The American Journal of Pathology, Vol. 174, No. 3, March 2009

DOI: 10.2353/ajpath.2009.080612

Vascular Biology, Atherosclerosis and Endothelium Biology

Semicarbazide-Sensitive Amine Oxidase/Vascular Adhesion Protein-1 Deficiency Reduces Leukocyte Infiltration into Adipose Tissue and Favors Fat Deposition

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Obesity is associated with low-grade inflammation and leukocyte infiltration in white adipose tissue (WAT) and is linked to diabetic complications. Semicarbazide-sensitive amine oxidase, also known as vascular adhesion protein-1 (SSAO/VAP-1), is a membrane protein that is highly expressed in adipocytes and is also present on the endothelial cell surface where it is involved in leukocyte extravasation. We studied fat deposition and leukocyte infiltration in WAT of mice with a null mutation in the amine oxidase copper-containing-3 (AOC3) gene encoding SSAO/VAP-1. Both epididymal and inguinal WATs were larger in 6-month-old AOC3-KO males than in age-matched wild-type controls. However, WAT from AOC3-KO mice contained lower CD45 mRNA levels and fewer $CD45^+$ leukocytes. Subpopulation analyses revealed a diminished infiltration of WAT by T cells, macrophages, natural killer, and natural killer T cells. A decrease in leukocyte content in WAT was also detected in female AOC3-KO mice as early as 2 months of age, whereas increased fat mass was evident by 6 months of age. Reduced $CD45^+$ populations in WAT of AOC3-KO mice was not rescued by human SSAO/VAP-1 expression on adipocytes under the control of aP2, suggesting the importance of vascular AOC3 in leukocyte entrance into fat. Our results indicate that SSAO/VAP-1 is instrumental for the presence of leukocytes in WAT. Therefore, AOC3-KO mice present a unique model of mild obesity, characterized by increased WAT devoid of low-grade inflammation. ***(Am J Pathol 2009, 174:1075–1083; DOI: 10.2353/ajpath.2009.080612)***

Anatomical and functional relationships exist between the immune system and adipose tissue. Lymph nodes (LNs) are present in inguinal fat depots,[1] and their surrounding adipocytes produce fatty acids, adipokines (tumor necrosis factor-α, interleukin-1, interleukin-6, leptin, interferon-γ), and chemokines (MCP-1, MIP-1) that can influence immune cells.[2] On the other hand, macrophages and lymphocytes are also present within white adipose tissue (WAT).[3–6] These immune cells belong to a WAT fraction called the stroma-vascular fraction (SVF). The SVF contains all of the cell types found in adipose tissue (endothelial cells, fibroblasts, preadipocytes, immune cells) except the lipid-laden adipocytes.

Several recent studies, in different mouse models of nutritional or genetic obesity, have demonstrated that the low-grade inflammation observed in obesity is associated with macrophage infiltration into the WAT, and is linked with the development of insulin resistance.[7–9] Macrophages have been observed in crown-like structures aggregating around adipocytes.[10,11] Furthermore, a correlation between adipocyte size or fat mass and macrophage marker expression has been found in adipose depots of different murine models of obesity.[9] In humans, body mass index and macrophage number in the SVF of WAT are correlated.[6,12,13] A reduction of macrophage amount in the subcutaneous adipose tissue of obese patients has also been associated with body weight reduction.[14]

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Accepted for publication November 20, 2008.

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Lymphocytes represent ~10% of SVF cells found in mouse EPI, or in inguinal subcutaneous fat depot (ING) when cleared from the LNs it contains.[5] Our previous results have demonstrated by flow cytometric analyses that, in EPI, lymphocytes display an ancestral immune system phenotype (up to 70% of all lymphocytes were natural killer (NK), $\gamma\delta^+$ T, and NKT cells), whereas the immune system presents more adaptive characteristics (high levels of $\alpha\beta^+$ T and B cells) in ING fat pads. Furthermore, an increase of T cells was reported in the fatter WAT of diet-induced obese mice.[15,16]

SSAO/VAP-1 (semicarbazide-sensitive amine oxidase/vascular adhesion protein-1) is abundantly present on adipocytes.[17] This membrane protein is encoded by the AOC3 gene (amine oxidase copper containing-3) and is also expressed on pericytes and vascular endothelium, where it is involved in leukocyte extravasation to inflamed tissues in different models.[18] SSAO/VAP-1 protein located on the endothelial cell surface is implicated in rolling, firm adhesion, and transmigration of different types of leukocytes through the endothelial cell barrier to penetrate into tissues.[19] Mice were recently created in which the AOC3 gene was genetically invalidated. They are devoid of VAP-1 protein, SSAO activity, and leukocyte extravasation to diverse sites of experimental inflammation is impaired.[19] Here we asked whether SSAO/VAP-1 could be involved in leukocyte infiltration in WAT since we recently observed that AOC3-KO mice are moderately overweight and have enlarged adipose tissues when compared with their age-matched wild-type controls.[20] We used wild-type, AOC3-deficient and newly generated fat cell-specific AOC-3 transgenic mice to study the infiltration of leukocytes into WAT. The following results indicate that SSAO/VAP-1 contributes to leukocyte extravasation at the level of adipose tissue because there were fewer immune cells in the WAT of mice invalidated for SSAO/VAP-1, despite their moderate overweight. Therefore, AOC3-deficient mice may serve as a new model of mild obesity, in which fat accumulation is not associated with increased inflammatory cell infiltration. Moreover, they may be valuable tools for future studies on the obesity-related development of diabetes.

Materials and Methods

Mice and in Vivo *Analyses*

Mice deficient in SSAO/VAP-1 were produced on a pure 129 background by replacing a portion of the first exon of the mouse AOC3 gene with a neomycin-resistance cassette.[19] AOC3-KO mice, homozygous for the null mutation, were backcrossed onto C57BL/6 for eight generations and compared with wild-type mice with a C57BL/6 background. The aP2hVAP-1TG/KO line was created by crossing the aP2hVAP-1 TG (FBV/n mice expressing human VAP-1 on adipocytes)[21] to AOC3-KO mice on C57BL/6 background for eight generations. The aP2hVAP-1 transgene, mouse VAP-1 mutant allele (null), and endogenous mouse VAP-1 allele (wt) were all identified by polymerase chain reaction (PCR) screening of purified genomic DNA with specific primers and verified immunohistochemically with human and mouse SSAO/VAP-1 antibodies and enzymatic assays (Amplex Red-based fluorometric detection) as previously described.[19] All of the mice used for this study were handled in accordance with the European Communities Council Directives for experimental animal care. They were housed under specific pathogen-free conditions with constant temperature (20 to 22°C) and with a 12-hour light-dark cycle. All mice had free access to food and water.

Glucose tolerance tests were performed on 5-hour-fasted conscious male mice (20 to 25 weeks of age) and glucose was measured on arterio-venous blood from tail vessels using an Accu-Chek glucometer from Roche Diagnostics (Meylan, France). To measure total body fat and lean mass by nuclear magnetic resonance, living female mice were placed into a Plexiglas tubular holder inserted into an Echo MRI NMR machine (100TM3; Echo Medical Systems, Houston, TX) and measured during a period of less than 1 minute. All other comparisons between genotypes were made on mice sacrificed after overnight fasting, at the age of 6 months, unless otherwise stated. Blood samples were immediately used for cell count analyses in an automated analyzer (MICROS-60; Horiba-ABX, Montpellier, France).

Tissue Dissection and Preparation of SVF Preparation

Epididymal (EPI) and inguinal (ING) WATs were dissected and visible blood vessels were removed. LNs were carefully isolated from ING adipose tissue and used for further analysis. Adipose tissues and LNs were immediately processed for cell isolation or frozen in liquid nitrogen and stored at −80°C until RNA extraction. Adipose tissues were digested in Dulbecco's modified Eagle's medium/F12 (1:1) medium (Invitrogen, Cergy-Pontoise, France) containing 2% bovine serum albumin and 2 mg/ml collagenase (type II collagenase; Sigma-Aldrich, St. Quentin Fallavier, France) for 30 minutes at 37°C. Digested tissues were filtered through a 25-μm mesh nylon membrane. After centrifugation (630 × *g* for 10 minutes), the pellet from adipose tissues contained a heterogeneous cell population called SVF. Buoyant adipocytes were discarded because their large cell size and fragility and were incompatible with cell sorter analyzer performances. Pelleted cells were washed in Dulbecco's modified Eagle's medium/F12 (1:1) containing 10% newborn calf serum (Sigma-Aldrich). Digested LNs were passed twice through a needle (0.8 mm) before washing. Isolated LNs and cells forming SVF were suspended in Dulbecco's modified Eagle's medium F12 10% newborn calf serum. Red cells were lysed in a buffer containing 155 mmol/L ammonium chloride, 20 mmol/L Tris, pH 7.6, for 5 minutes. Cells were then centrifuged (560 × *g* for 5 minutes) and resuspended in supernatant from hybridoma-secreting murine monoclonal CD16/32 antibodies (clone 2-4G-2) to block nonspecific Fc receptors before counting on a Coulter counter (Beckman-Coulter, Roissy, France).

Flow Cytometric Analyses

Staining of lymphocytes and macrophages were performed as previously described.[5,22] Briefly, cells were incubated with conjugated anti-mouse monoclonal antibodies for 20 minutes at room temperature in the dark. Fluorescein isothiocyanate-conjugated anti-CD3 antibodies, phycoerythrin-conjugated NK1.1 and CD115 antibodies, peridinin-chlorophyll-protein complex-conjugated anti-CD45 antibodies, and allophycocyanin-conjugated anti-CD11b monoclonal antibodies were purchased from Becton Dickinson (Le Pont-de-Claix, France). Phycoerythrin-conjugated anti-CD19 monoclonal antibody was obtained from Immunotech (Marseille, France). Cells were then washed in phosphate-buffered saline and analyzed on a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson). Subpopulations and total lymphocytes were identified by appropriate marker combinations and light scatter properties. Briefly, the combination of phycoerythrin-conjugated anti-NK1.1 and fluorescein isothiocyanate-conjugated anti-CD3 allowed differentiation of three cell populations: $NK1.1^{-}$ $CD3^{+}$, T cells; $NK1.1^{+}CD3^{-}$, NK cells; and $NK1.1^{+}CD3^{+}$, NKT cells. Anti-CD19 was used to sort B cells and CD11b, CD115 to differentiate subsets of macrophages. Data acquisition and analysis were performed with Cell Quest software (Becton Dickinson).

RNA Extraction, Reverse Transcription, and Real-Time PCR

Four hundred mg of ING WAT were homogenized in 2 ml of a highly denaturing guanidine-thiocyanate-containing buffer supplied by the RNeasy mini kit (Qiagen, Courtaboeuf, France) plus 20 μl of β-mercaptoethanol. After lipid elimination by chloroform extraction, total RNA was extracted according to the supplier's instructions. Then, 0.5 μg of total RNA was reverse-transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). A reaction was performed in parallel without reverse transcriptase (RT−) to estimate genomic DNA contamination. Real-time PCR was performed starting with 6.25 ng of cDNA and both sense and antisense oligonucleotides in a final volume of 20 μl using the SYBR Green TaqMan Universal PCR Mastermix (Eurogentec, Angers, France). Fluorescence was monitored in GeneAmp 7500 detection system instrument (Applied Biosystems, Foster City, CA). Oligonucleotide primers were designed using Primer Express (Perkin-Elmer Life Sciences, Courtaboeuf, France) and verified on Blast Nucleotide software. Primer specificity was checked by the occurrence of a single peak of expected size during dissociation experiments. Analysis of the 18S ribosomal RNA was performed using the Ribosomal RNA Control TaqMan assay kit (Applied Biosystems) to normalize gene expression. Results were expressed as arbitrary units relative to 18S expression, as already reported[23] using the following equation: $2^{(Ct18S\ -\ Ct\ gene\ "RT+")} \times [1\ -\ 1/(2^{(Ct\ gene\ "RT-"\ -\ Ct\ gene\ "RT+")})]$, where Ct corresponds to the number of cycles needed to generate a fluorescence threshold.

Immunohistochemistry

For immunohistological analysis, WAT was fixed in zinc buffer and paraffin-embedded sections were prepared. The sections were first incubated with 1 μg/ml of rat anti-mouse CD45, rat anti-VAP-1 (TK10-79 mAb recognizing both human and mouse VAP-1) or class-matched negative control antibody followed by biotinylated anti-rat IgG (Vector Laboratories, Peterborough, UK) in 5% normal mouse serum and streptavidin horseradish peroxidase (Vector Laboratories). 3,3′-Diaminobenzidine was used as a chromogen. The sections were counterstained with hematoxylin. For immunofluorescence stainings the frozen sections were fixed with acetone and stained with monoclonal antibodies recognizing mouse VAP-1 (clone 7-106),[24] human VAP-1 (JG2.10),[21] and a negative control antibody followed by fluorescein isothiocyanate anti-rat IgG (Sigma, St. Louis, MO) containing 5% normal mouse serum.

Statistics

Statistical significance was assessed by the use of nonparametric Kruskal-Wallis one-way analysis for the nonnormal data of flow cytometry, which are reported as mean ± SD. For all other parameters, means are given ± SEM and Student's *t*-test was used to compare genotypes. A P value of less than 0.05 was considered significant for all tests; n denotes the number of cases.

Results

AOC3-KO Mice Have Enlarged Fat Depots

The body weights of 28-week-old males were significantly higher for AOC3-KO mice than that for wild-type C57BL/6 mice (KO: 37.4 ± 1.2 g, $n = 15$; and wild-type: 32.4 ± 0.8 g, $n = 12$; $P < 0.001$). An increase in epididymal (EPI) and inguinal (ING) WAT weight was found in male AOC3-KO mice when compared with wild-type mice (Table 1). In ING, the enlarged tissue was characterized by a higher level of lipid content (KO: 728 ± 12 mg lipid/g tissue versus wild-type: 661 ± 18 mg lipid/g tissue, $n = 8$; $P < 0.01$) and a reduced protein content (KO: 13.1 ± 1.6 mg protein/g tissue versus wild-type: 19.3 ± 1.6 mg protein/g tissue, $n = 8$; $P < 0.05$), as generally observed in most forms of murine obesities.[25] As a consequence, the lipid-to-protein ratio was significantly increased in ING depot of AOC3-KO mice (58.3 ± 5.0 versus 35.9 ± 2.9 for KO and wild-type mice, respectively; $n = 8$, $P < 0.02$). Although fatter, the AOC3-KO mice were normoglycemic (fasting blood glucose was 108 ± 5 mg/dl, ie, not significantly different from wild type: 98 ± 5 mg/dl, $n = 12$) and did not exhibit alteration of glucose tolerance (area under the curve of the hyperglycemic response to glucose challenge (1 g/kg, i.p.) was 4379 ± 575 and 4920 ± 157 arbitrary units in KO

Table 1. Adipose Depot Weight and CD45-Positive Cells in Stroma-Vascular Fraction of Wild-Type (WT) or AOC3-KO Male Mice

	WT		AOC3-KO	
	Weight (g)	$CD45^+$ (cells/g)	Weight (g)	$CD45^+$ (cells/g)
EPI WAT	1.04 ± 0.12	416,182 ± 105,539	1.44 ± 0.08*	79,166 ± 11,074*
ING WAT	1.05 ± 0.12	566,346 ± 124,636	1.85 ± 0.20†	183,490 ± 26,915*

The number of $CD45^+$ cells was expressed per g of tissue. Means ± SEM of 5 to 15 control (WT) and 4 to 12 AOC3-KO mice.
*$P < 0.05$ and †$P < 0.001$ for the difference between WT and AOC3-KO mice.

and wild type, respectively; $n = 12$). Accordingly, we have previously reported that the dose-response curves to insulin stimulation of glucose transport are superimposable in adipocytes isolated from KO and wild-type mice.[20]

AOC3-KO Mice Contain Reduced Numbers of Leukocytes within Adipose Tissues

Among the cells that constitute the SVF of WAT (endothelial cells, fibroblasts, leukocytes, preadipocytes), we focused on leukocytes (CD45-positive cells) and compared them to the cell population (mainly lymphocytes) of the inguinal LN as a reference immune organ. CD45-positive cells ($CD45^+$) were analyzed by flow cytometry according to their size and granulosity. As shown in Figure 1A, $CD45^+$ cells present in SVF exhibited heterogeneous size and granulosity whereas $CD45^+$ cells from LN were more homogeneous. In control mice, cells expressing CD45 represented 39 ± 6% and 56 ± 7% of the SVF cells counted in EPI and ING fat tissues, respectively (Figure 1B). These proportions were strongly reduced in AOC3-KO male mice because $CD45^+$ cells represented only 9 ± 1% and 16 ± 2% of SVF cells in EPI and ING. By contrast, in both genotypes, the leukocytes accounted for more than 96% of the cells found in LN (Figure 1B). The reduction of the abundance of $CD45^+$ cells in WAT from AOC3-KO mice was also marked when results were expressed as number of cells per g of tissue, as shown in Table 1. Among SVF cells, two CD45-positive populations with different fluorescence intensities for CD45 could be distinguished (Figure 1C). This allowed us to define two regions, R1 and R2, corresponding, respectively, to CD45-high and CD45-low populations. As previously described, gate R1 was defined according to size and granulosity of cells present in LN and mainly contains lymphocytes.[5] Macrophages are present in the gate R2.[22] The two populations were found in all adipose depots from wild-type or AOC3-KO mice. Noteworthy, the proportions of both lymphocytes (R1) and macrophages (R2) were strongly reduced in the SVF of ING and EPI WAT of AOC3-KO male mice (Figure 1C). In whole blood, the white blood cell number remained unchanged between wild-type and AOC3-KO (6.4 ± 0.6 and 5.8 ± 0.8 10^3 cells/μl, respectively; $n = 8$).

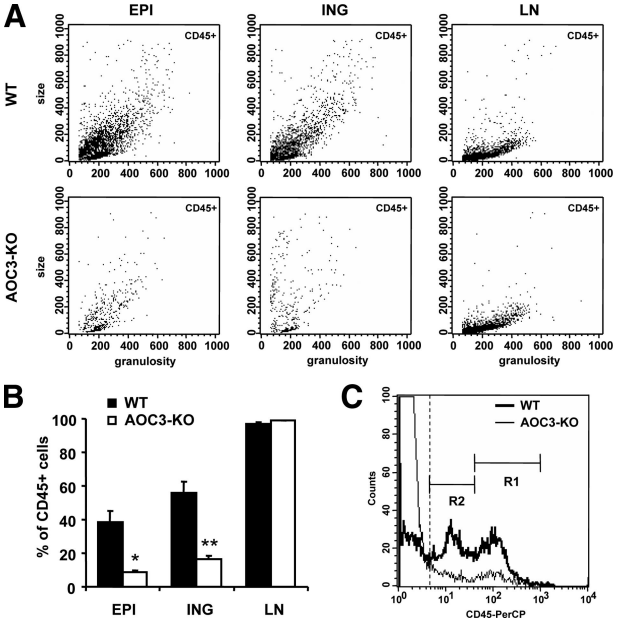

Figure 1. Flow cytometric analysis of SVF and LN lymphocytes from adipose tissue of wild-type (WT) or AOC3-KO mice**. A:** Representative profiles of $CD45^+$ cell populations according to cell size and granulosity criteria in SVF from epididymal (EPI) and inguinal (ING) adipose tissues, or LNs contained in inguinal adipose tissue (LN) from 28-week-old wild-type or AOC3-KO mice. **B:** Percentage of $CD45^+$ cells contained in total SVF or LNs. Means ± SD of nine wild-type and four AOC3-KO mice. Different from control (wild-type) group at *$P < 0.05$, **$P < 0.01$. **C:** Representative flow cytograms of cells labeled with anti-CD45 antibody in EPI SVF from wild-type and AOC3-KO mice. Vertical dotted line separates nonspecific signal from higher intensity labeling. Bold curve represents fluorescence intensity in a preparation from a wild-type mouse, thin curve from an AOC3-KO mouse, determined under identical conditions. R1 delimits the lymphocyte gate and R2 the macrophage gate taken into account for further analyses. Similar flow cytograms were observed on ING SVF.

Reduced Content of Leukocytes within Adipose Tissue of Overweight AOC3-KO Female Mice

A small but significant weight increase also occurred in AOC3-KO female mice because the body mass was 21.6 ± 0.6 g and 24.6 ± 1.2 g in 6-month-old wild-type and AOC3-KO, respectively ($n = 11$ and 9, $P < 0.05$). All of the dissected AOC3-KO fat depots tended to be heavier although significance was not reached in any, eg, 0.25 ± 0.05 g versus 0.59 ± 0.15 g for parametrial WAT or 0.29 ± 0.04 g versus 0.61 ± 0.18 g for ING ($n = 11$ and 9). Body composition measurements using nuclear magnetic resonance indicated that total fat mass increased from 8.9 ± 1.0% in wild-type to 11.4 ± 0.7% in AOC3-KO when expressed as percentage of body mass ($n = 11$ and 17, $P < 0.05$). A reduction of the abundance of $CD45^+$ cells also occurred in WAT from SSAO/VAP-1-deficient female mice, decreasing from 42 ± 2% in wild-type to 15 ± 4% in AOC3-KO. A decrease of lymphocyte abundance (R1) was detected in ING of 9-week-old AOC3-KO females (12 ± 1% versus 39 ± 2% in age-

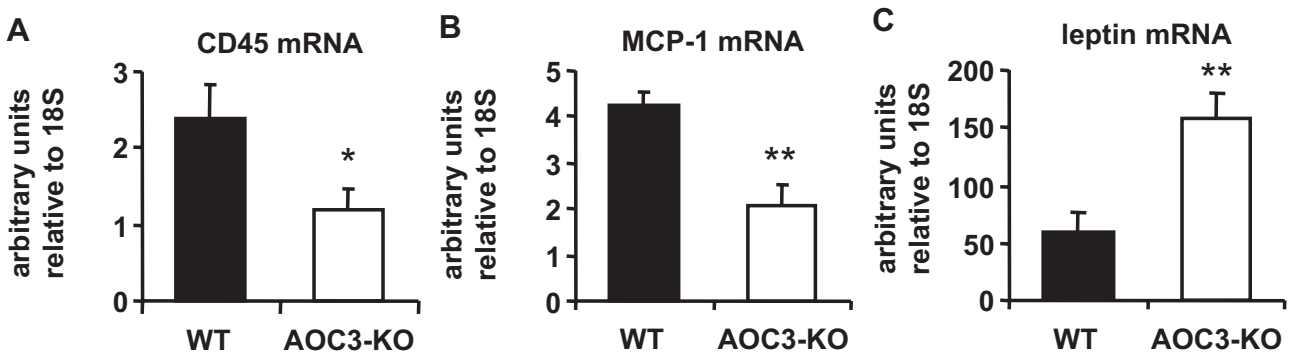

Figure 2. Decreased CD45 and MCP-1 expression and increased leptin mRNA abundance in ING WAT from AOC3-KO mice. mRNA levels were compared between control (wild-type) and AOC3-KO mice by real-time PCR in subcutaneous adipose tissue. **A:** CD34. **B:** MCP-1. **C:** Leptin. Means ± SEM of arbitrary units obtained from five to seven wild-type (WT) and six to eight AOC3-KO mice as detailed in the Materials and Methods. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus WT mice.

matched control; $n = 6$, $P < 0.001$). Taken together, these observations indicated that a defect in leukocyte presence occurred in WAT of AOC3-KO mice independent of age or gender.

CD45 and MCP-1 Expressions Are Decreased and Leptin Expression Is Increased in the Subcutaneous Adipose Tissue of AOC3-KO Mice

A complete lack of AOC3 expression was found in the ING WAT by real-time PCR experiments (not shown), therefore confirming the total disruption of the gene encoding SSAO/VAP-1. A reduction of CD45 expression was observed in the mRNAs prepared from ING adipose tissue of 28-week-old male AOC3-KO mice (Figure 2A). This was in accordance with the reduction of leukocyte number in adipose depot SVF reported above. The expression of the chemoattractant protein MCP-1 was reduced by 51% in the subcutaneous adipose tissue of AOC3-KO mice, when compared with wild-type mice (Figure 2B). The expression of leptin, an adipokine known to possess pro-inflammatory action exhibited a clear increase in ING WAT of AOC3-KO mice (Figure 2C). This increased leptin expression is in agreement with the enhanced fat deposition found in these mice. The fact that AOC3-KO mice demonstrated reduced leukocyte infiltration suggests that the adhesion defects in the absence of SSAO/VAP-1 dominate over the recognized pro-inflammatory functions of leptin.

Respective Role of Adipocyte and Vascular SSAO/VAP1 for the Presence of Leukocytes in WAT

The reduction of leukocyte presence in adipose tissue of AOC3-KO mice could be linked to a reduction in their extravasation from blood to tissue. This mechanism seems to be the consequence of the lack of vascular SSAO/VAP-1, known to be involved in the adhesion of circulating leukocytes. Alternatively, the lack of adipose SSAO/VAP-1 could also influence the presence of leukocytes in adipose tissue via changes in chemotactic signals released by adipocytes, as indicated by the reduced MCP-1 mRNA levels in ING of AOC3-KO mice.

To directly test whether lack of AOC3 in adipocytes or vasculature was responsible for the reduced leukocyte number in fat tissue despite the mild obesity, we created mice expressing human AOC3 only on adipocytes on the KO background as shown in Figure 3 by immunostaining with monoclonal antibodies selective for the mouse[24] and human[21] forms of SSAO/VAP-1. The transgene in these mice was enzymatically active because adipose tissue revealed SSAO activity of 1452 ± 171 pmol/hour/mg protein ($n = 6$), whereas the AOC3-KO mice completely lacked the activity. In these studies we analyzed mice 24 to 33 weeks of age. There were no statistically significant differences either in the number of adipocytes or in the number of $CD45^{+}$ cells in WAT between the genotypes (Figure 4, A–C). This suggests that lack of AOC3 in the vasculature is behind the reduced number of leukocytes in fat of AOC3-KO mice.

It must be emphasized that the observed reduction of leukocyte content in adipose tissue takes place in overweight mice. This contrasts with the widely recognized increase of macrophage and lymphocyte presence within adipose tissue in murine[7–9,16] and human obesi-

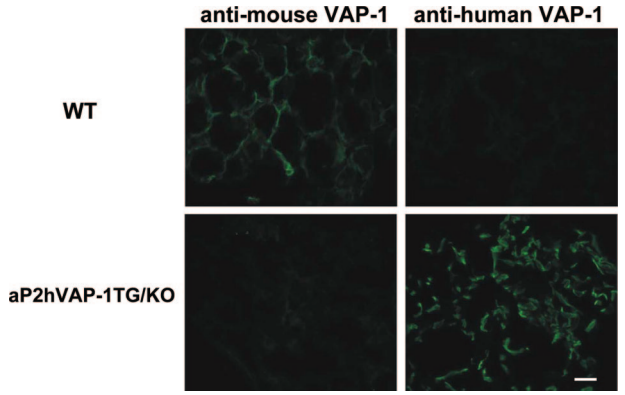

Figure 3. aP2hVAP-1TG/KO mice express human AOC3 but lack endogenous murine AOC3 in adipose tissue. Murine and human AOC3 are detected by species-specific antibodies recognizing mouse (7-106) and human VAP-1 (JG2.10). Stainings of wild-type (WT) adipose tissue are shown as controls. Scale bar = 50 μm.

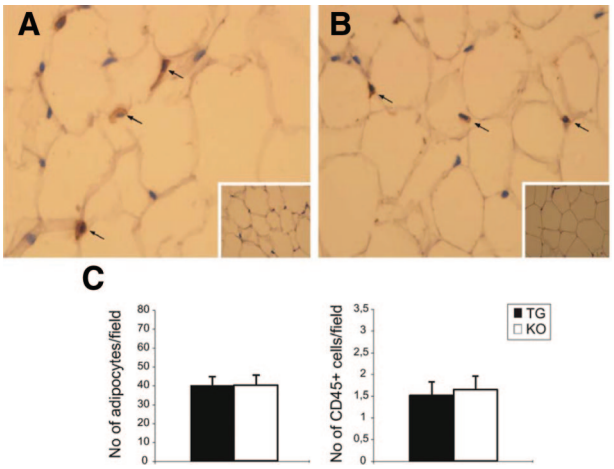

Figure 4. Endothelial AOC3 rather than adipose AOC3 is responsible for reduced leukocyte number in adipose tissue of AOC3-KO mice. Examples of the scarce expression of CD45-positive cells (**arrows**) in AOC3-KO mice (**A**) and aP2hVAP-1TG/KO mice (**B**), stainings with negative control antibodies are shown in four fold reduced insets. Original magnifications × 400. **C:** Number of adipocytes and $CD45^+$ cells in sections of paraffin-embedded and zinc-fixed adipose tissue from aP2hVAP-1TG/KO and AOC3-KO mice. Mean ± SEM of counts in high-power field ($n = 5$ in each group).

ty[6,12] and suggests that leukocyte infiltration into the adipose tissue is not directly linked to obesity as such.

Phenotype of Lymphocytes and Macrophages in the Enlarged WAT of AOC3-KO Mice

Several cell surface marker combinations were then used to distinguish the lymphocyte subtypes and to delineate by flow cytometric analysis the activation state of leukocytes remaining in the WAT of AOC3-KO mice. CD19 is a B-cell-specific antigen receptor accessory molecule. CD3 is expressed by all T cells and is associated with the T-cell antigen receptor. The surface receptor NK1.1 is expressed by NK lymphocytes. The combination of these markers allowed us to differentiate T non-NKT ($CD3^+$, $NK1.1^-$), NK ($CD3^-$, $NK1.1^+$), and NKT ($CD3^+$, $NK1.1^+$) cells. Macrophages were characterized by the expression of the specific marker CD11b, or by CD115, a receptor for the chemokine M-CSF. Despite the strong decrease in $CD45^+$ cell number found in the WAT of AOC3-KO mice, an attempt to analyze the phenotype of the remaining lymphocytes and macrophages in SVF of EPI and ING WAT was conducted by expressing results as percentages of $CD45^+$ cells present in the previously defined R1 or R2 fractions. The respective proportion of each subset of immune cells could therefore be compared between wild-type and AOC3 KO mice without influence of the reduction of leukocyte number (Figure 5, A and B).

Regarding lymphocytes, the T-cell subpopulation was the predominant cell type in both adipose depots and its proportion relative to the total lymphocyte population was not significantly changed in AOC3-KO male mice. A slight increase in the proportion of B lymphocytes was observed in EPI of AOC3-KO mice whereas this proportion (representing ~30% of the lymphocytes) remained unchanged in ING (Figure 5, A and B). In EPI, there was

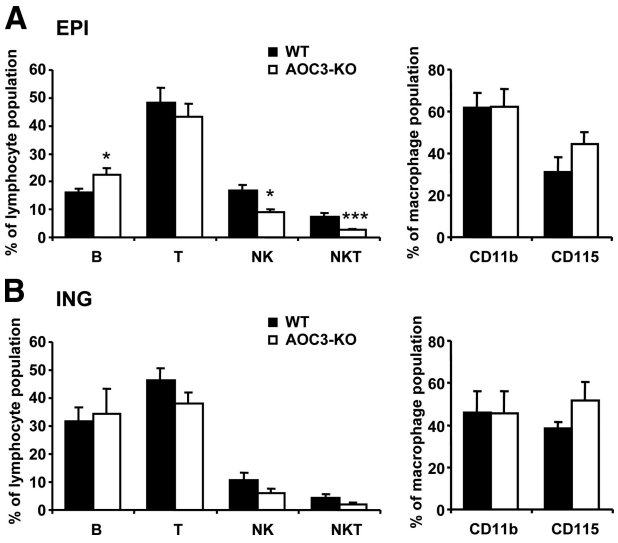

Figure 5. Flow cytometric analysis of different lymphocyte and macrophage populations in EPI (**A**) and ING (**B**) adipose tissues of wild-type (WT) and AOC3-KO mice. Subclasses of lymphocytes represented in the **left** panel were determined by staining with a combination of antibodies to differentiate B ($CD19^+$), T non-NKT ($CD3^+$, $NK1.1^-$), NK ($CD3^-$, $NK1.1^+$), and NKT ($CD3^+$, $NK1.1^+$) cells. Results are expressed as percentage of lymphocyte population ($CD45^+$ of R1 gate). Means ± SD of nine wild-type and four AOC3-KO mice. Subclasses of macrophages represented in the **right** panel were determined by the presence of antigens recognized by anti-CD11b or anti-CD115 antibodies. Results as percentage of macrophage population ($CD45^+$ of R2 gate). Means ± SD of four wild-type and four AOC3-KO mice. Different from wild-type group at $^*P < 0.05$, $^{***}P < 0.001$.

a clear reduction of the proportion of NK and NKT cells in AOC3-KO (both proportions decreased by approximately one half) (Figure 5A). In ING of AOC3-KO mice, the subpopulations of NK and NKT cells also tended to be lower (Figure 5B), but their respective reductions did not reach statistical significance. Despite all these changes in the relative proportions of lymphocyte subtypes, the results indicated that a reduction in the number of T cells was the major cause of the overall decrease in $CD45^+$ cells found in fat depots of AOC3-KO mice. Regarding the macrophages remaining in SVF from EPI- and ING-enlarged fat depots of AOC3-KO mice, their phenotype did not appear to be modified because the proportions of cells expressing $CD11b^+$ or CD115 remained unchanged when compared with wild-type.

Discussion

Taken together, our results clearly demonstrate a reduced proportion of the infiltrated or resident immune cells in the adipose depots of AOC3-KO mice, despite their increased body and adipose tissue weights. The reduction of macrophage and lymphocyte number could be explained by the known role of vascular SSAO/VAP-1 in leukocyte extravasation. Therefore, AOC3 gene invalidation results in a loss of SSAO/VAP-1 that leads to the generation of a unique model of mild obesity that lacks low-grade inflammation in the adipose tissue.

The reduction of immune cells in the WAT of AOC3 KO mice occurred without modification of leukocyte content in inguinal LNs, and without change in the white blood

cell number. This suggested that, as previously stated for this model,[19] invalidation of SSAO/VAP-1 impaired the extravasation of leukocytes rather than immune lineage maturation. The complete lack of AOC3 mRNA and the absence of SSAO activity or VAP-1 immunoreactivity found in adipose tissue confirmed the total invalidation for SSAO/VAP-1 already reported in AOC3-KO mice.[19,20] Our present observations also confirm the overweight status already observed in this model.[20] The mechanism underlying the enlargement of fat stores in AOC3-KO mice remains unknown, but intriguingly recalls the obese phenotype observed in mice invalidated for other adhesion molecules such as ICAM-1 and Mac-1.[26] However, the strong reduction in leukocyte presence in adipose depots reported in AOC3-KO mice hardly can be considered as directly responsible for increased fat accretion since it has been stated that obesity is associated with increased accumulation of lymphocytes and macrophages in WAT.[15,16] Moreover, the decline in leukocyte abundance resulted from a reduction of both lymphocytes and macrophages, the two cell populations of the immune system mainly constituting $CD45^+$ cells present in SVF whereas, in WAT, it is essentially an increase of macrophages that has been repeatedly reported to be involved in obesity-related insulin resistance.[9,27] Indeed, the AOC3-KO mouse model reveals that fat accretion can occur without any increase of leukocyte number in WAT. Thus, fat store hypertrophy is not sufficient to trigger leukocyte infiltration in WAT in the absence of SSAO/VAP-1.

The reduced proportion of $CD45^+$ cells sorted from stroma-vascular preparations of AOC3-KO adipose depots was in accordance with the reduction of CD45 abundance in mRNAs prepared from ING and with the alteration of $CD45^+$ cell presence in WAT sections of AOC3-KO mice. Because SSAO/VAP-1 is highly expressed in adipocytes, and because this ectoenzyme is involved in lymphocyte adhesion,[19] its deletion could have disabled the direct binding of leukocytes to fat cells. Alternatively, SSAO/VAP-1 deficiency could have modified chemokine expression by adipocytes themselves, leading to a lower presence of chemoattractive signals[28] rather than impairing vascular SSAO/VAP-1 and leukocyte extravasation.

The use of aP2hVAP-1TG/KO transgenic mice allowed to re-establish a targeted SSAO/VAP-1 activity in adipocytes, but was insufficient to increase the proportion of $CD45^+$ cells in WAT. The reduction of leukocytes within the adipose tissue of AOC3-KO mice therefore appeared linked to a reduction in their extravasation from blood to tissue. This mechanism may be the consequence of the lack of vascular SSAO/VAP-1, known to be involved in leukocyte adhesion.[18]

Nevertheless, it cannot be excluded that alteration of adipocyte SSAO/VAP-1 could also participate in the presence of leukocytes in adipose tissue via changes in chemotactic signals released by adipocytes, as indicated by the reduced monocyte chemoattractant protein-1 (MCP-1) mRNA levels in ING of AOC3-KO mice. However, MCP-1, which is able to attract monocytes and other immune cells such as T and NK cells,[29,30] is not only abundantly expressed by adipocytes[31–34] but could also be produced directly by SVF cells.[8] In addition, it has been reported that medium conditioned by macrophages induces MCP-1 secretion by adipocytes, increasing monocyte adhesion to adipocytes and maintaining inflammation.[35,36] Therefore, it was difficult to conclude whether the decrease in MCP-1 mRNA levels was a cause or a consequence of decreased leukocyte infiltration in adipose tissue of AOC3-KO mice.

The enhanced fat deposition of AOC3-KO mice was associated with an increase in leptin expression. This well-known adipokine exhibits proinflammatory properties[37,38] and should have participated in increasing leukocyte infiltration in adipose tissue. Despite that, a decrease of leukocyte abundance was fascinatingly observed in the fat depots of AOC3-KO mice therefore evidencing that SSAO/VAP-1 plays a pivotal role in leukocyte recruitment.

Our analysis showed that two lymphocyte subpopulations, NK and NKT cells, belonging to ancestral immunity and specifically present in adipose tissue and liver, were affected in AOC3-KO mice. The reduced NK cell distribution in EPI of AOC3-KO mice is in accordance with the previous results obtained from high-fat diet fed overweight mice.[15] However, a difference between the two models appeared regarding NKT cells, the proportion of which was clearly diminished in AOC3-KO mice, whereas no change was reported for adipose depots of high-fat fed mice.[15,16] Another contrast between the two models is that the number of T cells increased in the EPI under diet-induced obesity,[15] but was reduced in AOC3-KO mice. Definitively, the net reduction in the number of leukocytes in the vicinity of adipocytes of AOC3-KO mice, and the modification of lymphocyte phenotype are totally different from the changes in the immune cell subpopulations observed in adipose depots of diet-induced obese rodents.[5,15] Whether these differences in the inflammation state of adipose depots are related to the differences observed in insulin responsiveness (unaltered in KO-AOC3 mice and impaired by HFD) appears likely but remains to be definitively established.

In the literature, the link between SSAO/VAP-1 and obesity or diabetes is essentially explored by clinical studies focusing on a truncated, soluble form of the protein circulating in blood. It is widely recognized that this plasma SSAO/VAP-1 increases with type 1 or 2 diabetes[39] but does not increase with body mass index in nondiabetic patients.[40,41] It has been demonstrated that both vascular and adipose tissues are sources of the soluble form of SSAO/VAP-1,[21] the function of which remains obscure and may probably be deleterious for endothelial integrity. Here, the late occurrence of a modest obesity in SSAO-deficient mice cannot be explained by the currently known functions of SSAO/VAP-1 in adipocytes, because this enzyme, once activated by its substrates, reproduces insulin effects such as lipogenesis activation and lipolysis inhibition.[17] The fact that the modest obesity of AOC3-KO mice is not associated with glucose intolerance completely agrees with the deleterious role of adipose tissue low-grade inflammation reported to link obesity and metabolic syndrome.[8,35] Lastly, the invalidation of SSAO/VAP-1 results in substantial changes in the leukocyte presence and phenotype

within WAT that perfectly fit with the role of vascular SSAO/VAP-1 in white blood cell extravasation. Although SSAO/VAP1 is only one among the numerous adhesion molecules involved in leukocyte diapedesis, we propose that SSAO/VAP1 plays a pivotal role this phenomenon in WAT and deserves to be further considered in studies investigating the onset of obesity and its diabetic complications.

Acknowledgments

We thank Estelle Wanecq, Sandra Grès, Suvi Nevalainen, and Katriina Salmivirta for their technical assistance and advice; and the animal facilities in Toulouse (IFR BMT) and Turku.

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