

# Osteopontin is a key player for local adipose tissue macrophage proliferation in obesity



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## ABSTRACT

**Objective:** Recent findings point towards an important role of local macrophage proliferation also in obesity-induced adipose tissue inflammation that underlies insulin resistance and type 2 diabetes. Osteopontin (OPN) is an inflammatory cytokine highly upregulated in adipose tissue (AT) of obese and has repeatedly been shown to be functionally involved in adipose-tissue inflammation and metabolic sequelae. In the present work, we aimed at unveiling both the role of OPN in human monocyte and macrophage proliferation as well as the impact of OPN deficiency on local macrophage proliferation in a mouse model for diet-induced obesity.

**Methods:** The impact of recombinant OPN on viability, apoptosis, and proliferation was analyzed in human peripheral blood monocytes and derived macrophages. Wild type (WT) and OPN knockout mice (SPP1KO) were compared with respect to *in vivo* adipose tissue macrophage and *in vitro* bone marrow-derived macrophage (BMDM) proliferation.

**Results:** OPN not only enhanced survival and decreased apoptosis of human monocytes but also induced proliferation similar to macrophage colony stimulating factor (M-CSF). Even in fully differentiated monocyte-derived macrophages, OPN induced a proliferative response. Moreover, proliferation of adipose tissue macrophages in obese mice was detectable in WT but virtually absent in SPP1KO. In BMDM, OPN also induced proliferation while OPN as well as M-CSF-induced proliferation was similar in WT and SPP1KO.

**Conclusions:** These data confirm that monocytes and macrophages not only are responsive to OPN and migrate to sites of inflammation but also they survive and proliferate more in the presence of OPN, a mechanism also strongly confirmed *in vivo*. Therefore, secreted OPN appears to be an essential player in AT inflammation, not only by driving monocyte chemotaxis and macrophage differentiation but also by facilitating local proliferation of macrophages.

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**Keywords** OPN; Obesity; Inflammation; Adipose tissue; Adipose tissue macrophage

## 1. INTRODUCTION

Macrophage infiltration of the adipose tissue (AT) is a hallmark of the so-called obesity-associated low-grade inflammation that occurs in obesity and drives insulin resistance and development of type 2 diabetes. *In situ* proliferation of adipose tissue macrophages (ATMs) has been shown to take place at early stages of obesity and is associated with different cytokines [1,2]. This topic has become in vogue in recent years as an increasing number of studies described ATMs accumulation as the main driver of obesity-associated inflammation. Macrophages were shown to proliferate in atherosclerotic plaques [3], another inflammation-driven disorder. They also manifest increased proliferation in AT in response to cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 4 (IL-4) [1,4]. However, prerequisite mediators for AT macrophage proliferation in obesity have not yet been found.

Osteopontin (OPN) is a secreted glycoprotein involved in a wide variety of physiological and pathological conditions, including inflammatory processes [5–7]. OPN was found to be expressed in different cell types such as activated macrophages and T-cells, epithelial cells, and osteoclasts [8,9]. It contributes to mineralization of bones and kidney, tumor development and metastasis, and atherosclerosis [10]. OPN is actively expressed and secreted in macrophages at sites of inflammation, playing an important role in cell-mediated immunity [11,12]. An intracellular variant has also been described in cytoplasm and nucleus, with biological functions different from the secreted form and involved in signaling transduction pathways and cytoskeletal rearrangements [13,14]. OPN is also described as a migratory cytokine for monocytes and macrophages [15] and has also been shown to act as a survival factor for monocytes [16], while neutralizing of OPN resulted in increased macrophage apoptosis in AT and liver of obese animals [17]. A link between OPN and inflammation, obesity, and insulin resistance

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became well described during recent years [18,19]; however a putative involvement of OPN in macrophage proliferation remains unexplored. In the present work, we aimed at identifying the effects of OPN on monocyte and macrophage proliferation and their relevance in the context of obesity-driven AT inflammation. We focused at the beginning on proliferation and survival in human monocytes, discovering that OPN-treated cells outnumbered controls while diminishing apoptosis in the same experimental set up. Furthermore, OPN enhanced proliferation rates not only in human peripheral blood monocytes but also in *in vitro* differentiated macrophages. Notably, macrophages expressing proliferation marker Ki67 were virtually absent in genetically OPN-deficient (SP1KO) obese mice. Hence, local macrophage proliferation in obese AT is facilitated by OPN, thereby pointing to a novel mechanism that might trigger and maintain low-grade inflammation in obesity.

## 2. MATERIALS AND METHODS

### 2.1. Isolation and culture of human monocyte and macrophages

Monocytes were obtained from peripheral blood of healthy individuals by using a density gradient centrifugation (GE Healthcare, Little Chalfont, United Kingdom) and separated with a CD14-positive magnetic activated cell sorting — MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol. Human monocytes were differentiated to macrophages for 6 days in presence of 50 ng/ml M-CSF [20,21] (PeproTech, Rocky Hill, NJ, USA). The study was approved by the local ethics committee (EK 1241/2015).

### 2.2. Viability and apoptosis assays

Survival of monocytes was determined by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Wisconsin, USA). Briefly,  $1 \times 10^5$  cells were seeded in a 96-well plate, incubated with either OPN (1  $\mu\text{g/ml}$ ) or 50 ng/ml M-CSF as viability control or left untreated for 48 h. A total of 50  $\mu\text{l}$  buffers were added, according to manufacturer's instructions. The plate was then shaken for 2 min and luminescence was detected by a plate reader (EnSpire, Perkin Elmer). To test for apoptosis, freshly isolated monocytes were seeded in a concentration of  $1 \times 10^6$ /well into 24-well plates. Cells were incubated with either 1  $\mu\text{g/ml}$  OPN, 50 ng/ml M-CSF or left untreated for 24–48 h. Apoptosis was determined by TUNEL (Terminal deoxynucleotidyl transferase deoxyuracil triphosphate nick end labeling) assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). Cell suspensions were analyzed in flow cytometry quantifying integrated Fluorescein-dUTPs.

### 2.3. Proliferation assay

Monocytes were seeded in 24-well cell plates with normal RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h; the following day, they were stimulated with 1  $\mu\text{g/ml}$  of recombinant full length OPN (PeproTech). After 24 and 48 h stimulation, cells were harvested and prepared for CFSE Cell trace proliferation kit (Thermo Fisher Scientific). Briefly, cells were centrifuged, washed twice with cold PBS, resuspended in PBS with a final concentration of CFSE cell trace of 1  $\mu\text{M}$ , and incubated for 1 h at 37 °C. Afterwards, cells were washed twice with cold PBS and resuspended in warm DMEM (Thermo Fisher Scientific) for 10 min at RT in the dark and washed and resuspended again in cold PBS and proceeded to flow cytometric analysis. Flow cytometry was performed with BD FACSCanto™ II and BD FACSDiva™ software (Becton Dickinson New Jersey, USA). For human macrophages, proliferation was followed up to 48 h with a live cell movie analyzer (Juli Br, NanoEnTeck Inc., Seoul, Korea), which

took pictures of the indicated area every 5 min, generating a video and quantitative output. Control, untreated wells were compared and recorded at the same time with the OPN stimulated ones (1  $\mu\text{g/ml}$ ).

### 2.4. Diet induced obesity mouse study

Eight male WT (C57BL/6J) and eight male OPN-knockout mice (SPP1KO; B6.Cg-Spp1tm1Blh/J) were purchased by Charles River (Sulzfeld, Germany) and fed a high-fat diet (HFD, 60 kcal %, D12492; Research Diets, New Brunswick, NJ, USA) for 8 or 12 weeks. After the indicated time, mice were sacrificed and gonadal white adipose tissue (GWAT) was collected. The protocol was approved by the local ethics committee for animal studies.

### 2.5. Immunohistochemistry

Formalin-fixed GWAT was sectioned, de-paraffinized, and blocked for 60 min in blocking buffer (PBS, 5% normal goat serum, 0.3% Triton™ X-100). Blocking buffer was aspirated and sections were incubated overnight at 4 °C with 1:200 dilution of the monoclonal rat anti-mouse MAC-2 antibody (Cedarlane labs, Burlington, Canada) in PBS with 5% normal goat serum (Dako, Glostrup Municipality, Denmark). Slides were washed three times in PBS and incubated for 1 h at RT with 1:500 dilution of the monoclonal rabbit anti-mouse Ki67 antibody (Abcam, Cambridge, United Kingdom) in PBS with 5% normal goat serum (Dako). Slides were then washed three times in PBS and incubated for 1 h at RT in the darkness with 1:500 dilution of the Alexa Fluor 488 goat anti rat IgG (Thermo scientific). The process was repeated with Alexa Fluor 594 goat anti rabbit IgG for Ki67 (Thermo scientific). Nuclei were counterstained with DAPI (1  $\mu\text{g/ml}$  in ddH<sub>2</sub>O) for 10 min, washed three times in PBS, and mounted (VECTASHIELD® Mounting Medium for fluorescence, Vector Laboratories, Burlingame, CA USA) for microscope analysis (EVOS® FLoid® Cell Imaging Station) [22].

### 2.6. Bone marrow derived macrophages isolation

In order to isolate bone marrow derived macrophages (BMDMs), 6 WT and 6 SPP1KO mice were sacrificed, disinfected; femurs were extracted and cleaned with bone cleansing solution (PBS, 1% BSA, 100  $\mu\text{g/ml}$  streptomycin, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  Amphotericin B) and seeded in a 12 well plate at a concentration of  $10 \times 10^4$  cells per well for 6 days with M $\phi$  diff. medium (DMEM, 10% FBS, L929 conditioned medium, 100  $\mu\text{g/ml}$  streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 50  $\mu\text{M}$   $\beta$ -mercaptoethanol). On day 6, medium was changed to normal medium with either 50 ng/ml murine M-CSF (PeproTech) or murine OPN 1  $\mu\text{g/ml}$  (Sigma—Aldrich, St. Louis, Missouri, USA). The following day, cells were harvested and prepared for CFSE Cell trace proliferation kit as previously described (Thermo Fisher Scientific).

### 2.7. Statistics

Data are presented as mean values  $\pm$  standard error of the mean (SEM), and significance was assessed by Student's t-test. Dunnett-T *post-hoc* testing was employed to compare 2 different treatments to the same control. A p-value  $<0.05$  was considered statistically significant. All statistics were calculated using SPSS 22.0 software (Chicago, IL, USA).

## 3. RESULTS

### 3.1. Human primary monocytes proliferate in presence of OPN

Monocytes are recruited to inflamed tissues such as obese AT and differentiate into macrophages in response to several stimuli [23]. OPN was previously described to be an anti-apoptotic factor for human

monocytes [16]. To further evaluate the effects of OPN on monocyte fate, we cultivated human peripheral monocytes in presence of OPN. The number of viable cells, as determined by the amount of adenosine triphosphate (ATP), was significantly increased by OPN in a dose-dependent manner as shown in Figure 1A. Simultaneously, the percentage of apoptotic cells was reduced by OPN and M-CSF with similar efficiency (Figure 1B and C). Since not only apoptosis but also proliferation could contribute to the increased macrophage number, we performed a proliferation assay, which clearly demonstrated that human monocytes proliferated in response to OPN after 24 and 48 h, similar to induction by M-CSF (Figure 2A).

### 3.2. OPN induces proliferation in mature human macrophages

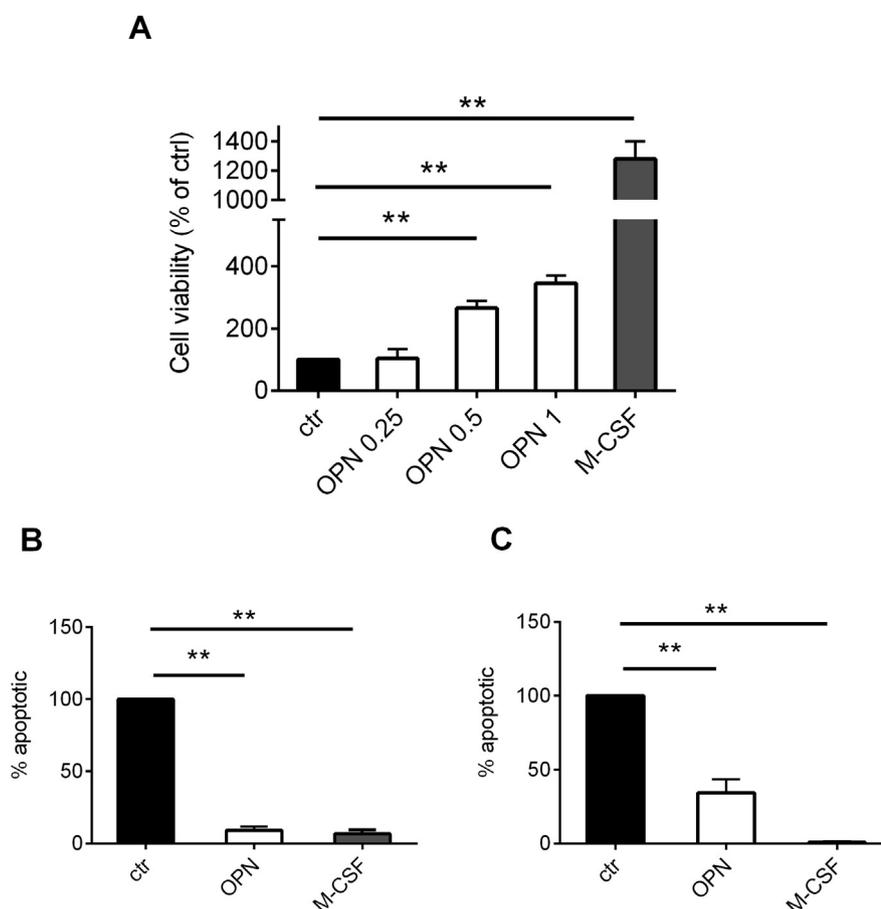
Since previous data [24] indicated that OPN acts locally in AT rather than systemically and because monocytes recruited to the tissue differentiate to macrophages, we addressed the question whether OPN also has a proliferative effect on mature macrophages. After differentiating peripheral blood monocytes into macrophages, we stimulated the cells with OPN and followed cell numbers with a motion camera. In the presence of OPN, monocyte-derived human macrophages increased cell motility and, strikingly, the cell number was increased indicating cell proliferation (Figure 2B).

### 3.3. ATMs proliferation is increased in WT compared to SPP1KO after 12 weeks of HFD

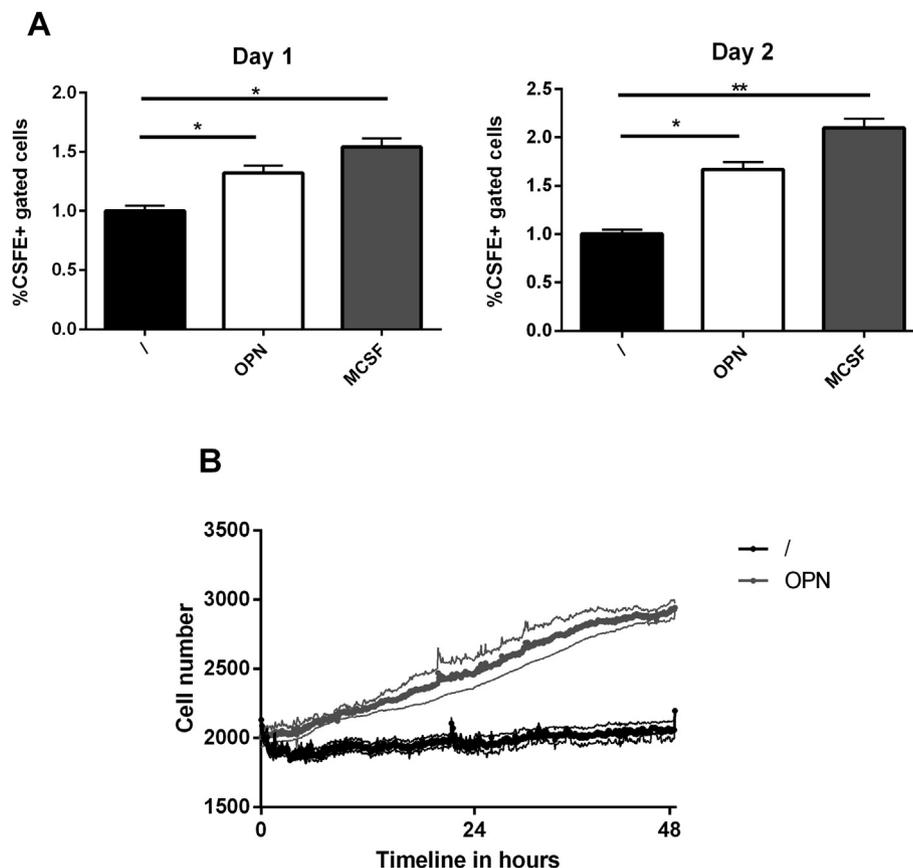
In order to confirm whether our results on human cells *in vitro* also apply *in vivo*, we performed a diet-induced obesity experiment using WT and SPP1KO mice. The average weight of mice was  $47.2 \pm 2.2$  g and  $47.9 \pm 1.7$  g for WT and SPP1KO, respectively, after eight weeks on HFD; and  $51.5 \pm 1.9$  g and  $50.7 \pm 2.6$  g, respectively, after 12 weeks, without significant differences between genotypes. We found proliferating macrophages in WT mice residing mainly in crown-like structures formed around adipocytes (Figure 3). Notably, significantly less proliferating macrophages were detected in obese SPP1KO compared to WT mice, particularly after 12 weeks HFD, whereas almost no proliferation at all could be observed in SPP1KO (Figure 3E and F).

### 3.4. OPN augments proliferation in murine bone marrow derived macrophages (BMDMs) independently of the genotype

*In vitro*, OPN increased BMDM proliferation after 24 h of treatment (Figure 4). Interestingly, there was no difference between the WT and SPP1KO genotypes, indicating that exogenous OPN is required for the proliferative response while intracellular OPN is not required for macrophage proliferation.



**Figure 1:** OPN increases human monocyte survival and strongly diminishes apoptosis. (A) Peripheral blood monocyte viability was determined by using Cell Titer-Glo Luminescent Cell Viability Assay. Cells were treated with 0.25/0.5/1  $\mu\text{g/ml}$  OPN, 50 ng/ml M-CSF or left untreated for 48 h. For the apoptosis assay, peripheral blood monocytes were stimulated for either (B) 24 h or (C) 48 h with 1  $\mu\text{g/mg}$  OPN, 50 ng/ml M-CSF or left untreated (ctr), apoptosis was determined with TUNEL assay and flow cytometric analysis. Results shown as mean of 4 independent experiments performed in duplicates  $\pm$  SEM. \*\*,  $p < 0.01$ .



**Figure 2:** Murine BMDMs and human macrophages proliferate in response to OPN. (A) BMDM proliferation was assayed with CSFE proliferation kit. After 6 days of differentiation, macrophages were treated with 1  $\mu\text{g}/\text{mg}$  OPN or 50  $\text{ng}/\text{ml}$  MCSF for 24 h, followed by assessment of BMDM proliferation using a CSFE kit. Results are shown as mean  $\pm$  SEM of 6 independent experiments performed in duplicates. \*\*,  $p < 0.01$ . (B) Human peripheral blood monocytes were differentiated to macrophages for 6 days with M-CSF. At the 6th day of differentiation, medium was changed to fresh RPMI1640 with or without 1  $\mu\text{g}/\text{mg}$  OPN. Proliferation rate was measured with live cell counting Juli Br ( $n = 3$ ). Results shown as mean of 3 independent experiments  $\pm$  SD depicted as thinner lines above and below the means.

#### 4. DISCUSSION

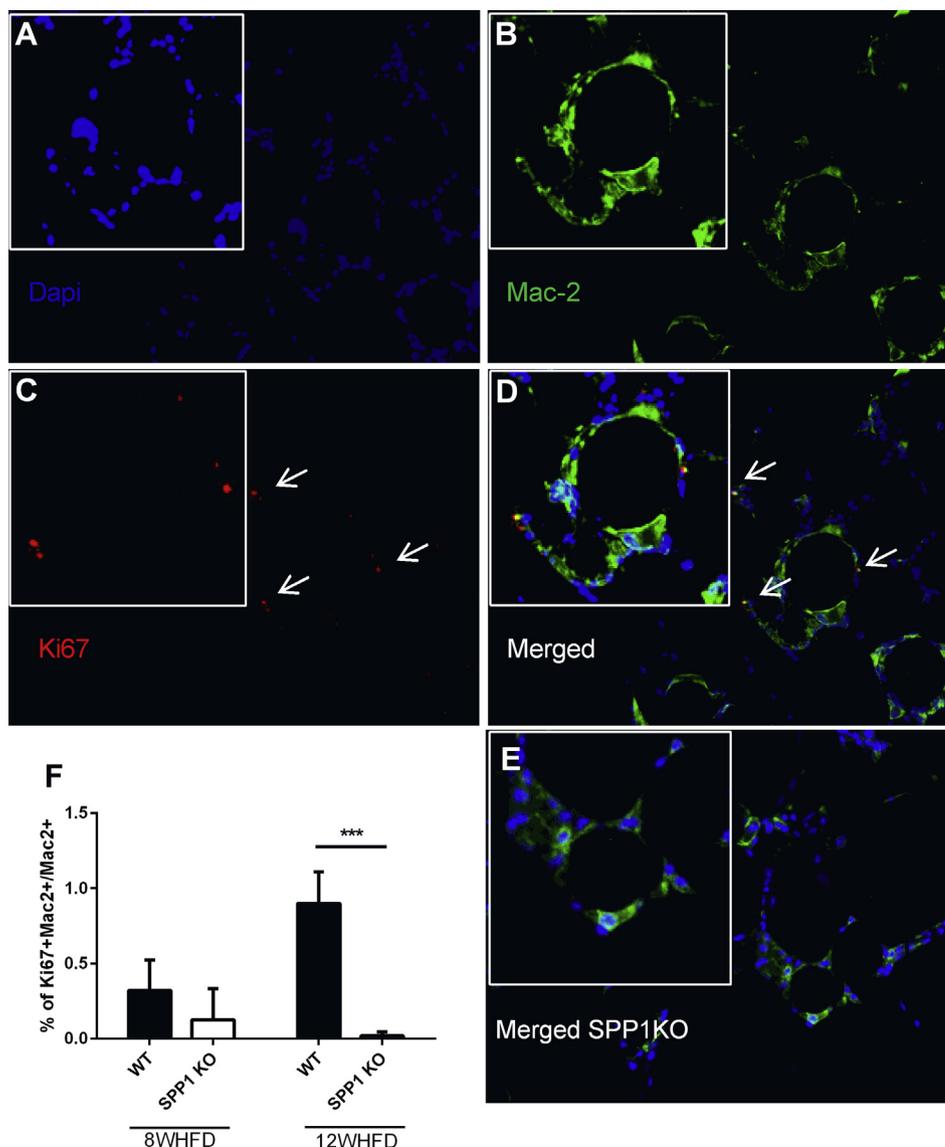
OPN is highly upregulated in mice after HFD and activates the inflammation cascade in monocytes and macrophages [25,26]. Activated macrophages produce OPN, which is also a strong chemotactic stimulus for recruitment and differentiation of blood monocytes into ATMs [17,25,27,28]. Here, we describe a novel aspect of OPN in promoting AT inflammation, namely by facilitating local macrophage proliferation in obesity.

Cell cycle progression involves upregulation of cyclins and down-regulation of cyclin-dependent kinase (Cdk) inhibitors, which are in turn regulated by two families of proteins, Inhibitors of CDK4 (INK4) family of tumor suppressor genes (p15, p16, p18, p19) and Cip/Kip family of Cdk inhibitor (p21, p27, p57) [29]. Some evidence suggests that p21 and p57 are downregulated whereas cyclin D is upregulated in adipose tissue macrophages in animals fed with high fat diet [30,31]. Although this is not well understood, it might be a molecular mechanism involved in OPN-induced proliferation of ATMs.

Cytokines such as MCP-1 and IL-4 were recently shown by other groups to be important in driving ATM proliferation [1–4]. The effect of these cytokines was demonstrated to be independent of the number of circulating monocytes and specific for AT, since other

organs such as liver or spleen did not show an increase in macrophage numbers in obesity [1]. These data raised some questions on the impact of monocyte recruitment to promote ATM accumulation in obesity [2]. The majority of ATMs are bone marrow derived, as elegantly shown by bone marrow transplantation and irradiation experiments [26]. However, others highlighted the importance of recruitment-independent mechanisms such as apoptosis, proliferation, and retention, which appear to be crucial factors in ATM-driven inflammation as well [32].

In obese AT, a resident [24] population of anti-inflammatory macrophages taking part in AT homeostasis could shift into an inflammatory macrophages in response to AT expansion, cytokine production, and adipocyte apoptosis. The total number of macrophages was shown to increase in mouse AT in response to diet-induced obesity [28]. This was explained by an augmented accumulation of macrophages due to an influx of bone marrow-derived precursors. However, these results were challenged in a recent publication [2], showing that ATM proliferation occurs independently of monocyte recruitment. According to these data, proliferation of resident macrophages appears to precede monocyte recruitment during obesity development. In mice, a heterogeneous population of local ATMs and recruited monocyte-derived macrophages have been shown to reside and proliferate locally in AT [2].



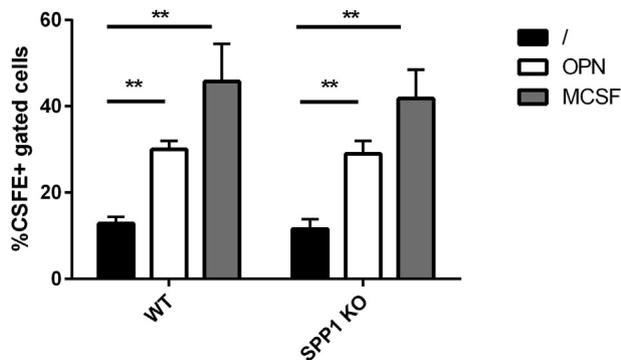
**Figure 3:** Murine AT macrophages proliferate more in obese WT compared to obese SPP1KO. WT and SPP1KO mice were fed a HFD for 8 and 12 weeks (8–12 WHFD) to induce obesity. Paraffin-embedded GWAT sections from WT mice were stained with specific antibodies against Mac-2 (green; B, D) and Ki67 (red; C, D) and nuclei counterstained with DAPI (blue; A, D), with merged stainings given in D and E (SPP1KO section shown as comparison) and higher magnification shown in the insets. (F) Quantification of Ki67<sup>+</sup>Mac2<sup>+</sup> double positive cells. Results shown as the mean percentage of Ki67<sup>+</sup>Mac2<sup>+</sup> cells of all Mac2<sup>+</sup> macrophages  $\pm$  SEM, of 8 obese WT and 8 obese SPP1KO mice (5 fields per slide). \*\*\*,  $p < 0.001$ .

Our studies reveal a crucial impact of extracellular OPN on local proliferation of ATM as shown by a variety of methods including human and murine experimental setups. On the one hand, human monocyte-derived macrophages showed robust proliferation in response to exogenous OPN treatment as assessed by ATP production. In addition, proliferating mouse ATMs undergoing cell cycle progression were detectable during diet-induced obesity (12 weeks). When OPN was deprived in the knock out model (SPP1KO), ATMs completely lost their proliferative behavior, highlighting the critical importance of OPN in obesity-driven AT inflammation. Notably, the proliferative action of exogenous OPN on murine macrophages was independent of endogenous OPN expression, indicating that intracellular OPN is not involved.

## 5. CONCLUSIONS

In conclusion, our data indicate that local macrophage proliferation in obese AT *in situ* is OPN dependent. Still to be explored is the relative contribution of other cytokines such as MCP-1 and IL-4 in this scenario. Nonetheless, we demonstrated that OPN is a substantial driver of macrophage, and particularly ATM, proliferation and is one of the most important cytokines in the onset and propagation of AT inflammation. Targeting and neutralizing OPN may represent an effective therapeutic option in the prevention and treatment of obesity-associated chronic inflammation and its clinical sequelae, namely type 2 diabetes and cardiovascular disease.

## Brief Communication



**Figure 4:** Mouse BMDMs show increased proliferation in response to exogenous OPN and M-CSF, regardless of endogenous OPN expression. BMDM proliferation was assayed with CFSE proliferation kit. After 6 days of differentiation, macrophages were treated with 1  $\mu$ g/mg OPN or 50 ng/ml MCSF for 24 h, followed by assessment of BMDM proliferation using a CFSE kit. Results are shown as mean  $\pm$  SEM of 6 independent experiments performed in duplicates. \*\*,  $p < 0.01$ .

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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