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Abstract

An effect of the vitamin A metabolite all-*trans*-retinoic acid (ATRA) on body weight regulation and adiposity has been described, but little is known about its impact on obesity-associated inflammation. Our objective was to evaluate the overall impact of this metabolite on inflammatory response in human and mouse adipocytes, using high-throughput methods, and to confirm its effects in a mouse model. ATRA (2 μM for 24 h) downregulated the mRNA expression of 17 chemokines in human adipocytes, and limited macrophage migration in a TNFα-conditioned 3T3-L1 adipocyte medium (26.3%, $p < 0.05$). These effects were confirmed in mice (n = 6-9 per group) subjected to oral gavage of ATRA (5 mg/kg of body weight) and subsequently injected intraperitoneally with lipopolysaccharide. In this model, both systemic and adipose levels of inflammatory markers were reduced. The anti-inflammatory effect of ATRA was associated with a reduction in the phosphorylation levels of IκB and p65 (~ 50%, $p < 0.05$), two subunits of the NF-κB pathway, probably mediated by PGC1α, in 3T3-L1 adipocytes. Taken together, these results show a significant overall anti-inflammatory effect of ATRA on proinflammatory cytokine and chemokine production in adipocyte and adipose tissue and suggest that ATRA supplementation may represent a strategy of preventive nutrition to fight against obesity and its complications.

Keywords: Inflammatory response; ATRA; adipocyte; chemokines; macrophage migration, NF-κB

Introduction

The inflammatory response triggered by obesity involves many components of the classical inflammatory response to pathogens, and is thought to play a major role in the onset of insulin resistance [1]. The main source of pro-inflammatory cytokines in obesity is adipose tissue [2]. Various products of adipose tissue have been characterized. Among the soluble factors it produces are the adipokines: these include leptin, adiponectin, tumor necrosis factor-alpha (TNFα), interleukin-6 (IL6), interleukin-1β (IL1β), and chemokines [3]. These cytokines are mainly produced by infiltrating macrophages, although adipocytes play a role [4].

Adipose tissue is also considered as the second most important storage site for retinol after the liver [5, 6]. Other retinoids including several isomers of retinoic acid, of which all-*trans*-retinoic acid (ATRA) and retinaldehyde, have been detected in this tissue [7, 8].

Many studies indicate that vitamin A inadequacy is associated with obesity, and that vitamin A may play an important role in body weight regulation and adiposity in humans (for a review [9]). Many animal and *in vitro* studies have demonstrated the role of vitamin A metabolites in the control of adiposity (for a review [10]). Notably, the action of ATRA has been linked to increased oxidative metabolism and energy expenditure in different tissues including white adipose tissue (WAT) [10, 11], and could be related to the ability of ATRA to impact oxidative phosphorylation (OXPHOS) capacity and mitochondrial content in adipocytes [12].

The *per se* anti-inflammatory effect of ATRA has not yet been thoroughly investigated in adipocytes. A few studies have reported that ATRA can regulate the production of several adipokines linked to inflammatory tone, including leptin, adipsin and resistin. An inhibitory effect of ATRA on leptin expression has been described [13, 14]. ATRA also suppressed the adipose production of resistin [15] *in vivo* and in adipocyte cell models. Similarly, adipsin was downregulated by ATRA in adipocyte cell culture [16]. Our group has shown that ATRA is able to limit the expression of pro-inflammatory markers (IL6 and IL1β) in murine adipocytes pretreated with ATRA and incubated with TNFα [17].

The aim of the present study was to extend current knowledge on the impact of the active form of vitamin A (ATRA) on inflammatory responses in human and mouse adipocytes, using high-throughput methods, and to confirm its effects in the adipose tissue of an inflammatory animal model.

1. Materials and Methods

1.1. Reagents - Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Cergy-Pontoise, France). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Les Mureaux, France). Isobutylmethylxanthine, dexamethasone and insulin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). TRIzol reagent, random primers and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Saint-Aubin, France). SYBR Green reaction buffer was purchased from Eurogentec (Angers, France).

1.2. Cell culture - Macrophage and adipocyte cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. The 3T3-L1 preadipocytes (ATCC, Manassas, VA; passage 10) were seeded in 3.5 cm diameter dishes at a density of 15×10^4 cells/well, and grown in DMEM supplemented with 10% FBS, at 37 °C, as previously described [18]. To induce differentiation, two-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM isobutylmethylxanthine, 0.25 µmol/L dexamethasone and 1 µg/mL insulin in DMEM supplemented with 10% FBS. The cultures were then treated with DMEM supplemented with 10% FBS and 1 µg/ml insulin (complete medium). In most cases, adipocytes were preincubated with all-*trans*-retinoic acid (ATRA) (0.2 and 2 µM; dissolved in absolute ethanol) for 24 h and incubated with TNFα (15 ng/mL) for an additional 24 h. To identify the signaling pathway involved in chemokine regulation, 3T3-L1 cells were treated with a specific inhibitor of NF-κB signaling (BAY 117082, 10 µM) for 1 h, and then stimulated with TNFα (15 ng/mL) for 24 h. To examine ATRA effects on NF-κB signaling, adipocytes were incubated with TNFα (15 ng/ml) for 5 min. All treatments were performed on day 8. Each experiment was reproduced in triplicate, at least 3 independent times. To examine the impact of PGC1α and PGC1β on NF-κB signaling, adipocytes were transfected with pCMX-PGC1α or pCMX-PGC1β for 24 h, and then incubated with TNFα (15 ng/ml) for 5 min.

Raw 264.7 macrophages (ECACC, Salisbury, UK; passage 8) were seeded in 3.5 cm diameter dishes and grown in DMEM supplemented with 10% FBS, 2% HEPES and 1% antibiotics.

The human preadipocytes (five independent cultures) were supplied by Promocell (Heidelberg, Germany) and cultured according to the company's instructions. The mature adipocytes (day 15) were incubated with

ATRA (2 µM, 24 h) followed by a 24 h incubation with TNFα (15 ng/mL). Experiments were performed in triplicate, on 3 independent times.

1.3. Microarray hybridization and data analysis - RNA was extracted from human adipocyte cultures (three independent cultures), and RNA quality control was performed on an Agilent 2100 Bioanalyzer (Massy, France), according to the manufacturer's instructions. RNA was hybridized to the Agilent Whole Human Genome 8×60K microarray (Massy, France). For each independent culture, treated conditions (ATRA + TNFα) were labeled with cyanine 5, and control conditions (TNFα alone) with cyanine 3. In addition, a treated pool and a control pool made from an equal amount of each independent control and treated condition were made up and labeled likewise (treated pool with cyanine 5 and control pool with cyanine 3). The resulting four pairs of samples (three independent pairs of samples plus one pair of pooled samples) were further hybridized. All labeling, hybridization, washing and scanning were performed as described in the manufacturer's protocol and as previously described [17, 19]. The same procedure was applied for 3T3-L1 adipocytes treated only with ATRA.

The arrays were scanned using an Agilent Scanner (Massy, France). The data were extracted using Agilent Feature Extraction v10.5.1.1 software and analyzed with Agilent GeneSpring GX v11.0.2 software (Massy, France). The analyses were performed using GSEA (http://www.broadinstitute.org/gsea) (Gene Set Enrichment Analysis) and DAVID (Database for Annotation, Visualization, and Integrated Discovery) software as previously described [20]. A false discovery rate $q < 0.25$ for normalized enrichment score was considered significant.

1.4. RNA isolation and qPCR - Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA) as previously described [21]. For each condition, the expression was quantified in duplicate, and the ribosomal protein 18S mRNA was used as the endogenous control in the comparative cycle threshold (C_T) method. The sequences of the primers used for qPCR determination of gene expression are displayed in Table S1.

1.5. Chemokine quantification in cell culture supernatants – A Proteome Profiler™ Array Human Chemokine Array Kit, Catalog Number ARY017 (R&D Systems (Lille, France)) was used to quantify 31 chemokines in human adipocyte cell culture supernatants according to the manufacturer's instructions. Pixel density was quantified with Image J software. A Luminex screening assay (R&D Systems (Lille, France)) was also used to quantify CXCL10 with the Luminex 200 platform.

1.6. Chemokine/cytokine quantification in plasma and epididymal adipose tissue samples – Ccl2 and Ccl5 were quantified with mouse Ccl2 and Ccl5 DuoSet ELISA from R&D Systems. Tnfα and Il6 were quantified with Ready-SET-Go! ELISA from eBioscience.

1.7. Macrophage migration assay - Migration assays were performed using cell culture inserts of 3 µm membrane pore size (Transwell Millipore, Molsheim, France). The 3T3-L1 adipocytes were preincubated with or without various concentrations of ATRA (0.2 and 2 µM) for 24 h. The adipocytes were then incubated with TNFα (15 ng/mL) for 24 h. The 3T3-L1 conditioned media were transferred to plates containing inserts. RAW 264.7 macrophages (ECACC, Salisbury, UK) were seeded on these inserts at a density of 900 cells/cm^2. After migration for 4 h at 37 °C, macrophages in the lower compartment were fixed with 2.5% glutaraldehyde for 15 min, and counted as previously described [22-24].

1.8. NF-κB activation – To examine ATRA effects on NF-κB signaling, adipocytes were preincubated with ATRA (0.2 and 2 µM) for 24 h and incubated with TNFα (15 ng/mL) for an additional 5 min. Phosphorylation levels of p65 (Ser536) and IκBα (Ser32/36) were quantified with an ELISA Instant One kit according to the manufacturer's instructions (eBiosciences SAS, Paris, France).

1.9. Transfection experiments – For RNA interference experiments, 3T3-L1 differentiated cells were transfected with siRNA targeted against Ppargc1α or Ppargc1β (encoding peroxisome proliferator-activated receptor gamma coactivator (PGC)1α or PGC1β, respectively) or a nontargeting siRNA according to the manufacturer's instructions (Polyplus). Briefly, the cells were transfected overnight using a mixture of 10

μM siRNA and 2.8 μL of interferin reagent per well. The media were then replaced with complete medium. Adipocytes were then treated with ATRA for 24 h, and incubated for a further 24 h.

For overexpression experiments, 3T3-L1 adipocytes were transfected using Lipofectamine LTX Plus Reagent (Life Technologies) with empty or PGC1α-containing pCMX plasmid (pCMX-PGC1α; Addgene plasmid # 6 [25]). After 24 h incubation with transfection mixes (1 μg of plasmid, 0.9 μL of Lipofectamine LTX and 1 μL of Plus Reagent per well, according to the manufacturer's instructions), the medium was replaced, and cells were incubated with TNFα for 24 h. RNA was isolated from treated cells, and residual plasmidic DNA contamination was removed by DNase I digestion (Sigma-Aldrich (Saint-Quentin-Fallavier, France)). PGC1α expression was quantified by qPCR.

1.10. Animal experiment – The protocol was approved by the local ethics committee. Six-week-old male C57BL/6J mice were obtained from Janvier (Le Genest-Saint-Isle, France). Mice were fed *ad libitum* (chow diet A04, Safe, Augy, France), with full access to drinking water. The animals were maintained at 22 °C under a 12 h light – 12 h dark cycle at 50% humidity.

To assess the impact of vitamin A on acute inflammation, the mice received by gavage (n = 6–9 per group) the active form of vitamin A (ATRA) (5 mg/kg of body weight; Sigma-Aldrich, Saint-Quentin-Fallavier, France) or vehicle alone (olive oil), once a day for 4 days. On the fifth day, the mice were injected intraperitoneally with saline or *Escherichia coli* lipopolysaccharide (LPS) (4 mg/kg; serotype O111:B4, Sigma-Aldrich). The mice were sacrificed 4 h after LPS injection, and epididymal adipose tissue was dissected and stored at −80 °C.

1.11. Statistical analysis - The data are expressed as means ± SEM. Significant differences between the control and treated group were determined using the ANOVA analysis followed by the Tukey-Kramer *post hoc* test using Statview software: $p < 0.05$ was considered statistically significant.

2. Results

2.1. ATRA modulates the transcriptome of human adipocytes in inflammatory conditions.

To study the impact of ATRA on chemokines under inflammatory conditions, these cells were preincubated with ATRA, or untreated, and then incubated with TNFα. The transcriptomic approach was applied in cells treated with TNFα compared with cells pretreated with ATRA before incubation with TNFα. The impact of these treatments on the transcriptome was evaluated with a fold change filter of 1.3. Examination of the gene list by DAVID (Database for Annotation, Visualization, and Integrated Discovery) software revealed that the "chemokine family" was strongly impacted by the ATRA / TNFα treatment compared with TNFα alone (P-Value = 0.018) (Table S1). These data were confirmed by other software, GSEA (Gene Set Enrichment Analysis), with which analysis according to gene ontology terms highlighted inflammation related processes such as "G protein coupled receptor binding", "defense response", "locomotory behavior", "cytokine activity" and "chemokine activity". The chemokine activity was impacted by the ATRA / TNFα treatment compared with TNFα alone (NES = −1.757, $p < 0.05$ and FDR $q < 0.25$; the ten most frequently represented gene sets are given in Table S2). It is noteworthy that all these gene sets presented a negative enrichment score, meaning that chemokines were downregulated by ATRA pretreatment in comparison with TNFα (Table S2). As seen in Table S3, 17 chemokines identified by GSEA software were regulated by ATRA pretreatment in human adipocytes. Interestingly, we also observed that five chemokine receptors were downregulated by ATRA pretreatment (Table S4). To confirm our observations, we evaluated the gene expression profiles of six chemokines (randomly chosen, CCL5, CCL19, CX3CL1, CXCL1, CXCL5 and CXCL10) in human adipocytes incubated with ATRA (2 μM) for 24 h followed or not by incubation with TNFα (15 ng/mL) for a further 24 h. A significant increase in expression was observed in all the genes investigated: CCL5, CCL19, CXCL1, CX3CL1, CXCL5 and CXCL10 were increased 1611%, 2194%, 1865%, 993%, 3221% and 493%, respectively, in TNFα condition *vs*. control (Figure 1A). Interestingly, the incubation with ATRA reduced the mRNA expression levels of these chemokines 57.7%, 92.6%, 816.5%, 67.8%, 55.5% and 54.4%, respectively, compared withTNFα alone.

Very similar results were obtained when murine 3T3-L1 differentiated adipocytes were treated: the mRNA expression levels of Ccl5, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were significantly increased by TNFα 34163%, 3489%, 729%, 211%, and 1221%, respectively. By contrast, ATRA incubation significantly reduced chemokine expression in comparison with the TNFα condition 49%, 92.3%, 60.6%, 80.1% and 20%, respectively (Figure 1B).

The preventive effect of ATRA on the TNFα-mediated proinflammatory cytokine expression was also evaluated in the human adipocyte culture supernatants. An increase in protein secretion was obtained for CXCL1, CXCL10 and midkine under TNFα treatment (168%, 173% and 118%, respectively). Their levels were significantly decreased in adipocytes pretreated with ATRA followed by TNFα incubation compared with TNFα alone (18%, 68% and 23.7%, respectively) (Figure 1C).

2.2. ATRA supplementation limits cytokine/chemokine expression induced by LPS in mice adipose tissue.

The effects of ATRA on cytokine/chemokine expression were studied in a model of acute inflammation, consisting of an intraperitoneal injection of LPS for 4 h in mice. As shown in Figure 2A, a significant increase in the mRNA expression levels of Il6 (8667%), Tnfα (1180%) and Il1β (360%) was observed in epididymal fat pads under the LPS effect, whereas a 4-day ATRA supplementation prior to LPS stimulation significantly reduced inflammation in white adipose tissue 37.4%, 38.5% and 43.9%, respectively (Figure 2A). Chemokine mRNA including Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were also quantified. Their expression was significantly increased by LPS (4412%, 7097%, 547%, 956%, 3150%, 320%, 31944%, respectively) and reduced by 4 days of ATRA supplementation (55.3%, 56.3%, 54%, 66%, 36.2%, 75.7% and 66.6%, respectively) (Figure 2A).

We confirmed the preventive effect of ATRA on the LPS-mediated inflammation in the plasma mice. An increase in protein secretion was obtained for Il6, TNFα and Ccl5 after LPS intraperitoneal injection in mice (288.4%, 404% and 5597%, respectively) (Figure 2B). Their levels were significantly decreased in the plasma of mice that received ATRA by gavage followed by LPS injection compared with LPS alone (46.2%, 14% and 13.5%, respectively) (Figure 2B). We obtained similar results in the epididymal adipose tissue of these mice for Ccl2 and Ccl5, which increased in LPS-injected mice (275% and 30%, respectively)

(Figure 2C). Interestingly, their levels were significantly decreased in epididymal adipose tissue of mice that received ATRA by gavage followed by LPS injection compared with LPS alone (24.2% and 15.8%, respectively) (Figure 2C).

2.3. ATRA limits RAW 264.7 macrophage migration in 3T3-L1 conditioned medium.

To examine the potential of ATRA to limit the migration of macrophages, 3T3-L1 cells were preincubated with ATRA (0.2 and 2 μM) prior to TNFα incubation, and compared with cells incubated with TNFα alone. The resulting 3T3-L1 conditioned medium was used to study the RAW 264.7 macrophage migration. As expected, 3T3-L1 conditioned medium under TNFα pro-inflammatory stimulus induced a significant migration of macrophages (approximately 100%, Figure 3). Interestingly, the preincubation of 3T3-L1 with 2 μM of ATRA significantly reduced the migration of macrophages 26.3% (Figure 3).

2.4. ATRA limits NF-κB activation in 3T3-L1 adipocytes.

As previously demonstrated, the expression of several chemokines is upregulated in inflammatory conditions, mainly via the NF-κB signaling pathway in human adipocytes [4], and in 3T3-L1 adipocytes [23]. Since NF-κB activation is central in the induction of chemokines, we next examined whether the effect of ATRA on chemokine production could be mediated through limitation of NF-κB signaling in murine adipocytes. Cells were pretreated with ATRA for 24 h followed by a 5 min incubation with TNFα. As expected, phosphorylation levels of p65 and IκB, two subunits of the NF-κB pathway, were significantly increased in the inflammatory condition induced by TNFα (414% and 360%, respectively). On the other hand, preincubation of cells with ATRA strongly limited the phosphorylation of p65 and IκB, suggesting that ATRA was able to reduce NF-κB activation in 3T3-L1 adipocytes (Figure 4).

2.5. ATRA modulates NF-κB signaling and cytokine/chemokine expression through PGC1α in 3T3-L1 adipocytes.

To identify the molecular mechanisms involved in ATRA-mediated NF-κB signaling deactivation, we hypothesized the involvement of PGC1α and/or β, since we had recently shown that ATRA induced the

283 expression of PGC1α and PGC1β in 3T3-L1 adipocytes [12], and these transcription factors are known to
284 reduce NF-κB activity in muscle cells [26].
285 The involvement of PGC1α and/or β on cytokine/chemokine modulation by ATRA was investigated in
286 3T3-L1 adipocytes transfected with siRNA designed against PGC1α or PGC1β. The cells were then
287 preincubated with ATRA for 24 h followed by an incubation with TNFα for 24 h (Figure 5A). As expected,
288 TNFα treatment significantly increased the mRNA expression levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and
289 Cxcl10 (521%, 424%, 4420%, 3791%, 232% and 2787%, respectively), whereas ATRA incubation
290 significantly decreased them (48.4%, 35%, 17%, 41.8%, 26.8% and 43%, respectively) compared with the
291 TNFα condition (Figure 5A). Interestingly, in the 3T3-L1 adipocytes transfected with PGC1α siRNA, there
292 was a modulation in the expression of these inflammatory markers (Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10,
293 211.9%, 160%, 154%, 189%, 160% and 196%, respectively compared with the ATRA+TNFα condition).
294 However, in the 3T3-L1 adipocytes transfected with PGC1β siRNA, no difference in Tnfα, Ccl2, Ccl5 and
295 Cxcl10 expression was observed compared with the ATRA+TNFα condition, whereas the Il6 and Ccl11
296 decrease mediated by ATRA was slightly blunted (Figure 5A). These results suggest that the
297 cytokine/chemokine downregulation mediated by ATRA in inflamed adipocytes is mainly PGC1α-
298 dependent.
299 To further investigate the involvement of PGC1α in these regulations, we studied the effect of PGC1α
300 overexpression on TNFα-mediated cytokine/chemokine expression (Figure 5B). PGC1α overexpression
301 induced a significant decrease in Tnfα, Ccl2, Ccl11 and Cxcl10 expression (46.3%, 14.6%, 33.3% and
302 13.3% compared with pCMX in the TNFα condition). However, in the case of Ccl5 and Il6 expression, no
303 effect of PGC1α was observed. These data suggest that the upregulation of some cytokine/chemokine
304 expression mediated by TNFα can be partly blunted by PGC1α.
305 To confirm the effect of PGC1α on the NF-κB signaling pathway, cells transfected with pCMX-PGC1α
306 were incubated with or without TNFα for 5 min, and IκB phosphorylation was evaluated. As expected,
307 TNFα treatment significantly increased the IκB phosphorylation by 30% (Figure 6). Interestingly, PGC1α
308 overexpression in adipocytes significantly decreased IκB phosphorylation by 18.4% compared with TNFα-
309 treated adipocytes, suggesting that PGC1α overexpression is able to deactivate NF-κB signaling in
310 adipocytes (Figure 6).

3. Discussion

Here we studied the putative role of ATRA on inflammatory marker expression in adipocytes and in adipose tissue, together with the molecular mechanisms involved

To evaluate the impact of ATRA on the adipocyte inflammatory response, we used both human and murine mature adipocyte cultures subjected to TNFα incubation (to induce a low-grade inflammation). Analyses were conducted using high-throughput methods (microarrays and proteome profiler) combined with bioinformatic analysis of gene set enrichment. This approach demonstrated the ability of ATRA treatment to downregulate the expression of TNFα-mediated chemokine expression (at mRNA level and at protein level in some cases). Numerous genes coding for chemokines were impacted, among which Ccl2 [27], Ccl5 [28, 29], C-x-c motif chemokine ligand 5 (Cxcl5 [30]), Cxcl12 [31], Cxcl14 [32], and Ccl20, which are known to be involved in leukocyte recruitment in adipose tissue [33]. Several chemokine receptors were also downregulated, which may add to the physiological consequences of ATRA-mediated chemokine downregulation. The impact of these regulations was also evaluated in terms of leukocyte chemotaxis [34], since chemokines mediate this process, and we found that ATRA limited macrophage migration induced by adipocyte-conditioned medium, which gives our results a functional validation.

To confirm the physiological relevance of these regulations, mice underwent oral gavage of ATRA for 4 days and were injected with LPS to induce inflammation, both at the systemic level and in adipose tissue. In these conditions, not only were adipose tissue mRNA levels of inflammatory markers reduced, but so also was adipose tissue protein expression (Ccl2 and Ccl5). It is also of note that this improvement in adipose tissue inflammatory status was associated with a reduced systemic inflammatory tone, as shown by the reduction of several inflammatory markers, such as Il6, Tnfα and Ccl5. Surprisingly, our results diverge from the recent finding of a moderate increase in proinflammatory markers (Il18 and MIP-1γ) in male mice subjected to a moderate vitamin A supplementation [35]. This discrepancy may derive from the major differences between the two experimental protocols (use of vitamin A or ATRA, duration of treatment,

concentrations of substances used, etc.). However, our results seem to be in line with the suspected role of vitamin A deficiency in elevated proinflammatory cytokines and T-cell activation [36, 37].

From a molecular point of view, it is well-established that NF-κB controls the transcription of proinflammatory cytokines and chemokines in many cell types, including preadipocytes and adipocytes [1, 4, 38]. Thus we evaluated the ability of ATRA to blunt NF-κB-mediated chemokine expression. First we demonstrated that ATRA displayed a strong inhibitory effect on NF-κB signaling in 3T3-L1 adipocytes, characterized by reduced phosphorylation levels of IκB and p65 (two main intermediates in the NF-κB signaling pathway).

Similar inhibition of NF-κB signaling has already been reported in various cell models [39], and in transgenic NF-κB reporter mice [40]. To pursue the identification of the precise molecular mechanisms, we hypothesized the involvement of PGC1α and/or PGC1β. Peroxisome proliferator-activated receptor γ coactivator 1 α and β are members of the PGC1 family of transcriptional coactivators [41]. PGC1α is mainly involved in adaptive thermogenesis, glucose disposal and mitochondrial biogenesis [42], whereas PGC1β regulates saturated fatty acid-induced hepatic triglyceride synthesis and hyperlipidemia [43], and mitochondrial fatty acid β oxidation [44]. In addition, these two transcription factors are able to reduce phosphorylation of the NF-κB family member p65 and thereby its transcriptional activation potential in muscle cells [26], and we recently showed that ATRA upregulated the two transcription factors in 3T3-L1 adipocytes [12]. To confirm the involvement of PGC1α and/or β in cytokine/chemokine downregulation, siRNA designed against PGC1α and PGC1β was used. Results clearly show that inflammatory marker limitation through ATRA was primarily mediated by PGC1α, the ATRA downregulation of chemokines being abolished in the presence of siRNA directed against PGC1α. Furthermore, the ability of PGC1α to repress the TNFα-mediated upregulation of chemokine mRNA levels together with NF-κB signaling, and notably IκB phosphorylation, was confirmed in overexpression experiments. Taken together, these findings strongly support the involvement of the ATRA-mediated upregulation of PGC1α in the inhibition of NF-κB signaling and subsequent limitation of chemokine expression. However, other putative mechanisms cannot be excluded, notably the ATRA-mediated upregulation of specific phosphatases leading to NF-κB signaling deactivation, similar to the anti-inflammatory effect of the $1,25(OH)_2D$, which is mediated in

particular via an induction of Dusp10 [24], a phosphatase involved in stress-activated kinase dephosphorylation.

Data related to the impact of retinoic acid on inflammation in humans are particularly scant, but it is noteworthy that in a recent prospective study, an inverse association between retinoic acid (RA) with several inflammatory and oxidative stress biomarkers such as 8-iso-prostaglandin F2α, C Reactive Protein and IL6 was reported in a Chinese metabolic syndrome population [45]. Even if our results seem to be encouraging, and in agreement with a clinical study, our data present several limitations due to the supraphysiological doses of ATRA used both in vitro and in vivo, and to the fact that extrapolation from mice to human is sometimes hazardous.

In conclusion, the results of the present study show that ATRA reduces chemokine expression in adipocytes and macrophage migration *in vitro*. We confirmed these results in mice adipose tissue. These effects may be associated with the ability of ATRA to limit the activation of the NF-κB signaling pathway, probably through PGC1α.

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Legends for figures

Figure 1. ATRA decreases chemokine expression levels in adipocytes. Cells were preincubated with ATRA (2 μM) for 24 h and incubated with TNFα (15 ng/mL) for 24 h. **A.** The mRNA levels of CCL5, CCL19, CXCL1, CX3CL1, CXCL5 and CXCL10 were quantified through qPCR in human adipocytes. The values are presented as means ± SEM. Experiments were performed in triplicate, on 3 independent cultures. **B.** The mRNA levels of Ccl5, Ccl19, Cxcl1, Cx3xl1 and Cxcl10 were quantified through qPCR in 3T3-L1 adipocytes. The values are presented as means ± SEM. Experiments were performed in triplicate, on 3 independent cultures. **C.** CXCL10, CXCL1 and Midkine secretion in cell culture supernatants of human adipocytes was quantified by Luminex technology and Proteome Profiler Array. n.d.: not detected. Bars not sharing the same letters are significantly different, $p < 0.05$.

Figure 2. ATRA decreases chemokine expression levels in LPS-mediated inflammation in mice epididymal adipose tissue. A. In an acute inflammation model (LPS injection for 4 h), the mRNA levels of Il6, Tnfα, Il1β, Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were quantified through qPCR in epididymal adipose tissue of mice (n = 6–9 per group), and expressed relative to 18S ribosomal RNA. The data are expressed as relative expression ratios. The values are presented as means ± SEM. Bars not sharing the same letters are significantly different, $p < 0.05$. **B.** In the LPS inflammation model, protein amounts of Il6, Tnfα and Ccl5 secreted were quantified by ELISA. The data are expressed as relative expression ratios. The values are presented as means ± SEM. Bars not sharing the same letters are significantly different, $p < 0.05$. **C.** In the LPS inflammation model, protein amounts of Ccl2 and Ccl5 in epididymal adipose tissue of mice were quantified by ELISA. The data are expressed as relative expression ratios. The values are presented as means ± SEM. Bars not sharing the same letters are significantly different, $p < 0.05$.

Figure 3. ATRA decreases macrophage migration in 3T3-L1 adipocytes. 3T3-L1 cells were preincubated with ATRA (0.2 and 2 μM) for 24 h, and incubated with TNFα (15 ng/mL) for 24 h. RAW 264.7 macrophage migration in 3T3-L1 conditioned media (4 h, 37 °C) was then performed. Data are expressed as relative expression ratio. Values are presented as means ± SEM. Bars not sharing the same

letter are significantly different, $p < 0.05$. Experiments were performed in triplicate, on 3 independent cultures.

Figure 4. ATRA limits NF-κB activation. Cells were preincubated with ATRA (1 and 2 μM) for 24 h dose-dependently, and incubated with TNFα (15 ng/mL) for 5 min. Phosphorylation levels of the NF-κB subunits (p65 and IκB) were evaluated by ELISA. Data are expressed as relative expression ratio. Values are presented as means ± SEM. Bars not sharing the same letter are significantly different, $p < 0.05$. Experiments were performed in triplicate, on 3 independent cultures.

Figure 5. ATRA modulates NF-κB signaling and cytokine/chemokine expression through PGC1α in 3T3-L1 adipocytes. A. Cells were transfected with siRNA designed against PGC1α or PGC1β or with nontargeted siRNA. The cells were then preincubated with ATRA for 24 h followed by an incubation with TNFα for 24 h. The mRNA levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through qPCR. The values are presented as means ± SEM. **B.** Cells were transfected with pCMX plasmids, empty or containing PGC1α (pCMX-PGC1α). After 24h incubation, cells were incubated with TNFα for 24 h. The mRNA levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through qPCR. Values are presented as means ± SEM. Bars not sharing the same letter are significantly different, $p < 0.05$. Each experiment was reproduced in triplicate, at least 3 independent times.

Figure 6. PGC1α limits IκB phosphorylation in 3T3-L1 adipocytes. Cells were transfected with pCMX plasmids, empty or containing PGC1α (pCMX-PGC1α). After 24 h incubation, cells were incubated with TNFα for 5 min. Phosphorylation levels of IκB were evaluated by ELISA. Data are expressed as relative expression ratio. Values are presented as means ± SEM. Bars not sharing the same letter are significantly different, $p < 0.05$. Experiments were performed in triplicate, on 3 independent cultures.

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