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1 **All-*trans*-retinoic acid represses chemokine expression in adipocytes and adipose tissue by inhibiting**  
2 **NF- $\kappa$ B signaling**

3

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10 **Running title:** All-*trans*-retinoic acid limits chemokine expression

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17 **Conflict of interest statement:** The authors have nothing to disclose.

18 **Abstract**

19

20 An effect of the vitamin A metabolite all-*trans*-retinoic acid (ATRA) on body weight regulation and  
21 adiposity has been described, but little is known about its impact on obesity-associated inflammation. Our  
22 objective was to evaluate the overall impact of this metabolite on inflammatory response in human and  
23 mouse adipocytes, using high-throughput methods, and to confirm its effects in a mouse model. ATRA (2  
24  $\mu$ M for 24 h) downregulated the mRNA expression of 17 chemokines in human adipocytes, and limited  
25 macrophage migration in a TNF $\alpha$ -conditioned 3T3-L1 adipocyte medium (26.3%,  $p < 0.05$ ). These effects  
26 were confirmed in mice ( $n = 6-9$  per group) subjected to oral gavage of ATRA (5 mg/kg of body weight)  
27 and subsequently injected intraperitoneally with lipopolysaccharide. In this model, both systemic and  
28 adipose levels of inflammatory markers were reduced. The anti-inflammatory effect of ATRA was  
29 associated with a reduction in the phosphorylation levels of I $\kappa$ B and p65 (~ 50%,  $p < 0.05$ ), two subunits  
30 of the NF- $\kappa$ B pathway, probably mediated by PGC1 $\alpha$ , in 3T3-L1 adipocytes. Taken together, these results  
31 show a significant overall anti-inflammatory effect of ATRA on proinflammatory cytokine and chemokine  
32 production in adipocyte and adipose tissue and suggest that ATRA supplementation may represent a  
33 strategy of preventive nutrition to fight against obesity and its complications.

34

35 **Keywords:** Inflammatory response; ATRA; adipocyte; chemokines; macrophage migration, NF- $\kappa$ B

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37

## 38 **Introduction**

39

40 The inflammatory response triggered by obesity involves many components of the classical inflammatory  
41 response to pathogens, and is thought to play a major role in the onset of insulin resistance [1]. The main  
42 source of pro-inflammatory cytokines in obesity is adipose tissue [2]. Various products of adipose tissue  
43 have been characterized. Among the soluble factors it produces are the adipokines: these include leptin,  
44 adiponectin, tumor necrosis factor-alpha (TNF $\alpha$ ), interleukin-6 (IL6), interleukin-1 $\beta$  (IL1 $\beta$ ), and  
45 chemokines [3]. These cytokines are mainly produced by infiltrating macrophages, although adipocytes  
46 play a role [4].

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

50 Many studies indicate that vitamin A inadequacy is associated with obesity, and that vitamin A may play  
51 an important role in body weight regulation and adiposity in humans (for a review [9]). Many animal and  
52 *in vitro* studies have demonstrated the role of vitamin A metabolites in the control of adiposity (for a review  
53 [10]). Notably, the action of ATRA has been linked to increased oxidative metabolism and energy  
54 expenditure in different tissues including white adipose tissue (WAT) [10, 11], and could be related to the  
55 ability of ATRA to impact oxidative phosphorylation (OXPHOS) capacity and mitochondrial content in  
56 adipocytes [12].

57 The *per se* anti-inflammatory effect of ATRA has not yet been thoroughly investigated in adipocytes. A  
58 few studies have reported that ATRA can regulate the production of several adipokines linked to  
59 inflammatory tone, including leptin, adiponectin and resistin. An inhibitory effect of ATRA on leptin  
60 expression has been described [13, 14]. ATRA also suppressed the adipose production of resistin [15] *in*  
61 *vivo* and in adipocyte cell models. Similarly, adiponectin was downregulated by ATRA in adipocyte cell culture  
62 [16]. Our group has shown that ATRA is able to limit the expression of pro-inflammatory markers (IL6 and  
63 IL1 $\beta$ ) in murine adipocytes pretreated with ATRA and incubated with TNF $\alpha$  [17].

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

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92 **1. Materials and Methods**

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94 **1.1. Reagents** - Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies  
95 (Cergy-Pontoise, France). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Les Mureaux,  
96 France). Isobutylmethylxanthine, dexamethasone and insulin were purchased from Sigma-Aldrich (Saint-  
97 Quentin-Fallavier, France). TRIzol reagent, random primers and Moloney murine leukemia virus reverse  
98 transcriptase were obtained from Life Technologies (Saint-Aubin, France). SYBR Green reaction buffer  
99 was purchased from Eurogentec (Angers, France).

100

101 **1.2. Cell culture** - Macrophage and adipocyte cells were grown at 37 °C in a 5% CO<sub>2</sub> humidified  
102 atmosphere. The 3T3-L1 preadipocytes (ATCC, Manassas, VA; passage 10) were seeded in 3.5 cm  
103 diameter dishes at a density of  $15 \times 10^4$  cells/well, and grown in DMEM supplemented with 10% FBS, at  
104 37 °C, as previously described [18]. To induce differentiation, two-day postconfluent 3T3-L1 preadipocytes  
105 (day 0) were stimulated for 72 h with 0.5 mM isobutylmethylxanthine, 0.25 µmol/L dexamethasone and 1  
106 µg/mL insulin in DMEM supplemented with 10% FBS. The cultures were then treated with DMEM  
107 supplemented with 10% FBS and 1 µg/ml insulin (complete medium). In most cases, adipocytes were  
108 preincubated with all-*trans*-retinoic acid (ATRA) (0.2 and 2 µM; dissolved in absolute ethanol) for 24 h  
109 and incubated with TNFα (15 ng/mL) for an additional 24 h. To identify the signaling pathway involved in  
110 chemokine regulation, 3T3-L1 cells were treated with a specific inhibitor of NF-κB signaling (BAY  
111 117082, 10 µM) for 1 h, and then stimulated with TNFα (15 ng/mL) for 24 h. To examine ATRA effects  
112 on NF-κB signaling, adipocytes were incubated with TNFα (15 ng/ml) for 5 min. All treatments were  
113 performed on day 8. Each experiment was reproduced in triplicate, at least 3 independent times. To examine  
114 the impact of PGC1α and PGC1β on NF-κB signaling, adipocytes were transfected with pCMX-PGC1α or  
115 pCMX-PGC1β for 24 h, and then incubated with TNFα (15 ng/ml) for 5 min.

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

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

120 ATRA (2  $\mu$ M, 24 h) followed by a 24 h incubation with TNF $\alpha$  (15 ng/mL). Experiments were performed  
121 in triplicate, on 3 independent times.

122

123 **1.3. Microarray hybridization and data analysis** - RNA was extracted from human adipocyte cultures  
124 (three independent cultures), and RNA quality control was performed on an Agilent 2100 Bioanalyzer  
125 (Massy, France), according to the manufacturer's instructions. RNA was hybridized to the Agilent Whole  
126 Human Genome 8 $\times$ 60K microarray (Massy, France). For each independent culture, treated conditions  
127 (ATRA + TNF $\alpha$ ) were labeled with cyanine 5, and control conditions (TNF $\alpha$  alone) with cyanine 3. In  
128 addition, a treated pool and a control pool made from an equal amount of each independent control and  
129 treated condition were made up and labeled likewise (treated pool with cyanine 5 and control pool with  
130 cyanine 3). The resulting four pairs of samples (three independent pairs of samples plus one pair of pooled  
131 samples) were further hybridized. All labeling, hybridization, washing and scanning were performed as  
132 described in the manufacturer's protocol and as previously described [17, 19]. The same procedure was  
133 applied for 3T3-L1 adipocytes treated only with ATRA.

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

140

141 **1.4. RNA isolation and qPCR** - Total cellular RNA was extracted using TRIzol reagent according to the  
142 manufacturer's instructions. cDNA was synthesized from 1  $\mu$ g of total RNA using random primers and  
143 Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses were  
144 performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA) as previously described  
145 [21]. For each condition, the expression was quantified in duplicate, and the ribosomal protein 18S mRNA  
146 was used as the endogenous control in the comparative cycle threshold ( $C_T$ ) method. The sequences of the  
147 primers used for qPCR determination of gene expression are displayed in Table S1.

148

149 **1.5. Chemokine quantification in cell culture supernatants** – A Proteome Profiler™ Array Human  
150 Chemokine Array Kit, Catalog Number ARY017 (R&D Systems (Lille, France)) was used to quantify 31  
151 chemokines in human adipocyte cell culture supernatants according to the manufacturer's instructions.  
152 Pixel density was quantified with Image J software. A Luminex screening assay (R&D Systems (Lille,  
153 France)) was also used to quantify CXCL10 with the Luminex 200 platform.

154

155 **1.6. Chemokine/cytokine quantification in plasma and epididymal adipose tissue samples** – Ccl2 and  
156 Ccl5 were quantified with mouse Ccl2 and Ccl5 DuoSet ELISA from R&D Systems. Tnf $\alpha$  and Il6 were  
157 quantified with Ready-SET-Go! ELISA from eBioscience.

158

159 **1.7. Macrophage migration assay** - Migration assays were performed using cell culture inserts of 3  $\mu$ m  
160 membrane pore size (Transwell Millipore, Molsheim, France). The 3T3-L1 adipocytes were preincubated  
161 with or without various concentrations of ATRA (0.2 and 2  $\mu$ M) for 24 h. The adipocytes were then  
162 incubated with TNF $\alpha$  (15 ng/mL) for 24 h. The 3T3-L1 conditioned media were transferred to plates  
163 containing inserts. RAW 264.7 macrophages (ECACC, Salisbury, UK) were seeded on these inserts at a  
164 density of 900 cells/cm<sup>2</sup>. After migration for 4 h at 37 °C, macrophages in the lower compartment were  
165 fixed with 2.5% glutaraldehyde for 15 min, and counted as previously described [22-24].

166

167 **1.8. NF- $\kappa$ B activation** – To examine ATRA effects on NF- $\kappa$ B signaling, adipocytes were preincubated  
168 with ATRA (0.2 and 2  $\mu$ M) for 24 h and incubated with TNF $\alpha$  (15 ng/mL) for an additional 5 min.  
169 Phosphorylation levels of p65 (Ser536) and I $\kappa$ B $\alpha$  (Ser32/36) were quantified with an ELISA Instant One  
170 kit according to the manufacturer's instructions (eBiosciences SAS, Paris, France).

171

172 **1.9. Transfection experiments** – For RNA interference experiments, 3T3-L1 differentiated cells were  
173 transfected with siRNA targeted against Ppargc1 $\alpha$  or Ppargc1 $\beta$  (encoding peroxisome proliferator-activated  
174 receptor gamma coactivator (PGC)1 $\alpha$  or PGC1 $\beta$ , respectively) or a nontargeting siRNA according to the  
175 manufacturer's instructions (Polyplus). Briefly, the cells were transfected overnight using a mixture of 10



176  $\mu$ M siRNA and 2.8  $\mu$ L of interferin reagent per well. The media were then replaced with complete medium.  
177 Adipocytes were then treated with ATRA for 24 h, and incubated for a further 24 h.  
178 For overexpression experiments, 3T3-L1 adipocytes were transfected using Lipofectamine LTX Plus  
179 Reagent (Life Technologies) with empty or PGC1 $\alpha$ -containing pCMX plasmid (pCMX-PGC1 $\alpha$ ; Addgene  
180 plasmid # 6 [25]). After 24 h incubation with transfection mixes (1  $\mu$ g of plasmid, 0.9  $\mu$ L of Lipofectamine  
181 LTX and 1  $\mu$ L of Plus Reagent per well, according to the manufacturer's instructions), the medium was  
182 replaced, and cells were incubated with TNF $\alpha$  for 24 h. RNA was isolated from treated cells, and residual  
183 plasmidic DNA contamination was removed by DNase I digestion (Sigma-Aldrich (Saint-Quentin-  
184 Fallavier, France)). PGC1 $\alpha$  expression was quantified by qPCR.

185

186 **1.10. Animal experiment** – The protocol was approved by the local ethics committee. Six-week-old male  
187 C57BL/6J mice were obtained from Janvier (Le Genest-Saint-Isle, France). Mice were fed *ad libitum* (chow  
188 diet A04, Safe, Augy, France), with full access to drinking water. The animals were maintained at 22 °C  
189 under a 12 h light – 12 h dark cycle at 50% humidity.

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

196

197 **1.11. Statistical analysis** - The data are expressed as means  $\pm$  SEM. Significant differences between the  
198 control and treated group were determined using the ANOVA analysis followed by the Tukey-Kramer *post*  
199 *hoc* test using Statview software:  $p < 0.05$  was considered statistically significant.

200

## 201 2. Results

202

### 203 2.1. ATRA modulates the transcriptome of human adipocytes in inflammatory conditions.

204 To study the impact of ATRA on chemokines under inflammatory conditions, these cells were preincubated  
205 with ATRA, or untreated, and then incubated with TNF $\alpha$ . The transcriptomic approach was applied in cells  
206 treated with TNF $\alpha$  compared with cells pretreated with ATRA before incubation with TNF $\alpha$ . The impact  
207 of these treatments on the transcriptome was evaluated with a fold change filter of 1.3. Examination of the  
208 gene list by DAVID (Database for Annotation, Visualization, and Integrated Discovery) software revealed  
209 that the “chemokine family” was strongly impacted by the ATRA / TNF $\alpha$  treatment compared with TNF $\alpha$   
210 alone (P-Value = 0.018) (Table S1). These data were confirmed by other software, GSEA (Gene Set  
211 Enrichment Analysis), with which analysis according to gene ontology terms highlighted inflammation  
212 related processes such as “G protein coupled receptor binding”, “defense response”, “locomotory  
213 behavior”, “cytokine activity” and “chemokine activity”. The chemokine activity was impacted by the  
214 ATRA / TNF $\alpha$  treatment compared with TNF $\alpha$  alone (NES = -1.757,  $p < 0.05$  and FDR  $q < 0.25$ ; the ten  
215 most frequently represented gene sets are given in Table S2). It is noteworthy that all these gene sets  
216 presented a negative enrichment score, meaning that chemokines were downregulated by ATRA  
217 pretreatment in comparison with TNF $\alpha$  (Table S2). As seen in Table S3, 17 chemokines identified by GSEA  
218 software were regulated by ATRA pretreatment in human adipocytes. Interestingly, we also observed that  
219 five chemokine receptors were downregulated by ATRA pretreatment (Table S4). To confirm our  
220 observations, we evaluated the gene expression profiles of six chemokines (randomly chosen, CCL5,  
221 CCL19, CX3CL1, CXCL1, CXCL5 and CXCL10) in human adipocytes incubated with ATRA (2  $\mu$ M) for  
222 24 h followed or not by incubation with TNF $\alpha$  (15 ng/mL) for a further 24 h. A significant increase in  
223 expression was observed in all the genes investigated: CCL5, CCL19, CXCL1, CX3CL1, CXCL5 and  
224 CXCL10 were increased 1611%, 2194%, 1865%, 993%, 3221% and 493%, respectively, in TNF $\alpha$  condition  
225 *vs.* control (Figure 1A). Interestingly, the incubation with ATRA reduced the mRNA expression levels of  
226 these chemokines 57.7%, 92.6%, 816.5%, 67.8%, 55.5% and 54.4%, respectively, compared with TNF $\alpha$   
227 alone.

228 Very similar results were obtained when murine 3T3-L1 differentiated adipocytes were treated: the mRNA  
229 expression levels of Ccl5, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were significantly increased by TNF $\alpha$  34163%,  
230 3489%, 729%, 211%, and 1221%, respectively. By contrast, ATRA incubation significantly reduced  
231 chemokine expression in comparison with the TNF $\alpha$  condition 49%, 92.3%, 60.6%, 80.1% and 20%,  
232 respectively (Figure 1B).

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

238

## 239 **2.2. ATRA supplementation limits cytokine/chemokine expression induced by LPS in mice adipose** 240 **tissue.**

241 The effects of ATRA on cytokine/chemokine expression were studied in a model of acute inflammation,  
242 consisting of an intraperitoneal injection of LPS for 4 h in mice. As shown in Figure 2A, a significant  
243 increase in the mRNA expression levels of Il6 (8667%), Tnf $\alpha$  (1180%) and Il1 $\beta$  (360%) was observed in  
244 epididymal fat pads under the LPS effect, whereas a 4-day ATRA supplementation prior to LPS stimulation  
245 significantly reduced inflammation in white adipose tissue 37.4%, 38.5% and 43.9%, respectively (Figure  
246 2A). Chemokine mRNA including Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were also  
247 quantified. Their expression was significantly increased by LPS (4412%, 7097%, 547%, 956%, 3150%,  
248 320%, 31944%, respectively) and reduced by 4 days of ATRA supplementation (55.3%, 56.3%, 54%, 66%,  
249 36.2%, 75.7% and 66.6%, respectively) (Figure 2A).

250 We confirmed the preventive effect of ATRA on the LPS-mediated inflammation in the plasma mice. An  
251 increase in protein secretion was obtained for Il6, TNF $\alpha$  and Ccl5 after LPS intraperitoneal injection in  
252 mice (288.4%, 404% and 5597%, respectively) (Figure 2B). Their levels were significantly decreased in  
253 the plasma of mice that received ATRA by gavage followed by LPS injection compared with LPS alone  
254 (46.2%, 14% and 13.5%, respectively) (Figure 2B). We obtained similar results in the epididymal adipose  
255 tissue of these mice for Ccl2 and Ccl5, which increased in LPS-injected mice (275% and 30%, respectively)

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

259

### 260 **2.3. ATRA limits RAW 264.7 macrophage migration in 3T3-L1 conditioned medium.**

261 To examine the potential of ATRA to limit the migration of macrophages, 3T3-L1 cells were preincubated  
262 with ATRA (0.2 and 2  $\mu$ M) prior to TNF $\alpha$  incubation, and compared with cells incubated with TNF $\alpha$  alone.  
263 The resulting 3T3-L1 conditioned medium was used to study the RAW 264.7 macrophage migration. As  
264 expected, 3T3-L1 conditioned medium under TNF $\alpha$  pro-inflammatory stimulus induced a significant  
265 migration of macrophages (approximately 100%, Figure 3). Interestingly, the preincubation of 3T3-L1 with  
266 2  $\mu$ M of ATRA significantly reduced the migration of macrophages 26.3% (Figure 3).

267

### 268 **2.4. ATRA limits NF- $\kappa$ B activation in 3T3-L1 adipocytes.**

269 As previously demonstrated, the expression of several chemokines is upregulated in inflammatory  
270 conditions, mainly via the NF- $\kappa$ B signaling pathway in human adipocytes [4], and in 3T3-L1 adipocytes  
271 [23]. Since NF- $\kappa$ B activation is central in the induction of chemokines, we next examined whether the effect  
272 of ATRA on chemokine production could be mediated through limitation of NF- $\kappa$ B signaling in murine  
273 adipocytes. Cells were pretreated with ATRA for 24 h followed by a 5 min incubation with TNF $\alpha$ . As  
274 expected, phosphorylation levels of p65 and I $\kappa$ B, two subunits of the NF- $\kappa$ B pathway, were significantly  
275 increased in the inflammatory condition induced by TNF $\alpha$  (414% and 360%, respectively). On the other  
276 hand, preincubation of cells with ATRA strongly limited the phosphorylation of p65 and I $\kappa$ B, suggesting  
277 that ATRA was able to reduce NF- $\kappa$ B activation in 3T3-L1 adipocytes (Figure 4).

278

### 279 **2.5. ATRA modulates NF- $\kappa$ B signaling and cytokine/chemokine expression through PGC1 $\alpha$ in 3T3- 280 L1 adipocytes.**

281 To identify the molecular mechanisms involved in ATRA-mediated NF- $\kappa$ B signaling deactivation, we  
282 hypothesized the involvement of PGC1 $\alpha$  and/or  $\beta$ , since we had recently shown that ATRA induced the

283 expression of PGC1 $\alpha$  and PGC1 $\beta$  in 3T3-L1 adipocytes [12], and these transcription factors are known to  
284 reduce NF- $\kappa$ B activity in muscle cells [26].

285 The involvement of PGC1 $\alpha$  and/or  $\beta$  on cytokine/chemokine modulation by ATRA was investigated in  
286 3T3-L1 adipocytes transfected with siRNA designed against PGC1 $\alpha$  or PGC1 $\beta$ . The cells were then  
287 preincubated with ATRA for 24 h followed by an incubation with TNF $\alpha$  for 24 h (Figure 5A). As expected,  
288 TNF $\alpha$  treatment significantly increased the mRNA expression levels of Tnf $\alpha$ , 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 TNF $\alpha$  condition (Figure 5A). Interestingly, in the 3T3-L1 adipocytes transfected with PGC1 $\alpha$  siRNA, there  
292 was a modulation in the expression of these inflammatory markers (Tnf $\alpha$ , Il6, Ccl2, Ccl5, Ccl11 and Cxcl10,  
293 211.9%, 160%, 154%, 189%, 160% and 196%, respectively compared with the ATRA+TNF $\alpha$  condition).  
294 However, in the 3T3-L1 adipocytes transfected with PGC1 $\beta$  siRNA, no difference in Tnf $\alpha$ , Ccl2, Ccl5 and  
295 Cxcl10 expression was observed compared with the ATRA+TNF $\alpha$  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 PGC1 $\alpha$ -  
298 dependent.

299 To further investigate the involvement of PGC1 $\alpha$  in these regulations, we studied the effect of PGC1 $\alpha$   
300 overexpression on TNF $\alpha$ -mediated cytokine/chemokine expression (Figure 5B). PGC1 $\alpha$  overexpression  
301 induced a significant decrease in Tnf $\alpha$ , Ccl2, Ccl11 and Cxcl10 expression (46.3%, 14.6%, 33.3% and  
302 13.3% compared with pCMX in the TNF $\alpha$  condition). However, in the case of Ccl5 and Il6 expression, no  
303 effect of PGC1 $\alpha$  was observed. These data suggest that the upregulation of some cytokine/chemokine  
304 expression mediated by TNF $\alpha$  can be partly blunted by PGC1 $\alpha$ .

305 To confirm the effect of PGC1 $\alpha$  on the NF- $\kappa$ B signaling pathway, cells transfected with pCMX-PGC1 $\alpha$   
306 were incubated with or without TNF $\alpha$  for 5 min, and I $\kappa$ B phosphorylation was evaluated. As expected,  
307 TNF $\alpha$  treatment significantly increased the I $\kappa$ B phosphorylation by 30% (Figure 6). Interestingly, PGC1 $\alpha$   
308 overexpression in adipocytes significantly decreased I $\kappa$ B phosphorylation by 18.4% compared with TNF $\alpha$ -  
309 treated adipocytes, suggesting that PGC1 $\alpha$  overexpression is able to deactivate NF- $\kappa$ B signaling in  
310 adipocytes (Figure 6).

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### 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 TNF $\alpha$  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 TNF $\alpha$ -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, Tnf $\alpha$  and Ccl5. Surprisingly, our results diverge from the recent finding of a moderate increase in proinflammatory markers (Il18 and MIP-1 $\gamma$ ) 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,

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

341  
342 From a molecular point of view, it is well-established that NF- $\kappa$ B controls the transcription of  
343 proinflammatory cytokines and chemokines in many cell types, including preadipocytes and adipocytes [1,  
344 4, 38]. Thus we evaluated the ability of ATRA to blunt NF- $\kappa$ B-mediated chemokine expression. First we  
345 demonstrated that ATRA displayed a strong inhibitory effect on NF- $\kappa$ B signaling in 3T3-L1 adipocytes,  
346 characterized by reduced phosphorylation levels of I $\kappa$ B and p65 (two main intermediates in the NF- $\kappa$ B  
347 signaling pathway).

348 Similar inhibition of NF- $\kappa$ B signaling has already been reported in various cell models [39], and in  
349 transgenic NF- $\kappa$ B reporter mice [40]. To pursue the identification of the precise molecular mechanisms, we  
350 hypothesized the involvement of PGC1 $\alpha$  and/or PGC1 $\beta$ . Peroxisome proliferator-activated receptor  $\gamma$   
351 coactivator 1  $\alpha$  and  $\beta$  are members of the PGC1 family of transcriptional coactivators [41]. PGC1 $\alpha$  is mainly  
352 involved in adaptive thermogenesis, glucose disposal and mitochondrial biogenesis [42], whereas PGC1 $\beta$   
353 regulates saturated fatty acid-induced hepatic triglyceride synthesis and hyperlipidemia [43], and  
354 mitochondrial fatty acid  $\beta$  oxidation [44]. In addition, these two transcription factors are able to reduce  
355 phosphorylation of the NF- $\kappa$ B family member p65 and thereby its transcriptional activation potential in  
356 muscle cells [26], and we recently showed that ATRA upregulated the two transcription factors in 3T3-L1  
357 adipocytes [12]. To confirm the involvement of PGC1 $\alpha$  and/or  $\beta$  in cytokine/chemokine downregulation,  
358 siRNA designed against PGC1 $\alpha$  and PGC1 $\beta$  was used. Results clearly show that inflammatory marker  
359 limitation through ATRA was primarily mediated by PGC1 $\alpha$ , the ATRA downregulation of chemokines  
360 being abolished in the presence of siRNA directed against PGC1 $\alpha$ . Furthermore, the ability of PGC1 $\alpha$  to  
361 repress the TNF $\alpha$ -mediated upregulation of chemokine mRNA levels together with NF- $\kappa$ B signaling, and  
362 notably I $\kappa$ B phosphorylation, was confirmed in overexpression experiments. Taken together, these findings  
363 strongly support the involvement of the ATRA-mediated upregulation of PGC1 $\alpha$  in the inhibition of NF-  
364  $\kappa$ B signaling and subsequent limitation of chemokine expression. However, other putative mechanisms  
365 cannot be excluded, notably the ATRA-mediated upregulation of specific phosphatases leading to NF- $\kappa$ B  
366 signaling deactivation, similar to the anti-inflammatory effect of the 1,25(OH) $_2$ D, which is mediated in

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

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370 Data related to the impact of retinoic acid on inflammation in humans are particularly scant, but it is  
371 noteworthy that in a recent prospective study, an inverse association between retinoic acid (RA) with several  
372 inflammatory and oxidative stress biomarkers such as 8-iso-prostaglandin F2 $\alpha$ , C Reactive Protein and IL6  
373 was reported in a Chinese metabolic syndrome population [45]. Even if our results seem to be encouraging,  
374 and in agreement with a clinical study, our data present several limitations due to the supraphysiological  
375 doses of ATRA used both *in vitro* and *in vivo*, and to the fact that extrapolation from mice to human is  
376 sometimes hazardous.

377 In conclusion, the results of the present study show that ATRA reduces chemokine expression in adipocytes  
378 and macrophage migration *in vitro*. We confirmed these results in mice adipose tissue. These effects may  
379 be associated with the ability of ATRA to limit the activation of the NF- $\kappa$ B signaling pathway, probably  
380 through PGC1 $\alpha$ .

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392 **Legends for figures**

393

394 **Figure 1. ATRA decreases chemokine expression levels in adipocytes.** Cells were preincubated with  
395 ATRA (2  $\mu$ M) for 24 h and incubated with TNF $\alpha$  (15 ng/mL) for 24 h. **A.** The mRNA levels of CCL5,  
396 CCL19, CXCL1, CX3CL1, CXCL5 and CXCL10 were quantified through qPCR in human adipocytes. The  
397 values are presented as means  $\pm$  SEM. Experiments were performed in triplicate, on 3 independent cultures.  
398 **B.** The mRNA levels of Ccl5, Ccl19, Cxcl1, Cx3x11 and Cxcl10 were quantified through qPCR in 3T3-L1  
399 adipocytes. The values are presented as means  $\pm$  SEM. Experiments were performed in triplicate, on 3  
400 independent cultures. **C.** CXCL10, CXCL1 and Midkine secretion in cell culture supernatants of human  
401 adipocytes was quantified by Luminex technology and Proteome Profiler Array. n.d.: not detected. Bars not  
402 sharing the same letters are significantly different,  $p < 0.05$ .

403

404 **Figure 2. ATRA decreases chemokine expression levels in LPS-mediated inflammation in mice**  
405 **epididymal adipose tissue. A.** In an acute inflammation model (LPS injection for 4 h), the mRNA levels  
406 of Il6, Tnf $\alpha$ , Il1 $\beta$ , Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were quantified through qPCR in  
407 epididymal adipose tissue of mice ( $n = 6-9$  per group), and expressed relative to 18S ribosomal RNA. The  
408 data are expressed as relative expression ratios. The values are presented as means  $\pm$  SEM. Bars not sharing  
409 the same letters are significantly different,  $p < 0.05$ . **B.** In the LPS inflammation model, protein amounts of  
410 Il6, Tnf $\alpha$  and Ccl5 secreted were quantified by ELISA. The data are expressed as relative expression ratios.  
411 The values are presented as means  $\pm$  SEM. Bars not sharing the same letters are significantly different,  $p <$   
412  $0.05$ . **C.** In the LPS inflammation model, protein amounts of Ccl2 and Ccl5 in epididymal adipose tissue of  
413 mice were quantified by ELISA. The data are expressed as relative expression ratios. The values are  
414 presented as means  $\pm$  SEM. Bars not sharing the same letters are significantly different,  $p < 0.05$ .

415

416 **Figure 3. ATRA decreases macrophage migration in 3T3-L1 adipocytes.** 3T3-L1 cells were  
417 preincubated with ATRA (0.2 and 2  $\mu$ M) for 24 h, and incubated with TNF $\alpha$  (15 ng/mL) for 24 h. RAW  
418 264.7 macrophage migration in 3T3-L1 conditioned media (4 h, 37  $^{\circ}$ C) was then performed. Data are  
419 expressed as relative expression ratio. Values are presented as means  $\pm$  SEM. Bars not sharing the same

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

422

423 **Figure 4. ATRA limits NF- $\kappa$ B activation.** Cells were preincubated with ATRA (1 and 2  $\mu$ M) for 24 h  
424 dose-dependently, and incubated with TNF $\alpha$  (15 ng/mL) for 5 min. Phosphorylation levels of the NF- $\kappa$ B  
425 subunits (p65 and I $\kappa$ B) were evaluated by ELISA. Data are expressed as relative expression ratio. Values  
426 are presented as means  $\pm$  SEM. Bars not sharing the same letter are significantly different,  $p < 0.05$ .  
427 Experiments were performed in triplicate, on 3 independent cultures.

428

429 **Figure 5. ATRA modulates NF- $\kappa$ B signaling and cytokine/chemokine expression through PGC1 $\alpha$  in**  
430 **3T3-L1 adipocytes. A.** Cells were transfected with siRNA designed against PGC1 $\alpha$  or PGC1 $\beta$  or with  
431 nontargeted siRNA. The cells were then preincubated with ATRA for 24 h followed by an incubation with  
432 TNF $\alpha$  for 24 h. The mRNA levels of Tnf $\alpha$ , Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through  
433 qPCR. The values are presented as means  $\pm$  SEM. **B.** Cells were transfected with pCMX plasmids, empty  
434 or containing PGC1 $\alpha$  (pCMX-PGC1 $\alpha$ ). After 24h incubation, cells were incubated with TNF $\alpha$  for 24 h. The  
435 mRNA levels of Tnf $\alpha$ , Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through qPCR. Values are  
436 presented as means  $\pm$  SEM. Bars not sharing the same letter are significantly different,  $p < 0.05$ . Each  
437 experiment was reproduced in triplicate, at least 3 independent times.

438

439 **Figure 6. PGC1 $\alpha$  limits I $\kappa$ B phosphorylation in 3T3-L1 adipocytes.** Cells were transfected with pCMX  
440 plasmids, empty or containing PGC1 $\alpha$  (pCMX-PGC1 $\alpha$ ). After 24 h incubation, cells were incubated with  
441 TNF $\alpha$  for 5 min. Phosphorylation levels of I $\kappa$ B were evaluated by ELISA. Data are expressed as relative  
442 expression ratio. Values are presented as means  $\pm$  SEM. Bars not sharing the same letter are significantly  
443 different,  $p < 0.05$ . Experiments were performed in triplicate, on 3 independent cultures.

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