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**CD40L-expressing CD4<sup>pos</sup> T cells prime adipose-derived stromal cells  
to produce inflammatory chemokines**

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**Short title:** CD40 signaling and ASC immune functions

## ABSTRACT

The therapeutic potential of culture-adapted adipose-derived stromal cells (ASCs) is largely related to their production of immunosuppressive factors that are inducible *in vitro* by the priming with inflammatory stimuli, in particular tumor necrosis factor- $\alpha$  (TNF) and interferon- $\gamma$  (IFN). *In vivo*, obesity is associated with a chronic inflammation of white adipose tissue, including accumulation of neutrophils, infiltration by IFN/TNF-producing immune cells, and ASC dysfunction. In the current study we identified in obese patients a simultaneous upregulation of *CD40L* within adipose tissue stroma vascular fraction (AT-SVF), correlated with Th1 gene signature, and an overexpression of CD40 by native ASCs. Moreover, activated CD4<sup>pos</sup> T cells upregulated CD40 on culture-expanded ASCs and triggered their production of IL-8 in a CD40L-dependent manner, leading to an increased capacity to recruit neutrophils. Finally, activation of ASCs by sCD40L or CD40L-expressing CD4<sup>pos</sup> T cells rely on both canonical and non-canonical NF- $\kappa$ B pathways, and *IL-8* was found co-regulated with NF- $\kappa$ B family members in AT-SVF. These data identify CD40-CD40L axis as a priming mechanism of ASCs, able to modulate their crosstalk with neutrophils in inflammatory context, and their functional capacity for therapeutic applications.

**KEYWORDS:** Adipose tissue, obesity, CD40, neutrophils, mesenchymal stromal cells, IL-8

## INTRODUCTION

White adipose tissue (WAT) contains large amounts of adipose progenitor cells considered as a valuable source of clinical-grade stromal cells after *in vitro* expansion. Culture-adapted adipose-derived stromal cells (ASCs) are a promising therapeutic alternative in a broad range of clinical applications, including inflammatory diseases and chronic degenerative disorders, owing to their anti-inflammatory and immunosuppressive properties [1]. In fact, *in vitro*-expanded ASCs and bone marrow-derived mesenchymal stromal cells (BM-MSCs), despite sharing inhibitory activity towards all components of innate and adaptive immunity, display distinctive phenotypic, transcriptomic, and functional features, and ASCs have recently emerged as easier to harvest, more immunosuppressive, and less immunogenic than BM-MSCs [2][3][4]. Such specificities are shared by their native counterparts, highlighting an imprinting of tissue source on cultured MSC immune properties [2]. Yet, the main driver of MSC immune functions is their response to inflammatory stimuli, with tumor necrosis factor- $\alpha$  (TNF) and interferon- $\gamma$  (IFN) acknowledged as key inducers of the complex pattern of MSC immunosuppressive determinants *in vitro* [5][6]. How such factors, produced by activated immune cells, impact the immune properties of MSCs *in vivo* remains largely underexplored.

Obesity is characterized by a chronic inflammation of WAT, associated with remodeling of immune cell landscape, including recruitment of pro-inflammatory and pro-fibrotic macrophages together with type 1 immune cells such as CD8<sup>pos</sup> T cells, IFN-producing Th1 CD4<sup>pos</sup> T cells, or NK cells [7][8][9]. Even if their role in obesity is less well studied, neutrophils accumulate early upon high-fat diet feeding in mice [10] and are the most important fat myeloid subset in morbidly obese patients, strongly decreasing after losing weight [11]. In obesity, adipocytes themselves are dysfunctional and display proinflammatory properties [12]. Furthermore, ASCs expanded from obese patients exhibit altered transcriptomic profile, with upregulation of adipogenic and inflammatory genes, including CXCL8/IL8 and CCL2 chemokines [13]. Moreover, they display superior migratory but weaker immunosuppressive capacities compared to ASCs expanded from lean donors, and IL-1 $\beta$  was proposed as partly involved in obesity-related ASC immune dysfunctions [14][15][16]. However, the mechanisms involved in the interplay between native and culture-adapted ASCs and immune cells are poorly described.

The CD40-CD40L pathway has been involved in WAT inflammation during obesity [17]. CD40L is produced by activated CD4<sup>pos</sup> T cells, and its circulating soluble form (sCD40L) is increased in obese patients and correlated to body mass index (BMI) [18][19]. Moreover, mature adipocytes express CD40 and respond to CD40L activation by upregulating IL-6, CCL2, and TNF proinflammatory factors [20]. Whereas CD40 is detectable on the surface of native BM-MSCs [21] and is upregulated by inflammatory stimuli on culture-adapted BM-MSCs and ASCs [4], no study has explored whether CD40 is expressed on native ASC and could directly affect their immune functions. In the current work, we studied CD40-CD40L axis deregulation in adipose tissue from obese *versus* lean patients and evaluated the mechanisms and consequences of CD4<sup>pos</sup> T cell-derived CD40L stimulation in ASCs, with a particular interest for its impact on the crosstalk between ASCs and neutrophils.

## MATERIAL AND METHODS

### *ASC and immune cell production*

Donor recruitment followed institutional review board approval and written informed consent process according to the revised Declaration of Helsinki was obtained. Adipose tissue stromal vascular fractions (AT-SVF) were obtained from obese (BMI, median [min-max]: 39.6 [35.5-46]) and lean (24.4 [21.7-25]) WAT donors (**Supplementary Table S1**). Lipoaspirates were harvested at the beginning of abdominoplasty using the traditional suction-assisted lipectomy, and were then digested with 200 IU/mL CLS4 collagenase (Worthington), 1.6 IU/mL NPRO dispase (Worthington), and 10 IU/mL DNase (Pulmozyme®, Roche) for 45 min at 37°C, filtrated, centrifuged, and resulting ATSVF were frozen. Thawed AT-SVF were then used for sorting DAPI<sup>neg</sup>CD45<sup>neg</sup>CD11b<sup>neg</sup>CD235a<sup>neg</sup>CD31<sup>neg</sup>CD146<sup>neg</sup>CD34<sup>pos</sup> viable native ASCs using a FACS Aria II (BD Biosciences) as previously described [2] or seeded at 1,000 cells/cm<sup>2</sup> in  $\alpha$ MEM (Invitrogen) supplemented with 10% fetal calf serum (FCS, Biosera), 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin (Invitrogen), and 1ng/mL fibroblast growth factor  $\beta$  (Cellgenix). Culture medium was renewed every 3-4 days until the cells reached near confluence (end of passage 0 (P0)). ASCs were then detached using trypsin, and used at P1 for phenotypic and functional experiments. The lack of hematopoietic and endothelial cells within DAPI<sup>neg</sup> viable cells was validated by flow cytometry using PE-conjugated anti-CD90, BUV395-conjugated anti-CD45, BV786-conjugated anti-CD31 (all from Becton Dickinson), and APC-conjugated anti-CD73 (Miltenyi Biotec) monoclonal antibodies (mAbs) and their isotypic controls (**Supplementary Figure S1**).

CD4<sup>pos</sup> T cells and monocytes were isolated from peripheral blood mononuclear cell (PBMC) and polynuclear neutrophils from blood samples using CD4<sup>pos</sup> T cell isolation kit, pan monocyte isolation kit, and whole blood CD15 MicroBeads (Miltenyi Biotec) respectively.

### *Gene expression profiling of AT-SVF*

RNA was extracted from AT-SVF using the Nucleospin RNA XS Micro kit (Macherey Nagel) and gene expression level was assessed using the Fluidigm BioMark HD system. Briefly, cDNAs were obtained using the Fluidigm reverse transcription Master Mix and were then pre-amplified for 14 cycles in the presence of Pre-Amp Master Mix and pooled Taqman assay mix (**Supplementary Table S2**). Gene expression was

then measured with the TaqMan Gene Expression Master Mix (ThermoFischer) on a 48.48 Dynamic Array IFC. After quality control check, gene expression was calculated with the  $\Delta$ CT calculation method and *CDKN1B*, *EIF2B1*, and *PUM1* as housekeeping genes. Principal Component Analysis (PCA) was done on the 41 expressed genes with the `prcomp()` R function and visualized using the `Factoextra` R package (v1.0.7). Eigengene values of the first dimension of PCA (DIM1) were then visualized through a dotplot. We then used the function `fviz_contrib()` in order to identify the genes that contributed more than average to the DIM1 axis. Levels of expression of these 23 genes were then visualized through an heatmap with the package `pheatmap` (v1.0.12).

### *ASC activation*

For T-cell activation, purified CD4<sup>pos</sup> T cells were cultured in RPMI-10% human AB serum (Eurobio), 10 IU/mL recombinant human IL2 (rhIL2, Proleukin<sup>®</sup>, Clinigen), and 5  $\mu$ g/mL concanavalin A (Sigma Aldrich). At day 3, 20 IU/mL rhIL2 were added and at day 7, T cells were restimulated by 0.1  $\mu$ M phorbol 12-myristate 13-acetate and 10  $\mu$ M ionomycin (Sigma Aldrich) for the last 5 hours. CD40L protein membrane expression was then checked by flow cytometry using PE-conjugated anti-CD40L mAb (Beckman Coulter) or appropriate isotype control.

Confluent monolayers of P1 ASCs were treated or not with 100 IU/mL IFN, 50 ng/mL TNF (Bio-technie), soluble CD40L (Immunex, 1.5  $\mu$ g/ml for evaluation of NF- $\kappa$ B pathway activation or 300 ng/ml for functional experiments) or activated third-party CD4<sup>pos</sup> T cells (1 T to 4.5 ASC ratio) for the indicated time. Supernatant was frozen and ASCs were collected for flow cytometry and RNA extraction. In case of coculture experiments with activated CD4<sup>pos</sup> T cells, ASCs were sorted as CD45<sup>neg</sup>CD105<sup>pos</sup> cells using FITC-conjugated anti-human CD45 (Beckman Coulter) and PE-conjugated anti-human CD105 (Beckman Coulter) mAbs prior to RNA extraction. In some experiments, ASCs were transfected before stimulation with control, *CD40* (3 different siRNA, 50nM each), *RELA* (3 different siRNA, 5nM each), or *NFKB2* (3 different siRNA, 50nM each) siRNA (Life Technologie) using HiPerfect (Qiagen) following manufacturer instruction. Efficacy of siRNA was evaluated by QPCR for corresponding genes (**Supplementary Figure S2**).

### *Evaluation of CD40 and chemokine expression in ASCs*

After *ex vivo* expanded ASC activation, CD40 surface expression was evaluated by flow cytometry on CD45<sup>neg</sup>CD105<sup>pos</sup> cells using PE-conjugated anti-CD40 mAb (Diaclone) and appropriate isotype control and analysis on a Fortessa X20 (Becton Dickinson). Moreover, *CCL2*, *CCL5*, *IL8*, *CD40*, and *IL6* were quantified by RT-QPCR. Briefly, cDNA synthesis was performed with the Superscript II reverse-transcriptase (Invitrogen) and assay-on-demand primers and probes, and Taqman Universal MasterMix were used to run QPCR on a StepOnePlus Real time PCR System (Applied Biosystems). PCR data were normalized to the geometric mean of three housekeeping genes (*PUM1*, *CDKN1B* and *EIF2B1*). Results were further standardized by comparison to gene expression of a pool of five MSCs when mentioned. Similarly, *CD40* expression was quantified by RT-QPCR on sorted native ASCs using the same housekeeping genes.

CCL2, CCL5 and IL8 protein levels were also quantified in ASC supernatants by ELISA (Bio-Techne) according to the manufacturer instructions. The minimum value was the detection threshold of the method.

### *NF $\kappa$ B pathway activation study*

NF $\kappa$ B pathway activation was evaluated using the Nuclear Extract kit and the TransAM<sup>®</sup> NF $\kappa$ B Family Kits (Active Motif) according to manufacturer instructions using a Varioskan (ThermoFisher) to read absorbance. NF $\kappa$ B1 and RELA activation were evaluated after 1 hour of stimulation, and NF $\kappa$ B2 and RELB after 5 hours of stimulation by 1.5  $\mu$ g/mL CD40L.

RELA nuclear translocation was evaluated by immunofluorescence on a SP5 microscope (Leica) after 30min of stimulation by 1.5  $\mu$ g/mL CD40L. After 15 min of methanol fixation at -20°C, cells were incubated with PBS Bovine Serum Albumine (BSA; Sigma Aldrich) during 1 hour at 37°C, before adding anti-RELA antibody (1/400; Cell signaling) overnight at 4°C and secondary antibody goat anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. After washing, cells were mounted with Moviol containing sytox blue (Thermofisher) for nuclear labelling. The colocalization between Nucleus and RELA staining was evaluated using the plugin Colocalization Threshold in FIJI software.



### *Neutrophil and monocyte migration*

After 3 days of ASC stimulation (300 ng/mL sCD40L or coculture with activated CD4<sup>pos</sup> T cells), the medium was replaced by a migration medium (RPMI-0.1% human serum albumin (Vialebex<sup>®</sup>, LFB) for neutrophils and RPMI-1% FCS for monocytes [22]) for 3 days before supernatant collection.

Neutrophils and monocytes were added at 10<sup>5</sup> cells / 100 µL in migration medium to the upper compartment of Transwell chambers with 5-µm pore filters (Corning Costar) with lower chambers containing ASC-conditioned migration medium. Supernatants from ASC/T-cell coculture experiments were diluted 1/3 before their use in migration assay. When indicated, 10 µg/mL anti-CXCR1 blocking antibody or isotype control (Bio-Techne) were added in the lower chamber. The absolute number of viable DAPI<sup>neg</sup>CD45<sup>pos</sup> neutrophils or DAPI<sup>neg</sup>CD14<sup>pos</sup> monocytes in the lower chamber was quantified with FlowCount beads (Beckman Coulter). The percentage of cell migration was determined by comparing the number of viable cells in the lower compartment after 2 hours of migration with the initial number of cells seeded in the upper compartment.

### *Neutrophil survival*

After 3 days of ASC stimulation (300 ng/mL sCD40L), purified neutrophils were co-cultured with or without ASCs (neutrophil-to-ASC ratio 2.5/1). The absolute number of viable DAPI<sup>neg</sup>CD45<sup>pos</sup> neutrophils was evaluated by flow cytometry with FlowCount beads after 2 days of culture.

### *Statistical analysis*

Statistical analyses were performed with Prism software version 9.1.2 (GraphPad Software) and R version 3.6.0 ([www.R-project.org](http://www.R-project.org)). The Wilcoxon test was used for matched pairs, the Mann-Whitney U-test for non-paired samples. When indicated, the normality of the distribution was tested by a Shapiro-wilk test before using paired t-test. The fold change is the ratio of median values.

The co-expression of *NFKB1*, *NFKB2*, *RELA*, *RELB*, and *CXCL8* as well as the correlation between *TBX21*, *CD40LG* and *IFNG* were evaluated by Pearson correlation analysis between two variables and visualized on a matrix with package R corrplot v0.84.

## RESULTS

### *Inflammation induces CD40 on ASCs*

In order to explore the inflammatory context in obese patients, we analyzed by QPCR the expression of 44 inflammation-related genes in AT-SVF obtained from 10 obese and 9 lean patients. Gene expression profile of the 41 detectable genes was analyzed in a non-supervised manner through principal component analysis (PCA) (**Figure 1A**). Interestingly, AT-SVF samples coming from obese *versus* lean patients were differentially organized along the first dimension of the PCA (DIM1,  $P < 0.05$ ) (**Figure 1B**). The 23 genes contributing more than average to PCA DIM1 (**Supplementary Figure S3A**) identified a pattern of genes overexpressed in obese patients (**Figure 1C**) and essentially included T-cell and myeloid cell markers. *CCL2*, *CCL5*, and *CXCL8*, three inflammatory chemokines involved in the recruitment of myeloid cells belonged to this 23-gene panel together with the *CD68* and *CSF1R* macrophage markers. In agreement, *ADIPOQ*, the gene coding for the anti-inflammatory factor adiponectin known to suppress M1 macrophage activation [23], was significantly reduced in obese patients (**Supplementary Figure S3B**). Moreover, both *TBX21* and *GATA3*, the drivers of Th1 versus Th2 T-cell differentiation, were found differentially expressed between obese and lean patients ( $P < 0.05$ ) and *TBX21* expression was found strongly correlated with *IFNG* and *CD40LG* ( $P = 0.002$  and  $P = 2.97 \times 10^{-5}$ ; respectively, **Figure 1D**), suggesting an enrichment for functional Th1 cells in obese patients and highlighting for the first time an overexpression of *CD40LG* in obese AT-SVF ( $P < 0.01$ , **Figure 1E**). We thus evaluated *CD40* expression by purified ASCs and revealed its overexpression in a series of obese ( $n = 5$ ) versus lean ( $n = 5$ ) native ASCs ( $P < 0.01$ , **Figure 1F**). Interestingly, analysis of public single cell RNAseq data obtained from frozen WAT [24] identified native ASCs (corresponding to preadipocyte and adipose precursor cell clusters) as the main *CD40*-expressing cells within AT-SVF (**Supplementary Figure S4A-B**). These data suggest that *CD40-CD40L* pathway could be active in WAT from obese patients and do not only involve mature adipocytes but also *CD40*-expressing ASCs.

In order to better understand the signals triggered by *CD40* upregulation on ASCs, we stimulated short-term expanded ASCs by various inflammatory stimuli produced by activated Th1 cells, including soluble *CD40L* (s*CD40L*), TNF, and IFN, or by *CD4*<sup>pos</sup> T cells activated *in vitro* to express *CD40L* (**Supplementary Figure S4C**). Whereas

sCD40L did not increase CD40, IFN and TNF induced CD40 overexpression on ASCs as single agents, at both mRNA and protein levels (**Figure 1 G-H and Supplementary Figure S4D-E**). A CD40 induction similar to that obtained in ASCs co-culture with activated CD4<sup>pos</sup> T cells was reached when combining IFN, TNF, and sCD40L with about 65-fold increase of CD40 expression compared to resting ASCs. Overall, we confirmed that inflammatory cytokines but also direct contact with activated CD4<sup>pos</sup> T cells upregulate CD40 expression on *in vitro*-expanded ASCs, like in ASCs coming from obese patients.

#### *CD40L triggers inflammatory chemokine overexpression in ASCs*

Following the demonstration of CD40 induction on ASCs exposed to inflammatory stimuli *in vitro* and *in vivo*, we decided to investigate the impact of CD40 activation on ASC properties, with a particular interest for their production of chemokines, a key step in MSC immunosuppressive properties [25]. Stimulation of *in vitro*-expanded ASCs by sCD40L upregulated their expression of CCL2, CCL5, and IL-8 at the mRNA and protein levels (**Figure 2 A-B**). Given the huge infiltration of WAT from obese patients by neutrophils [11], we further explored IL-8 production by primed ASCs. Activated CD4<sup>pos</sup> T cells strongly increased IL-8 production by ASCs and this induction was significantly reduced by decreasing ASC CD40 expression by a CD40 siRNA (**Figure 2C**), demonstrating a role for CD40L-CD40 pathway in T-cell dependent ASC activation. We next explored the ability of the IL-8 produced by ASCs to recruit neutrophils. For that purpose, we quantified by flow cytometry the number of neutrophils migrating towards supernatants from unstimulated and sCD40L-stimulated ASCs through Transwell chambers (**Figure 2D**). Interestingly, sCD40L-stimulated ASCs were more potent to recruit neutrophils than unstimulated ASCs and neutrophil migration was completely abrogated by blocking the IL-8 receptor CXCR1 by an antagonist antibody, demonstrating that neutrophil recruitment by resting and CD40L-primed ASCs was dependent on IL-8. To further investigate the relevance of these data, we next assessed whether supernatants from ASC co-cultured with activated CD4<sup>pos</sup> T cells were able to trigger the same effect on neutrophil migration (**Figure 2E**). Of note, diluted supernatant (1/3) from T-cell primed ASCs were used to obtain the maximum neutrophil migration (data not shown). Neutrophil migration was higher in response to CD4<sup>pos</sup> T cell-stimulated ASCs than resting ASCs and was suppressed when ASCs have been transduced by CD40 siRNA prior to coculture with T cells,

indicating that CD4<sup>pos</sup> T cells increased the capacity of ASCs to recruit neutrophils through a CD40-CD40L dependent IL-8 induction. BM-MSCs are well known to protect neutrophils from apoptosis [22][26] and we thus evaluated whether ASCs displayed the same properties and how it could be affected by CD40 stimulation. Interestingly, resting and sCD40L-primed ASCs strongly and similarly decreased neutrophil spontaneous cell death *in vitro* (**Figure 2F**). Of note, despite the slight upregulation of CCL2 production in sCD40L-activated ASCs (**Figure 2A-B**), the resulting increase of monocyte migration remained nonsignificant (**Figure 2G**), highlighting neutrophils as a major target of CD40 signaling in ASCs.

#### *NF-κB mediates ASC activation by T-cell derived CD40L*

CD40 activity has never been studied on ASCs and we decided to explore the molecular mechanisms underlying its activation by CD40L. Since both canonical (*NFKB1*) and non-canonical (*RELB*, *NFKB2*) genes participated to the 23-gene pattern overexpressed in obese AT-SVF (**Figure 1C**), and given the well-known activation of both NF-κB pathways in immune cells by CD40L [27], we evaluated the activation of NF-κB by sCD40L in ASCs. ELISA-based transcription factor activation assays highlighted activation of both canonical and non-canonical NF-κB pathways in ASCs after exposure to sCD40L (**Figure 3A**). Moreover, we confirmed by confocal microscopy the translocation of the NF-κB subunit RelA to the nucleus after CD40 engagement on ASCs (**Figure 3B**). We then assessed whether activation of canonical and non-canonical NF-κB pathway contributed to IL-8 production triggered by sCD40L and activated CD4<sup>pos</sup> T cells in ASCs using validated (**Supplementary Figure S2**) siRNAs targeting *RELA* and *NFKB2*. Interestingly, IL-8 production was significantly decreased by these 2 siRNA, independently of the source of CD40L (sCD40L or activated CD4<sup>pos</sup> T cells) (**Figure 3C**), leading to a decrease of neutrophil recruitment (**Figure 3D**). Finally, *IL-8* was found co-regulated with NF-κB family members in WAT, as highlighted by a Pearson correlation performed on our series of 19 AT-SVF (**Supplementary Figure S5**), supporting a role for NF-κB in IL-8 expression in adipose tissue. Overall, these data support the hypothesis that CD4<sup>pos</sup> T cells induce IL-8 production and neutrophil recruitment by ASCs through the activation of both canonical and non-canonical NF-κB pathways by CD40-CD40L interaction therefore participating to the inflammatory context seen in AT-SVF coming from obese patients.

## DISCUSSION

Accumulated evidence suggests that MSC immunosuppressive activity is essentially inducible by inflammatory stimuli but, besides well-described TNF, IFN, and toll like receptor (TLR) ligands, few data are available on the impact of other immune cell-derived factors on MSC immunoregulatory functions. In the current study, we identified CD40L-CD40 axis as a new pathway involved in T-cell/ASC crosstalk and demonstrated that it could modulate the capacity of native and culture-adapted ASCs to interact with neutrophils.

Mature adipocytes have been already shown to express CD40 [20], similarly to BM-MSCs that display increased CD40 expression in response to *in vitro* inflammatory stimuli [28]. We reported here that ASCs also expressed CD40 in particular within inflamed WAT of obese patients and under stimulation by activated CD4<sup>pos</sup> T-cell signaling *in vitro*. A role for CD40-CD40L axis in the interplay between stromal cells and CD4<sup>pos</sup> T cells has been reported for murine fibroblastic reticular cells (FRC), the specialized lymph node stromal cells regulating immune response initiation, maintenance, and control. In this context, activated T cells increase production of inflammatory chemokines, including CCL5, by FRCs, in a CD40L-dependant manner [29]. Interestingly, BM-MSCs activation by CD40L-expressing mast cells contributes to disease progression in splenic marginal zone lymphoma [30] and mast cells accumulate in WAT from obese patients [8] suggesting they could participate in CD40-dependent activation of ASCs. Of note, activated neutrophils could express CD40L [31] and CD40 [32] making them able to directly interact with both CD40-expressing ASCs and CD40L-expressing CD4<sup>pos</sup> T cells, thus reinforcing this ASC-T cell-neutrophil activation loop.

MSC immune functions involve early interaction with neutrophils. BM-MSCs recruit neutrophils, in particular after priming by inflammatory stimuli, and sustain their survival through IL-6 production [26]. Interestingly, CD40 activation did not upregulate *IL-6* in ASCs (**Supplementary Figure S6**) in agreement with their similar capacity to inhibit neutrophil apoptosis *in vitro* compared to resting ASCs. BM-MSCs also downregulate oxidative burst through ICAM1-dependent neutrophil engulfment and extracellular release of the antioxidant superoxide dismutase 3, while inhibiting the formation of neutrophil extracellular trap (NET) and preventing neutrophil-related tissue damage [33][34]. Similarly, amniotic membrane MSCs decrease NET release as well as the production of reactive oxygen species through producing TNF-stimulated gene 6

protein (TSG-6) [35]. Few data are available on the inhibition of neutrophil activity by ASCs but their clinical efficacy in cornea repair was attributed to their capacity to trigger neutrophil clearance in a preclinical mouse model [36]. In our study, the use of frozen AT-SVF precluded any analysis of neutrophil infiltration and activation *in situ*. How ASC inhibitory functions towards neutrophils could reduce inflammatory response in obese patient remains thus to be explored. Of note, IL-1 $\beta$  was recently shown to collaborate with TNF/IFN to upregulate IL-8 production and resulting neutrophil migration in BM-MSCs in a NF- $\kappa$ B dependent manner [37]. These data suggest that other NF- $\kappa$ B-activating stimuli could synergize with CD40L in triggering neutrophil recruitment by primed MSCs. IFN is produced by CD40L-expressing Th1 cells and is overexpressed in obese WAT, while TNF upregulates IFN- $\gamma$ R [37] and CD40 (our study) on MSCs underlying the capacity of MSCs to respond to complex inflammatory molecular patterns.

Culture-adapted ASCs are intrinsically more efficient than BM-MSCs to recruit neutrophils, and overexpress TSG-6, involved in the early inhibition of neutrophil and macrophage activity, a specific feature imprinted by their tissue origin [2]. ASCs also overexpress NF- $\kappa$ B family members compared to their BM counterpart and are considered as FRC precursors [38]. It is thus tempting to speculate that expression of functional CD40 should thus be taken into account when generating ASCs for clinical application, in particular considering the huge amount of sCD40L present in platelet lysate containing medium frequently used to produce clinical-grade ASCs [39]. Given the major role of MSC-neutrophil interaction in MSC immunosuppressive activity, and the increasing interest for ASCs in MSC-based clinical trials at the expense of their BM counterpart, our work could pave the way for an integration of CD40-CD40L as a MSC priming pathway with physiopathological and clinical impact.

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**AUTHOR CONTRIBUTIONS**

JD, SLo: designed and performed experiments, analyzed data, and contributed writing; DRos, SLe: performed statistical analyzes; NBes, IB, ML, CM: provided technical assistance; DM: contributed to the study design; NBer: provided adipose tissue; DRou: analyzed data and contributed writing; KT: designed and supervised research, analyzed data, and wrote the paper.

All authors have approved the final manuscript.

**CONFLICT-OF-INTEREST DISCLOSURE**

Authors have no competing interests to declare.

## FIGURE LEGENDS

### Figure 1. Inflammatory context induces CD40 on ASC *in vitro* and *in vivo*.

**(A-E)** Adipose Tissue Stromal Vascular Fraction (AT-SVF) from obese (n= 10) and lean (n=9) donors were studied by RT-QPCR for a panel of inflammation-related genes. A) Unsupervised analysis of the gene expression profile of the 41 expressed genes by Principal Component Analysis; B) Scatter plot visualization of the first dimension of the PCA (DIM1) eigengene value of each AT-SVF sample. \* $p < 0.05$ ; C) Heatmap representation of the 23 genes contributing more than average to PCA DIM1; D) Correlation between *TBX21* and *IFNG* and *TBX21* and *CD40LG* across the 19 AT-SVF samples; E) Expression of *CD40LG*. \*\*  $p < 0.01$ .

**(F)** DAPI<sup>neg</sup>CD45<sup>neg</sup>CD11b<sup>neg</sup>CD235a<sup>neg</sup>CD31<sup>neg</sup>CD146<sup>neg</sup>CD34<sup>pos</sup> viable native ASCs were sorted from obese (n=10) and lean (n=9) obese patients and studied for CD40 expression by RT-QPCR \*\*  $p < 0.01$

**(G-H)** *In vitro*-expanded ASCs were activated by soluble CD40L (sCD40L), TNF- $\alpha$  (TNF), IFN- $\gamma$  (IFN), or an activation cocktail containing TNF, IFN, and sCD40L. ASCs were also stimulated by activated CD40L-expressing CD4<sup>pos</sup> T cells (Act CD4<sup>pos</sup> T cells) before sorting (for RNA extraction) or gating (for flow cytometry experiments) of CD45<sup>neg</sup>CD105<sup>pos</sup> primed ASCs. G) *CD40* mRNA expression was measured by RT-QPCR after 24 hours of ASC activation (n=6 ASC batches), normalized to three housekeeping genes, and represented in arbitrary units obtained by assigning the value of 1 to non-stimulated ASC (dotted lines); H) CD40 protein membrane expression was also quantified by flow cytometry on after 2 days (n=6 ASC batches). The ratio of CD40 mean fluorescence intensity (MFI) to isotype control MFI (rMFI) was normalized to 1 for the rMFI obtained with non-stimulated ASC (dotted line) and the relative rMFI (rrMFI) of CD40 between stimulated and non-stimulated ASC are represented. Bars: medians from independent experiments. \*  $p < 0.05$ , ns: not significant.

### Figure 2. CD40L triggers chemokine overexpression and neutrophil recruitment by ASCs.

**(A-B)** ASCs were cultured with (black symbols) or without (opened symbols) soluble CD40L (sCD40L). A) *CCL2*, *CCL5* and *IL-8* were quantified by RT-QPCR after 24 hours of culture (n=13 ASC batches), normalized to three housekeeping genes, and



represented in arbitrary units obtained by assigning the value of 1 to a pool of MSCs. B) CCL2, CCL5 and IL-8 protein levels were quantified in cell supernatant by ELISA after 3 days of culture (n=10, n=8, n=12 ASC batches; respectively).

**(C)** ASCs were transfected with control (CTL) or CD40 siRNA (3 different siRNA; 50nM each) before coculture with activated CD4<sup>pos</sup> T cells (n=5 ASC batches). IL-8 protein level was quantified in the supernatant by ELISA after 3 days of culture. Dotted line represents the average of IL-8 levels found in the supernatant of ASCs cultured without activated CD4<sup>pos</sup> T cells. Each symbol corresponds to an individual experiment. Statistical analysis was performed using paired t-test after testing the normality of the distribution.

**(D)** ASCs were cultured during 3 days with or without (medium) soluble CD40L (sCD40L) before medium replacement. Supernatants were then collected and tested for their capacity to trigger neutrophil recruitment in 2 hours through 5- $\mu$ M Transwell. Anti-CXCR1 blocking monoclonal antibody (a-CXCR1; 10 $\mu$ g/mL, green square) or its isotype control (CTL, 10 $\mu$ g/mL, pink circle) was added in the lower chamber.

**(E)** ASCs were cultured during 3 days with or without (medium) or activated CD4<sup>pos</sup> T cells before medium replacement. Supernatants were then collected and tested for their capacity to trigger neutrophil recruitment through 5- $\mu$ M Transwell. When indicated, ASCs were transfected with control (CTL siRNA, pink circle) or CD40 (CD40 siRNA, green square) siRNAs before coculture.

**(F)** ASCs were cultured during 3 days with or without (medium) soluble CD40L (sCD40L) before coculture with neutrophils (neutrophil-to-ASC ratio: 2.5/1) for 2 days. The number of viable neutrophils was determined by flow cytometry. Shown are the data obtained using 8 ASC batches and neutrophils purified from 3 donors.

**(G)** ASCs were cultured during 3 days with or without (medium) soluble CD40L (sCD40L) before medium replacement. Supernatants were then collected and tested for their capacity to trigger monocyte recruitment through 5- $\mu$ M Transwell. Each symbol corresponds to an individual experiment (n=8 ASC batches). Statistical analysis was performed using Wilcoxon matched-pairs test.

**Figure 3. CD40 functional signaling in ASCs is mediated through NF- $\kappa$ B pathway**

**(A)** ASCs were treated with (grey bars) or without (medium, opened bars) soluble CD40L (sCD40L). NF- $\kappa$ B activation was evaluated after 1 hour (NF- $\kappa$ B1 and RELA) or

5 hours (NF- $\kappa$ B2 and RELB) of culture by an ELISA specific for activated DNA-binding forms of each NF- $\kappa$ B factor (n=3 ASC batches).

**(B)** ASCs were cultured on glass coverslides and treated with or without (medium) soluble CD40L (sCD40L). RELA nuclear translocation was evaluated by immunofluorescence after 30 min of stimulation with anti-RELA antibody (green) and nucleus labelling (Sytox blue, blue). The scale bar represents 100 $\mu$ m. The blank dots represent colocalization. Shown is one representative experiment out of 3.

**(C)** ASCs were transfected with control, RELA (pink circle), or NF- $\kappa$ B2 (green square) siRNAs before culture with activated CD4<sup>pos</sup> T cells (n=3 ASC batches) or soluble CD40L (sCD40L, n=3 ASC batches). IL-8 level was quantified in the supernatant by ELISA after 2 days (sCD40L) or 3 days (activated CD4<sup>pos</sup> T cells) of culture and normalized to 100% for the IL-8 quantification obtained with control siRNA transfected ASCs (dotted lines)

**(D)** ASCs were transfected with control, RELA (pink circle), or NF- $\kappa$ B2 (green square) siRNAs before culture with activated CD4<sup>pos</sup> T cells (n=3 ASC batches). Supernatants were then collected and tested for their capacity to trigger neutrophil recruitment in 2 hours through 5- $\mu$ M Transwell. The percentage of migrating neutrophils was normalized to 100% for the migration measured with supernatants from control siRNA transfected ASCs (dotted lines).

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**Figure 1**

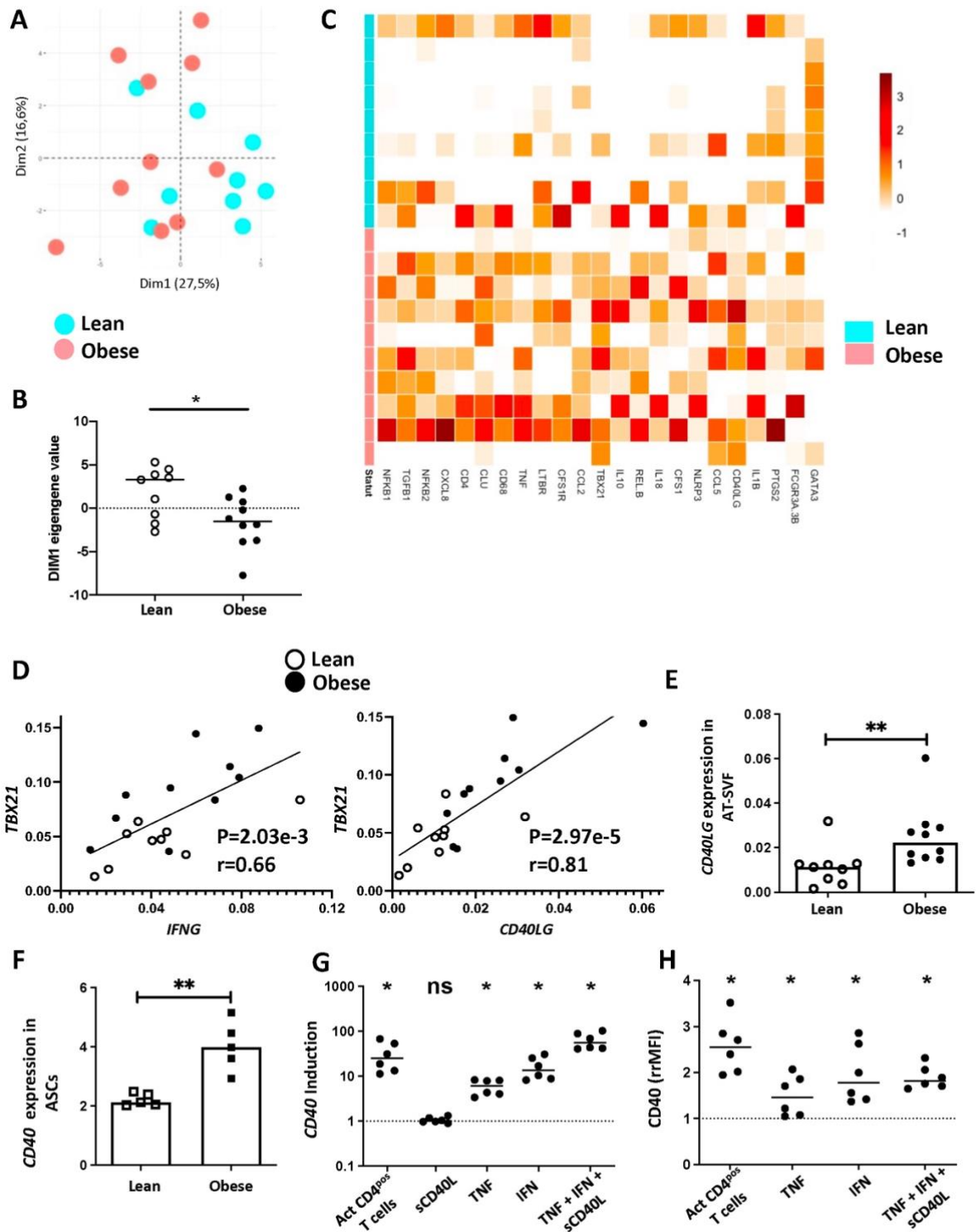
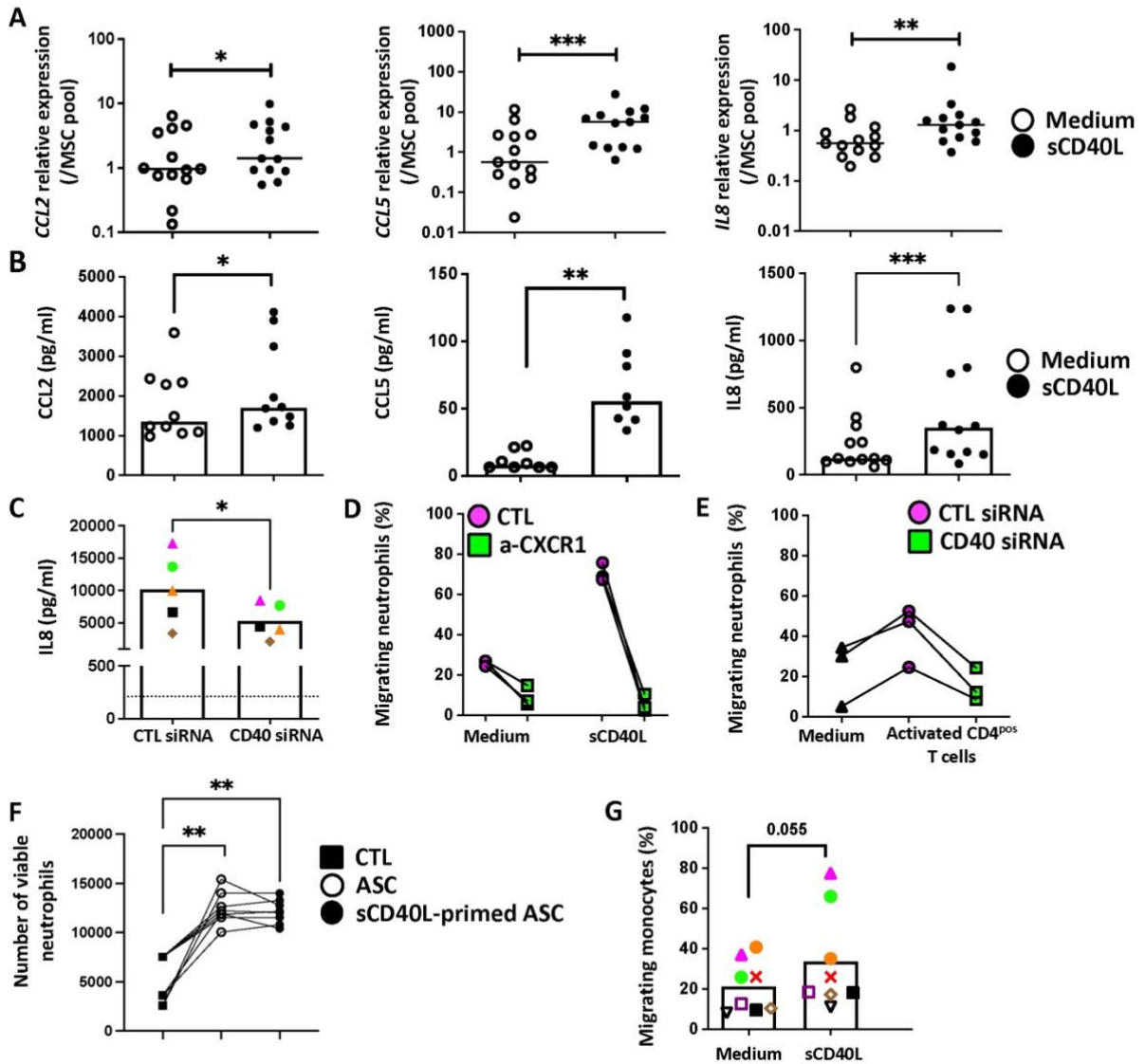


Figure 2





**Figure 3**

