Vitamin D Limits Chemokine Expression in Adipocytes and Macrophage Migration In Vitro and in Male Mice

Esma Karkeni, Julie Marcotorchino, Franck Tourniaire, Julien Astier, Franck Peiretti, Patrice Darmon, and Jean-François Landrier

Institut National de Recherche Agronomique, Unité Mixte de Recherche 1260; Inserm, Unité Mixte de Recherche 1062, Nutrition, Obésité et Risque Thrombotique; and Faculté de Médecine, Aix-Marseille Université, F-13385 Marseille Cedex 05, France

Vitamin D (VD) displays immunoregulatory effects and reduces adipocyte inflammation, which may participate to a reduction of adipose tissue macrophage infiltration in the context of obesity-associated low-grade inflammation. These observations have been described mainly in vitro, through the evaluation of a limited number of inflammatory markers. Here, we studied the effects of 1,25 dihydroxy-VD on chemokine network expression in adipocytes (by transcriptomic approach), and we confirm the physiological relevance of these data in vivo, by demonstrating the effect of VD on cytokine and chemokine gene expression as well as on macrophage infiltration in adipose tissue. 1,25 dihydroxy-VD down-regulated (1.3- to 10.8-fold) the mRNA expression of 29 chemokines and limited macrophage migration in TNFα-conditioned adipocyte medium (1.5-fold; \(P < 0.05\)). This effect was associated with a reduction in p65 and IκB (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) phosphorylation (2-fold compared with TNFα; \(P < 0.05\)). The effects of VD were confirmed in mice injected ip with lipopolysaccharide (acute inflammation) and diet-induced obese mice (metabolic inflammation), where the levels of mRNA encoding proinflammatory cytokines and chemokines (2-fold) were reduced in adipocytes (acute and metabolic inflammation) and adipose tissue and that macrophage infiltration was also inhibited in the adipose tissue of obese mice (metabolic inflammation). Altogether, these results showed that VD displayed a global immunoregulatory impact on adipocytes, notably via the inhibition of chemokine expression and macrophage infiltration in inflamed adipose tissue. (Endocrinology 156: 1782–1793, 2015)

Obesity is characterized by an excess of fat mass corresponding with the expansion of adipose tissue, associated with adipocyte hypertrophia and/or hyperplasia (1). Obesity has been strongly associated with chronic low-grade or metabolic inflammation, characterized by the activation of inflammatory signaling pathways and the abnormal secretion of a large set of immune response mediators (2, 3), known as adipokines (4, 5), including leptin, adiponectin, resistin, and visfatin, and cytokines, such as TNFα, IL-6, IL-1β, and chemokines. Indeed, previous studies (6–8) have demonstrated that adipose tissue and adipocytes express a large number of chemokines involved in the chemotaxis of most immune cells during inflammation, reflecting the increased number of infiltrated immune cells (natural killers, lymphocytes, macrophages) (9) observed in obese adipose tissue.

In parallel to the development of low-grade inflammation, vitamin D (VD) (cholecalciferol) insufficiency has been well documented in obesity. This insufficiency could influence the development of obesity-related diseases, including inflammation and insulin resistance (10–12). Indeed, several studies have reported the impact of VD on...
inflammation, with some discordance. Initial studies investigating the effects of VD on adipocytes (from murine and human origin) reported a proinflammatory effect (13, 14), whereas several other studies have reported an anti-inflammatory effect of VD on adipocytes (15–18), consistent with the antiinflammatory effect of VD in many other cell types (19).

Indeed, 1,25 dihydroxy-VD (1,25(OH)2D) (the active form of VD) significantly decreased the release of IL-8, CCL2, and IL-6 in human preadipocytes (16) and CCL2 in human adipocytes (15) induced through proinflammatory stimuli, such as macrophage-conditioned medium, TNFα and IL-1β. In addition, 1,25(OH)2D decreased the preadipocyte-induced monocyte migration of THP-1 monocytes (16). In parallel, we previously showed that 1,25(OH)2D displayed an antiinflammatory effect through a reduction in proinflammatory cytokine and chemokine expression in 3T3-L1 and human adipocytes under both basal and TNFα-stimulated conditions (18). We also demonstrated that the VD receptor, the NF-κB signaling pathway, and p38 MAPKs were involved in the down-regulation of proinflammatory cytokine and chemokine expression in 3T3-L1 cells (18), consistent with the conclusion of Mutt et al (17), who demonstrated that the antiinflammatory effect of 1,25(OH)2D was mediated through the inhibition of the NF-κB signaling pathway. More recently, it had been demonstrated that 1,25(OH)2D displays an inhibitory effect on the activation of the NF-κB and MAPK signaling pathways in human adipocytes, leading to a reduction in the gene transcription and protein synthesis of proinflammatory chemokines/cytokines, such as IL8, Ccl2, Ccl5, and Il6, leading to a decrease of THP-1 monocyte migration (20). Similar results, i.e., inhibition of proinflammatory cytokine expression through 1,25(OH)2D, were obtained using cultured human adipose tissue biopsies. Indeed, the preincubation of the biopsies with 1,25(OH)2D reduced the mRNA levels of CCL2, IL-6, and IL-8 and the secretion of IL-8 mediated through IL-1β incubation (21). However, these authors failed to demonstrate the similar adipose tissue-specific antiinflammatory properties of oral VD supplementation in obese subjects. Notably, the antiinflammatory effect of VD in adipose tissue in vivo has not been confirmed. Studies have only shown that dietary treatment with 1,25(OH)2D reduced the IL-6 protein content in the epididymal adipose tissue of diet-induced obese mice (22).

The aim of the present study was to extend the current knowledge of the impact of 1,25(OH)2D on inflammatory responses in human and mouse adipocytes, using high-throughput methodology (transcriptomic), evaluate the functional consequences of gene expression regulation in terms of macrophage migration, unveil the molecular mechanisms underlying gene expression regulation, and confirm in vivo the adipose tissue antiinflammatory potential of VD in acute and chronic mice models of inflammation.

Materials and Methods

Reagents

DMEM was purchased from Life Technologies, and fetal bovine serum (FBS) was obtained from PAA Laboratories. Isobutyrylcholine, dexamethasone, and insulin were purchased from Sigma-Aldrich. TRIZol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies. SYBR Green reaction buffer was purchased from Eurogentec. Antibodies were purchased from eBioscences SAS, R&D Systems, and Santa Cruz Biotechnology, Inc.

Cell culture

Macrophage and adipocyte cells were grown at 37°C in a 5% CO2 humified atmosphere. The 3T3-L1 preadipocytes (American Type Culture Collection) were seeded onto 3.5-cm dishes at a density of 15 × 10⁴ cells/well and grown in DMEM supplemented with 10% FBS at 37°C as previously reported (23). To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (d 0) were stimulated for 72 hours with 0.5mM isobutyrylcholine, 0.25 μg/mL dexamethasone, and 1-μg/mL insulin in DMEM supplemented with 10% FBS. The cultures were subsequently treated with DMEM supplemented with 10% FBS and 1-μg/mL insulin. In most cases, the adipocytes were preincubated with 1,25(OH)2D, the active form of VD (1nM and 100nM) for 24 hours and incubated with TNFα (15 ng/mL) for an additional 24 hours. 3T3-L1 adipocytes were also exposed to 25(OH)D (100nM) for 24 hours and incubated with TNFα (15 ng/mL) for an additional 24 hours. To identify the signaling pathway implicated in chemokine regulation, 3T3-L1 cells were treated with specific inhibitors of NF-κB signaling (10μM BAY 117082) for 1 hour and subsequently stimulated with TNFα (15 ng/mL) for 24 hours. To examine the effects of 1,25(OH)2D on NF-κB signaling, adipocytes were pretreated with 1,25(OH)2D (10nM and 100nM) and incubated with TNFα (15 ng/mL) for 5 minutes. All treatments were performed on day 8 of 3T3-L1 differentiation. Each experiment was reproduced in triplicate, at least 3 independent times.

Raw 264.7 macrophages (ECACC) were seeded onto 3.5-cm dishes and grown in DMEM supplemented with 10% FBS and 2% HEPES as previously reported (24).

The human preadipocytes (3 independent cultures) were obtained from Promocell and cultured according to the manufacturer’s instructions. The mature adipocytes (d 15) were incubated with 1,25(OH)2D (100nM, 24 h) followed by a 24-hour incubation with TNFα (15 ng/mL). Experiments were performed in triplicate, on 3 independent cultures.

Hybridization arrays and microarrays data analysis

RNA were extracted from human adipocytes cultures (3 independent cultures) and RNA quality control was performed on an Agilent 2100 Bioanalyzer, according to the manufacturer’s
instructions. RNA was hybridized to the Agilent Whole Human Genome 8x60K. For each independent culture, treated conditions (1,25(OH)2D + TNFα) were labeled with cyanine 5, and control conditions (TNFα alone) were labeled with cyanine 3. In addition, a treated pool and a control pool resulting of an equal amount of each independent control and treated conditions were realized and were labeled as previously described (treated pool labeled with cyanine 5 and control pool labeled with cyanine 3). The resulting 4 couples of samples (3 independent couple of samples plus pool couple of samples) were further hybridized. All labeling, hybridization, washing, and scanning were performed as described in the manufacturer’s protocol and as previously reported (25, 26). The arrays were scanned using an Agilent Scanner. The data were extracted using Agilent Feature Extraction v10.5.1.1 software and analyzed with Agilent GeneSpring GX v11.0.2 software. The analyses were performed using Gene Set Enrichment Analysis (GSEA) (http://www.broadinstitute.org/gsea) and Database for Annotation, Visualization, and Integrated Discovery software as previously described (27). A false discovery rate q < 0.25 for normalized enrichment score was considered significant.

RNA isolation and qPCR
Total cellular RNA was extracted using TRIzol reagent according to the manufacturer’s instructions. cDNAs were synthesized from 1 μg of total RNA using random primers and Mlooney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene) as previously described (28). 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 method (29). The next primer sequences were used for qPCR determination: mIl6_F_ acaagtcggaggcttaattacat, R_ttgccattgcttaagctgc; mIl10_F_cacaaagcagccttgcagaa, R_ agagcagagtgctcattttctgc; mTnf_F_ acaagtcggaggcttaattacat, R_ttgccattgcttaagctgc; mIl6_F_ acaagtcggaggcttaattacat, R_ttgccattgcttaagctgc.

Chemokine quantification in cell culture supernatants
A Luminex screening assay (R&D Systems) was used to quantify CCL2, CCL5, CXCL1, and CXCL10 in human adipocyte cell culture supernatants with the Luminex 200 platform.

Analysis of plasma samples
Plasma 25(OH)D concentration was measured using an ELISA kit (Promokine, Promocell). Calcium concentration in plasma was evaluated using Calcium Colorimetric Assay kit (Biovision).

Macrophages migration assay
Migration assays were performed using cell culture inserts with a 3-μm membrane pore size (Transwell Millipore). The 3T3-L1 adipocytes were preincubated with or without various concentrations of 1,25(OH)2D (1nM and 100nM) for 24 hours. Subsequently, the adipocytes were incubated with TNFα (15 ng/mL) for 24 hours. The 3T3-L1-conditioned media was transferred to plates containing inserts. RAW 264.7 macrophages were seeded onto these inserts at a density of 900 cells/cm². After migration for 4 hours at 37°C, the macrophages in the lower compartment were fixed with 2.5% glutaraldehyde for 15 minutes and counted as previously reported (24).

To validate the results of the migration experiments, neutralizing antibodies against Ccl2 (AF-479-NA; 0.5, 1, 2.5, and 5 μg/mL; R&D Systems) and Ccl5 (AF478; 0.07, 0.2, 0.3, and 0.6 μg/mL; R&D Systems) were added to 3T3-L1 adipocyte-conditioned media (with or without 1,25(OH)2D [100nM] for 24 h), and RAW 264.7 macrophages were seeded onto the inserts. Goat IgG (SC-2028; Santa Cruz Biotechnology, Inc) was used as a negative control in each case.

NF-κB activation
The levels of p65 (Ser536) and IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) (Ser32/36) phosphorylation were quantified using the ELISA Instant One kit according to the manufacturer’s instructions (eBioSciences SA).

Animal experiments
The protocol was approved through the local ethics committee. Six-week-old male C57BL/6j mice were obtained from Janvier. The mice were fed ad libitum (chow diet A04; Safe), with full access to drinking water. The animals were maintained at 22°C under a 12-hour light, 12-hour dark cycle with a 50% humidity level.

To assess the impact of VD on acute inflammation, the mice received by gavage (n = 6–9 per group) the native form of VD (cholecalciferol) (15 000 IU/kg of body weight, ie, ~300 IU/mouse; Sigma-Aldrich) or vehicle alone (olive oil) once a day for 4 days. On the fifth day, the mice were ip injected with saline or Escherichia coli lipopolysaccharide (LPS) (4 mg/kg, serotype O111:B4; Sigma-Aldrich). At 4 hours after LPS injection, the mice were sacrificed, and epididymal adipose tissue was dissected and stored at −80°C.

To examine the impact of VD (cholecalciferol) on chronic inflammation, 3 experimental groups of mice were fed ad libitum with a low (10% of total energy as fat; TD06416; Harlan) or
Human adipocytes primocultures were preincubated or not with 1,25(OH)₂D (100nM) for 24 hours, followed by incubation with TNFα (15 ng/mL) for an additional 24 hours. The transcriptome of cells incubated with TNFα was compared with the transcriptome of cells pretreated with 1,25(OH)₂D before incubation with TNFα. The data were filtered (P < .05 and fold-change ≥ 1.3 or ≤ −1.3) and subsequently analyzed using GSEA software. This analysis revealed that “chemokine activity” was the most overrepresented and strongly impacted gene set after treatment with 1,25(OH)₂D/TNFα compared with TNFα alone (normalized enrichment score = −1.992; the first 10 most-represented gene sets are shown in Supplemental Table 1). Several linked gene sets were also strongly impacted after treatment, namely “chemokine receptor binding,” “G protein-coupled receptor binding,” “cytokine activity,” “defense response,” or “cellular defense response” (Supplemental Table 1). Notably, these gene sets presented a negative enrichment score, suggesting that chemokines were down-regulated after 1,25(OH)₂D pretreatment compared with TNFα treatment (Supplemental Table 1). Interestingly, similar results were confirmed using another software, Database for Annotation, Visualization, and Integrated Discovery software, showing the overrepresentation of chemokines in regulated genes (Supplemental Table 2). Based on the GSEA gene sets, we established a list of 29 chemokines regulated through 1,25(OH)₂D pretreatment in human adipocytes (Supplemental Table 3). Interestingly we also observed that 10 chemokines receptors were impacted by 1,25(OH)₂D pretreatment (Supplemental Table 4). Most of these compounds were down-regulated after treatment. The regulation of some genes was confirmed using RT-qPCR. The mRNA expression of ten chemokines was evaluated (CCL2, CCL5, CCL13, CCL19, CCL20, CX3CL1, CXCL1, CXCL5, CXCL10, and CXCL11) in human adipocytes incubated with 1,25(OH)₂D (100nM) for 24 hours, followed or not by incubation with TNFα (15 ng/mL) for an additional 24 hours. As shown in Figure 1A, the inflammatory response induced through TNFα significantly increased the mRNA expression levels of CCL2, CCL5, CCL13, CCL19, CCL20, CXCL1, CX3CL1, CXCL5, CXCL10, and CXCL11 9.5-, 16.1-, 1.91-, 21.9-, 82-, 18.6-, 9.9-, 32.2-, 4.9-, and 86.9-fold, respectively. As anticipated from the microarray experiments, incubation with 1,25(OH)₂D significantly reduced the mRNA expression levels of most of these chemokines (CCL5, CCL13, CCL19, CCL20, CXCL1, CX3CL1, CXCL5, and CXCL11) 2.9-, 6.8-, 16.6-, 2-, 4.3-, 3-, 1.7-, and 2.63-fold, respectively), whereas CCL2 and CXCL10 decrease did not reach statistical significance, compared with TNFα alone.

Results

1,25(OH)₂D limits chemokine expression in human adipocyte primocultures

To examine, in detail, the impact of 1,25(OH)₂D on the modulation of TNFα-mediated inflammatory responses in adipocytes, microarray experiments were performed. Human adipocytes primocultures were preincubated or not with 1,25(OH)₂D (100nM) for 24 hours, followed by incubation with TNFα (15 ng/mL) for an additional 24 hours. The transcriptome of cells incubated with TNFα was compared with the transcriptome of cells pretreated with 1,25(OH)₂D before incubation with TNFα. The data were filtered (P < .05 and fold-change ≥ 1.3 or ≤ −1.3) and subsequently analyzed using GSEA software. This analysis revealed that “chemokine activity” was the most overrepresented and strongly impacted gene set after treatment with 1,25(OH)₂D/TNFα compared with TNFα alone (normalized enrichment score = −1.992; the first 10 most-represented gene sets are shown in Supplemental Table 1). Several linked gene sets were also strongly impacted after treatment, namely “chemokine receptor binding,” “G protein-coupled receptor binding,” “cytokine activity,” “defense response,” or “cellular defense response” (Supplemental Table 1). Notably, these gene sets presented a negative enrichment score, suggesting that chemokines were down-regulated after 1,25(OH)₂D pretreatment compared with TNFα treatment (Supplemental Table 1). Interestingly, similar results were confirmed using another software, Database for Annotation, Visualization, and Integrated Discovery software, showing the overrepresentation of chemokines in regulated genes (Supplemental Table 2). Based on the GSEA gene sets, we established a list of 29 chemokines regulated through 1,25(OH)₂D pretreatment in human adipocytes (Supplemental Table 3). Interestingly we also observed that 10 chemokines receptors were impacted by 1,25(OH)₂D pretreatment (Supplemental Table 4). Most of these compounds were down-regulated after treatment. The regulation of some genes was confirmed using RT-qPCR. The mRNA expression of ten chemokines was evaluated (CCL2, CCL5, CCL13, CCL19, CCL20, CX3CL1, CXCL1, CXCL5, CXCL10, and CXCL11) in human adipocytes incubated with 1,25(OH)₂D (100nM) for 24 hours, followed or not by incubation with TNFα (15 ng/mL) for an additional 24 hours. As shown in Figure 1A, the inflammatory response induced through TNFα significantly increased the mRNA expression levels of CCL2, CCL5, CCL13, CCL19, CCL20, CXCL1, CX3CL1, CXCL5, CXCL10, and CXCL11 9.5-, 16.1-, 1.91-, 21.9-, 82-, 18.6-, 9.9-, 32.2-, 4.9-, and 86.9-fold, respectively. As anticipated from the microarray experiments, incubation with 1,25(OH)₂D significantly reduced the mRNA expression levels of most of these chemokines (CCL5, CCL13, CCL19, CCL20, CXCL1, CX3CL1, CXCL5, and CXCL11) 2.9-, 6.8-, 16.6-, 2-, 4.3-, 3-, 1.7-, and 2.63-fold, respectively), whereas CCL2 and CXCL10 decrease did not reach statistical significance, compared with TNFα alone.
were preincubated with 1,25(OH)2D (100nM) for 24 hours and incubated with TNFα (15 ng/mL) for 24 hours. A, The mRNA levels of CCL2, CCL5, CCL13, CCL19, CCL20, CCL11, CXCL1, CXCL5, CXCL10, and CXCL11 were quantified through qPCR. The data are expressed as relative expression ratios. The values are presented as the mean ± SEM. Experiments were performed in triplicate, on 3 independent cultures. B, CCL2, CCL5, CCL1, and CXCL10 secretion in cell culture supernatants was quantified by Luminex technology. n.d., not detected. Bars not sharing the same letters are significantly different, P < .05.

A parallel increase in protein secretion in cell culture supernatants of human adipocytes was obtained for CCL2, CCL5, CCL1, and CXCL10 under TNFα treatment (6.57-, 3.18-, 16.9-, and 1.73-fold, respectively). CCL2, CCL1, and CXCL10 levels were significantly decreased in adipocytes incubated with 1,25(OH)2D followed by TNFα incubation compared with TNFα condition (1.5-, 3.18-, and 5.24-fold, respectively). CCL5 secretion was also decreased in the culture medium of adipocytes submitted to inflammation and pretreated with 1,25(OH)2D, but it did not reach statistical significance (Figure 1B).

1,25(OH)2D and 25(OH)D limit chemokine expression in 3T3-L1 adipocytes

To confirm the effect of 1,25(OH)2D on the modulation of TNFα-mediated chemokine regulation in murine adipocytes and to evaluate the effect of 25(OH)D, 3T3-L1 adipocytes were incubated with 1,25(OH)2D (100nM) or 25(OH)D (100nM) for 24 hours, followed by incubation with TNFα (15 ng/mL) for an additional 24 hours, and the mRNA expression of some chemokines was evaluated by RT-qPCR. As expected, TNFα treatment significantly decreased the mRNA expression levels of Ccl11, Ccl19, Cxcl1, Cx3c1, and Cxcl10 2.87-, 59.21-, 167.34-, 1.81-, and 29.83-fold, respectively, whereas 1,25(OH)2D incubation significantly decreased 2.8-, 15-, 8.4-, 2.5-, and 2.8-fold, respectively, compared with TNFα-treated adipocytes (Figure 2). Interestingly, 25(OH)D incubation also significantly reduced the chemokines mRNA levels 5.8-, 5.4-, 3.5-, 1.57-, and 1.51-fold, respectively, compared with TNFα condition (Figure 2).

1,25(OH)2D limits macrophages migration induced with 3T3-L1-conditioned medium

To examine the functional consequences of chemokine expression modulation under 1,25(OH)2D treatment, macrophage migration experiments were performed, using adipocyte-conditioned media. 3T3-L1 cells were preincubated with 1,25(OH)2D (1nM and 100nM), followed by incubation or not with TNFα. The resulting 3T3-L1-conditioned medium was used to examine RAW264.7 macrophage migration. Expectedly, in presence of TNFα, 3T3-L1-conditioned medium induced the significant migration of macrophages (~2-fold) (Figure 3A). The preincubation of 3T3-L1 with 100nM of 1,25(OH)2D significantly reduced macrophages migration 1.5-fold, whereas the lowest concentration of 1,25(OH)2D (1nM) was ineffective (Figure 3A) under both basal and TNFα conditions.

In subsequent experiments, neutralizing antibodies directed against Ccl2 and/or Ccl5 were added to 3T3-L1 adipocyte-conditioned media incubated with or without 1,25(OH)2D (100nM for 24 h). Preliminary experiments were conducted to determine the effective dose of antibodies to limit macrophage migration (Supplemental Figure 1, A and B). This effect was dose dependent for both Ccl2 and Ccl5 antibodies, with a plateau phase at 2.5 μg/mL for the anti-Ccl2 antibody and 0.3 μg/mL for the anti-Ccl5 antibody. These concentrations were used for subsequent macrophage migration assays (Figure 3, B and C). In addition, other validations, such as the use of irrelevant IgG and heated conditioned media, were performed to demonstrate the specificity of the experimental conditions (Supplemental Figure 2).

The addition of anti-Ccl2 or anti-Ccl5 antibodies to conditioned medium preincubated with 1,25(OH)2D and incubated (Figure 3C) or not (Figure 3B) with TNFα sig-
significantly reduced macrophage migration (~20% under basal conditions, ie, without TNFα, or 200% under TNFα-stimulated conditions). The combination of the 2 antibodies did not markedly decrease macrophage migration compared with anti-Ccl2 or anti-Ccl5 alone (Figure 3, B and C).

1,25(OH)2D limits NF-κB activation in 3T3-L1 adipocytes

Chemokine expression under TNFα stimulation has previously been examined in human adipocytes, and NF-κB was identified as a major signaling pathway in this regulation (8). To confirm the role of NF-κB in 3T3-L1 adipocytes, the cells were incubated with a specific NF-κB inhibitor, followed by TNFα stimulation. As shown in Figure 4A, the inhibition of the NF-κB signaling pathway significantly reduced the expression of Ccl5, Cx3cl1, and Cxcl10 2.9-, 6.5-, and 1.5-fold, respectively, suggesting that chemokine regulation is primarily NF-κB dependent in 3T3-L1 cells, as previously reported in human adipocytes (8).

Thus, we examined the impact of 1,25(OH)2D preincubation (10nM and 100nM) for 24 hours followed by a 24-hour incubation with TNFα on NF-κB signaling. To this end, the phosphorylation levels of p65 and IκB were quantified through ELISA. Expectedly, the phosphorylation levels of p65 and IκB were significantly increased after 3T3-L1 adipocyte stimulation through TNFα (4.1- and 3.6-fold, respectively), whereas incubation with 1,25(OH)2D strongly limited the phosphorylation of p65 and IκB, suggesting that 1,25(OH)2D reduced NF-κB activation in 3T3-L1 adipocytes (Figure 4B).

Vitamin D3 supplementation limits LPS-mediated inflammatory response in epididymal white adipose tissue

To examine the effects of vitamin D3 (VD) on inflammatory responses in adipose tissue in vivo, a model of acute inflammation based on the ip injection of LPS for 4 hours in mice was implemented. To confirm the efficiency of VD supplementation, 25(OH)D was measured in the serum of mice at the end of the experiment, before LPS injection. As expected, a higher plasma concentration of 25(OH)D was observed in mice submitted to VD gavage (122.4 ± 7.7 ng/mL) compared with control mice (35.6 ± 7.2 ng/mL, P = .01), whereas no modification of calcemia was observed (control mice, 2.52 ± 0.08 mmol/L vs VD-supplemented mice, 2.36 ± 0.22 mmol/L; P > .05). As reported in Figure 5, a significant increase in the mRNA expression levels of Il6 (86.7-fold), Tnfa (11.8-fold), and Il1β (3.8-fold) was observed in epididymal fat pads after LPS treatment, whereas 4-day VD supplementation, before LPS stimulation, significantly reduced the expression of these genes in white adipose tissue 1.8-, 1.6-, and 1.5-fold, respectively (Figure 5A). Chemokine mRNA (Figure 5, B–D), including Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1, and Cxcl10, was also quantified, and the expression of these molecules was significantly increased after
LPS treatment (44.1-, 71-, 5.4-, 9.5-, 31.5-, 3.2-, and 319.4-fold, respectively) and reduced after a 4-day VD supplementation (1.9-, 2.2-, 2.3-, 2.4-, 2-, 1.6-, and 2.1-fold, respectively).

Vitamin D3 supplementation limits obesity-mediated inflammatory responses in epididymal white adipose tissue

To further examine the antiinflammatory effect of VD on inflammatory responses in vivo, a model of chronic inflammation induced through a HF diet was used. Indeed, obesity induced through a HF diet has been associated with low-grade inflammation in adipose tissue, marked by the induction of proinflammatory cytokine and chemokine expression and leukocyte infiltration (2). To confirm the efficiency of VD supplementation, 25(OH)D was measured in the serum of mice at the end of the regimen. As expected, a higher plasma concentration of 25(OH)D was observed in the VD-supplemented HF-fed mice (65.66 ± 7.8 ng/mL), whereas the HF-fed mice displayed a reduction of 25(OH)D (7.14 ± 2.02 ng/mL) compared with control mice (38.5 ± 5.56 ng/mL), whereas no modification of calcemia was observed as previously reported (30).

The induction of the mRNA levels of proinflammatory cytokines (Il6 and Tnfa), except Il1β, was observed after 10 weeks of HF diet (by 3.1- and 14.8-fold) compared with control mice. Interestingly, VD supplementation for 10 weeks strongly limited proinflammatory cytokine expression in epididymal adipose tissue (~3-fold) (Figure 6A). The mRNA levels of Ccl2, Ccl5, and Ccl11 were increased in the white adipose tissue of HF-fed mice (1.6-, 1.8-, and 1.4-fold, respectively), and the expression of these cytokines was inhibited after VD supplementation for 10 weeks (Figure 6B). The mRNA levels of some chemokines, such as Ccl19 and Cxc11, were not modified through HF diet or VD supplementation, whereas the mRNA levels of other chemokines, including Cx3cl1 and Cxcl10, were decreased in the WAT of HF-fed mice and reinduced (Cxc110) or not after VD supplementation (Cx3cl1) (Figure 6B).

To confirm the specific involvement of adipocytes in chemokine regulation, adipocytes were isolated from the white adipose tissue of HF-fed mice, and the chemokine expression was measured. The mRNA levels of Ccl2, Ccl5, Ccl19, Cxc11, Cx3cl1, and Cxc10 were significantly increased in the isolated adipocytes obtained HF-fed mice compared with standard diet-fed mice (2.4-, 1.8-, 4.1-, 1.6-, 1.5-, and 1.5-fold, respectively), and the expression of these molecules was limited after VD supplementation (1.7-, 2-, 2.1-, 1.9-, 1.5-, and 2.5-fold, respectively) (Figure 6C), supporting a specific role for adipocytes in the adipose tissue regulation of chemokines through VD supplementation.

To confirm the potential impact of chemokine regulation on leukocytes infiltration in adipose tissue, the expression of several macrophage markers, such as F4/80,
CD11c (M1 marker), beta-N-acethylhexosaminidase, and IL-10 (M2 markers), was quantified in the epididymal fat pads of mice, as well as CD3, a marker of T lymphocytes.

Discussion

In the present study, we used a transcriptomic approach to examine the role of VD supplementation on chemokine expression induced through inflammation in human adipocytes. Chemokines are defined as “cytokines with selective chemotaxic properties,” and these molecules coordinate leukocyte movement to sites of inflammation or injury (31). In the context of obesity, chemokines are involved leukocytes recruitment in adipose tissue (9). Here, we showed that in addition to several previously reported chemokines, such as Ccl2, Il8, and Ccl5 (20), many other chemokines are also down-regulated through 1,25(OH)2D. Indeed, we identified 29 down-regulated chemokines after 1,25(OH)2D treatment compared with inflammatory conditions. For some chemokines, both mRNA and protein levels were affected, whereas for other chemokines, only mRNA or protein levels were impacted, reflecting the fact that mRNA and protein levels may present slight discrepancies. These data are consistent with recently published data demonstrating the in-

Figure 4. 1,25(OH)2D limits NF-κB activation. A, The NF-κB signaling inhibitor modulates chemokine expression. 3T3-L1 cells were treated with a specific inhibitor of NF-κB signaling (10μM BAY 117082) for 1 hour and subsequently stimulated with TNFα (15 ng/mL) for 24 hours. B, The cells were preincubated with 1,25(OH)2D (10nM and 100nM) for 24 hours in dose-dependent manner and incubated with TNFα (15 ng/mL) for 24 hours. The phosphorylation levels of the NF-κB subunits (p65 and IkB) were evaluated using EUSA. The data are expressed as relative expression ratios. The values are presented as the mean ± SEM. Bars not sharing the same letters are significantly different, P < .05. Experiments were reproduced in triplicate, at least 3 independent times.

Figure 5. Effects of vitamin D3 on LPS-mediated inflammation in mice epididymal adipose tissue. In an acute inflammation model (LPS injection for 4 h), the mRNA levels of Il6, Tnfα and Il1β (A) and Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3c11, and Cxcl10 (B–D) were quantified through qPCR in mice (n = 6–9 per group) epididymal adipose tissue and expressed relative to 18S ribosomal RNA. The data are expressed as relative expression ratios. The values are presented as the mean ± SEM. Bars not sharing the same letters are significantly different, P < .05.
We have shown that 1,25(OH)₂D has a limiting effect on macrophage migration in the conditioned medium of adipocytes. These results are consistent with the previously reported antiinflammatory effect of 1,25(OH)₂D on adipocytes (20). Indeed, Ding et al (20) showed that VD decreases the expression of some chemokines (Ccl2, Il8, and Ccl5) and decreases macrophages migration in the conditioned medium of human adipocytes. In addition, we used neutralizing antibodies directed against Ccl2 and Ccl5 in 3T3-L1 adipocytes to demonstrate the involvement of these chemokines in macrophage migration. The results showed that 1,25(OH)₂D incubation and neutralizing antibodies did not completely block macrophage migration in adipocyte-conditioned medium, suggesting that the inhibition of macrophage migration does not depend uniquely on single chemokines, such as Ccl2 or Ccl5. Consistently, in the case of Ccl2, previous studies have shown that the inhibition of Ccl2 through gene knockout or chemical blockade diminishes macrophage infiltration but does not completely block this activity (32, 41, 42), suggesting the involvement of other chemokines. In addition, in support of the global chemokine network involvement rather than the specific contribution of a unique chemokine, we recently showed that human adipocytes express a large number of chemokines under inflammatory conditions (34 chemokines are expressed in human adipocytes under TNFα treatment) (8). The expression of a large number of chemokines suggests that macrophage and global leukocyte infiltration into obese adipose tissue is not only dependent on 1 or a few proteins, such as Ccl2 or Ccl5, but rather depends on the complete network.

We also evaluated the effect of VD on the phosphorylation of 2 intermediates of the NF-κB signaling pathway (p65 and IkB). NF-κB controls the transcription of proinflammatory cytokines in many cell types, including preadipocytes and adipocytes (2, 43). In the present study, we demonstrated that 1,25(OH)₂D has a strong limiting effect on NF-κB signaling in 3T3-L1 adipocytes through the reduction of the phosphorylation levels of p65 and IkB. These data are consistent with previous studies demonstrating the inhibition of IkB protein expression in 3T3-L1 adipocytes.
A

B

Figure 7. Effects of vitamin D₃ on leukocyte infiltration in epididymal adipose tissue of mice. **A**, Gene expression of leukocyte and T cell markers (M1, M2, and CD3) in the white adipose tissue measured through qPCR and expressed relative to 18S ribosomal RNA in mice submitted to standard, HF, or VD-supplemented HF diets for 10 weeks (n = 10). The data are expressed as relative expression ratios. The values are presented as the mean ± SEM. Bars not sharing the same letters are significantly different, P < .05. **B**, Immune cell accumulation in epididymal adipose tissue. Fluorescence-activated cell sorting analysis of the stromal vascular fraction from the epididymal adipose tissue of mice fed standard (white bars), HF (black bars), or vitamin D₃-supplemented HF (gray bars) diets for 10 weeks.

In vivo, we evaluated the impact of VD supplementation in a chronic context of inflammation induced through a HF diet. As expected, we observed that supplemented mice displayed a 25(OH)D plasma concentration higher than control mice, whereas HF diet strongly decreased 25(OH)D plasma concentration in mice. Such decrease in HF condition could be due to the sequestration of VD in adipose tissue (45) or to volumetric dilution of VD (46). Interestingly, compared with HF diet-fed mice, we observed a reduction in the expression of inflammatory markers, including Il6, Il1β, and Tnfa, and some chemokine expression in adipose tissue during obesity. However, it cannot be excluded that the decreased inflammatory status of adipose tissue might reflect the reduction of fat mass, because the relationship between these 2 events has been previously documented (47), and we recently reported that VD supplementation reduced body weight gain induced through a HF diet (30). However, similar results, ie, a decrease in chemokine and cytokine gene expression in adipose tissue, were obtained under acute inflammation induced using the bacterial endotoxin LPS. Indeed, short-term injections induce the expression of proinflammatory cytokines and chemokines in adipose tissue (48). The fact that similar inhibition of chemokine expression was observed in mice supplemented with VD for 4 days, followed by the induction of acute inflammatory stress using LPS, strongly supports a direct role for VD in the inhibition of chemokine expression, independently of body weight modulation, because no modification in the body weight was observed at 4 hours after LPS treatment. However, further studies are needed to determine the respective contribution of fat mass decrease and direct antiinflammatory effects of VD in dedicated animal models.

The increase of 25(OH)D in vivo, which parallels with the decrease in chemokine gene expression in mice adipose...
tissue, together with inhibitory effect of 25(OH)D on chemokine expression in 3T3-L1 adipocytes, strongly suggest that 25(OH)D may produce the antiinflammatory effects reported in vivo, probably via a production of 1,25(OH)2D in adipose tissue. Altogether, these data may support a potential role of VD status (ie, 25(OH)D plasma concentration) on the control of adipose tissue inflammation.

The clinical data related to the effect of VD supplementation on adipose tissue inflammation are still unclear. Wamberg et al (21) studied the inflammation reduction in adipose tissue under VD supplementation in obese subjects with low plasma levels of 25-hydroxy-VD. No improvement in the inflammatory status was observed; however, in the same study, these authors reported that incubation with 1,25(OH)2D reduced the mRNA levels of inflammatory markers in vitro (21). Clearly, more clinical studies are needed to demonstrate the role of VD in the prevention of obesity-associated inflammation in adipose tissue.

In conclusion, the results of the present study show that VD and 1,25(OH)2D reduce chemokine expression in adipocytes and macrophage migration in vitro. We have also provided the first evidence of this effect in mice adipose tissue. These effects might be associated with the ability of 1,25(OH)2D to limit the activation of the NF-κB signaling pathway. This antiinflammatory effect of VD, if confirmed in humans, could provide major health benefits, particularly for obesity-associated pathologies.

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Address all correspondence and requests for reprints to: Jean-François Landrier, UMR 1260 INRA/1062 Inserm/Université d’Aix-Marseille, 27 Boulevard Jean Moulin, F-13385 Marseille Cedex 05, France. E-mail: jean-françois.landrier@univ-amu.fr.

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