Distinct Roles of Endothelial and Adipocyte Caveolin-1 in Macrophage Infiltration and Adipose Tissue Metabolic Activity

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OBJECTIVE—Defective caveolin-1 expression is now recognized as a cause of lipoatrophic diabetes in patients, due to primary caveolin gene mutations or secondary caveolin deficiency caused by *PTRF/cavin* gene defects. The goal of this study was to establish the relative contribution of endothelial cells and adipocytes, both highly expressing caveolin-1 to the lipoatrophic phenotype of mice with global *caveolin-1* gene invalidation (Cav1-KO).

RESEARCH DESIGN AND METHODS—We compared adipose tissue development and metabolic phenotype of wild-type (WT), lipoatrophic Cav1-KO, and a murine model with specific rescue of caveolin-1 expression in endothelial cells (caveolin-1-reconstituted [Cav1-RC]).

RESULTS—Defective adipose tissue development, reduced adipocyte size, and global alteration in adipose tissue gene expression that characterize lipoatrophic caveolin-1 null mice were still observed in Cav1-RC, indicating a prominent role of adipocyte-derived caveolin in lipoatrophy. We also observed that Cav1-KO adipose tissue contained an increased proportion of infiltrated macrophages compared with control mice, mostly with an alternate activation M2 phenotype. In contrast with defective lipid storage and lipoatrophy, macrophage infiltration was normalized in Cav1-RC mice, pointing to caveolin-1-dependent endothelium permeability as the causing factor for adipose tissue macrophage infiltration in this model.

CONCLUSIONS—This is the first report of a specific role for adipocyte caveolin expression in lipid storage. Our study also shows that endothelium caveolin critically participates in the control of macrophage extravasation from the blood into adipose tissue, therefore establishing distinct roles depending on topology of caveolin expression in different cell types of adipose tissue. ***Diabetes*** **60:448–453, 2011**

Lipoatrophic diabetes is primarily a result of defective adipose tissue lipid storage resulting in severe dyslipidemia and insulin resistance. Multiple alterations can cause lipoatrophic diabetes, and rare single gene defects have been identified in patients (1). The recent discovery of mutations in the human *caveolin-1* gene in Berardinelli-Seip congenital lipodystrophy, and mutations in polymerase I and transcript release factor (PTRF)/cavin causing secondary deficiency of caveolins and generalized lipodystrophy, brings into focus caveolin proteins in the pathogeny of lipoatrophic diabetes in humans (2,3).

Caveolins form a conserved family of membrane-associated proteins generated from three genes with tissue-specific expression. Caveolin-1 and -2 are coexpressed and mostly abundant in endothelial cells, adipocytes, and type II pneumocytes, whereas caveolin-3 is restricted to muscle. A striking trait of caveolins is to induce the formation of caveolae, small (50–100 nm) invaginated pits on the cell surface. Caveolae represent well-defined membrane subdomains with specific lipid composition closely related to lipid rafts, coated with oligomerized caveolins on their cytoplasmic face (4). It is no longer accepted that caveolins are the only specific proteins required for caveolae formation since an additional group of structurally related proteins, called cavins, was recently identified (5–7). Despite this recent progress, precise roles of caveolae still remain unclear. Furthermore, it is not well established whether caveolins can exert functions outside of caveolae.

Murine models with targeted gene invalidation for *caveolin-1* and *PTRF/cavin* are available, and almost recapitulate the human phenotype, developing a lipoatrophic syndrome with only residual fat at higher ages, hyperlipidemia, and glucose intolerance (6,8). However, phenotypic alterations in caveolin-1 null mice appear more severe than that observed in patients, since they include additional vascular abnormalities. Exaggerated vascular relaxation, related to increased endothelial nitric oxide synthase activity, that deteriorates toward pulmonary dysfunction and cardiac hypertrophy has been described in caveolin-1 null mice (9,10). Noteworthy, all these alterations are rescued by cell-specific re-expression of caveolin-1 in endothelium, underlying a prominent role for this protein in the physiology of endothelial cells (11).

The question of whether lipoatrophy could be related to disrupted endothelial and/or adipocyte caveolin expression is presently unanswered. Adipocytes as well as endothelial cells highly express caveolin-1, and a close interconnection with vascular network is of crucial importance for adipose tissue metabolic function, particularly nutrient storage. In this study, we took advantage of a murine model with tissue-specific caveolin deficiency to dissect the roles of caveolin in fat and endothelium and their respective contribution to the lipoatrophic phenotype seen in the total absence of caveolin.

We show that a lack of caveolin in adipocytes but not in endothelial cells accounts for lipoatrophy. Interestingly, we also observed that endothelial caveolin regulate macrophage infiltration and extravasation of immune cells into adipose tissue, thus unraveling distinct roles of endothelial and adipocyte caveolins.

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RESEARCH DESIGN AND METHODS

Animal studies were conducted according to French guidelines. Caveolin-1 reconstituted (Cav1-RC) mice (11) were generated on the caveolin-1 KO genetic background (10). Mice were killed at 6 months of age by cervical dislocation. Serum cytokines and insulin were measured using a milliplex kit (Millipore).

Adipose tissues were processed for immunohistochemistry as described (12). For immunohistofluorescence, adipose tissue sections were incubated in antigen retrieval solution (Dako) and perilipin antibody (Progen) in PBS 2% FCS and 1% BSA. Fluorescein isothiocyanate (FITC)-coupled guinea pig antibody (JacksonImmunoresearch) was used, and slides were mounted in Fluoromount-G (SouthernBiotech).

Adipose and stroma-vascular fractions were isolated as described previously (13). Briefly, fat depots were digested in Krebs-Ringer bicarbonate (pH 7.4) containing 1% BSA and 1 mg/mL collagenase A (Roche). After filtration (180-μm nylon mesh), adipocyte fraction was washed twice in DMEM (GIBCO, Invitrogen). Adipocyte diameters were measured using Perfect Image 6.10 (Clara Vision).

Total RNA was extracted from frozen adipose tissue by cesium chloride centrifugation and analyzed by real-time PCR using MyiQ real-time thermal cycler (Bio-Rad) with Mesa green qPCR kit (Eurogentec) as described previously (14). Melt curve analysis was conducted to check single products, and 18 S mRNA was used for normalization.

mRNA preparations (six mice/group) were used to obtain labeled cRNA and hybridized on Agilent microarrays at the Functional Genomics Center of Zurich (ETH, Zurich). Expression values were imported into Gene-spring 7.3 (Agilent Technologies).

Results are presented as means ± SE. The data were statistically analyzed by ANOVA or Student *t* test.

RESULTS

Caveolin rescue in endothelium does not reverse lipoatrophy. It is not known whether the lipoatrophic phenotype described in Cav1-KO mice (11), and also found in human patients with caveolin deficiency (2,3), can be alleviated by endothelium caveolin re-expression. In murine adipose tissue extracts, caveolin is expressed in both mature adipocyte and endothelial containing stroma-vascular cell fractions, whereas it is found exclusively in the stroma-vascular fraction in endothelium-specific Cav1-RC mice (Fig. 1*A*). Caveolin-1 distribution in adipose tissue sections indicated Cav1 labeling of both blood vessel endothelium and surrounding adipocytes in wild-type (WT) mice and a complete absence of signal in Cav1-KO adipose tissue. As expected, Cav1 labeling was restricted to blood vessels and absent from adipocytes in Cav1-RC (Fig. 1*B*). Blood levels of glucose, insulin, and free fatty acids were not different between Cav1-KO and Cav1-RC mice (Fig. 1*C*), and the three groups of mice have comparable body weight at 6 months of age (data not shown). However, subcutaneous and perigenital fat in Cav1-KO and Cav1-RC is drastically decreased compared with controls, in both males and females (Fig. 2*A*). Poor adipose tissue development in Cav1-KO and Cav1-RC is associated with a decreased size of adipocytes on adipose tissue sections (Fig. 2*B*), confirmed by direct measurement of adipocyte diameters after collagenase digestion of fat pads (Fig. 2*C*). Thus persistent reduction of adipocyte fat storage in Cav1-RC and Cav1-KO mice compared with WT controls indicates that specific endothelium caveolin-1 rescue is ineffective to normalize adipose tissue growth.

Lipoatrophic phenotype in caveolin-1 null mice is therefore linked to specific failure within adipocytes. To further investigate adipocyte function, we assess global

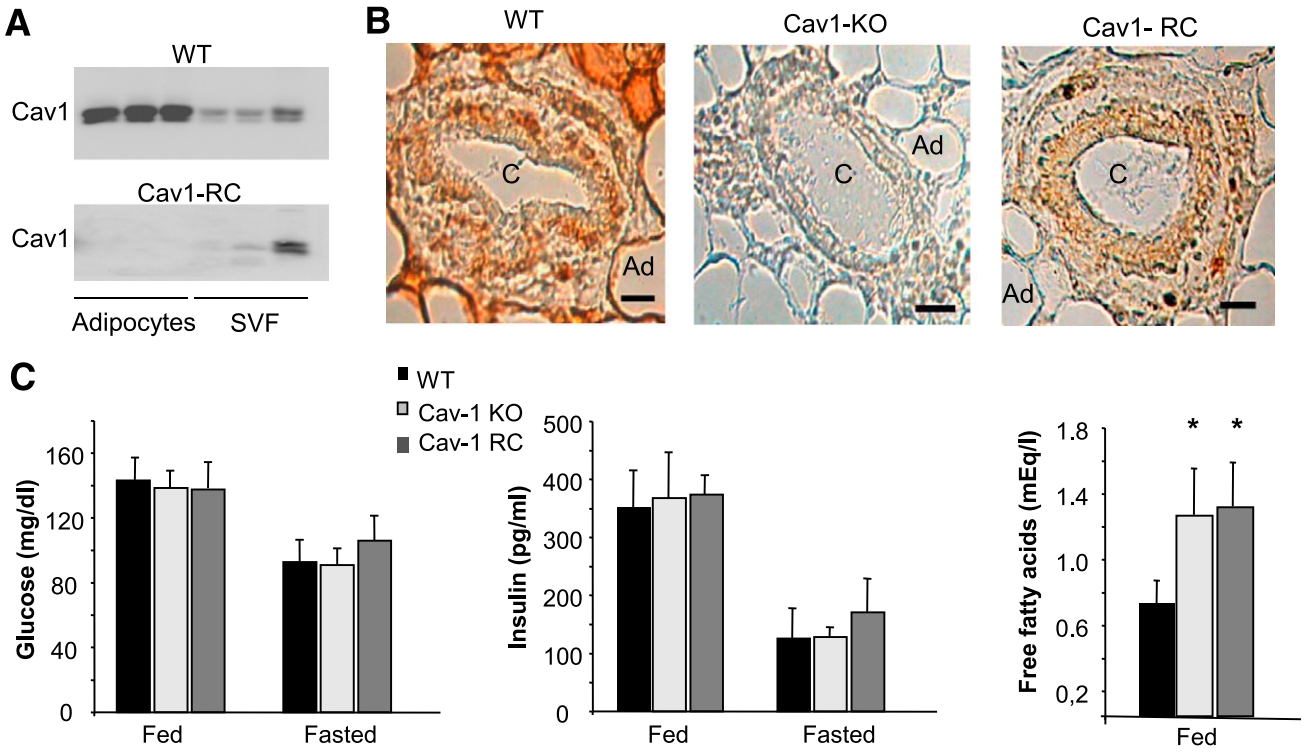

FIG. 1. Topology of caveolin-1 expression in WT mice, Cav1-KO, and Cav1-RC. *A*: Western blot analysis of caveolin-1 expression after collagenase digestion of adipose tissue and fractionation according to cell density. SVF and floating adipocytes were considered. Three independent cell preparations are shown, each obtained from adipose tissues of four pooled mice. Time exposure is different for WT (30 s) and Cav1-RC samples (5 min). Incomplete recovery of Cav1 expression in Cav1-RC SVF is likely because of the fact that in WT SVF, Cav1 expression is not restricted to endothelial cells (macrophages, fibroblasts). *B*: Immunostaining of subcutaneous inguinal adipose tissue. Caveolin-1 labeling was revealed with a polyclonal antibody (BD Transduction Laboratories). Scale bar is 20 μm. C, capillary lumen; Ad, adipocyte. *C*: Glycemia, insulinemia, and free fatty acids levels were assessed in the postabsorptive state (fed) or after an overnight fast in 6-month-old mice. In each group, 5–9 mice were measured. (A high-quality digital representation of this figure is available in the online issue.)

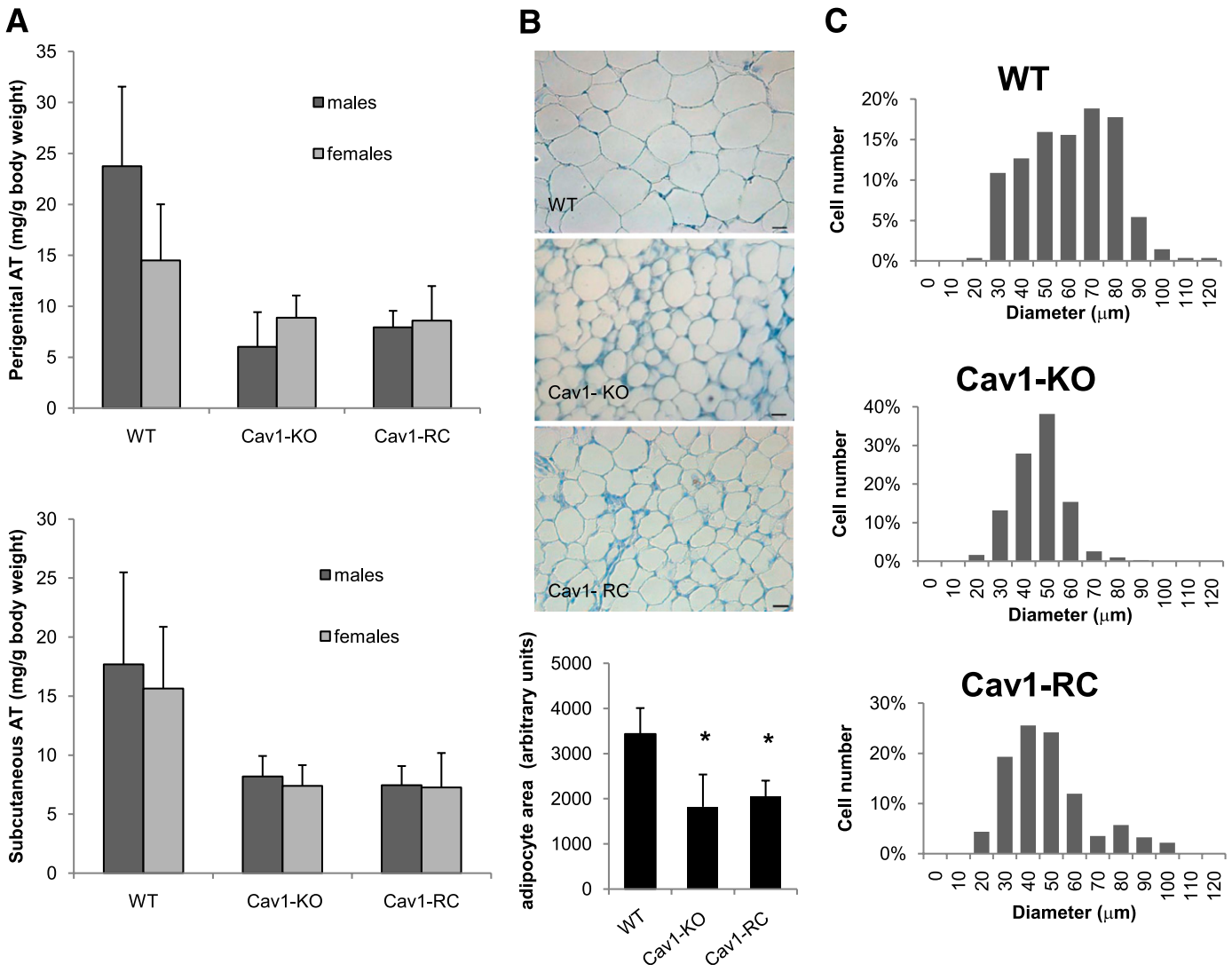

FIG. 2. Persistence of lipoatrophic phenotype in Cav1-RC mice. ***A*****: Adipose tissue weights in perigenital (*top*) and subcutaneous (*bottom*) locations in 6-month-old male and female mice. Bars represent mean values ± SE, with at least eight individual mice in each group. WT mice were significantly different from other groups by ANOVA, $P < 0.01$.** ***B*****: Hemalun staining of subcutaneaous adipose tissue sections from sex-matched 6-month-old mice. Scale bar is 20 μm. Adipocyte area quantification was performed using ImageJ software. Values are means ± SE obtained from at least five different mice in each group. *Indicates a significant difference ($P < 0.05$) vs. WT by Student *t* test.** ***C*****: Measurements of isolated adipocyte diameters from sex-matched 6-month-old mice. The size of at least 200 individual adipocytes was determined from scaled images using Perfect Image 6.10 (Clara Vision software). Each plot represents a distribution of an individual adipocyte population in diameter classes. Each distribution was obtained from pooled perigenital adipose tissues of three mice in each group. (A high-quality color representation of this figure is available in the online issue.)**

changes in gene expression by microarray experiments on epididymal adipose tissue of Cav1-RC and WT mice. Classification of differentially expressed genes was based on gene ontology (GO) annotation using FunNet software (15). Relevant biological themes, annotating differentially expressed genes, are indicated by significantly overrepresented categories from the GO Biological Process ontologies (Supplementary Fig. 1 and Supplementary Table 1). The genes upregulated in Cav1-RC adipose tissue were annotated mainly by functional themes associated with the cellular membrane and cell signaling (cell surface linked receptor signal transduction, cell adhesion). Not surprisingly, these pathways connect to a well-established role of caveolin as a general suppressor of signal transduction. More interestingly, downregulated genes were annotated mostly by themes related to intracellular metabolism such as transport, metabolic process, generation of precursor metabolites and energy, and carbohydrate and lipid metabolic process. Thus caveolin absence in adipocytes is shown here to profoundly affect gene expression toward reduced metabolic activity, which fits with the persistence of lipoatrophic phenotype in Cav1-RC mice.

Endothelium caveolin deficiency promotes infiltration of noninflammatory M2 macrophages in adipose tissue. Careful inspection of histological images revealed that additional changes in cellular composition of the adipose tissue were also present in these mice. DAPI and perilipin staining indicated increased nuclei-to-adipocytes ratio specifically in totally deficient animals (Cav1-KO) and not in Cav1-RC or WT (Fig. 3*A*). Because macrophage infiltration in adipose tissue is now well-established as a process related to changes in fat mass (12,16), we sought to examine adipose tissue sections for macrophage markers. F4/80 or Mac2 labeling revealed few positive cells

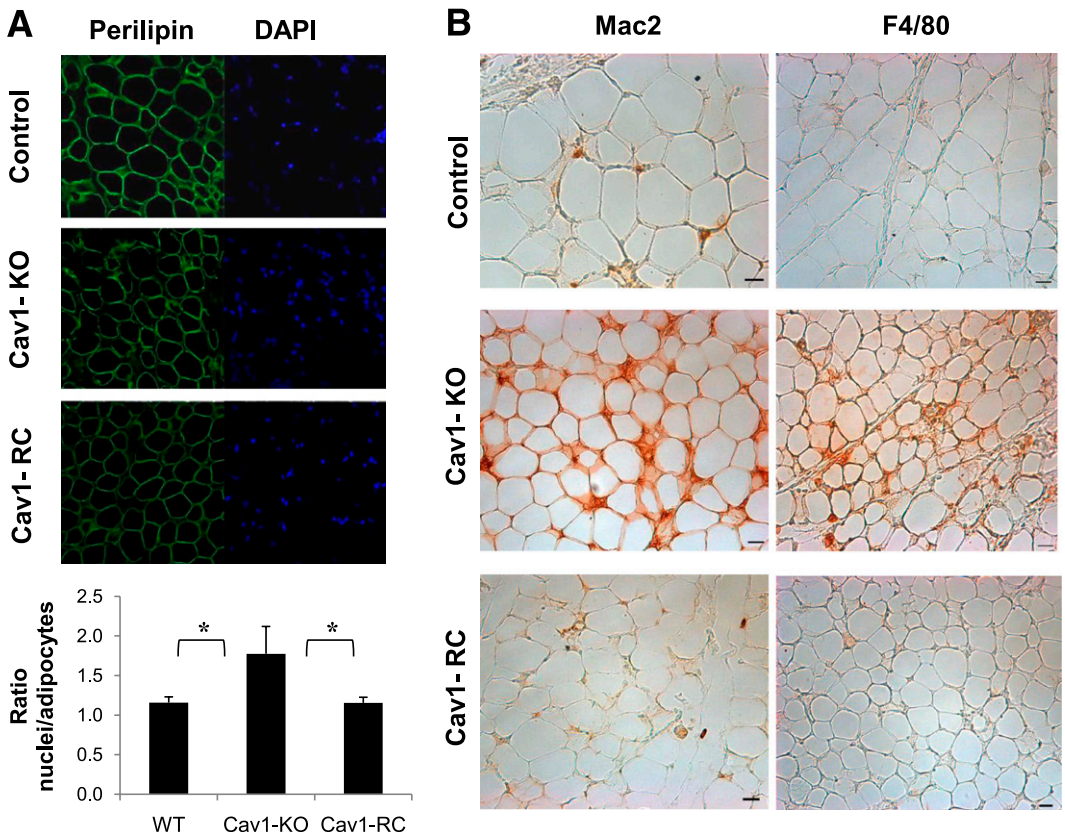

FIG. 3. Altered cellular composition of adipose tissue in Cav1-KO but not Cav1-RC mice. Inguinal adipose tissue sections of WT mice, Cav1-KO, and Cav1-RC (at least five mice/group) were labeled for nuclei with DAPI and for lipid droplet-associated protein perilipin (*A*) or with macrophage surface markers (*B*). Ratios of DAPI positive nuclei to perilipin positive adipocytes are quantified. Significant differences by Student *t* test are noted (*). (A high-quality digital representation of this figure is available in the online issue.)

in WT, reflecting the presence of a small proportion of resident macrophages (Fig. 3*B*). F4/80 or Mac2 positive cells increased in Cav1-KO, indicating that caveolin-deficient adipose tissue was infiltrated by macrophages. On the contrary, specific endothelium caveolin re-expression in adipose tissue limited macrophage presence with identical F4/80 or Mac2 staining between Cav1-RC and WT (Fig. 3*B*). Therefore, these data suggest a critical role for endothelial caveolin-1 in the control of adipose tissue macrophage infiltration.

We also compared gene expression of macrophage markers in adipose tissue of the three mice strains. Emr1 (F4/80) and cd68 expression increased threefold in Cav1-KO adipose tissue in comparison with WT (Fig. 4*A*). Both Emr1 and cd68 mRNA expression are normalized in Cav1-RC. All together, mRNA expression and immunostaining confirm the increased presence of macrophages in adipose tissue of Cav1-KO mice, which can be reversed by specific re-expression of caveolin-1 in endothelial cells.

Adipose tissue is normally hosting a small number of resident macrophages, which display a reparative phenotype (M2 or alternative activation) (17), characterized by the expression of proteins implicated in tissue remodeling as arginase-1 (Arg-1), mannose receptor C type 2 (Mrc-2), or macrophage galactose *N*-acetyl-galactosamine-specific lectins (Mgl-1). Upon obesity, adipose tissue macrophage infiltration increases, and they switch to a proinflammatory phenotype (M1 or classical activation) leading to production of proinflammatory cytokines like monocyte chemoattractant protein-1 (Mcp-1), tumor necrosis factor-α (TNF-α), or interleukin (IL)-6. Relative mRNA expression (Fig. 4*B*) and blood levels (Fig. 4*C*) of macrophage markers defining M1 and M2 states (Fig. 4*E*) indicated no change for M1 markers in WT, Cav1-KO, and Cav1-RC, whereas M2 markers were increased in Cav1-KO mice compared with WT and normalized by specific endothelial caveolin re-expression (Cav1-RC). A noticeable exception to this scheme is plasminogen activator inhibitor 1 (PAI-1) mRNA and blood levels (Fig. 4*D*), which remained elevated in Cav1-KO and Cav1-RC mice, possibly because PAI-1 mRNA is mainly expressed in mature adipocytes (data not shown), both lacking caveolin in Cav1-KO and Cav1-RC mice. Altogether, these data demonstrate that endothelium caveolin-1 specifically regulates extravasation of macrophages into adipose tissue.

DISCUSSION

We show here that defective adipose tissue growth and lipoatrophy in mice with global caveolin gene invalidation cannot be rescued by specific re-expression of caveolin in endothelial cells. Among multiple alterations described so far in caveolin-deficient mice, like cardiac and pulmonary defects, lipoatrophy is the first phenotype that cannot be ameliorated by endothelial caveolin rescue. Even a complex phenotype such as progression of atherosclerosis, potentially involving interactions between multiple cell types within the vessel wall, was recently shown to critically depend on endothelial caveolin expression (18). Thus

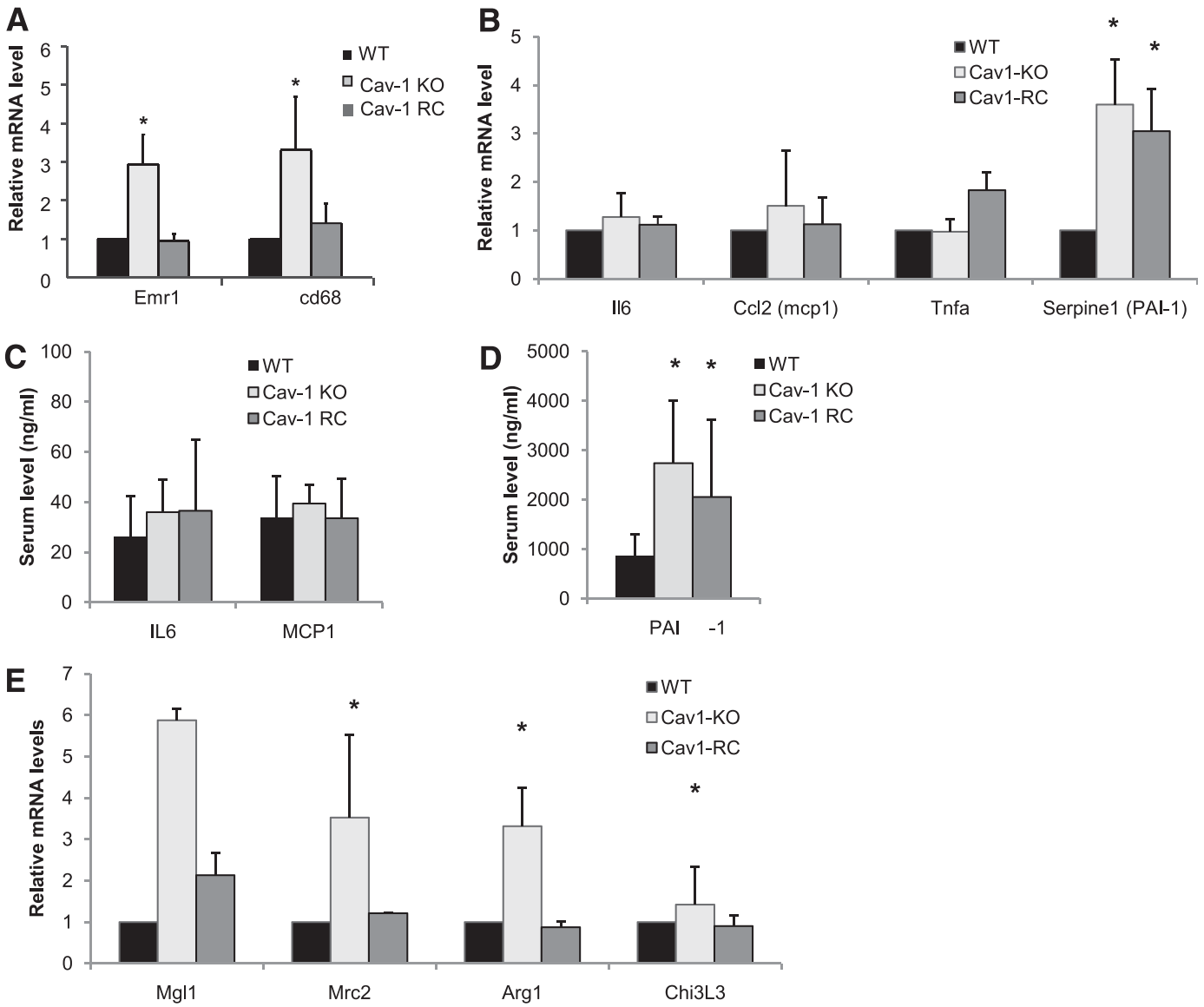

FIG. 4. Macrophage marker analysis in WT mice, Cav1-KO, and Cav1-RC. *A*, *B*, and *E*: mRNA were extracted from frozen individual perigenital adipose tissues (at least five mice in each group) and used as templates for cDNA synthesis. mRNA were measured by real-time RT-PCR using primers validated for PCR efficiency and single product amplification. For each gene, mRNA levels were normalized to 18S, and values obtained in WT mice were set to 1. *C* and *D*: Circulating IL6, MCP-1, and PAI-1 in 6-month-old mice were measured in serum (5–8 mice/group). Statistically significant differences are indicated (*$P < 0.05$).

our present observation is the first evidence for a functional role of caveolin adipocyte, and not endothelial caveolin in a metabolic phenotype.

The reasons why adipocytes remain atrophic in the total absence of caveolin-1 have not been related to overt alterations in food intake, nutrient absorption, or energy expenditure (8,19). They might be linked to global reduction in adipocyte metabolic activity revealed here by gene expression defects in microarray analysis. Accordingly, autophagic degradation was recently reported in caveolin-deficient adipocytes (20), as well as compromised lipid droplet growth and maturation defects (21).

A second interesting finding is the implication of endothelial caveolin in adipose tissue infiltration by macrophages. Indeed, it has been shown that both transcellular and paracellular pathways, which participate in endothelial barrier function, implicate caveolins (9,22,23).

Macrophage infiltration of adipose tissue is linked to obesity (12,16) and could play a major role in insulin resistance through proinflammatory cytokine production. Previous reports show that macrophage polarity in obesity is mainly M1 proinflammatory, whereas alternatively activated M2 macrophages are usually found in lean (24). In agreement with a previous report on a different lipodystrophic murine model (25), we show an increased number of macrophages in caveolin-1-deficient mice adipose tissue mostly resembling a M2 state. In the complex cascade leading to macrophage recruitment, rescue of adipose tissue macrophage content in Cav1-RC, despite persistent lipoatrophy, suggests a regulatory role for endothelium. In this context, endothelial caveolin-1 thus appears as a new actor for macrophage entry into adipose tissue. This highlights endothelium permeability as a potential target to control obesity-related macrophage infiltration.

In conclusion, our present study identifies distinct roles for caveolin-1, linked to its presence in different cell types within adipose tissue. Adipocyte-derived caveolin is shown here to play a crucial role in lipid storage and global fat cell function, whereas endothelium-derived caveolin can modulate tissue cell composition and macrophage infiltration.

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N.B. researched data, reviewed and edited the article, and contributed to discussion. S.L.L. researched data and wrote the article. W.C.S. and P.F. reviewed and edited the article and contributed to discussion. I.D. researched data and wrote the article.

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