** <sup>UZH</sup>

**Zurich Open Repository and  
Archive**

University of Zurich  
University Library  
Strickhofstrasse 39  
CH-8057 Zurich  
[www.zora.uzh.ch](http://www.zora.uzh.ch)

---

Year: 2013

---

### **The effects of vitamin A on cells of innate immunity in vitro**

Wojtal, Kacper A ; Wolfram, Lutz ; Frey-Wagner, Isabelle ; Lang, Silvia ; Scharl, Michael ; Vavricka, Stephan R ; Rogler, Gerhard

DOI: <https://doi.org/10.1016/j.tiv.2013.03.013>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-92842>

Journal Article

Accepted Version

Originally published at:

Wojtal, Kacper A; Wolfram, Lutz; Frey-Wagner, Isabelle; Lang, Silvia; Scharl, Michael; Vavricka, Stephan R; Rogler, Gerhard (2013). The effects of vitamin A on cells of innate immunity in vitro. *Toxicology in Vitro*, 27(5):1525-1532.

DOI: <https://doi.org/10.1016/j.tiv.2013.03.013>

## The Effects of Vitamin A on Cells of Innate Immunity In Vitro

Kacper A Wojtal PhD, Lutz Wolfram PhD, Isabelle Frey-Wagner PhD, Silvia Lang,  
Michael Scharl MD, Stephan R Vavricka MD, Gerhard Rogler MD, PhD\*

*Division of Gastroenterology and Hepatology, Department of Internal Medicine,  
University-Hospital of Zürich, Switzerland*

\*Corresponding author:

Prof. Dr. med. Dr. phil. Gerhard Rogler

Division of Gastroenterology and Hepatology, Department of Internal Medicine,  
University-Hospital of Zürich, Rämistrasse 100, CH-8091 Zürich, Switzerland

E-mail: gerhard.rogler@usz.ch

Telephone: +41-(0)44-255-9477

Fax: +41-(0)44-255-9497

### Abbreviations used:

13-cis-RA = 13-cis-retinoic acid; 4-oxo-13-cis-RA = 4-oxo-13-cis-retinoic acid; ATRA = all-trans retinoic acid; Caco-2 = human endothelial colorectal adenocarcinoma; CD = Crohn's disease; DSS = dextran sodium sulphate; EDTA = ethylenediaminetetraacetic acid; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; GI = gastrointestinal; GM-CSF = granulocyte-macrophage colony-stimulating factor; IBD = irritable bowel disease; ICAM-1 = intercellular adhesion molecule-1; IFN = interferon; IL = interleukin; IL-1RA, interleukin-1 receptor antagonist; *iv*DCs = *in vitro*-differentiated human dendritic cells; *iv*MACs = *in vitro*-

differentiated human macrophages; LPS = lipopolysaccharide; MCP = monocyte chemotactic protein; MIP = macrophage inflammatory protein; MMP = matrix metalloproteinase; TGF = transforming growth factor; THP = monocytic leukemia, TNBS = trinitrobenzene sulfonic acid; TNF = tumor necrosis factor; PBMCs = peripheral blood mononuclear cells; PBS = phosphate buffered saline; RH = relative humidity; UC = ulcerative colitis; VEGF = vascular endothelial growth factor

## ABSTRACT

Retinoid treatment is suggested to promote development of inflammatory bowel disease, although preclinical studies are not supportive. We evaluated the effect of retinoids on cytokine response in *in vitro*-differentiated human dendritic cells (*iv*DCs) and macrophages (*iv*MACs) derived from healthy human donors and in cultured human THP-1 cells. Effect on human intestinal epithelial cell integrity was also assessed. Each cell type was incubated ( $\pm$  lipopolysaccharide [LPS]) with all-trans retinoic acid (ATRA), 13-cis-RA (isotretinoin) and 4-oxo-13-cis-RA. Cytokine analysis was performed by array analysis. Cultured human endothelial colorectal adenocarcinoma (Caco-2) cells were incubated with these retinoids and media analyzed for leakage by spectrofluorometric analysis. ATRA consistently and significantly inhibited LPS-induced release of the pro-inflammatory cytokines tumour necrosis factor, interleukin (IL)-6, macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$ . All retinoids tested stimulated release of the anti-inflammatory cytokines granulocyte-macrophage colony-stimulating factor and IL-10, and also monocyte chemoattractant protein-1, vascular endothelial growth factor and eotaxin-1. Incubation with retinoids did not significantly alter the permeability of Caco-2 monolayers. Pre-treatment of each cell type with retinoids promoted an anti-inflammatory cytokine profile with only minimal effect on intestinal epithelial cell permeability; consistent with *in vivo* studies.

**Word count (max 200):** 186

**Keywords:** cytokines, inflammation in IBD, dendritic cells in IBD, macrophages in IBD.

## 1. Introduction

There is currently much debate as to whether vitamin A and associated retinoid derivatives are beneficial or harmful to the gastrointestinal (GI) tract, a situation primarily driven by clinical case reports claiming a putative causal relationship between retinoid treatment with 13-cis-retinoic acid (13-cis-RA, isotretinoin) and the occurrence of ulcerative colitis (UC) and Crohn's disease (CD), i.e. two forms of chronic inflammatory bowel disease (IBD) (Crockett et al., 2010; Reddy et al., 2006). Contrary to this, key basic research data do, in fact, support anti-inflammatory effects of retinoids on the GI tract (Bai et al., 2009; Iwata and Yokata, 2011). Nevertheless, the case for retinoids being beneficial or harmful to the GI tract has only infrequently been based on robust scientific evidence and, thus far, it has not been possible to confirm or refute a causative relationship (Crockett et al., 2009). Ideally, further prospective or well-designed retrospective pharmacoepidemiological studies are needed to definitively establish causality (Rogler et al., manuscript in preparation).

Understanding of the pathophysiology of IBD has markedly increased recently with a number of pre-disposing genetic risk factors identified for CD and (to a lesser extent) UC, along with a number of environmental triggers considered as potential key mediators of disease development (Rogler, 2011). Although more risk factors are expected to follow (Barrett et al., 2008; Latella et al. 2010; Nguyen et al., 2006), the role of many these in the pathophysiology of CD, for example, is unclear (Mathew, 2008) and, nevertheless, account for only a fraction of observed CD incidence (Torkamani et al., 2008). Key environmental triggers include dietary factors, food additives or drugs (Cosnes, 2010; Hou et al., 2011a; Hou et al., 2011b;

Järnerot et al., 1983; Katschinski et al., 1988; Martini and Brandes, 1976; Silkoff et al. 1980; Thornton et al., 1979), and cigarette smoking (Avidan et al., 2005; Cosnes et al., 2001; Cosnes et al., 1996; Kane et al., 2005) while psychological factors may influence disease course (Cámara et al., 2010; Danese et al., 2004; Levenstein, 2002). Significantly, the innate immune system and epithelial barrier function are critical to the pathophysiology of both UC and CD; thus, environmental triggers that influence the intestinal microbiota and modify intestinal barrier function would appear particularly relevant.

Recent studies in experimental models of IBD and in human colonic biopsy samples have shown retinoids to be potentially anti-inflammatory; for example, all-trans-retinoic acid (ATRA, tretinoin) and transforming growth factor (TGF)- $\beta$ 1 promoted differentiation of FOXP3<sup>+</sup> regulatory T-cell subsets (Benson et al., 2007; Iwata and Yokota, 2011) and prevented differentiation of pro-inflammatory interleukin (IL)-17-secreting Th17 cells (Bai et al., 2009; Hundorfean et al., 2012; Nikoopour et al., 2008; Reifen et al., 2002). Notably, observations of lower levels of pro-inflammatory cytokines (tumor necrosis factor [TNF]- $\alpha$ , subsequently referred to as TNF, IL-1 $\beta$ , IL-17), increased levels of regulatory cytokines (IL-10, TGF- $\beta$ ), and a dose-dependent amelioration of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis by ATRA were sufficiently strong for the authors to suggest ATRA as having therapeutic potential in IBD (Bai et al., 2009). Moreover, ATRA has been shown to be a critical regulator for barrier protection during mucosal injuries via up-regulation of tight-junction proteins and cyclo-oxygenase (Osanai et al., 2007). ATRA has also been shown to up-regulate the expression of gut-homing receptors e.g., integrin  $\alpha$ 4 $\beta$ 7 and C-C chemokine receptor type 9 on T-cells in vitro, which, upon binding to mucosal vascular addressin cell adhesion molecule 1 and chemokine (C-C motif)

ligand 25, respectively, mediate the migration of Th17 cells and regulatory T cells to the gut mucosa (Iwata et al., 2004).

Nevertheless, data from studies in primary human cells and intestinal cell lines as to the effects of retinoids remain limited. In this in-vitro study we evaluated the effects of retinoid derivatives of vitamin A – ATRA (tretinoin, the major metabolic derivative of vitamin A), 13-cis-RA (isotretinoin) and 4-oxo-13-cis-retinoic acid (4-oxo-13-cis-RA, the primary stable metabolite of isotretinoin) – on lipopolysaccharide (LPS)-induced cytokine release from differentiated monocytic dendritic cells and macrophages, and from cultured human acute monocytic leukaemia (THP)-1 cells, and also their effects on human intestinal epithelial cell integrity. The effect of retinoids in *in vivo* animal models has been investigated also (data to be published separately).

## 2. Materials and methods

### 2.1. Retinoids

ATRA, 13-cis-RA and 4-oxo-13-cis-RA (RO22-6595, Roche, Switzerland) were dissolved in dimethylsulfoxide (40 mg/mL), diluted in phosphate buffered saline (PBS), and tested at final concentrations of 0.01–5.0 µg/mL.

### 2.2. Human donors

Peripheral blood from healthy donors (two males and five females, aged 25–43 years) was obtained after oral consent, and in accordance with the guidelines of the ethical committee of Canton Zurich.

### 2.3. Isolation of human peripheral blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from 80 mL of peripheral blood according to the Ficoll<sup>®</sup> gradient centrifugation method. PBMCs were incubated with magnetic microbeads (130-091-153, Monocyte Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's protocol, and final isolation of monocytes was achieved using a magnetic cell sorter (AutoMACS, Miltenyi Biotec, Germany).

### 2.4. Generation of in vitro-differentiated dendritic cells (ivDCs)

PBMCs were differentiated into dendritic cells using an established protocol (Rogler et al., 1998); monocytes were cultivated in flasks for 1 week under optimal conditions (37°C, 5% CO<sub>2</sub>, 95% relative humidity [RH]) with 5 ng/mL IL-4 and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).



### 2.5. Generation of *in vitro*-differentiated macrophages (ivMACs)

As described above for *ivDCs*, peripheral blood monocytes were differentiated into macrophages based on the established protocol cited, with monocytes being cultivated in Teflon bags for 1 week under optimal conditions (37°C, 5% CO<sub>2</sub>, 95% RH). Differentiated macrophages were detached from the Teflon bags by incubation at 4°C.

### 2.6. Cell culture (THP-1 and Caco-2 cells)

The monocytic/macrophage-like THP-1 cell line was cultivated in Roswell Park Memorial Institute medium containing 10% fetal calf serum (FCS), supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) under standard conditions (37°C, 5% CO<sub>2</sub>, 95% RH). Human epithelial colorectal adenocarcinoma (Caco-2) cells were grown in high glucose Dulbecco's Modified Eagle's Medium containing 10% FCS, supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) under recommended conditions (37°C, 10% CO<sub>2</sub>, 95% RH). For both cell lines, passaging was carried out according to the guidelines of the American Type Culture Collection (ATCC 2012).

### 2.7. Stimulation of cytokine response

The influence of retinoids on the LPS-induced cytokine response of *ivDCs*, *ivMACs* and THP-1 cells was evaluated in each cell type using the same experimental methodology. Primary cells were adjusted to a density of  $1 \times 10^6$  cells/mL and plated onto 96-well plates (100 µL/well). THP-1 cells were incubated in six-well plates at a density of  $7 \times 10^5$  cells/mL. Retinoids were added to the medium (0.01, 0.1, 1.0 and 5.0 µg/mL) for *ivDCs*, *ivMACs* and THP-1 cells, and pre-incubated

for 1 hour prior to stimulation with LPS (to a final concentration of 100 ng/ml) for a further 48 hours at 37°C.

Incubation medium was collected and processed for cytokine analysis (Rules-Based Medicine, Austin, Texas, USA) using Human Cytokine MAP A 1.0<sup>®</sup> array. Levels of IL-15 were below the detection limit of the assay and were excluded from the analysis.

For studies in ivDCs, cytokine response data shown are based on at least six (LPS-induced) and at least four (no LPS) independently performed experiments, each corresponding to a different donor. In ivMACs, these data (both LPS-induced and no LPS) were each based on at least four independently performed experiments, each corresponding to a different donor. Data shown for cytokine response in THP-1 cells are based on three independent experiments.

### *2.8. Intestinal epithelial cell permeability*

To investigate a potential role of retinoid derivatives of vitamin A on intestinal permeability, the diffusion of fluorescently-labelled dextran was measured across Caco-2 monolayers pre-incubated with retinoid. Caco-2 cells were grown onto trans-well inserts of 0.4 µm pore size for 3 weeks to reach maximum confluency. Cells were subsequently pre-incubated with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL) for 48 hours. Caco-2 monolayers were washed once with PBS and fluorescein isothiocyanate (FITC)-labelled 10 kDA dextran (Sigma-Aldrich, St Louis, USA) and added to the apical chambers at a final concentration of 0.2 mg/mL. Ethylenediaminetetraacetic acid (EDTA) 0.1 mM was used in parallel as a positive control. Following overnight incubation, media from the basal chambers were collected and analysed for FITC-dextran leakage using spectrofluorometric analysis

(Biotek, Winooski, USA). Data are provided based on mean values from two independent representative experiments.

### *2.9. Statistical analysis*

Based on a paired analysis of LPS-induced responses, statistical significance was determined using a one-way analysis of variance with Tukey's multi-comparison post-test using Graph Pad Prism 5 software (GraphPad Software, La Jolla, California, USA).

### 3. Results

#### 3.1. Cytokine response

##### 3.1.1. *ivDCs*

In the presence of LPS, ATRA significantly inhibited the LPS-induced release of pro-inflammatory cytokines such as TNF, IL-6, macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$  from *ivDCs* (Fig. 1); data were consistent across all retinoid concentrations tested (0.01, 0.1, 1.0 and 5.0  $\mu\text{g/mL}$ ) and, for clarity, only 1  $\mu\text{g/mL}$  data are shown. Additionally, ATRA and its derivatives significantly stimulated the production of monocyte chemotactic protein (MCP)-1 and vascular endothelial growth factor (VEGF), and also the anti-inflammatory cytokine IL-10 (Fig. 1).

Although incubation of *ivDCs* with retinoids affected the LPS-induced release of several other cytokine targets implicated in the inflammatory response, none of these changes were significant (Supplementary Fig. I).

In the absence of LPS, incubation with ATRA and 13-cis-RA each induced increases in GM-CSF, MCP-1 and VEGF from *ivDCs*, which were significant at the highest doses tested; a similar but non-significant trend being evident for 4-oxo-13-cis-RA (Fig. 2). There was little or no change in the cytokine response for IL-1 $\alpha$ , IL-1 receptor antagonist (IL-1RA), IL-4, and IL-18. Although there was a tendency for the retinoids tested to induce the release of intercellular adhesion molecule-1 (ICAM-1), interferon (IFN)- $\gamma$ , IL-1 $\beta$ , lymphotoxin- $\alpha$ , matrix metalloproteinase (MMP)-2 and stem cell factor, and to also inhibit the release of IL-10, IL-6, MIP-1 $\alpha$ , MIP-1 $\beta$  and TNF, these changes were modest and in all cases not statistically significant (Supplementary Fig. II).

### 3.1.2. *ivMACs*

In the presence of LPS, similarly significant increases were seen in the release of MCP-1, eotaxin-1, and VEGF following incubation of *ivMACs* with each retinoid (Fig. 3, consistent responses were again evident across all retinoid concentrations and, for clarity, only 1  $\mu\text{g/mL}$  data are shown). Additionally, significant reductions were seen in the LPS-induced release of the pro-inflammatory cytokines TNF, IL-6, MIP-1 $\alpha$  and MIP-1 $\beta$  (Fig. 3), i.e. responses consistent with those seen for *ivDCs*. Incubation of *ivMACs* with retinoids also tended to promote increased LPS-induced release of IL-8, IL-10, IL-1 $\beta$  and IL-1RA and reduction in the release of IL-1 $\alpha$ , but these changes were not statistically significant (Supplementary Fig. III). Moreover, no changes were evident in the responses for ICAM-1, IL-18, and MMP-3.

In the absence of LPS, comparable responses to those observed for *ivDCs* were seen in that the retinoids tested induced the release of MCP-1, eotaxin-1, IL-8 and VEGF; for eotaxin-1 and VEGF, responses appeared dose dependent (albeit non-significant) for all retinoids tested (Fig. 4). There was little or no change in the cytokine response for ICAM-1, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6. Although there was a tendency for the retinoids tested to induce the release of IL-10, IL-18 and MIP-1 $\alpha$  as well as inhibit the release of pro-inflammatory IFN- $\gamma$ , IL-1RA, MIP-1 $\beta$ , MMP-3 and TNF, these changes were modest and in all cases not statistically significant (Supplementary Fig. IV).

### 3.1.3. *THP-1 cells*

The effects of retinoids on LPS-induced cytokine response from THP-1 cells were generally similar to those observed for both *ivDCs* and *ivMACs*. Pre-incubation of THP-1 cells with retinoids resulted in reduced LPS-induced release of IL-6 and

increased release of IL-8 and to a minor extent -MCP-1; these responses were evident for each of the retinoids tested (Fig. 5) and generally consistent with responses seen in *ivDCs* and *ivMACs* (Figs. 1–4).

### *3.2. Intestinal permeability*

Incubation of human Caco-2 cells with different concentrations each of ATRA, isotretinoin and 4-oxo-cis-RA resulted in no significant change in permeability of Caco-2 monolayers at all doses tested (Supplementary Fig. V). FITC-labeled dextran was observed to be translocated effectively in EDTA-treated monolayers, a finding consistent with the known potent adverse effect of this compound on tight-junction integrity (Tomita et al., 1994).

## 4. Discussion

Retinoid treatment has recently been suggested to play a pathophysiological role in the development of chronic IBD, a contention based essentially on several case reports (Crockett et al., 2009; Shale et al., 2009). However, key basic research data appear to contradict this in showing retinoids to be mainly associated with anti-inflammatory activity (Bai et al., 2009; Straus and Glass, 2007) and, for example, substitution of vitamin A in a TNBS rat model of colitis was found to ameliorate colitis according to histological scores and weight curves (Bai et al., 2009; Reifen et al., 2002). While the molecular effects of vitamin A and its retinoid derivatives are well understood based on studies in multiple in-vitro settings (Amann et al., 2011; Delacroix et al., 2010; Li et al., 2006; Norris et al., 1987; Wada et al., 2009), the data available from intestinal cell lines and primary human cells are generally limited; a situation that has led to speculation recently as to whether retinoids are harmful or beneficial to the GI tract (Crockett et al., 2010; Reddy et al., 2006), and suggested need for further study (Rogler et al., 2012, unpublished observation, manuscript in preparation).

The findings from this *in vitro* study, designed to evaluate the effect of retinoids on cytokine release and suppression, and GI integrity in various human immune cell types, clearly demonstrate that pre-treatment of *iv*DCs, *iv*MACs and cultured human THP-1 cells with ATRA, or the derivatives tested, promotes an anti-inflammatory pattern of cytokine release with little or no change in epithelial cell line integrity. This specifically relates to significant inhibition of LPS-induced release of pro-inflammatory cytokines such as TNF and IL-6, and also of MIP-1 $\alpha$  and MIP-1 $\beta$ . These observations, and also the fact that all retinoids tested stimulated the release

of the anti-inflammatory cytokine IL-10 from *i*MACs and *i*DCs, collectively confirm that retinoids promote a pattern of cytokine release that is more anti-inflammatory than pro-inflammatory. Such a pattern is, in fact, consistent with recent *in vitro* and *in vivo* studies. For example, retinoids such as ATRA play a crucial role in the differentiation of T-cells by inducing the differentiation of gut-homing FOXP3<sup>+</sup> regulatory T-cells and preventing the differentiation of pro-inflammatory IL-17-secreting Th17 cells (including in human colonic biopsies) (Bai et al., 2009; Iwata and Yokota, 2011); they also promote the homing of Th17 cells and regulatory T cells to the GI mucosa and stimulation of antigen-presenting cells to secrete IL-10 (Benson et al., 2007; Crockett et al., 2009; Hundorfean et al., 2012; Nikoopour et al., 2008). Additionally, ATRA treatment has been shown to reduce inflammation, mucosal damage and myeloperoxidase activity in the mouse TNBS colitis model. In this study, lamina propria mononuclear cells from ATRA-treated animals were reported to produce lower levels of pro-inflammatory TNF, IL-1 $\beta$ , and IL-17 and release more regulatory cytokines (IL-10 and TGF- $\beta$ ) (Bai et al., 2009).

In the absence of LPS, incubation with each of the retinoids tested was similarly associated with little, or no, effect on the release of inflammatory mediators from all cell types; an effect that was observed over a broad range of retinoid concentrations (0.01, 0.1, 1.0 and 5  $\mu$ g/mL). Under these conditions, the retinoids tested induced the release of eotaxin-1, MCP-1 and IL-8, i.e. chemokine targets involved in the migration of immune cells, and also GM-CSF and VEGF, from *i*DCs and *i*MACs. Perhaps most notable is the markedly increased release of GM-CSF, MCP-1 and VEGF in response to retinoids alone. There is now strong evidence, including studies both in GM-CSF knockout mice (Xu et al., 2008) and in human subjects (Goldstein et al., 2011), to support a key role of GM-CSF in activating and



maintaining intestinal innate immune barrier function. Collectively, such findings have fostered the emergence of CSFs as a potential tool for the treatment of IBD (Barahona-Garrido and Yamamoto-Furusho, 2008) and, in fact, recent controlled clinical trials have shown treatment with recombinant human GM-CSF to decrease disease severity and improve the quality of life of patients with active CD (Goldstein et al., 2011; Korzenik et al., 2005). It follows, therefore, that the retinoid-induced release of GM-CSF reported here, as distinct from LPS-induced responses, would provide potential benefit to the GI environment, particularly in pathological states such as IBD.

A similar view could be taken regarding the observed changes in MCP-1. This key target, together with IL-10, is crucial for the regulation of immune responses against commensal bacteria by intestinal macrophages (Takada et al., 2010) and has been shown also to exert a beneficial effect on dextran sodium sulphate (DSS)-induced colitis in mice (Maharshak et al., 2010). Thus, as for GM-CSF, the retinoid-induced release of MCP-1 seen in this study, both in the presence and absence of LPS, may similarly preclude a beneficial effect of this chemokine in steady-state gut homeostasis. In contrast, however, overexpression of VEGF-A has been shown to be associated with deterioration in disease status in mice with DSS-induced colitis, levels correlating with increased angiogenesis and leukocyte adhesion in the intestine (Scaldaferri et al., 2009), while increased levels of VEGF are usually observed in human subjects with IBD (Tsiolakidou et al., 2008). The release of VEGF might, therefore, be expected to convey potentially negative effects on intestinal immunology. To counterbalance this argument, VEGF has also been observed to inhibit the apoptosis of intestinal epithelial cells – thus preventing bacterial translocation across ileal mucosa (Nakajima et al., 2007) – while levels of

VEGF expression are reported as not being associated with disease activity in patients with IBD (Alkim et al. 2012). Nevertheless, until more data become available relating to the effect of VEGF on maintenance of gut homeostasis, it is perhaps prudent that caution is exercised in assessing the overall effect of this cytokine target on the intestinal milieu.

All retinoids tested were also found to have little or no adverse effect on the permeability of Caco-2 monolayers. This was also evident at all doses tested and is in apparent conflict with a relatively early *in vitro* study, which showed that the permeability of the Caco-2 monolayer, as measured by transepithelial electric resistance and [<sup>3</sup>H]-mannitol flux, was enhanced by ATRA. Given the known association between vitamin A deficiency and impairment in intestinal integrity, the authors considered this surprising and attributed increased permeability to an unknown mechanism(s) and not altered tight-junction protein expression (Baltes et al., 2004). The findings with regard to permeability are, however, consistent with reported protective effects of retinoids for the GI mucosa. For example, gastric mucosal protection (against indomethacin treatment) was seen in healthy persons and in patients with gastric ulcer and duodenal ulcer without any inhibition of gastric acid secretion (Mózsik et al., 2001), while increased mucin production in the presence of retinoids was considered to contradict any putative drying effect of retinoid analogues on the intestinal epithelium as a causal contributor of IBD (Gray et al., 2001; Tan et al., 2007).

In summary, these *in vitro* findings confirm that retinoid derivatives of vitamin A provoke an LPS-induced cytokine response from human immune cells consistent with an anti-inflammatory pattern and with little or no adverse effect on intestinal

epithelial permeability. As such, these studies do not support retinoids as presenting a metabolic milieu dangerous to the GI tract. These findings are consistent with studies in *in vivo* animal models of colitis (to be published separately).

## Acknowledgements

This study was supported by F. Hoffmann-La Roche Ltd, Basel, Switzerland and also by research grants from the Swiss National Science Foundation to SRV (Grant No 320000-114009/3 and 32473B\_135694/1), to GR (Grant No. 310030-120312), and by the Swiss IBD Cohort (Grant No. 3347CO-108792) and by the Zurich Center for Integrative Human Physiology (ZIHP) of the University of Zurich.

The funding source had no influence on the study design, collection, analysis and interpretation of data, the writing of the report and in the decision to submit the paper for publication. Study design, collection, analysis and interpretation of data was exclusively performed by the authors.

The authors would like to thank Kirstin Atrott for technical support, and also Dr Harald Kropshofer and Dr Lutz Müller from Roche for helpful discussions and assistance during the course of these studies.

Medical writing support for this paper was provided by Carl V Felton PhD of Prime Healthcare, supported by Roche. Responsibility for opinions, conclusions and interpretation of data lies with the authors.

## References

Alkim, C., Sakiz, D., Alkim, H., Livaoglu, A., Kendir, T., Demirsoy, H., Erdem, L., Akbayir, N., Sokmen, M. Thrombospondin-1 and VEGF in inflammatory bowel disease. *Libyan J Med.* 2012;7:8942.

Amann. P.M., Eichmüller, S.B., Schmidt, J., Bazhin, A.V. Regulation of gene expression by retinoids. *Curr Med Chem.* 2011;18:1405–1412.

ATCC 2012,

[www.atcc.org/LinkClick.aspx?fileticket=iiksZHcllfQ%3D&tabid=1570&mid=5414](http://www.atcc.org/LinkClick.aspx?fileticket=iiksZHcllfQ%3D&tabid=1570&mid=5414)

Accessed 9 October 2012.

Avidan, B., Sakhnini, E., Lahat, A., Lang, A., Koler, M., Zmora, O., Bar-Meir, S., Chowers, Y. Risk factors regarding the need for a second operation in patients with Crohn's disease. *Digestion.* 2005;72:248–253.

Bai, A., Lu, N., Guo, Y., Liu, Z., Chen, J., Peng, Z. All-trans retinoic acid down-regulates inflammatory responses by shifting the Treg/Th17 profile in human ulcerative and murine colitis. *J Leukoc Biol.* 2009;86:959–969.

Baltes, S., Nau, H., Lampen, A. All-trans retinoic acid enhances differentiation and influences permeability of intestinal Caco-2 cells under serum-free conditions. *Dev Growth Differ.* 2004;46:503–514.

Barahona-Garrido, J., Yamamoto-Furusho, J.K. New treatment options in the management of IBD - focus on colony stimulating factors. *Biologics.* 2008;2:501–504.

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D., Brant, S.R., Silverberg, M.S., Taylor, K.D., Barmada, M.M., Bitton, A., Dassopoulos, T., Datta, L.W., Green, T., Griffiths, A.M., Kistner, E.O., Murtha, M.T., Regueiro, M.D., Rotter, J.I., Schumm, L.P., Steinhardt, A.H., Targan, S.R., Xavier, R.J.; NIDDK IBD Genetics Consortium, Libioulle, C., Sandor, C., Lathrop, M., Belaiche, J., Dewit, O., Gut, I., Heath, S., Laukens, D., Mni, M., Rutgeerts, P., Van Gossum, A., Zelenika, D., Franchimont, D., Hugot, J.P., de Vos, M., Vermeire, S., Louis, E.; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon, L.R., Anderson, C.A., Drummond, H., Nimmo, E., Ahmad, T., Prescott, N.J., Onnie, C.M., Fisher, S.A., Marchini, J., Ghorri, J., Bumpstead, S., Gwilliam, R., Tremelling, M., Deloukas, P., Mansfield, J., Jewell, D., Satsangi, J., Mathew, C.G., Parkes, M., Georges, M., Daly, M.J. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008;40:955–962.

Benson, M.J., Pino-Lagos, K., Roseblatt, M., Noelle, R.J. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med.* 2007;204:1765–1774.

Cámara, R.J., Bgré, S., von Känel, R. [The effect of stress-relieving interventions on inflammatory bowel disease: quality assessment of 10 therapeutic studies]. *Z Psychosom Med Psychother.* 2010;56:116–135.

Cosnes, J., Beaugerie, L., Carbonnel, F., Gendre, J.P. Smoking cessation and the course of Crohn's disease: an intervention study. *Gastroenterology.* 2001;120:1093–1099.

Cosnes, J., Carbonnel, F., Beaugier, L., Le Quintrec, Y., Gendre, J.P. Effects of cigarette smoking on the long-term course of Crohn's disease. *Gastroenterology*. 1996;110:424–431.

Cosnes, J. Smoking, physical activity, nutrition and lifestyle: environmental factors and their impact on IBD. *Dig Dis*. 2010;28:411–417.

Crockett, S.D., Gulati, A., Sandler, R.S. A causal association between isotretinoin and inflammatory bowel disease has yet to be established. *Am J Gastroenterol*. 2009;104: 2387–2393.

Crockett, S.D., Porter, C.Q., Martin, C.F., Sandler, R.S., Kappelman, M.D. Isotretinoin use and the risk of inflammatory bowel disease: a case-control study. *Am J Gastroenterol*. 2010;105:1986–1993.

Danese, S., Sans, M., Fiocchi, C. Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev*. 2004;3:394–400.

Delacroix, L., Moutier, E., Altobelli, G., Legras, S., Poch, O., Choukrallah, M.A., Bertin, I., Jost, B., Davidson, I. Cell-specific interaction of retinoic acid receptors with target genes in mouse embryonic fibroblasts and embryonic stem cells. *Mol Cell Biol*. 2010;30:231–244.

Goldstein, J.I., Kominsky, D.J., Jacobson, N., Bowers, B., Regalia, K., Austin, G.L., Yousefi, M., Falta, M.T., Fontenot, A.P., Gerich, M.E., Golden-Mason, L., Colgan, S.P. Defective leukocyte GM-CSF receptor (CD116) expression and function in inflammatory bowel disease. *Gastroenterology*. 2011;141:208–216.

Gray, T., Koo, J.S., Nettesheim, P. Regulation of mucous differentiation and mucin gene expression in the tracheobronchial epithelium. *Toxicology*. 2001;160:35–46.

Hou, J., El-Serag, H., Sellin, J., Thirumurthi, S. Inflammatory bowel disease characteristics and treatment in Hispanics and Caucasians. *Dig Dis Sci*. 2011b;56:1476–1481.

Hou, J.K., Abraham, B., El-Serag, H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Am J Gastroenterol*. 2011a;106:563–573.

Hundorfean, G., Neurath, M.F., Mudter, J. Functional relevance of T helper 17 (Th17) cells and the IL-17 cytokine family in inflammatory bowel disease. *Inflamm Bowel Dis*. 2012;18:180–186.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., Song, S.Y. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004;21:527–538.

Iwata, M., Yokota, A. Retinoic acid production by intestinal dendritic cells. *Vitam Horm*. 2011;86:127–152.

Järnerot, G., Järnmark, I., Nilsson, K. Consumption of refined sugar by patients with Crohn's disease, ulcerative colitis, or irritable bowel syndrome. *Scand J Gastroenterol*. 1983;18:999–1002.

Kane, S.V., Flicker, M., Katz-Nelson, F. Tobacco use is associated with accelerated clinical recurrence of Crohn's disease after surgically induced remission. *J Clin Gastroenterol*. 2005;39:32–35.



Katschinski, B., Logan, R.F., Edmond, M., Langman, M.J. Smoking and sugar intake are separate but interactive risk factors in Crohn's disease. *Gut*. 1988;29:1202–1206.

Korzenik, J.R., Dieckgraefe, B.K., Valentine, J.F., Hausman, D.F., Gilbert, M.J.; Sargramostim in Crohn's Disease Study Group. Sargramostim for active Crohn's disease. *N Engl J Med*. 2005;352:2193–2201.

Latella, G., Fiocchi, C., Caprili, R. News from the "5th International Meeting on Inflammatory Bowel Diseases" CAPRI 2010. *J Crohns Colitis*. 2010;4:690–702.

Levenstein, S. Psychosocial factors in peptic ulcer and inflammatory bowel disease. *J Consult Clin Psychol*. 2002;70:739–750.

Li, G., Yin, W., Chamberlain, R., Hewett-Emmett, D., Roberts, J.N., Yang, X., Lippman, S.M., Clifford, J.L. Identification and characterization of the human retinoid X receptor alpha gene promoter. *Gene*. 2006;372:118–127.

Maharshak, N., Hart, G., Ron, E., Zelman, E., Sagiv, A., Arber, N., Brazowski, E., Margalit, R., Elinav, E., Shachar, I. CCL2 (pM levels) as a therapeutic agent in Inflammatory Bowel Disease models in mice. *Inflamm Bowel Dis*. 2010;16:1496–1504.

Martini, G.A., Brandes, J.W. Increased consumption of refined carbohydrates in patients with Crohn's disease. *Klin Wochenschr*. 1976;54:367–371.

Mathew, C.G. New links to the pathogenesis of Crohn's disease provided by genome-wide association scans. *Nat Rev Genet*. 2008;9:9–14.

Mózsik, G., Bódis, B., Figler, M., Király, A., Karádi, O., Pár, A., Rumi, G., Sütő, G., Tóth, G., Vincze, A. Mechanisms of action of retinoids in gastrointestinal mucosal

protection in animals, human healthy subjects and patients. *Life Sci.* 2001;69:3103–3112.

Nakajima, T., Ueda, T., Takeyama, Y., Yasuda, T., Shinzeki, M., Sawa, H., Kuroda, Y. Protective effects of vascular endothelial growth factor on intestinal epithelial apoptosis and bacterial translocation in experimental severe acute pancreatitis. *Pancreas.* 2007;34:410–416.

Nguyen, G.C., Torres, E.A., Regueiro, M., Bromfield, G., Bitton, A., Stempak, J., Dassopoulos, T., Schumm, P., Gregory, F.J., Griffiths, A.M., Hanauer, S.B., Hanson, J., Harris, M.L., Kane, S.V., Orkwis, H.K., Lahaie, R., Oliva-Hemker, M., Pare, P., Wild, G.E., Rioux, J.D., Yang, H., Duerr, R.H., Cho, J.H., Steinhart, A.H., Brant, S.R., Silverberg, M.S. Inflammatory bowel disease characteristics among African Americans, Hispanics, and non-Hispanic Whites: characterization of a large North American cohort. *Am J Gastroenterol.* 2006;101:1012–1023.

Nikoopour, E., Schwartz, J.A., Singh, B. Therapeutic benefits of regulating inflammation in autoimmunity. *Inflamm Allergy Drug Targets.* 2008;7:203–210.

Norris, D.A., Osborn, R., Robinson, W., Tonnesen, M.G. Isotretinoin produces significant inhibition of monocyte and neutrophil chemotaxis in vivo in patients with cystic acne. *J Invest Dermatol.* 1987;89:38–43.

Osanai, M., Nishikiori, N., Murata, M., Chiba, H., Kojima, T., Sawada, N. Cellular retinoic acid bioavailability determines epithelial integrity: Role of retinoic acid receptor alpha agonists in colitis. *Mol Pharmacol.* 2007;71:250–258.

Reddy, D., Siegel, C.A., Sands, B.E., Kane, S. Possible association between isotretinoin and inflammatory bowel disease. *Am J Gastroenterol.* 2006;101:1569–1573.

Reifen, R., Nur, T., Ghebermeskel, K., Zaiger, G., Urizky, R., Pines, M. Vitamin A deficiency exacerbates inflammation in a rat model of colitis through activation of nuclear factor-kappaB and collagen formation. *J Nutr.* 2002;132:2743–2747.

Rogler, G., Hausmann, M., Vogl, D., Aschenbrenner, E., Andus, T., Falk, W., Andreesen, R., Schölmerich, J., Gross, V. Isolation and phenotypic characterization of colonic macrophages. *Clin Exp Immunol.* 1998;112:205-215.

Rogler, G. Interaction between susceptibility and environment: examples from the digestive tract. *Dig Dis.* 2011;29:136–143.

Scaldaferri, F., Vetrano, S., Sans, M., Arena, V., Straface, G., Stigliano, E., Repici, A., Sturm, A., Malesci, A., Panes, J., Yla-Herttuala, S., Fiocchi, C., Danese, S. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology.* 2009;136:585–95.e5.

Shale, M., Kaplan, G.G., Panaccione, R., Ghosh, S. Isotretinoin and intestinal inflammation: what gastroenterologists need to know. *Gut.* 2009;58:737–741.

Silkoff, K., Hallak, A., Yegena, L., Rozen, P., Mayberry, J.F., Rhodes, J., Newcombe, R.G. Consumption of refined carbohydrate by patients with Crohn's disease in Tel-Aviv-Yafo. *Postgrad Med J.* 1980;56:842–846.

Straus, D.S., Glass, C.K. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends Immunol.* 2007;28:551–558.

Takada, Y., Hisamatsu, T., Kamada, N., Kitazume, M.T., Honda, H., Oshima, Y., Saito, R., Takayama, T., Kobayashi, T., Chinen, H., Mikami, Y., Kanai, T., Okamoto, S., Hibi, T. Monocyte chemoattractant protein-1 contributes to gut homeostasis and intestinal inflammation by composition of IL-10-producing regulatory macrophage subset. *J Immunol.* 2010;184:2671–2676.

Tan, S., Cheng, P.W. Mucin biosynthesis: identification of the cis-regulatory elements of human C2GnT-M gene. *Am J Respir Cell Mol Biol.* 2007;36:737–745.

Thornton, J.R., Emmett, P.M., Heaton, K.W. Diet and Crohn's disease: characteristics of the pre-illness diet. *Br Med J.* 1979;2:762–764.

Tomita, M., Hayashi, M., Awazu, S. Comparison of absorption-enhancing effect between sodium caprate and disodium ethylenediaminetetraacetate in Caco-2 cells. *Biol Pharm Bull.* 1994;17:753–735.

Torkamani, A., Topol, E.J., Schork, N.J. Pathway analysis of seven common diseases assessed by genome-wide association. *Genomics.* 2008;92:265–272.

Tsiolakidou, G., Koutroubakis, I.E., Tzardi, M., Kouroumalis, E.A. Increased expression of VEGF and CD146 in patients with inflammatory bowel disease. *Dig Liver Dis.* 2008;40:673–679.

Wada, Y., Hisamatsu, T., Kamada, N., Okamoto, S., Hibi, T. Retinoic acid contributes to the induction of IL-12-hypoproducing dendritic cells. *Inflamm Bowel Dis.* 2009;15:1548–1556.

Xu, Y., Hunt, N.H., Bao, S. The role of granulocyte macrophage-colony-stimulating factor in acute intestinal inflammation. *Cell Res.* 2008;18:1220–1229.

**Fig. 1.** The influence of retinoids on LPS-induced release of inflammatory targets from *iv*DCs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with retinoids (1  $\mu$ g/mL) and stimulation with LPS (100 ng/mL). Bars represent mean  $\pm$ SEM values from at least six independent experiments corresponding to different donors.

\* $P < 0.05$ ; \*\*\* $P < 0.001$

**Fig. 2.** The influence of retinoids on the release of inflammatory targets from *i*DCs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL).

Bars represent mean +SEM values from at least four independent experiments corresponding to different donors.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

**Fig. 3.** The influence of retinoids on LPS-induced release of inflammatory targets from *iv*MACs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with retinoids (1  $\mu$ g/mL) and stimulation with LPS (100 ng/mL). Bars represent mean  $\pm$ SEM values from at least four independent experiments corresponding to different donors.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

**Fig. 4.** The influence of retinoids on the release of inflammatory targets from *i*MACs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL).

Bars represent mean +SEM values from at least four independent experiments corresponding to different donors.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$



**Fig. 5.** The influence of retinoids on the release of inflammatory targets from THP-1 cells

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL) either in the absence (light bars) or presence (dark bars) of LPS (1 µg/mL). Bars indicate mean values from three independent experiments.