IL-1β- and IL-4-polarized macrophages have opposite effects on adipogenesis of intramuscular fibro-adipogenic progenitors in humans

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Intramuscular fat deposition represents a negative prognostic factor for several myopathies, metabolic diseases and aging. Fibro-adipogenic progenitors (FAPs) are considered as the main source of intramuscular adipocytes, but the mechanisms controlling their adipogenic potential are still not elucidated in humans. The aim of this study was to explore the regulation of human FAP adipogenesis by macrophages. We found that CD140a-expressing FAPs were located close to CD68 positive macrophages in muscles from patients with Duchenne muscular dystrophy (DMD). This strongly suggests a potential interaction between FAPs and macrophages in vivo. Isolated human primary FAPs were then differentiated in the presence of conditioned media obtained from primary blood monocyte-polarized macrophages. Molecules released by IL-1β-polarized macrophages (M(IL-1β)) drastically reduced FAP adipogenic potential as assessed by decreased cellular lipid accumulation and reduced gene expression of adipogenic markers. This was associated with an increased gene expression of pro-inflammatory cytokines in FAPs. Conversely, factors secreted by IL-4-polarized macrophages (M(IL-4)) enhanced FAP adipogenesis. Finally, the inhibition of FAP adipocyte differentiation by M(IL-1β) macrophages requires the stimulation of Smad2 phosphorylation of FAPs. Our findings identify a novel potential crosstalk between FAPs and M(IL-1β) and M(IL-4) macrophages in the development of adipocyte accumulation in human skeletal muscles.

Under pathological conditions such as Duchenne muscular dystrophy (DMD)1,2, type II diabetes3,4, sarcopenia5, intramuscular adipocytes invade skeletal muscles and replace a large proportion of muscle fibers. This adipocyte infiltration leads to a poor quality of muscles and dysfunctional performances6. Fatty degeneration was shown to be an accurate measurement of the severity of DMD and a limit for the success of current cell and genetic therapies. Many can contribute to ectopic intramuscular adipocyte deposits including myoendothelial cells7,8, pericytes9, mesoangioblasts10,11 and PW1-expressing cells (PICs)12,13. Recent studies demonstrated that intramuscular adipocytes mainly emanated from a population of fibro-adipogenic progenitors (FAPs) that reside between muscle fibers14. Their role in muscle regeneration was in part elucidated in mice. After injury, FAPs proliferate, interact with myoblasts to promote the formation of new muscle fibers15, and eventually return to quiescent state or are cleared by apoptosis16. With impaired regeneration as in DMD, FAPs expand and differentiate to generate the components of the fibro-fatty tissue17,18. We and others have identified the presence of FAPs in human skeletal muscles by the expression of specific markers CD140a19, CD15 and CD3418,20. In vitro and in vivo, these progenitors differentiate into adipocytes and myofibroblasts in response to adequate stimuli18,19,20. While Uezumi et al. reported an inhibition of FAP adipogenesis by myoblasts in mice18,21, the regulation of FAP differentiation during muscle regeneration remains to be elucidated in humans.

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Among potential regulators of intramuscular adiposity are macrophages whose depletion alters muscle regeneration\(^\text{29,30}\). In particular, the ablation of CD11b-positive macrophages in mice affects the fat accumulation in injured muscles\(^\text{31}\). Conditioned medium from murine macrophages inhibits the adipogenic potential of a subpopulation of multipotent muscle stem cells\(^\text{32}\). Moreover, melatonin, a nitric oxide donating drug, modulates the number of FAPs committed into adipocytes compared to RM CM (Fig. 2A, top and middle panels). Indeed, lipid accumulation, as stained by oil red O, was decreased in the presence of M(IL-1β) CM whereas bigger adipocytes were observed, as represented by the presence of fibrillary collagen stained by SHG (second harmonic generation) in a DMD muscle section (Fig. 1A) and CD68\(^+\) macrophages (Supplementary Fig. S1A). Interestingly, CD140\(+\) macrophages (Fig. 1A) and CD68\(+\) adipocytes (Fig. 1B) were localized in the same regions where degenerating areas were observed, as represented by the presence of fibrillar collagen stained by SHG (second harmonic generation) in blue. To demonstrate a possible connection between FAPs and macrophages, DMD muscle sections were co-stained with anti-CD140a and anti-CD68 antibodies. Importantly, CD140\(+\) FAPs and CD68\(+\) macrophages were found in contact with FAPs (in green), whereas another CD68\(+\) macrophage (white arrow) was in contact with FAPs (in green), whereas another CD68\(+\) macrophage (red arrow) was located less than 20 \(\mu\)m from FAPs (Fig. 1C, right panel). The number of CD68\(+\) macrophages per section was approximately 12.4 \(\pm\) 6.85 and the number of CD68\(+\) macrophages in contact with FAPs per section was approximately 3 to 8 \(\pm\) 0.10. This results show for the first time in humans and particularly in DMD muscles, that FAPs and macrophages reside near to each other, suggesting a potential in vivo interaction between these two types of cells.

### Results

#### FAPs and macrophages closely located in human dystrophic muscles.

DMD muscles are characterized by a significant inflammatory reaction with an increased number of infiltrating macrophages\(^\text{36}\) and FAPs\(^\text{28}\). Indeed, DMD muscles presented numerous CD140\(+\) FAPs (Fig. 1A) and macrophages expressing CD68 (Fig. 1B), a surface marker expressed by all macrophage subtypes\(^\text{37}\). By contrast, healthy muscles displayed few CD140\(+\) FAPs (Supplementary Fig. S1A) and CD68\(+\) macrophages (Supplementary Fig. S1B). Interestingly, CD140\(+\) FAPs (Fig. 1A) and CD68\(+\) macrophages (Fig. 1B) were localized in the same regions where degenerating areas were observed, as represented by the presence of fibrillar collagen stained by SHG (second harmonic generation) in blue. To demonstrate a possible connection between FAPs and macrophages, DMD muscle sections were co-stained with anti-CD140a and anti-CD68 antibodies. Importantly, CD140\(+\) FAPs and CD68\(+\) macrophages were found in contact with FAPs (in green), whereas another CD68\(+\) macrophage (red arrow) was located less than 20 \(\mu\)m from FAPs (Fig. 1C, right panel). The number of CD68\(+\) macrophages per section was approximately 12.4 \(\pm\) 6.85 and the number of CD68\(+\) macrophages in contact with FAPs per section was approximately 3 to 8 \(\pm\) 0.10. This results show for the first time in humans and particularly in DMD muscles, that FAPs and macrophages reside near to each other, suggesting a potential in vivo interaction between these two types of cells.

#### Unlike fibrogenesis, adipocyte differentiation of human FAPs is affected by M(IL-1β) and M(IL-4) macrophage-secreted factors.

Before evaluating the effect of macrophage-derived factors on human FAP differentiation, we firstly validated the in vitro model of human primary macrophage polarization as well as the adipogenic potential of FAPs. Three distinct conditioned media (CM) from control unpolarized resting macrophages (RM), IL-1β-treated (M(IL-1β)) or IL-4-treated (M(IL-4)) macrophages were produced. IL-1β, but not IL-4, increased the gene expression of IL1B, IL6 and CCL2 in macrophages (Supplementary Fig. S2A). IL-4, but not IL-1β, stimulated the gene expression of MRC1, CD200R1, F13A1 and CCL18 (Supplementary Fig. S2B) in macrophages. According to the nomenclature of macrophage activation and polarization\(^\text{23}\), these data indicate that M(IL-1β) and M(IL-4) can be considered as pro-inflammatory and anti-inflammatory macrophages, respectively. RM CM was used as control CM for unpolarized RM macrophages. We also confirmed the high adipogenic potential of FAPs as shown by the presence of adipocytes with lipid droplets (Supplementary Fig. S3A) and the induction of adipocyte marker expression (Supplementary Fig. S3B) after 3, 8, 13, 17 and 20 days of differentiation in the presence of a pro-adipogenic medium.

To note, adipogenesis is conserved in FAPs isolated from skeletal muscles of DMD patients. Indeed, gene expression of adipogenic markers PLIN1, ADIPOQ and FABP4 was similar in DMD patients compared to healthy donors (Supplementary Fig. S3C). Then, confluent FAPs were induced to differentiate in a pro-adipogenic medium containing RM, M(IL-1β) or M(IL-4) CM (50/50), and adipogenic differentiation was measured 10 days later. Interestingly, CM from M(IL-1β) or M(IL-4) macrophages strongly affected FAP adipocyte differentiation compared to RM CM (Fig. 2A, top and middle panels). Indeed, lipid accumulation, as stained by oil red O, was lower in the presence of CM from M(IL-1β) macrophages, compared to RM CM. By contrast, lipid accumulation increased after incubation with CM from M(IL-4) macrophages. In line, the number of FAPs committed into adipogenesis decreased in the presence of M(IL-1β) CM but strongly increased with CM from M(IL-4) macrophages (Fig. 2B). Moreover, a smaller size was observed for adipocytes differentiated with M(IL-1β) CM whereas bigger...
adipocytes were detected in the presence of M(IL-4) CM, compared to RM CM (Fig. 2C). This observation correlated with the lower number of lipid droplets/adipocyte observed with M(IL-1β) CM than with CM from M(IL-4) macrophages (Fig. 2D).

**Figure 1.** FAPs closely localized with CD68⁺ macrophages within degenerating areas of DMD muscles. Frozen sections of DMD biopsies were stained with anti-CD140a for detection of FAPs (A) or anti-CD68 antibody for macrophages (B) or both (C). Fibrillar collagen was visualized in blue by second-harmonic generation imaging (SHG) and DNA was stained with DRAQ5 in (A, B) (C) Cell nuclei were visualized with DAPI (blue). White arrow shows one CD68⁺ cell in contact with one FAP and red arrow shows another CD68⁺ cell in proximity to one FAP. Myofibers are indicated by white asterisks. Scale bar: 20 µm. The right panels are a magnification of the merge panels. The analysis was performed on three different DMD biopsies. Representative views are shown.
As we recently published, FAPs isolated from healthy skeletal muscles have a fibrogenic potential. Thus, the effect of conditioned media on FAP fibrogenesis was assessed. After 10 days of differentiation in a pro-adipogenic medium, only few α-smooth muscle actin (αSMA)-positive myofibroblasts were observed in the presence of RM, M(IL-1β) and M(IL-4) CM (Fig. 2A, bottom panel), suggesting that the inflammatory status of macrophages did not control FAP differentiation into myofibroblasts.

M(IL-1β) and M(IL-4) macrophage-secreted factors alter gene expression in human FAPs. To elucidate the molecular mechanisms involved in the differential regulation of FAP adipogenesis by M(IL-1β) and M(IL-4) CM, gene expression of several specific adipose markers was analysed. Expression of the transcription factors peroxisome proliferator-activated receptor γ (PPARG) and CCAAT/enhancer-binding protein α (C/EBPα) (Fig. 3A) and their target genes perilipin 1 (PLIN1) and fatty acid binding protein 4 (FABP4) (Fig. 3B) strongly diminished in M(IL-1β) CM-treated FAPs while significantly increased in M(IL-4) CM-treated FAPs compared to RM CM-treated FAPs. However, the expression of C/EBPB was unchanged in the presence of M(IL-1β) or M(IL-4) CM (Fig. 3A). The expression of adiponectin (ADIPOQ) also decreased with M(IL-1β) CM while it increased upon M(IL-4) CM treatment (Fig. 3C). Of note, M(IL-1β) and M(IL-4) CM did not affect leptin (LEP) expression (Fig. 3C).

The effect of M(IL-1β) and M(IL-4) CM on the inflammatory status of FAPs after 10 days of differentiation was also investigated by measuring the expression of pro-inflammatory markers (Fig. 3D). Interestingly, only M(IL-1β) CM significantly induced IL6, C-X-C motif chemokine ligand 8 (CXCL8) and TNFA expression compared to RM or M(IL-4) CM. IL1β and C-C motif chemokine ligand 2 (CCL2) expression also tended to be more expressed in M(IL-1β) CM-treated FAPs but without reaching statistical significance.

Figure 2. Factors secreted by M(IL-1β) and M(IL-4) macrophages differently affect human FAP adipogenesis. FAPs were differentiated into adipocytes for 10 days in a pro-adipogenic medium containing conditioned medium (CM) from RM, M(IL-1β) or M(IL-4) macrophages. (A) Adipocytes were unstained (top panel) or stained by oil red O (middle panel). Pictures were captured under light microscopy (scale bar: 10 μm). In the bottom panel, FAP-derived myofibroblasts were visualized by αSMA (α-smooth muscle actin) immuno-staining (red). Nuclei were labelled with DAPI in blue (scale bar: 50 μm). The number of adipocytes per field (B), the adipocyte size (C), and the number of lipid droplets per adipocyte (D) were measured on oil red O-stained cells. One representative experiment of nine separate experiments was shown (3 blood donors and 3 muscle donors). Data are presented as means of triplicates ± SEM; * indicates statistically significant difference vs RM CM-treated FAPs; # indicates statistically significant difference vs M(IL-1β) CM-treated FAPs; *P < 0.05, **P < 0.01, *** or ###P < 0.001.
Finally, in line with the results of fibrogenesis (Fig. 2A bottom panel), expression of specific fibrogenic markers such as collagen type A1 (COL1A1), fibronectin 1 (FN1) and α actin 2 (ACTA2) did not vary between the three experimental conditions (Fig. 3E).

Therefore, M(IL-1β) macrophage-secreted factors inhibited adipogenesis and promoted the gene pro-inflammatory profile while M(IL-4) macrophage-secreted factors stimulated adipocyte differentiation of human FAPs.

Smad2 phosphorylation drives the reduced FAP adipogenesis by M(IL-1β) macrophages. Since an anti-adipogenic role of recombinant TGFβ1 has been previously shown in FAPs30, the effect of SB431542, a specific inhibitor of the TGFβ superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK731, was first examined on intrinsic FAP adipogenic differentiation. As expected, SB431542 stimulated FAP differentiation into adipocytes (Fig. 4A), an effect correlated with a three fold increase of PLIN1 gene expression (Fig. 4B). These data prompted us to hypothesize a potential role of the ALK signalling pathway in the inhibition of adipogenic differentiation of FAPs by M(IL-1β) macrophages. To verify this hypothesis, FAPs were...
differentiated in the presence of M(IL-1β) CM with or without SB431542 and the effect of the inhibitor on adipogenesis was evaluated 10 days later and was compared to M(IL-4) CM-treated FAPs. The addition of SB431542 counteracted the M(IL-1β) CM-dependent inhibition of FAP adipogenesis, as assessed by an increase of oil red O staining (Fig. 4C) and the expression of several adipocyte markers (Fig. 4D). Lipid accumulation, PPARγ and ADIPOQ gene expression indicated that adipogenic rate in FAPs treated with M(IL-1β) CM and SB431542 was lower than in M(IL-4) CM-treated FAPs. By contrast, FABP4 and CEBPΑ expression was higher. We concluded that activation of the ALK/Smad2 pathway by soluble molecules secreted by M(IL-1β) macrophages was required to inhibit FAP differentiation into adipocytes.

We then measured the expression and phosphorylation of Smad2, a downstream effector of ALK. A lower Smad2 phosphorylation was observed in FAPs cultured with M(IL-4) CM, compared to M(IL-1β) CM-treated FAPs (Fig. 4E,F). This indicates that the regulation of FAP adipogenesis by M(IL-1β) and M(IL-4) macrophages was associated with a differential activation of the ALK/Smad2 signalling pathway.

**FAP localise closed to alternative macrophages in DMD muscles.** Expression of mannose receptor C-type 1 (MRCl), that codes for the mannose receptor (MR), was strongly induced in macrophages by IL-4 but not by IL-1β (Supplementary Fig. 2B). This receptor is considered as an in vivo marker for alternative macrophages47. We found the presence of MR+ macrophages in DMD muscle sections (Fig. 5A) and in healthy muscles (Supplementary Fig. S4). Interestingly, MR+ macrophages were located in the same fibrotic regions (Fig. 5A) than CD140α+ FAPs (Fig. 1A) in DMD muscles. To assess a possible interaction between FAPs and MR+ macrophages in vivo, DMD muscle sections were co-stained with human anti-CD140a and anti-MR antibodies. Importantly, MR-positive macrophages were located near to the CD140α+ FAPs (Fig. 5B). Indeed, at the higher magnification, we found one MR+ macrophage (in red) in contact with FAPs (in green) (Fig. 5B, right panel). The number of MR+ macrophages per section was approximately 4.25 ± 1.73 and the number of MR+ macrophages in contact with FAPs per section was approximately 1/2 ± 0.18. These data strongly suggest that in vivo M(IL-4)-dependent alternative macrophages could interact with FAPs to control their adipogenic potential.

**Discussion**

We have earlier reported that FAPs located between myofibers in human skeletal muscles can differentiate into white adipocytes in vitro and in vivo34. The regulation of their adipogenesis during muscle regeneration was partly characterized in mice. Indeed, satellite cell-derived myofibers strongly inhibit adipogenesis of FAPs to prevent fatty infiltration in regenerating muscles toward successful repair19. In addition to myogenic progenitors and FAPs, macrophages are central actors of regeneration in healthy and dystrophic muscles48. However, to our knowledge, no studies on the control of FAP differentiation by macrophages have been performed in humans. Here, we report that IL-1β-activated macrophages and IL-4-polarized macrophages have opposite effects on FAP differentiation into adipocytes in vitro, and this was dependent on the Smad2 phosphorylation status in FAPs.

First, we show that CD140α+ FAPs localized closed to CD68+ macrophages in DMD muscles, thus strengthening our hypothesis that macrophages could regulate FAPs behavior in humans. To verify this hypothesis, we used an experimental model where human FAPs were differentiated in the presence of conditioned medium from human blood monocyte-derived macrophages. Monocytes were differentiated for 6 days into unpolarized (RM) macrophages or were polarized into anti-inflammatory macrophages with IL-4 (M(IL-4)) or into pro-inflammatory macrophages with IL-1β (M(IL-1β)). The choice of these stimuli is consistent with the high IL1β expression in the diaphragm of mdx mice41, an animal model to study DMD, and with the expression of IL-4 receptor by macrophages in quadriceps of these mice42. These results thus validate the pertinence of our in vitro model of macrophage polarization. We use CM from resting macrophages as control to assess the effect of macrophage inflammatory status on FAP differentiation and to get rid of the decrease in medium nutrients in M(IL-1β) and M(IL-4) CM.

Importantly, we demonstrated that factors released from M(IL-1β) macrophages inhibit the differentiation capacity of FAPs while M(IL-4) macrophage-secreted molecules have a pro-adipogenic effect. CM affect both the differentiation of FAPs into adipocytes, as indicated by the variation of adipocyte number, and the terminal adipogenic rate of FAPs. We can hypothesize that the M(IL-4)-dependent increase of adipocyte number can be explained by an enhancement of FAP commitment towards the adipogenic lineage. Interestingly, CEBPB, a master gene of adipocyte commitment49, was unaffected, suggesting that macrophages regulate events downstream to CEBPB expression.

In the last decade, several studies reported that human macrophage cell lines or primary monocyte-derived pro-inflammatory macrophages inhibit adipogenesis of adipose progenitors derived from human adipose tissue43,44. Similar to these results, gene expression of PPARγ and CEBPα, the two master regulators of adipocyte differentiation49, decreased in FAPs cultured with M(IL-1β) CM. By contrast, since CEBPB expression was unaffected, this suggests that this transcription factor is not required for the effect of macrophages on PPARγ and CEBPB gene expression at day 10 of differentiation. This was consistent with the reported anti-adipogenic effects of secreted factors from bacterial lipopolysaccharide (LPS)-activated human monocyte-derived macrophages on subcutaneous adipose tissue preadipocytes47. Conversely to Lacasa et al., leptin expression did not vary in our experiments in FAPs cultured with M(IL-1β) conditioned medium, suggesting a specificity of FAP-derived adipocytes. We had also shown that these adipocytes have the unexpected feature of being insulin-resistant28.

It was reported that M(IL-4) anti-inflammatory macrophages serve as an important source of catecholamine for beiging activation in mouse subcutaneous adipose tissue46–51. However, no expression of uncoupling protein 1 (UCP1), a marker of adipocyte beiging, was detected in human FAPs cultured with M(IL-4) conditioned medium (data not shown), suggesting that factors released by M(IL-4) macrophages stimulated the differentiation of FAPs into white adipocytes exclusively. This is in accordance with our previous work showing that human FAPs give rise to bona fide white adipocytes28.
Figure 4. The M(IL-1β)-dependent inhibition of FAP adipogenesis requires Smad2 phosphorylation. (A) FAPs were differentiated into adipocytes for 10 days in a pro-adipogenic medium with or without SB431542. Oil red O-stained adipocytes were visualized by light microscopy. Scale bar: 10 µm. (B) PLIN1 gene expression was measured by quantitative Q-PCR. Data are presented as means ± SEM of three separate experiments in duplicates; **P < 0.01 vs untreated FAPs. (C,D) FAPs were differentiated into adipocytes for 10 days in a pro-adipogenic medium containing M(IL-1β) CM with or without SB431542 or containing M(IL-4) CM. (C) Oil red O-stained adipocytes were visualized by light microscopy. Scale bar: 10 µm. (D) PLIN1, ADIPOQ, FABP4 and C/EBPA gene expression was determined by quantitative Q-PCR. Data are presented as means ± SD of one representative experiment performed in triplicates. * indicates statistically significant difference vs M1 CM-treated FAPs; # indicates statistically significant difference vs FAPs treated with M(IL-1β) CM and SB431542; * or P < 0.05, ** or 0.01 < P < 0.001, *** or 0.001 < P < 0.0001. (E,F) Confluent FAPs were treated with a pro-adipogenic medium containing M(IL-1β) CM or M(IL-4) CM and total proteins were extracted 1 hour later. (E) Protein expression of phosphorylated Smad2 (P-Smad2), total Smad2/3 and tubulin was assessed by western blot. (F) P-Smad2 band intensity was quantified and normalized to total Smad2/3 signals. **P < 0.01 vs M(IL-1β) CM-treated FAPs.
We also found that anti-adipogenic effect of M(IL-1β) macrophages is associated with the increase of IL-6, IL-8, TNFA, IL-1B and MCP-1 gene expression in FAPs after 10 days of culture in pro-adipogenic medium. Even if these results have to be confirmed at the protein level, the significance increase of pro-inflammatory cytokines in FAPs cultured with M(IL-1β) CM and their defective engagement toward adipogenesis needs to be understood, particularly during the muscle regeneration process and in DMD muscles characterized by macrophage infiltration in injured areas. Previous studies have reported that IL-6, MCP-1, TNFα and IL-1β are usually up-regulated in damaged muscle in the early phase of regeneration and in dystrophic muscles where an intramuscular accumulation of FAPs is observed. FAPs could be one of the sources of these cytokines in injured and diseased muscles and, thus, could mediate the inflammatory response. In addition to their role in inflammation, IL-6 and TNFα can also contribute to muscle homeostasis by controlling the proliferative and differentiation capacities of muscle stem cells. Interestingly, mouse FAPs provide a source of IL-6 that regulate satellite cell activity.

In addition to their adipogenic potential, *in vitro* human FAPs display fibrogenic capacity in response to TGFβ. We showed that M(IL-1β) and M(IL-4) macrophage-secreted factors did not affect extra-cellular matrix. In contrary to our results, it has been reported that inflammatory adipose tissue-derived preadipocytes exposed to macrophage-secreted factors overexpressed extra-cellular matrix genes. This discrepancy could be explained by the different cytokines secreted by macrophages employed in each study, which differ in their origin (isolated from blood or adipose tissue), and in their culture conditions (such as difference in stimuli used to induce differentiation). Moreover, our results were obtained in a pro-adipogenic medium, which is not
representative of the environment of fibrotic muscles, such as DMD. Further experiments using for example a pro-fibrogenic medium complemented with TGF\(\beta\)1, could allow concluding regarding a potential regulation of FAP fibrogenesis by macrophages.

Mechanistically, we show that M(IL-1\(\beta\)) macrophages-secreted factors highly phosphorylated Smad2 in FAPs, and this phosphorylation is mediated by ALK4/5/7 receptor activity. Human DMD muscles contain CD140a-expressing cells that are positive for phospho-Smad2/3, but are also invaded by adipocytes. These conflicting results could be explained by the difference in the inflammatory status between in vitro and in vivo macrophages. Members of the TGF\(\beta\) family, that are known to stimulate Smad2 phosphorylation via ALK4/5/7 receptor activity, are numerous such as activins, myostatin, GDF11 (growth differentiation factor 11) and TGF\(\beta\)56. Their implication in the inhibition of FAP adipogenesis by M(IL-1\(\beta\)) macrophages remains to be identified. Finally, we detected FAPs in proximity to MR-positive macrophages in human DMD muscles suggesting that these macrophages could promote the accumulation of intramuscular adipocytes by interacting with FAPs. Furthermore, healthy and DMD FAPs have similar adipogenic differentiation rate suggesting that DMD FAPs could be competent to respond to signals issued from MR-positive macrophages.

In summary, our results show that M(IL-1\(\beta\)) macrophages release cytokines that inhibit FAP adipogenesis via Smad2 phosphorylation, whereas M(IL-4) macrophages have a pro-adipogenic effect. These differential interplays between FAPs and macrophage subtypes provide new elements to help developing medical regenerative strategies to prevent adipocyte deposits in diseased muscles associated with a chronic inflammation such as DMD.

### Materials and Methods

#### Reagents and antibodies.

Cell culture media, serum, phosphate-buffered saline and trypsin were purchased from Lonza (Verviers, Belgium) and cell culture reagents were from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France).

Human antibodies for immunofluorescence analysis were purchased as indicated: anti-\(\alpha\)SMA (#A5228) from Sigma-Aldrich, anti-CD68 (#EBM11) from Dako (Glostrup, Denmark), anti-MR (#ab8918) from Abcam (Cambridge, United-Kingdom), anti-CD140a (#3174 T) from Cell Signaling (Ozyme, St Quentin en Yvelines, France). Human antibodies for western blot analysis were purchased as indicated: anti-phospho-Smad2 (Ser465/467, #3101) and anti-Smad2/3 (#3102) from Cell Signaling, and anti-\(\beta\)-tubulin from Sigma-Aldrich.

Human antibodies against CD56-APC (#341027) and CD140a-PE (#556002) were purchased from BD-Biosciences (Le Pont de Claix, France). Human recombinant IL-4 (#200-04) and IL-1\(\beta\) (#200-01B) were purchased from Peprotech (Neuilly-Sur-Seine, France) and used at 15 ng/ml. SB431542 (#S4317) was purchased from Sigma-Aldrich Chimie and used at 5 \(\mu\)M. DRAQ5 fluorescent probe (#62254) was purchased from Thermofisher Scientific and used at 20 \(\mu\)M.

Human skeletal muscle FAP isolation. Tissue samples were obtained as res nullius from surgeries or diagnostic biopsies from healthy donors aged from 1 to 8 years and with the informed consent of the parents. All protocols for healthy skeletal muscles were approved by the Centre Hospitalier Universitaire de Nice Review Board, according to the French Regulatory Health Authorities. Samples were placed in F10 medium and transferred to the laboratory. DMD biopsies were obtained from Myobank-AFM Institut de Myologie, Paris, France. Characteristics of healthy and DMD muscles are reported in Table 1. Skeletal muscle cells were isolated by a standard method28. Briefly, healthy skeletal muscles were minced into 1 mm\(^3\) fragments and digested at 37°C, first using liberase (Roche Diagnostics, Meylan, France) for 1 hour and then using 0.25% trypsin-EDTA (Lonza, Verviers, Belgium) for 20 minutes. The enzymatic reaction was stopped by adding 10% fetal bovine serum (FBS).

| Name | Gender | Age (year) | Muscle origin |
|------|--------|------------|---------------|
| Healthy muscles |
| 1 | male | 8 | paravertebral |
| 2 | male | 1 | paravertebral |
| 3 | male | 4 | paravertebral |
| 4 | female | 17 | paravertebral |
| 5 | male | 19 | paravertebral |
| 6 | male | 17 | gluteus maximus |
| 7 | female | 15 | paravertebral |
| 8 | female | 16 | paravertebral |
| DMD muscles |
| 1 | male | 14 | latissimus dorsi |
| 2 | male | 16 | gluteus maximus |
| 3 | male | 15 | deltoid |
| 4 | male | 14 | paravertebral |
| 5 | male | 13 | paravertebral |
| 6 | male | 11 | Tensor fasciae latae |
| 7 | male | 15 | paravertebral |
| 8 | male | 16 | paravertebral |

Table 1. Characteristics of healthy and DMD muscular biopsies.
The suspension was homogenized, cells pelleted by centrifugation and cultured in growth culture medium (Ham’s F10 medium supplemented with 20% FBS, 10^{-6} M dexamethasone, 2.5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin, and 100 mg/ml streptomycin).

FAP purification was performed as previously described. Adherent cells were sorted by flow cytometry with the BD FACSARIA II sorter equipped with 4 lasers and FACSDiva software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For CD140a-PE, fluorescence was excited with the 561 nm laser and measured with a 586/15 bandpass filter. For CD56-APC, fluorescence was excited with the 633 nm laser and measured with a 670/14 bandpass filter. Unlike myogenic progenitors, FAPs are negative for myogenic marker CD56 and positive for CD140a expression.

Human peripheral blood mononuclear cell isolation and culture. Blood mononuclear cells were isolated from healthy donors (French Blood Service, Marseille, France). After Ficoll gradient centrifugation, the monocytes were suspended in RPMI 1640 medium (Gibco, Thermofisher Scientific) containing 0.1 mg/mL gentamycin, 2 mM glutamine and 10% decomplemented human serum. Cells were cultured at a density of \(10^6\) cells/well in 100 mm plastic culture dishes (Corning® Primaria™). Resting macrophages (RM) were obtained from adherent monocytes cultured for 6 days. To induce pro-inflammatory (M(IL-1β)) or anti-inflammatory (M(IL-4)) macrophage phenotype, recombinant human IL-1β or IL-4 was added at the beginning of differentiation, respectively. Conditioned media (CM) from RM, M(IL-1β) or M(IL-4) macrophages were obtained by washing differentiated cells with PBS 1X and then by adding serum and cytokine-free RPMI 1640 medium for an additional 24 hour. At the end, RM, M(IL-1β) and M(IL-4) CM were collected, centrifuged at 1500 rpm for 15 minutes and stored at \(-80°C\) until use.

FAP and macrophage indirect co-cultures. FAP cells were seeded in 12 well-plastic culture dishes at a density of \(1\times 10^5\) cells/well in the growth medium. Two days later, adipogenic differentiation was induced on confluent FAPs by switching the growth medium to a pro-adipogenic differentiation medium (Ham’s F10/F12/low-glucose DMEM with 2 mM glutamine and 10% decomplemented human serum. Cells were cultured at a density of \(10^6\) cells/well in 100 mm plastic culture dishes (Corning® Primaria™). Resting macrophages (RM) were obtained from adherent monocytes cultured for 6 days. To induce pro-inflammatory (M(IL-1β)) or anti-inflammatory (M(IL-4)) macrophage phenotype, recombinant human IL-1β or IL-4 was added at the beginning of differentiation, respectively. Conditioned media (CM) from RM, M(IL-1β) or M(IL-4) macrophages were obtained by washing differentiated cells with PBS 1X and then by adding serum and cytokine-free RPMI 1640 medium for an additional 24 hour. At the end, RM, M(IL-1β) and M(IL-4) CM were collected, centrifuged at 1500 rpm for 15 minutes and stored at \(-80°C\) until use.

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Oil red O staining and adipocyte morphometry analyses. Differentiated adipocytes were PBS-washed, fixed in 4% parformaldehyde for 10 min, and treated for 30 min with oil red O (60% of a stock solution at 0.5% w/v in isopropanol and 40% distilled water). Cells were washed with isopropanol/water (6 volumes/1 volume) to remove the unspecific staining and then several times with water. Images were recorded with a TE-2000U bright-field optical microscope (Nikon, Tokyo, Japan). Lipid droplet areas were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The number of adipocytes per field was counted on at least 15 different fields. The number of lipid droplets was counted per adipocyte. Incomplete droplets located at the edge of the image were excluded. At least 30 adipocytes from each condition were measured.

### Table 2. Q-PCR primers.

| Name        | Forward primer | Reverse primer       |
|-------------|----------------|----------------------|
| ACTA2       | TGGCTGCTATGGGCAAGTGA | CTGGGGCACGGAAACGC   |
| ADIPOQ      | GCCAGTCGTCGTTCTCTGATCCATAC | GCCCTTGAGTGTTTGGTC  |
| CCL2        | CCAGCTACCTGGCTGTATAT | TGGAACTCTGAAACCTCTC |
| CCL18       | AGCTCTGGTCGCTCTGCTTAT | CCCACTCCTTAATTGGGTC  |
| CD200R1     | CATCTGTTGATATACCTCAGC | CTGGTTAGGGCACAATGC   |
| CD56        | AACCAAAGGACCATGCAATGG | GGCAGAGGGGAGACGAGGAG |
| COL1A1      | ACCCTGCTGCACCCGCTCA | CCGCCTACCTGAACTGTA   |
| CXCL8       | AGAGAAGAACACCGGAAGG | GGCCCCAACCTGACCTACA  |
| FIA1A       | CTGACCTCTGCTTGATTTTG | CTGGATGCTGTAACGGAGG  |
| FABP4       | ATGGGATGGAADDCAACCAA | TGCCTGCTAATACAGGAAA  |
| FN1         | TGGGCCGAATATACATTGAAA | CACCAGTGGGTACAGGAG  |
| IL1B        | AGCTGCGGAGTGAAATGATGG | CAGGTTCTGAAAGGACAGGCT |
| IL6         | GCCACTGTCGCAAGAACAAC | GCAAGTCCTCTGATGATCC  |
| LEP         | AGGAGGACCGAGGCCGTTTTC | TGCACTCCTCACACACCAACC |
| MRC1        | GACATCCGGGTTCTGCTGCG | CAGGGAAGAGGTTGCTTACC |
| PLIN1       | ACCATCTCACCCGGCTCTC | GATGGAAGGAGCTGATGCTT |
| PPARG       | AGGCTCATGAGAGGCTTCTCA | TCCGGGAAACCACTTCA   |
| TBP         | AGCGGACCTGCGGAGGCTTC | CAACCCGGCTGGATATGATTG |
| TNFA        | CCCCATGTGGTAGCAAAACC | TATCTCTACGCTCACAGCCA |

The suspension was homogenized, cells pelleted by centrifugation and cultured in growth culture medium (Ham’s F10 medium supplemented with 20% FBS, 10 mM Hepes, 10^{-6} M dexamethasone, 2.5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin, and 100 mg/ml streptomycin).

FAP purification was performed as previously described. Adherent cells were sorted by flow cytometry with the BD FACSARIA II sorter equipped with 4 lasers and FACSDiva software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For CD140a-PE, fluorescence was excited with the 561 nm laser and measured with a 586/15 bandpass filter. For CD56-APC, fluorescence was excited with the 633 nm laser and measured with a 670/14 bandpass filter. Unlike myogenic progenitors, FAPs are negative for myogenic marker CD56 and positive for CD140a expression.
Immunofluorescence and histological staining. Cryosections of human healthy and DMD muscles or cultured cells were fixed with Histoфикс 4% (Carl Roth, Lauterbourg, France) for 10 minutes, permeabilized and saturated with 0.1% Triton X-100/3% bovine serum albumin for 30 minutes, subsequently incubated over night at 4 °C with primary antibody, and then with secondary antibody Alexa Fluor 594 or 488 goat anti-mouse or anti-rabbit IgG (Molecular Probes) for 45 minutes at room temperature. Samples were finally mounted in Mowiol containing DAPI and visualized with an Axiovert microscope (Carl Zeiss, Le Pecq, France) under oil immersion. Images were captured and analysed with AxioVision software (Carl Zeiss; Le Pecq, France).

Second Harmonic Generation (SHG) imaging. Imaging was performed on an LSM 780 NLO inverted Axio Observer.Z1 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a Plan Apo 25X multi-immersion (oil, glycerol, water) NA 0.8 lens. Fluorescence images were acquired using a laser 561 nm for Alexa 594 and a laser 640 nm for DRAQ5. The SHG light source was a Mai Tai DeepSee (Newport Corp., Irvine, CA, USA) tuned at 880 nm. Forward SHG signals were detected with an Oil condenser (1.4 NA), 440/40 nm bandpass filter and transmission PMT. Backward SHG was collected on GaAsP (BIG) non-descanned module with 440/10 nm (internal control, not shown). 1 pxl = 168 nm.

RNA extraction and Reverse Transcription quantitative polymerase chain reaction (Q-PCR). Total RNA was extracted using TRI reagent (Euromedex, Souffelweyersheim, France). Methods of RNA extraction and quantitative Q-PCR were previously described24. TATA box-binding protein (TBP) was used as housekeeping gene. The sequences of primers used are listed in Table 2.

Western blot analysis. Cells were lysed in RIPA buffer consisting of 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM NaF, 2.5 mM Na3PO4, 1% NP40, 2 mM sodium vanadate and protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Cell lysates were centrifuged at 13 000 g for 10 minutes at 4 °C, the supernatants were recovered and the protein content determined (Pierce BCA Protein Assay Kit, ThermoScientific, Rockford, IL, USA; #23227). 10 μg of proteins were resolved by 7.5% SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). The membranes were probed with primary antibodies that were detected by horseradish peroxidase-conjugated secondary antibodies (Promega, Charbonnières-les-bains, France) and visualized with an electrochemical luminescence detection kit (Bio-Rad, Marnes-la-Coquette, France). Band intensities were measured by the Quantity One software (Bio-Rad).

Statistical analysis. Statistical differences between groups were evaluated using the two-tailed unpaired Student’s t-test. A P-value < 0.05 was considered as significant.

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Author Contributions
C.M., C.A.D., C.D. and G.C. designed research; C.M., J.R., S.R, S.S. and N.A. performed experiments; C.M., N.A., C.A.D., C.D. and G.C. analyzed data; C.M., C.D. and G.C. wrote the paper.

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