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THESIS TITLE

PRO-INFLAMMATORY EFFECT OF EXTRACELLULAR VESICLES RELEASED BY SENESCENT-LIKE C2C12

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ABSTRACT

Sarcopenia is a geriatric syndrome involving progressive and generalized loss of skeletal muscle mass and strength. Aged muscles are characterized by an altered mitochondrial function, increased protein degradation, inflammation, a reduction of satellite cell (SC) number and function. It has been suggested that an altered secretory pattern involving inflammatory, growth-promoting, and remodelling factors, called senescence-associated secretory phenotype (SASP), could induce sarcopenia. Noteworthy, growing evidence highlighted the role of extracellular vesicles (EVs) as key players in the senescent cell secretome of sarcopenic muscles.

This study aimed to investigate the role of senescent-like myocytes derived EVs in the fine tuning of the muscle niche. In particular, how the senescent-like myocytes EVs could affect the behaviour of myoblasts and macrophages. We focused our attention on two models of *in vitro* senescent-like C2C12 cells: long term cultured C2C12 (more than 40 passages, HP), or exposed to oxidative stress (OX). EVs have been purified by serial ultracentrifugation and density gradient, quantified through Nanoparticles Tracking Assay (NTA), characterized by Western Blot (WB), and administered to recipient cells. Expression analyses were performed using RT-qPCR and WB.

HP and OX myocytes displayed senescence-associated characteristics such as defective myogenic differentiation process and a significantly inflammatory status compared to non-senescent C2C12 cells (cultured in standard condition at low passages, LP).

The NTA analysis showed that HP myocytes released a higher amount of EVs compared to LP cells. Importantly, EV cargo of HP and OX cells was enriched of nucleic acids, especially DNA, compared to LP.

Senescent-like EVs induced a higher level of IL-6 in LP C2C12, and IL-1β and IFN-1β in RAW264.7 without affecting the DNA sensor protein STING. Interestingly, in RAW264.7 the induction of IFN-1β was significantly reduced when RAW264.7 cells were pre-exposed to chloroquine, an inhibitor of endosomal toll-like receptors (TLRs). These results suggested the involvement of TLRs pathway.

Therefore, the data herein reported indicated that EVs released by senescent-like myocytes are capable of induce a pro-inflammatory response in the muscle niche. This led us to hypothesize a

functional relationship with the derangement of the myogenic process found in HP differentiating myocytes. Importantly, DNA associated with EVs seems to play an important role in the triggering of the inflammation process as suggested by IFN-1β expression, a mechanism that is likely to take place in endosomal compartments by stimulating TLRs.

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1. INTRODUCTION

Aging is a time-dependent process which brings most of the living organisms to a functional decline. It is characterised by several features, such as altered intercellular communication, mitochondrial dysfunction, telomere attrition etc. Some of them have been proposed as aging hallmarks due to their importance in the study of the aging process and in the defining of the aging phenotype (Fig. 1). In order to be considered as a hallmark, each characteristic should occur during normal aging, its experimental worsening should accelerate aging and on the contrary, its experimental improvement should delay the normal aging process [1].

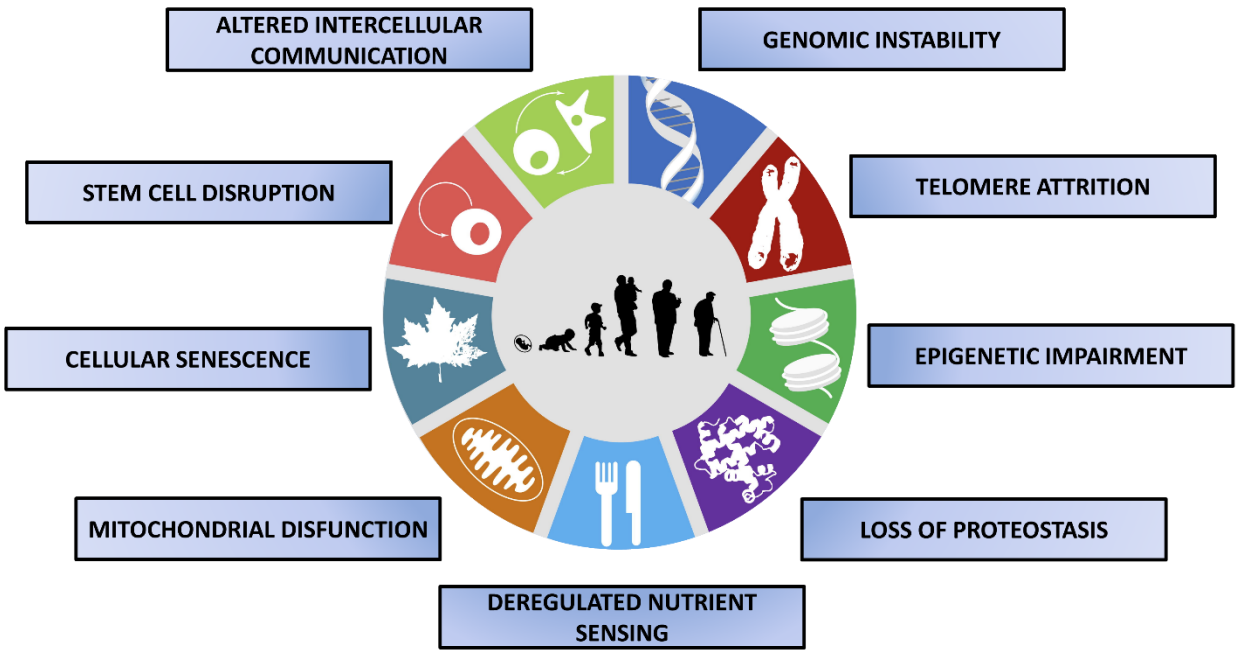


Figure 1. Scheme representing the major features of aging, which means genomic stability, telomere attrition, epigenetic impairment, loss of proteostasis, deregulated nutrient sensing, mitochondrial disruption, cellular senescence, stem cell alteration and impaired intercellular communication (adapted from [1]).

1.1. Main features of aging

Genomic instability

During aging, DNA is continuously subjected to exogenous or endogenous insults including physical, chemical and biological agents, and DNA replication errors, spontaneous hydrolytic reactions and the action of reactive oxygen species (ROS) [2].

Therefore, DNA accumulates several types of damages such as point mutations, translocations, chromosomal gains and losses, telomere shortening, and even gene disruption caused by the integration of viruses or transposons. To contrast the formation of these lesions, organisms evolved specific DNA repair mechanisms [3]: for example, to maintain the genome stability, there are enzymatic systems involved in the upkeep of telomere length and functionality and in the integrity of mitochondrial DNA (mtDNA) [4].

More in detail, during aging the cell genome (nuclear and mitochondrial DNA) is subjected to a specific type of insults.

It is reported that nuclear DNA is affected by the accumulation of somatic mutations [5] and other types of damage such as chromosomal aneuploidies, copy number variations [6] and chromosomal anomalies [7]. Therefore, essential genes and transcriptional pathways could be altered leading to a potential dysfunction of the whole cell which consequently has to be eliminated to prevent homeostasis alteration. This is particularly important especially with regard to stem cells since their impairment could affect the tissue renewal process [8]. It is reported that defects in the DNA repair mechanisms and the clearance of senescent cells promote the aging process and it has been correlated to several human progeroid syndromes [9].

mtDNA has been also implied in the aging process since over time it collects mutations and deletions due to the oxidative microenvironment to which it's subjected. The lack of protective histones and less efficient DNA repair mechanisms than nuclear DNA are likely the main reasons [10]. Indeed, different studies conducted on mice with a defective DNA polymerase γ and humans with multisystem disorders, reported that mutations and deletions in mtDNA lead to a premature aging and reduced lifespan [11]. Noteworthy, the studies on progeroid mice showed mitochondrial

dysfunction without an increase in ROS production, and their stem cells resulted impaired [12] as well.

Telomere Attrition

Telomeres are DNA regions located at the terminal ends of each chromosome and their main function is to protect essential genes during the replication mechanism. Indeed, replicative DNA polymerases are unable to completely replicate the terminal ends of linear DNA molecules, thus without these sequences, cells would go through a progressive loss of essential genetic material after each replication cycle. Although telomerases are the only DNA polymerases able to fulfil this function, most mammalian somatic cells do not express them. For this reason, telomeres are particularly subjected to age-related deterioration [13] since cells over time undergo the progressive and cumulative loss of telomere-protective sequences. In *in-vitro*-cultured cells this phenomenon results in a cell limited proliferative capacity defined as replicative senescence [14]. Moreover, due to a multiprotein complex called shelterin, telomeres are resistant to DNA repair systems otherwise DNA breaks would be fixed leading to chromosome fusion, but as a consequence DNA damage on telomeres are persistent and can induce cell senescence and/or apoptosis [15,16].

Epigenetic Alterations

Epigenetics consists in the whole of those mechanisms that influence the gene expression without alterations of DNA sequence. They involve multiple enzymatic systems including DNA methyltransferases, histone acetylases, deacetylases, methylases, demethylases, and protein complexes implied in chromatin remodelling. In aging these mechanisms could become deregulated resulting in the accumulation of several epigenetic alterations [17].

Histone modification

The main enzymatic involved in histone modification correlated to age include histone methylases, demethylases [18], members of the sirtuin family of NAD-dependent protein deacetylases and ADP ribosyltransferases. In mammals the sirtuins that have gained particular attention are SIRT1, SIRT3 and SIRT6 due to their role in the improvement of health and lifespan [19].

DNA methylation

DNA methylation process seems to be impaired in aging even if there is contrasting evidence: although it was observed an increase in global hypomethylation, some loci are reported to become instead hypermethylated [20].

Chromatin remodelling

Changes in chromatin architecture, such as global heterochromatin loss and redistribution, are considered characteristic features of aging [21]. The reason could be the above-mentioned alteration of DNA- and histone-modifying enzymes, but also key chromosomal proteins, including the HP1α (heterochromatin protein 1α), and chromatin remodelling factors, such as Polycomb group proteins or the NuRD complex, which decrease their expression in both normally and pathologically aged cells [22].

Furthermore, the alterations leading to the defects in chromatin remodelling could also be directly involved in the impairment of telomeres length, since mammalian telomeric and subtelomeric repeats are reported to be enriched in epigenetic modifications such as H3K9 and H4K20 trimethylation, HP1α binding, and DNA hypermethylation [23].

Transcriptional alterations

During aging an impairment of transcriptome occurs and it involves especially genes encoding key components of inflammatory, mitochondrial, and lysosomal degradation pathways [24], but also noncoding RNAs such as gero-miRs, a class of miRNAs associated with the aging process and that affects lifespan by targeting components of longevity networks or by regulating stem cell behaviour [25].

Loss of proteostasis

To survive, cells have to ensure that their proteome is stable and functional, in order to guarantee protein proper stability and folding. To maintain the protein homeostasis, or *proteostasis*, they take advantage of an array of quality control mechanisms, which consist in the heat-shock proteins family and mechanisms for the degradation of proteins by the proteasome or the lysosome [26]. Different studies suggest that during aging these systems are impaired: chaperones and HSPs result altered [27] and proteolytic pathways, such as the autophagy-lysosomal system and the ubiquitin-proteasome system, decline [28]. Consequently, the accumulation of damaged, unfolded or misfolded proteins occurs, promoting the arise of age-associated pathologies [29].

Deregulated Nutrient Sensing

The IIS, or "Insulin and IGF-1 signalling" pathway, comprises factors such as the growth hormone (GH) and the insulin-like growth factor 1 (IGF-1), which are involved in the regulation of metabolism and in nutrient sensing. It has been suggested that an alteration in the IIS pathway could play an important role in aging since IGF-1 and GH seem to decline in aging with a subsequent repression of age-associated target genes such as FOXO and mTOR [30].

Mitochondrial dysfunctions

Time-dependent mitochondrial deterioration has been classically considered as a driving cause of aging since it entails a progressive increase in electron leakage, a reduction in ATP generation and also an increase in ROS production which further promotes mitochondrial derangement and a global cellular damage. The latter is the basis of the mitochondrial free radical theory of aging [31]. Although there is multiple evidence in support of the pro-aging role of ROS, the most recent studies reported that increased ROS could instead not affect lifespan or even promote it [32]. In addition, it

has been suggested that ROS act triggering proliferation and survival in response to physiological signals and stress conditions [33]. Therefore, it has been hypothesized that in aging, ROS first are elicited to rescue the cell from mounting stress and damage, but if exceeded a certain threshold level they become detrimental and instead worsen the age-associated cell damage [34].

In addition, in aging mitochondria could become more permeable in response to stress signals favouring pro-apoptotic signal release [35]. In turn, increased permeabilization could induce an inflammatory ROS-mediated response which and/or due to the permeabilization-facilitated activation of inflammasomes [31]. Furthermore, in aging mitochondrial efficiency could decline also as a consequence of a progressive reduction in their biogenesis. Possible reasons could be the telomere attrition [36] or the disruption of Sirtuins which modulate mitochondrial biogenesis, removal of damaged mitochondria, energy metabolism and ROS rate production [37,38].

Cellular senescence

Cellular senescence, also defined as replicative senescence, was described for the first time by Hayflick in human fibroblasts serially passaged in culture [39]. It is a state in which cells exit the cell cycle permanently and acquire a specific stereotyped phenotype. In physiological conditions senescent cells are continuously removed, indeed it is thought that cellular senescence serves to eliminate cells severely damaged or oncogenic. Nevertheless, in aging senescent cells accumulate due to an increase in age-associated stressors and defects in clearance mechanisms. In addition, in aging cell turnover results impaired, thus the progressive accumulation of senescent cells is not even balanced with an efficient replacement of new cells [40].

Induction of cellular senescence largely depends on the activation of $p16^{INK4a}$/Rb and $p19^{ARF}$/p53 pathways. Indeed, telomere shortening, which is considered one of the main causes in inducing senescence, leads to the activation of the $p19^{ARF}$/p53 pathway. This occurs when telomeres reach a critical minimal length and lose their protective role leading to DNA damage and to the associated DNA damage response (DDR) which in turn leads to the activation of the $p19^{ARF}$/p53 pathway [41]. Alternatively, senescence could also be induced by multiple oncogenic or mitogenic insults, which to a large extent still involve the triggering of $p16^{INK4a}$/Rb or $p19^{ARF}$/p53 pathways [42].

Once initiated, cells take a while to become senescent stably and permanently. This process is further promoted by various intracellular signals such as ROS linked to DDRs, NF-kB, trans-forming growth factor-β (TGFβ), IL-1α, IL-6, and CCAAT enhancer binding protein-β (C/EBP-β) [43,44]. At the

end senescent cells show a specific phenotype characterised by the formation of the senescence-associated heterochromatin foci (SAHF), an increased cell size and protein content, and changes in cell and organelle shape [45]. Finally, they also show a senescence-associated secretory phenotype (SASP), that is an impaired secretory pattern linked to senescence [46].

Senescence-associated secretory phenotype (SASP)

The senescence-associated secretory phenotype (SASP) is an impaired secretory pattern occurring in cells when becoming senescent. It mainly consists in an increased secretion of inflammatory, growth-promoting, and remodelling factors such as TNFα, IL-6, matrix metalloproteinases (MMPs), monocyte chemoattractant protein-1 (MCP-1), and IGF binding proteins (IGFBPs) [47]. It is thought that there isn't a singular SASP, but rather that SASP composition could change depending on factors such as the stage reached in the senescence process, the main driving signal which has induced senescence, or the cell type [48]. In addition, SASP can exert different effects depending on the level and duration of expression of its components. Indeed, in aging for example it has been observed that SASP can carry out different functions in relation to different expression conditions. In younger individuals, where SASP is localized and reversible, it is involved in resolving tissue damage, for example limiting fibrosis after a lesion, or it can trigger the immune system to promote clearance of damaged or oncogenic cells [49]. Furthermore, SASP cytokines, IL-6 and IL-8, can reinforce the senescence growth arrest, further supporting the role of SASP in control of tumorigenesis [50]. In contrast, in older individuals, where SASP can be exacerbated and permanent, it can alter tissue architecture, stimulate surrounding malignant cells, and induce local or systemic inflammation, i.e. the age-associated low-grade inflammation [51]. Implied with the latter can be the age-associated decline in immune system because it involves that immune cells no longer respond efficiently to signals leading to the accumulation of senescent cells which in turn could themselves interfere with immune response [52].

Furthermore, there is evidence that SASP can spread in surrounding cells. This, in addition to the defective immune clearance mechanism, could further increase the senescent cells burden, the inflammation process, and chronic disease progression [53].

Stem cell disruption

As mentioned before, during aging, cell turnover results impaired and so does the whole tissue regeneration process. The main reason is the stem cell impairment which implies a decline in stem cell proliferation or a progressive exhaustion of the stem cell pool. The reduced stem cell proliferation and the decrease in cell cycle activity has been observed in various tissues [54]. An example is the decline in the hematopoietic stem cells (HSCs) which entail a decrease in hematopoiesis and it leads to a condition defined as “immunosenescence” [55]. It has been suggested that a main reason could be the accumulation of DNA damage and DDR due to telomere shortening which in turn activates cell-cycle inhibitory proteins such as $p16^{INK4a}$ [56].

On the other hand, defects in the regulation of the proliferation process could lead stem cells to proliferate excessively causing in the long term the exhaustion of the stem cell pool [57].

Noteworthy, several studies suggested that extracellular signals could play an important role in stem cell impairment since parabiosis experiments such as transplantation of tissues from young mice to old ones resulted to extend lifespan and improve degenerative changes in old mice. This amelioration has been observed even in tissues where donor cells were not detected suggesting that secreted factors could play an important role [58].

Altered Intercellular Communication

Another feature of aging is the impairment in intercellular communication, which is reported to involve different aspects such as endocrine, neuroendocrine, or neuronal [59]. Such alteration is characterised by changes in the composition of the peri- and extracellular environment and an increase in inflammation process.

Inflammation is a well-known age-associated trait and indeed it is used the term “inflammaging” which means the age-associated proinflammatory phenotype. This condition is due to different causes such as an excessive pro-inflammatory tissue damage, a defective immune system that doesn’t clear efficiently from pathogens and dysfunctional host cells, the above-mentioned accumulation of senescent cells which are likely to secrete proinflammatory cytokines, the enhanced activation of the NF-kB signalling pathway and/or a defective autophagy response. This scenario leads to the overexpression of pro-inflammatory cytokines such as interleukins, tumor necrosis factors and interferons [60].

Additionally, during aging occurs a condition defined as “immunosenescence”, which consists in the decline of the adaptive immune system, that contributes to worsen the inflammation process mainly because it leads to a less efficient clearance of senescent, infected, severely damaged or malignant cells [61].

Furthermore, other factors linking chronic inflammation to aging have been studied, such as AUF1 and sirtuins. AUF1 has an inhibitory effect on inflammation by mediating the degradation of pro-inflammatory cytokines mRNAs and inducing the expression of the telomerase catalytic subunit TERT, which guarantees the telomere length [62]. Likewise, sirtuins, in particular SIRT1, SIRT2 and SIRT6, down-regulate inflammatory response by acting on components of the inflammation signalling pathways, such as NF-kB factors or NF-kB target genes [63,64].

1.2. Sarcopenia: aging of muscle tissue

Sarcopenia, which literally means “poverty of flesh”, is a geriatric condition involving progressive and generalized loss of skeletal muscle mass and strength. It can lead to physical disability, poor quality of life and death [65]. Muscle strength does not depend exclusively on muscle mass; however, sarcopenia is always related to a condition of “dynapenia”, that is an age-related loss of muscle strength and function [66]. Immunological, metabolic, nutritional, and hormonal alterations are reported to be the main contributors to sarcopenia. Altogether they lead to a histological and morphological impairment of muscle tissue including denervation and neuromuscular remodelling, myofiber atrophy, increased heterogeneity in fiber size, and intramuscular infiltrations of connective and fat tissue. Moreover, sarcopenic muscles also show a reduced oxidative capacity associated with deficits in mitochondrial energy metabolism, increased protein degradation and inflammation [67,68]. Noteworthy, sarcopenia seems to exhibit a specific molecular profile that could be used to distinguish it from normal aging [69].

Furthermore, another important trait of sarcopenia is the loss in satellite cell number and function [70]. Therefore, since satellite cells are the muscle stem cells, their impairment can lead to a reduced regeneration capacity in muscle in case of injury or normal cell turnover [71].

1.3. Satellite Cells

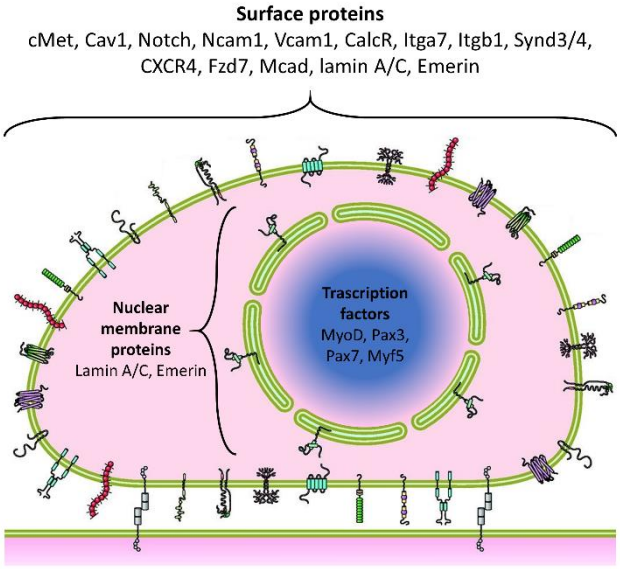


Figure 2. Schematic representation of a muscle satellite cell and the proteins and transcription factors generally expressed by this type of cell (adapted from [72])

Satellite cells (SCs) are the muscle stem cells, they are so called because of their anatomical location which is between sarcolemma and basal lamina of their associated muscle fiber. SCs in adult muscles are typically quiescent and can be activated by a stimulus such as a lesion. Once activated, SCs start to proliferate becoming myoblasts. Then, part of them returns quiescent in order to replenish activated SCs and thereby re-establishing the SC pool, part enters the myogenic program, known as myogenesis. These last ones can fuse with each other, giving rise to new myofibers, or to an existing muscle fiber [73]. It is thought that SC progression through myogenesis is mainly orchestrated by the paired box transcription factor 7 (Pax7) and the myogenic regulatory factors (MRFs) [74].

In the early stages of myogenesis SCs express MRFs, such as Myf5 and MyoD, the latter characterises most of the newly activated SCs [75]. Afterwards, in the late phase, myoblasts express mainly MRF4 and myogenin, while Pax7 becomes downregulated except for those myoblasts which return in quiescence in order to maintain SC pool; in this case Pax7 expression remains elevated [76].

Pax7 is also considered as the most reliable marker for the identification of SCs, at least in mouse models, even if there are other biomarkers such as the neural cell adhesion molecule (NCAM) or CD56, which however is not highly specific since it is also expressed in myoblasts, myotubes, and muscle fibers during development and/or regeneration, the tyrosine receptor kinase c-Met, which is likewise not considered very reliable, and M-cadherin (M-Cad) [77,78].

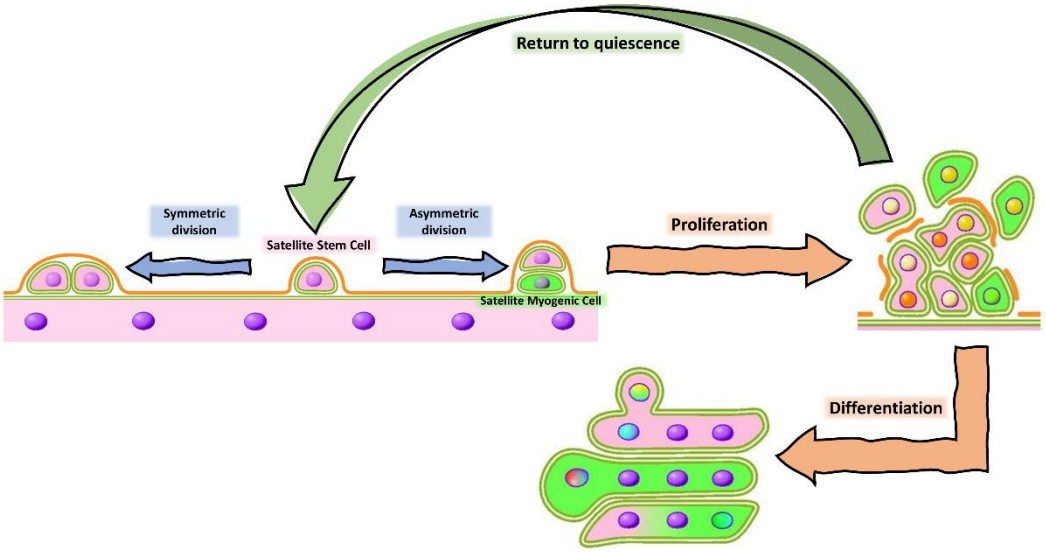


Figure 3. Schematic representation of the processes carried out by SCs in order to fulfil muscle regeneration and concurrently maintaining SCs pool homeostasis (adapted from [72]).

1.3.1. The satellite cell niche

The stem cell niche is a specific location or microenvironment where stem cells stably reside and where they can be activated when necessary, and proliferate in order to carry out regenerative processes, maintaining concurrently the stem cell pool in homeostasis fulfilling the self-renewal process. Here stem cells lay in specific extracellular matrices and are often anchored to stromal partner cells. Noteworthy, in the niche there are multiple factors involved in the regulation of the stem cell behaviour, which includes all those signals that can control processes like growth or differentiation. Furthermore, stem cell niches can re-acquire and maintain new introduced stem cells and can keep them at the optimal number [79].

Likewise, satellite cells have a proper niche which consists of a specific extracellular matrix (ECM), vascular and neural networks, different types of surrounding cells, and various diffusible molecules. In addition, all the molecules present in this highly specialized microenvironment can be released by the SCs on their own or by all the other cellular components of the niche and can exert a biological effect. These molecules have been somehow implied in the regulation of SC behaviours such as quiescence, self-renewal, proliferation, and differentiation [72].

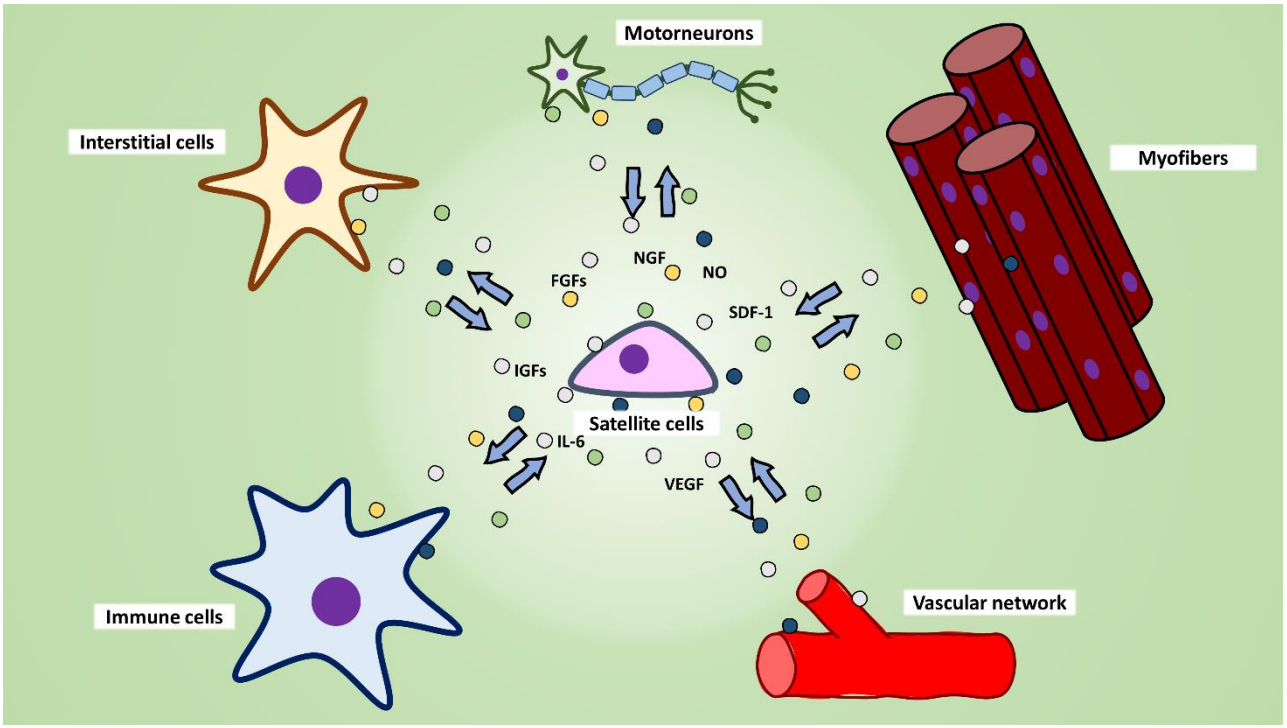


Figure 4. The satellite niche is a microenvironment composed by several cell and non-cell elements. The most relevant cell components are myofibers, motoneurons, interstitial and immune cells, and the local microvascular network. SCs and the other components of the niche communicate with each other releasing multiple molecular factors such as interleukins and growth factors.

The composition of the niche

As mentioned, SCs niche is a highly complex and specialized microenvironment composed by multiple cellular and non-cellular components acting together in order to ensure SCs and muscle homeostasis and at the same time allowing SCs to fulfil their function in muscle regeneration.

Myofibers

Myofibers are among the SCs closest cells being in direct contact with them and are reported to play an important role in modulating SCs quiescence either by their physical association or by releasing molecular signals [80]. An Important factor reported to play a role in such interplay is SDF-1, which binds the CXCR4 receptor on satellite cells promoting SCs migration [81]. In addition, myofibers are reported to display on the membrane the Notch ligand Delta that is overexpressed in particular after an injury and its overexpression leads to the activation and proliferation of SCs [82].

Interstitial cells and ECM associated factors

Along with myofibers, there are other cells not physically associated to SCs, but still considered part of the SCs niche since they release several factors that can affect SCs behaviour. Among these cells, fibroblasts are the major component of the interstitial stromal cell population [83]. Fibroblasts are reported to derive from SCs which undergo transdifferentiation, but also from fibrocyte/adipocyte progenitors (FAPs) which reside in the niche too. FAPs are also reported to differentiate into adipocytes and, although they do not show myogenic potential either in vivo or in vitro, it has been reported that undifferentiated FAPs can promote SCs myogenic differentiation probably through the secretion of Il-6. In degenerating muscles, instead, FAPs are induced to differentiate, in particular into adipocytes [84].

Fibroblasts play an important role in the secretion of numerous niche factors such as growth factors like the fibroblast growth factors (FGFs), but also collagen, laminin, fibronectin, heparan sulfate proteoglycans (HSPGs), tenascin, and neuronal cell adhesion molecules (NCAM), which all together compose the skeletal muscle ECM [85]. Nonetheless, ECM components can be secreted and remodelled also by myoblasts during muscle development and regeneration [86]. ECM plays an important role in the regulation of SCs behaviour, it forms the basal lamina where they reside, therefore it is in direct contact with SCs. In addition, it displays numerous binding sites for molecules, such as the integrins on the surface of SCs and myofibers, by which extracellular mechanical force is converted in intracellular signalling [87]. Furthermore, ECM proteoglycans on the surface of SCs act as receptors and can bind various inactive growth factor precursors, such as insulin-like growth factor isoforms (IGF-1, IGF-2), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and various Wnt glycoproteins [88,89], which in turn can be processed and hence activated by different proteolytic enzymes like MMPs, thrombin, and serine proteases. All these factors can be released by multiple niche residing cells, such as satellite cells, myofibers, interstitial cells, or can derive from the serum [90].

More in detail, HGF is involved in cell growth, migration, and organ morphogenesis through its mitogen, motogen, and morphogen activities [91]. To a large extent both inactive and active HGF forms are sequestered by HSPGs within the basal lamina and if necessary the active form can be released from the ECM without the need of proteolytic cleavage of pro-HGF [92]. In skeletal muscle it is implied in early phase of muscle regeneration and affects SCs and myoblast behaviour, by interacting with the c-Met receptor which in turn activates a signalling cascade involving the

repression of MRF/E-protein complexes, the inhibition of MyoD, the expression of $p27^{kip1}$, and sustained activation of MAPK/Erk signalling. The resulting effect is the induction of quiescent SCs to enter the cell cycle, myoblasts proliferation, and the inhibition of myogenic differentiation [93]. FGFs are a large family of mitogen factors implied in cell survival, growth, migration, and embryonic development. In muscle multiple FGFs have been found, but FGF-6 and FGF-2 are considered the most relevant because of their role in muscle regeneration, in particular in myoblast proliferation [94]. Moreover, they also promote muscle regeneration through their role in angiogenesis [95]. FGF signalling is reported to start when FGFs bind to one of the associated four known transmembrane tyrosine kinase receptors, defined as FGFR1–4, and proceed leading to the activation of the downstream Ras/MAPK pathway [96]. In addition, FGFs are thought to interact also with HSPGs, which act as low-affinity FGF receptors, and seem also to engage the FGF receptors, in order to promote ligand recognition. In this regard, quiescent and activated SCs express syndecan-3 and syndecan-4 [97].

Both IGF-1 and IGF-2, are other ECM associated factors involved in SCs regulation: IGF-1 is implied in anti-inflammatory processes, cell migration, and stimulation of both proliferation and differentiation in SCs, while IGF-2 is thought to only promote myogenic differentiation [98]. IGF signalling pathway consists in the binding of IGFs to their associated IGF receptors, in particular, in SCs the binding of IGF-1 to its receptor (IGF1R) leads to the induction of MRFs genes and hence to a mitogenic and myogenic response [99]. IGF1R can trigger two main intracellular pathways: the first causes the phosphorylation of insulin receptor substrate proteins (IRSs) and in turn to the activation of the phosphatidylinositol 3-kinase (PI3K), which initiate multiple processes, in particular cell proliferation through the AKT/mTOR pathway, or myoblast differentiation by triggering the activation of PI3K/AKT and p38-MAPK pathways [100,101]. The second pathway, instead, involves the activation of the Ras/Raf/extracellular response kinases (ERKs) cascade which also regulates SCs proliferation [102]. Furthermore, cultured myoblasts and activated SCs produce a family of secreted proteins that specifically bind IGFs, which are called insulin-like growth factor binding proteins (IGFBPs). IGFBPs have the main functions to carry IGFs in the circulation, increasing their half-life and regulating their turnover [103]. Of note, IGFBP-5 in muscle plays a critical role in growth and differentiation processes [104].

Finally, another important group of factors related to ECM are the MMPs, which are zinc-dependent enzymes involved in the degradation of ECM components like collagens, elastin, fibronectin,

laminin, and proteoglycans. In skeletal muscle MMPs are involved in satellite cell activation, migration, and differentiation during muscle regeneration [105]. In this context, MMP-2 and MMP-9 are the most relevant since they play a key role in ECM remodelling during the regeneration process. More in detail, MMP-2 is secreted by SCs and myofibers during both the degenerative and the regenerative stage, while MMP-9 is released by leukocytes and macrophages and is mainly involved in the degenerative stage [106]. During the degenerative stage the collagen IV forming the ECM is degraded allowing SCs to migrate across the basement membrane to the site of injury, whereas the regenerative stage takes place in order to re-establish the ECM previously degraded [107].

Motoneurons

Motoneurons are another type of cells strictly related to SCs, indeed during acute muscle denervation, SCs start to proliferate resembling a condition similar to the proliferation phase following a muscle injury. In addition, a long-term denervation leads to significant reduction of SCs number probably because of a decreased mitotic capacity and increased apoptosis processes [108]. Motoneurons appear to modulate SCs activity mostly releasing in the niche factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Both are suggested to bind to $p75^{NTR}$ receptor; NGF seems to promote myoblast differentiation *in vitro* stimulating the morphological changes needed for their fusion, whereas BDNF expression is associated with Pax3 expression and is significantly downregulated during the myogenic differentiation. Therefore, motoneurons through the secretion of these two factors appear to modulate SCs activity stimulating their activation or inducing them to quiescence [109,110].

The local vascular network

In muscle, SCs are also closely associated to the microvascular network which consists of endothelial cells that are not only involved in giving support to SCs by delivering nutrients, but they can also secrete in the niche multiple growth factors such as vascular endothelial growth factor (VEGF), IGF-1, hepatocyte growth factor (HGFs), FGFs and platelet-derived growth factor-BB (PDGF-BB). Such factors acquire particular relevance during muscle regeneration since it has been reported to proceed in concert with angiogenesis [111]. For example, VEGF, in addition to endothelial cells, can be secreted by satellite cells on their own, myoblasts and differentiating myofibers, and it can

stimulate proliferation, migration and differentiation processes, and prevent apoptosis [112]. Moreover, periendothelial cells, including smooth muscle cells and endomysial fibroblasts, are reported to induce a subset of myoblasts to return to the quiescent state favouring the self-renewal, a process that can take place when these cells release angiopoietin-1 which can trigger the Tie-2 receptor on SCs inducing in turn the ERK1/2 pathway [113].

Immune cells and factors deriving from the circulation

Immune cells are another important component of the niche, in physiological conditions only a few of them are present there, but upon injury they rapidly move to the site in order to remove necrotic tissue and secrete soluble factors which activate satellite cells [114]. Macrophages and monocytes are the main cells involved in this process and their depletion can alter muscle regeneration [115]. The interplay between SCs and immune cells takes place mainly through multiple chemokines, in particular pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα, which promote immune cell infiltration and function [116]. In detail, IL-6 is a ubiquitous pleiotropic cytokine, which can be released by many other cell types, such as fibroblasts, hematopoietic cells and vascular endothelial cells, besides the immune cells. In muscle IL-6 can induce SCs proliferation; it is implied in SCs-mediated muscle hypertrophy and regeneration, and it can interact with IL-6 receptors (IL-6Rα), present on myofiber sarcolemma, thereby activating the JAK/STAT3 signalling pathway and the associated target genes [117]. Moreover, during muscle regeneration, IL-6 is expressed in particular by SCs, myofibers and neutrophils and is modulated by signalling pathways involving NF-kB, AP-1, IL-1, NO and calcineurin-NFAT [118,119].

Nevertheless, in addition to pro-inflammatory cytokines, immune cells can secrete other diffusible factors involved in the niche regulation such as globular adiponectin, ECM components, and ECM remodelling MMPs. Immune cells can also make SCs more resistant to apoptosis through direct contact [120].

SCs activity can be regulated also by other factors deriving from the systemic milieu, such as hormones. Indeed, SCs for example express receptors for androgens (ARs), which play an important role in the regulation of muscle mass, strength and fiber size [121]. Androgens are able to promote SC activation and proliferation, probably modulating Notch signalling, and also myogenic differentiation [122]. Such hormones can increase the number of myonuclei and muscle hypertrophy due to the interplay between androgens and IGF-1 signalling [123]. In particular it can

take place for the interaction among androgens and IGF1R or by a mechanism involving an increase of the intracellular calcium and inositol 1,4,5-trisphosphate (IP_3) levels, which in turn can induce the phosphorylation of ERK1/2, or even counteracting the catabolic effect of glucocorticoid hormones [124].

Finally, another important factor, which is secreted by various cell types, but which also plays an important role in SCs activity regulation, is the nitric oxide (NO). NO is a small diffusible molecule, which is synthetized through the nitric oxide synthase (NOS) and it is produced by cells like epithelial cells, endothelial cells, fibroblasts, hepatocytes, macrophages, and skeletal muscle cells [125]. At present three NOS isoforms are known, which consist in endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [126]. In skeletal muscle the nNOS isoform is constitutively expressed within the sarcolemma of myofibers, whereas iNOS is induced mainly in response to muscle injury [127]. In muscle regeneration, during the degenerative phase, NO is reported to be implied in promoting the macrophage-mediated lysis of necrotic myofibers while reducing other inflammation-induced damage [128]. During the regenerative stage instead, NO appears to induce satellite cell activation, which can depend on the capacity of NO to induce the release of HGF from ECM, a process which involves the MMP-2 [129]. Furthermore, NO can induce SCs migration and prevent fibrosis by inhibiting TGFβ signalling [130].

Signalling pathways mainly involved in SCs regulation

Wnt signalling

Wnt signalling pathway is involved in the regulation of SC proliferation and differentiation, cell adhesion, polarity, and morphology. It is initiated when a member of the extracellular Wnt family glycoproteins binds to Frizzled receptors and low-density lipoprotein receptor-related proteins (LRP5 or LRP6). Once activated the Frizzled receptors can trigger two kinds of pathways, the canonical and non-canonical. In the canonical pathway Wnt/β-catenin, in unstimulated conditions, β-catenin is phosphorylated by a cytoplasmic destruction complex composed of Axin, Adenomatous Polyposis Coli (APC), Casein Kinase I (CKI) and Glycogen Synthase Kinase 3β (GSK3β) to be later ubiquitinated and degraded. After the binding of the Wnt ligands, instead, Axin is recruited to the phosphorylated tail of LRP5/6 by the scaffold protein Dishevelled (Dvl), as a result the destruction complex can’t form, β-catenin isn’t degraded and can accumulate in the cytoplasm. Therefore, it

can translocate to the nucleus and after being associated with the transcription factors TCF and LEF, it induces the expression of its target genes. The non-canonical pathways, instead, include the Planar Cell Polarity pathway Wnt/PCP and the calcium-dependent pathway Wnt/Ca^{+}. The Wnt/PCP starts when the frizzled receptors activate the small GTPase proteins Rho and Rac, which in turn trigger the Rho-associated protein kinase (ROCK) and the c-Jun N-terminal kinases (JNKs), and ends with cytoskeletal reorganization and/or activation of target genes. On the other hand, the Wnt/Ca^{+}, going through the induction of calcineurin (CaN), Ca^{2+} and calmodulin-dependent kinase (CAMKII), and protein kinase C (PKC), leads to the activation of the NFAT, CREB and NF-kB transcriptional regulators and then to the expression of target genes [131].

In muscle regeneration both canonical and non-canonical pathways have been implied by regulating SC differentiation and self-renewal [132]. The Wnt/β-catenin pathway acts in concert with notch signalling in order to modulate the transition of SCs from proliferation to differentiation [133]. In particular, initially low levels of Wnt signalling and high levels of Notch signalling are required because for example low levels of β-catenin are reported to prevent premature differentiation and allow cells to exit quiescence and proliferate [134]. Afterwards, in late stages of myogenesis occurs a switch, which means that Wnt/β-catenin becomes more activated than Notch signalling and as a result the myogenic differentiation is promoted. This can be due to the translocation of β-catenin into the nucleus and the following interaction with factors such as MyoD or Barx2 [135].

As mentioned before, also non-canonical pathways are involved in muscle regeneration, in particular the PCP pathway, which was mostly implicated in promoting SC self-renewal. The main contributor to this mechanism is reported to be the Wnt7a ligand which, interacting with the frizzled receptor Fzd7, induces the redistribution of Vangl2 and hence SC polarization which in turn leads to cell symmetric expansion [89]. In addition, Wnt7a also promotes SC migration and engraftment [136].

Furthermore, Wnt signalling pathways are triggered also by another class of activators, the R-spondins which have been implied in myoblast fusion and differentiation [137] and in muscle regeneration by regulating both canonical and non-canonical pathways. Indeed, in these conditions Rspo-1 was reported to promote myogenic differentiation dampening the PCP pathway and improving the Wnt/β-catenin signalling [72].

Notch signalling

Notch signalling modulates cell proliferation, differentiation, and cell fate determination. It is initiated when a ligand member of Delta and Jagged family binds to the Notch transmembrane receptors. This interaction induces the cleavage of the receptor and the consequent release of the Notch intracellular domain (NICD). Therefore, the NICD translocates to the nucleus and activates the CSL (CBF1, Suppressor of Hairless and Lag-1) family of transcription factors, which in turn induce target genes expression [72]. More in detail, in response to an injury SCs and myofibers overexpress the ligand Delta, which in turn interacts with Notch leading to the NICD release, and as a result SCs are stimulated to activate and proliferate. Notch signalling is also involved in the myogenic process since its inactivation is required at early stages of myogenesis for myoblast fusion, thus it has to be downregulated in order to allow myoblast differentiation [138]. To this end, two mechanisms exist: the first one implies the Notch signalling inhibitor Numb, which acts triggering NICD ubiquitination. Numb is asymmetrically partitioned during satellite cell/myoblast division and in the daughter cell inheriting Numb, it induces Desmin expression which in turn promotes the differentiation process. Therefore, this process promotes SC asymmetric division [139]. The second mechanism involves an interplay between Notch and Wnt signalling, namely Wnt signalling in differentiating myoblasts counteracts Notch signalling promoting terminal differentiation [133]. It is suggested that this mechanism could involve factors like Dishevelled, Presenilin, or β-catenin [140]. Furthermore, in SCs are expressed three Notch receptors, Notch1, Notch2, and Notch3, and Notch3 is reported to be a Notch1 repressor [141]. Finally, in addition to its function in muscle regeneration, Notch signalling plays also a key role in maintaining SC quiescence in physiological conditions [142].

Sphingolipid signalling

Sphingolipids, which are lipids characterized by a sphingoid backbone, are a large group of natural glycolipids that have been recently indicated as important bioactive signalling molecules involved in the regulation of cell proliferation, migration, death, and senescence [143]. Among them sphingomyelin is the most abundant component in the sphingolipid pathway and is also a precursor of other important sphingolipids like ceramide, sphingosine, and sphingosine-1-phosphate (S1P). In muscle sphingomyelin was reported to be enriched in plasma membranes of quiescent SCs and have been implicated in SC activation through a mechanism involving the biosynthesis of S1P [144]. S1P can induce SC activation through the triggering of downstream pathways with proliferative, pro-

inflammatory and antiapoptotic effects, and that it initiates these signalling cascades interacting with S1P receptors (S1PRs) on SCs [145].

1.3.2. Satellite cells and their niche in aging

A growing body of evidence suggests that in aging SC pool is reduced, impairing the overall muscle regenerative capacity [146]. The reasons for the SC loss are not clear but they seem to depend on extrinsic factors, i.e. both systemic factors and those deriving from the SC microenvironment, and intrinsic factors, which means SC autonomous alterations [147]. Indeed, the whole of these elements can negatively affect SC survival and self-renewal, for example leading to apoptosis or senescence [148].

The intracellular impairment

The SC autonomous derangement includes those alterations to which are subjected most of the cells in aging, namely genomic instability, DNA damage, oxidative damage, and a disrupted mitochondrial function. More in detail, although SCs appear to be more resistant to DNA damage and have more efficient DNA repair mechanisms compared with their committed progeny, in aging the persistent exposure to genotoxic stresses and the decline in antioxidant capacity still leads to a significant DNA damage and altered genomic integrity [149].

Furthermore, aged SCs show an altered expression profile involving myogenic differentiation-specific genes, atrophy-associated FoxO-regulated genes, and genes related to protein folding. Such alterations are related to SC age-associated traits like the defective differentiation program, the impaired antioxidant capacity, or their increased tendency to adopt fibroblastic and adipogenic fates [150]. Alterations in the epigenetic profile can contribute to this impairment, indeed for example the $p16^{INK4a}$, which is a marker of senescence, in aged SCs is reported to be induced. This can occur because the PRC1 (Polycomb Repressive Complex 1) repressive mark H2AUb, which inhibits $p16^{INK4a}$ by binding its promoter, results reduced [70]. Another age-associated epigenetic alteration affecting this promoter and that of $p21^{CIP1}$ involves the histone 3 lysine 4 trimethylation (H3K4me3) and H3K27me3 chromatin marks. This defect occurs in response to injury [151].

Moreover, some SC intracellular pathways are reported to be impaired in aging. The identified pathways are the p38α/β MAPK (mitogen-activated protein kinase) and JAK/STAT signalling. Both are induced, but the first promotes SC permanent cell cycle exit [152], while the second induces SCs to carry out asymmetric division [153]. In addition, the JAK/STAT signalling pathway can be induced also by the interleukin Il-6, which can activate STAT3 and in turn triggers MyoD, thereby promoting the myogenic program at the expense of the proliferation process [154].

The alteration of the extracellular environment

As mentioned, important contributors to SCs disruption in aging can be also extracellular cues deriving from the niche or the systemic milieu. Indeed, several heterochronic parabiosis studies reported that environmental factors can change during aging influencing SCs function [155]. Such studies reported also that old SCs, exposed to a young myogenic environment or to young systemic factors, showed an amelioration, while young SCs resulted negatively affected by aged serum [156]. Among the factors most implicated in muscle aging there is the FGF2 which is reported to disrupt SC quiescence inducing them to activate and proliferate in turn leading to a reduction of quiescent SC pool. Related to FGF2 there is the protein Sprouty1 which is an inhibitor of FGF signalling. In physiological conditions SCs, when depleted of Sprouty1, overexpressed FGF2 and are induced to exit quiescence promoting SC depletion. This evidence further suggests a key role for the FGF-Sprouty1 axis in SC pool disruption during aging [157]. In addition, FGF signalling could impact on SC survival since Sprouty1 deletion leads also to a persistent activation of the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling pathway, which impairs self-renewal through the prevention of a subset of SC progenitors to return in quiescence and inducing the SCs instead to go through apoptosis [158].

Beside FGF2, in aging SCs niche showed impaired levels of other relevant cues, for example aged animal serum displayed higher levels of Wnt ligands which negatively affect SCs and muscle regeneration through the promotion of myogenic-to-fibrogenic transition of muscle progenitors [159]. In addition, old myofibers are reported to also display elevated levels of TGFβ which has been related to SC function decline and reduced proliferation. The latter is due to a TGFβ-mediated induction of cyclin-dependent kinase (CDK) inhibitors. Such a process is countered by Notch signalling which acts by removing pSmad3 from the promoters of the CDK inhibitor genes. For this reason, given the relevance of Notch-smad3 signalling in maintaining SCfunction, an impairment of this pathway could play a role in aging. Indeed, Notch signalling-defective young SCs show a phenotype similar to that of old ones and a similar regenerative capacity. Thus, the re-establishment of Notch signalling is thought to be one of the mechanisms by which old SCs can recover their function when exposed to a young environment [156]. Notch signalling could be also related to aging because of its interaction with Wnt signalling: the latter promotes myogenesis through the inactivation of the glycogen synthase kinase3 beta (GSK3b) which in turn inhibits Notch pathway

[133]. In physiological conditions this mechanism is important to activate muscle regeneration by modulating SC activity, but in aging it could be a further mechanism by which Notch loss of function could lead to a SC depletion promoting differentiation at the expense of self-renewal [160]. In addition, SC niche could contribute to this process since myofibers does not express sufficient levels of Delta1 failing to properly induce Notch signalling in SCs [161].

Indeed, in this regard, the impairment of muscle niche in aging also involves a derangement of niche components such as myofibers, motoneurons, immune cells and cells related to the vascular network. Myofibers are reported to be less and with a reduced calibre, and in addition, as already mentioned, show an impaired expression of Delta ligand, which results at the same time reduced and unresponsive to muscle injury [161]. The alteration of myofibers was also associated with a decline in the vasculature: myofibers in aging tend to lose their close contact with the microvascular network leading to a decrease of VEGF levels [162]. In addition, endothelial cells display a decrease in the activity of eNOS [163]. Concerning motoneurons, the NMJ was found to have a reduced nerve terminal area, a decrease of mitochondria and synaptic vesicles, as well as an increase of denervated postsynaptic regions, which all together could contribute to disrupt the interplay between SCs and myofibers along with the promotion of progressive myofiber atrophy. Moreover, in aged muscle immune cells are impaired, in particular they display a reduced capacity of free radical generation, phagocytosis, and chemotaxis. Such alterations could imply a disruption in the interplay among SCs and immune cells, entailing defects of SC activation and migration [72].

In addition to the alteration of the components more closely linked to the niche, SC behaviour in aging result also affected by other impaired factors related to the systemic milieu. In this regard, chronic systemic inflammation has been associated with a chronic systemic increase of IL-6 levels, which leads to muscle atrophy and disease states [164]. Furthermore, plasma of aged animals shows also reduced levels of the growth differentiation factor 11 (GDF11) along with a decline of hormones, like oxytocin and androgens [151,165]. Concerning the decrease of androgens, it is reported to further promote loss in muscle mass versus a gain in fat mass [166].

1.4. Extracellular vesicles

According to the classical view, cells communicate through direct interactions or secreting soluble factors, which then can be uptaken by the cell itself, surrounding or distant cells; in other words, they act in an autocrine, paracrine or endocrine mode, respectively.

During the past decade it gained attention for another intercellular communication system based on extracellular vesicles (EVs). EVs are nanosized membranous structures observed for the first time in 1946 and originally they were considered equal to “platelet dust”. Nonetheless, further studies showed that EVs have actually a very complex structure and that they are present in almost all organisms, either prokaryotes or eukaryotes. EVs are secreted in order to transfer information between cells in the form of molecular signals which include all types of biological macromolecules, mainly proteins, lipids, nucleic acids. EV content, size and membrane composition are reported to be highly heterogeneous and dynamic as well as due to cellular source, condition and environmental status.

1.4.1. Structure, classification, and biogenesis

Based on their features, EVs are currently classified into 3 categories: apoptotic bodies (ABs), microvesicles or large EVs (lEVs), exosomes or small EVs (sEVs). ABs are the biggest with a size of 50-5000 nm; they derive from the blebbing of dying cells membrane occurring during apoptosis or programmed cell death. lEVs are the second largest type of EV with a mean diameter of 50-1000 nm; lEVs originate after the budding and the following fission of a cell membrane portion, i.e. the cell membrane forms a protrusion which is subsequently shedded. Finally, sEVs are the smallest with a size of 40-100 nm; they form initially during endocytosis as endocytic vesicles, which are then trafficked to the multivesicular bodies (MVBs) becoming intraluminal vesicles (ILVs); lastly ILVs fuse with the plasma membrane and are secreted as exosomes [167,168].

Each subgroup can be distinguished depending on physico-chemical, density, size, morphology and intracellular origin [169]. Moreover, EVs can be distinguished based on the different composition of protein content and the main biomarkers include tetraspanins (CD9, CD63, CD81 and CD82), 14-3-3 proteins, major histocompatibility complex (MHC) molecules and cytosolic proteins such as specific stress proteins (for example the heat shock proteins or HSPs), Tsg101, the Endosomal Sorting

Complex Required for Transport (ESCRT-3) and the related protein Alix [170]. However, none of these markers alone can be used to uniquely identify one of the EV subgroups. CD9, CD63 and CD81 initially were thought to be specific for sEVs, but further studies reported them to be expressed also in lEVs and ABs [171]. The lack of unique markers could be due in part to a lack of standardized protocols of isolation and characterization and in part to a difficulty in obtaining pure samples, which are mixtures of different types of EVs, and such difficulties can depend on the small differences in EV physical properties and composition. In addition, EVs are secreted as a very heterogeneous and dynamic population, indeed the same cell can secrete different subgroups of vesicles owing to environmental factors (e.g. oxygen tension), cell topography (e.g. from basolateral or apical cell surfaces) or activating stimulus (e.g. apoptosis or autophagy) [172]. Otherwise, different signals can induce the cell to release the same EV subgroup, but with a different protein content [173]. In addition, cells may show different kinds of MVBs with a different content of exosomes [174].

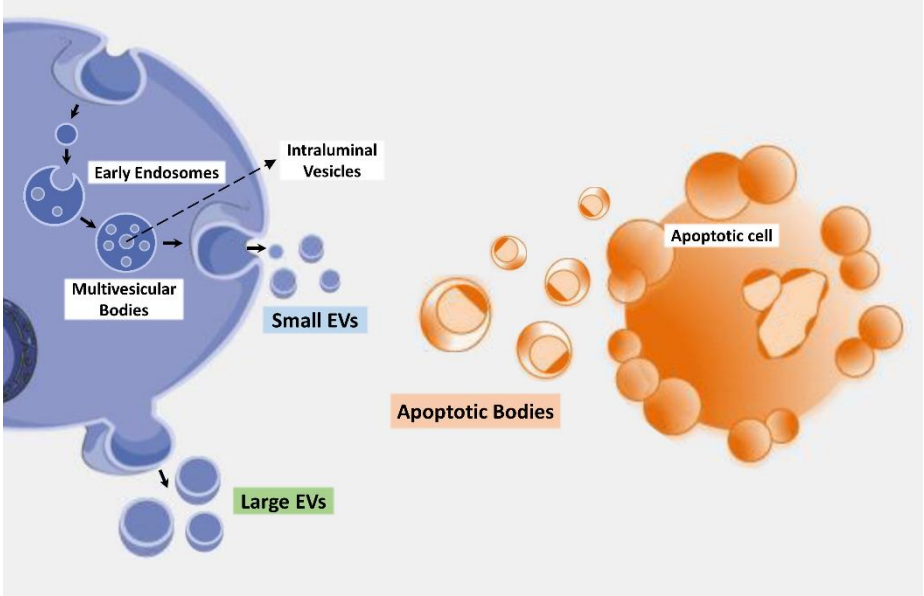


Figure 5. Representation of the three most studied classes of EVs and their corresponding biogenesis mechanism: apoptotic bodies, which are the biggest, are formed as a consequence of the blebbing of apoptotic cells' membranes; large EVs or microvesicles are released after the outward budding and fission of the plasma membrane; small EVs or exosomes originate in the endosomal network and are released after MVBs fuse with the plasma membrane (adapted from [167]).

EV membrane structure and molecule sorting

As mentioned above EVs are membranous structures which display a composition and an assembly similar to that of the cell membrane, but EVs result enriched in sphingomyelin, cholesterol, phosphatidylserine, and glycosphingolipids compared to their parent cells. Such composition is

likely to be mainly involved in promoting EV structural stability, indeed sphingolipids and cholesterol, for example, could give resistance to physiochemical changes and structural rigidity, and they seem to form domains, similar to cell membrane raft domains, which can confer to EVs resistance to detergents [175]. In addition, lipids are shown to be involved in EV formation: ceramide, for example, which is formed by the action of neutral sphingomyelinase 2 on sphingomyelin, has been proposed in the formation of ILVs within the MVBs [176]. EVs also show specific patterns of protein glycosylation, which differ from the native plasma membrane [177]. Studies on different cellular sources reported that these patterns mostly consist of highly mannosylated epitopes, including complex N-glycans, N-acetyl lactosamine, sialylated and fucosylated epitopes. In addition, most glycosylated proteins found on the studied EVs show lectin-binding patterns [178]. Such glyco-patterns are thought to be involved in several mechanisms such as EV sorting, EV uptake by recipient cell, and the specific effect induced on target cell [179].

The molecular components of EV membranes are assembled during EV maturation either on the cell plasma membrane or in the MVBs [180]. This sorting depends on the specific biochemical features of each molecule, which means that they move in membrane regions more energetically favourable thus determining membrane shape and size as well as local molecular composition [181]. Tetraspanins for example have been suggested to induce membrane curvature [182]. In addition, the interaction among multiple membrane components can lead to the formation of microdomains such as membrane rafts and tetraspanin-enriched microdomains [183]. The latter, along with lectins, has been implied in the recruitment of EV protein components, some of which have structural and shaping functions. Accordingly, tetraspanin-enriched microdomains incorporate membrane receptors, and besides, such a process has been implicated in the sorting of themselves to exosomes [184].

Important structural elements are also the BAR (Bin/Amphiphysin/Rvs) domain-containing proteins and the ESCRT proteins. The first drives the formation of tubular and vesicular membrane structures [185], whereas the ESCRT complex promotes the formation of the neck region and the following fission of the membrane protrusions [186]. It is also involved in the recruitment of exosomal cargo factors through the binding to ubiquitinated proteins [187].

1.4.2. EVs as carriers of molecular signals

EVs are carriers of molecular signals belonging to any class of biological macromolecules. EVs can induce a response in the recipient cells in different ways: through their internalization and subsequent release of their cargo, binding to a cell surface ligand or receptor, or also modifying the recipient cell phenotype transferring, for example, functionally active receptors [167]. Noteworthy, surface proteins play an important role in each of these mechanisms.

EV uptake can occur through processes of phagocytosis or macropinocytosis and implies the fusion of cell and EV membranes [188]. It largely depends on the type and phagocytic capabilities of the target cell by which it is internalized [189], but also on the biochemical features of the microenvironment. For example, direct membrane fusion needs that fusing membranes have similar fluidity, and in the case of EV and plasma membranes it occurs at acidic pH conditions, thus involving also electrostatic charges [190]. Therefore, it is likely that such microenvironmental conditions influence not only EV uptake, but also the functioning of the delivered molecules [191].
Furthermore, it has been shown that EVs can carry surface or soluble forms of proteolytically active metalloproteinases, which in turn may influence EV activity by altering its content, interacting with extracellular matrix proteins or with membrane-anchored receptors on target cells [192].

Protein signals

As mentioned above, EVs can influence target cells also through the interaction between ligands on the EV membrane and the associated receptors on cell surface. As a result, EVs can trigger various intracellular pathways in the recipient cell, such as integrins and calcium signalling, MAPK signalling, or natural killer group 2D (NKG2D) signalling (125, 126, 128).
Examples of EV surface ligands are MHC I and II, transferrin receptors, tetraspanins and some members of the HSPs, such as HSP60 and HSP70 [167]. On the other hand, among the best-known receptors triggered by EVs there are those associated with HSPs, namely CD14, CD91, Toll-like receptor (TLR)-2, TLR-4 and LOX-1, as well as CD94/CD56. Interestingly, EVs secreted from natural killer (NK) cells display fully functional ligands.

EV biodistribution implies how EVs are distributed inside the organism, how long is their half-life, and mostly may depend on parent cell source as well as the capacity of the target cell to internalize

the circulating EVs [167]. EV molecular content plays a key role in this mechanism, indeed despite circulating EV half-life is reported to be quite short, it is due mainly to the uptake and the retention by target organs, since circulating EVs display protection factors against complement-mediated lysis such as glycosylphosphatidylinositol (GPI)-anchored CD55 and CD59 [193,194]. Moreover, EVs show surface molecules which determine their targeting: as an example, EVs targeted to immune cells can display proteins modified with sialic acids which can be bonded by sialic-acid-binding immunoglobulin lectins (siglec) or CD169 (sialoadhesin), which are expressed from a variety of leukocytes [193].

EV-mediated signalling doesn't occur only through interaction with surface ligands since EVs carry also important soluble mediators such as cytokines. Among the most studied there are pro-inflammatory cytokines, such as some members of the interleukin-1 family, tumour necrosis factor (TNF), which can be released into EVs also as membrane-bound form, and Il-6 [195,196].
The vascular endothelial growth factor (VEGF) is another important soluble mediator found into EVs, it is released by platelets and it is also commonly present into tumour derived EVs [197]. Moreover, other examples of chemokines reported to be carried by EVs are IL-8 (CXCL8), the fractalkine CX3CL1, several chemokines derived from heat-stressed tumour cells [198]. Finally, also the Transforming Growth Factor β (TGFβ), which is a regulatory cytokine, has been suggested to be associated with thymus- and tumour- derived EVs [199].

RNA signals

Besides the protein content, EVs carry nucleic acids. Extracellular RNA can be found in different forms: it can be bound in protein complexes, as free circulating form, and enclosed into EVs. The latter was first observed in 2006 into murine stem cell derived EVs [200]. EVs are reported to contain different types of RNA such as intact or fragmented mRNAs, long non-coding RNAs, microRNAs (miRNAs), ribosomal RNA (rRNA) and transfer RNAs (tRNAs) [201,202]. Loading of RNA molecules into EVs is suggested to be an active mechanism which can depend on different factors, such as the presence of specific nucleotide motifs into the RNA sequence, the expression of cellular miRNAs or miRNA target sequences [203]. In addition, miRNA sorting is regulated also by hnRNPA2B1, a factor belonging to the heterogeneous nuclear ribonucleoproteins (hnRNPs) that is a family of ubiquitous proteins involved in RNA trafficking and function. hnRNPA2B1 acts recognizing the EXOmotif, a

GGAG tetranucleotide, within miRNA sequence. It requires a sumoylation as post-translational modification to fulfil its function [204]. Likewise, mRNAs targeted to EVs are reported to show, within the 3'-UTR region, a sequence of 25 nucleotides characterized by a short CTGCC core domain containing a mir-1289 binding site which in turn mediates mRNA enrichment into EVs [205].

The RNA loaded into EV seems to be involved mainly in the regulation of gene expression in the recipient cell. Indeed, EVs are reported to be enriched in 3'-UTR mRNA fragments rather than intact mRNA molecules. Such regions contain multiple sites for regulatory miRNA binding allowing these mRNA fragments to compete with cellular RNA for binding of miRNAs or RNA-binding proteins in the recipient cell [201]. However, several evidences show that EVs can contain and transfer also intact and functional mRNA molecules which are reported to be uptaken and translated by the recipient cell. Notably, the mRNA content into EVs was observed to vary depending on the physiological or stress condition of the source cell, thus it may be a mean to maintain homeostasis and synchronize the functional state of cells [206].

Furthermore, as mentioned above, EVs contain miRNAs, which are short RNA regulatory molecules of about 21 nucleotides, transcribed as hairpin precursors (pri-miRNAs), cleaved by Dicer (into pre-miRNAs), bound by Argonaute proteins (Ago) and loaded into the miRNA-induced silencing complex (miRISC) for mRNA target regulation. Besides, it has been suggested that EVs themselves can contribute to miRNA maturation through RISC proteins present inside EVs [207].

MiRNAs can be secreted both in EVs and in a non-vesicular form [208], however incorporation of miRNAs into EVs may allow them to circulate in the blood avoiding degradation from blood RNAse activity and thus cells could use them to regulate or alter gene expression of surrounding or distant recipient cells more quickly and efficiently [209].

Secretion of miRNAs into EVs was observed in a range of cells, such as immune cells, stem cells, blood cells or adipocytes and several evidences indicate that they have particular relevance in different pathological and physiological conditions [210,211]. In the immunological context, this mechanism is thought to be a rapid way to regulate recipient cell activity in lymphocyte activation when it is prompted by vaccination [212], but it is also involved in immune synapses formation since miRNAs enclosed into CD63+ EVs are transferred from T-cells to antigen-presenting cells (APCs) [211]. Moreover, cancer cells are reported to release EVs carrying miRNAs involved in the silencing of tumour suppressor miRNAs [213]. In the central nervous system EV miRNAs are involved in

neuron-to-astrocyte communication [214]. EV-mediated transfer of miRNAs was reported to be involved in muscle cell differentiation, follicular maturation, or osteogenic differentiation of human bone marrow-derived mesenchymal stem cells [215,216]. Furthermore, EVs carrying miR-126 has been implied in the regulation of hematopoietic stem/progenitor cell trafficking between the bone marrow and peripheral sites [217] while EVs released from embryonic stem cells were reported to carry a high quantity of miRNAs which could be transferred to mouse embryonic fibroblasts in vitro [218].

DNA signals

EVs can also carry DNA molecules, in particular they contain mitochondrial DNA (mtDNA), single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) and oncogene amplifications [219,220]. DNA into EVs reflects the genetic status of source cell, as in the case of tumour derived EVs which contain DNA resembling characteristics of the source cell such as oncogene c-Myc amplifications; otherwise, the DNA may also reflect the mutational status of the parental cell [221]. Alternatively, EV DNA could be a mean by which various pathological states could be transferred between cells, for example by mtDNA [220].

Lipid signals

In addition to their structural function, lipids are also reported to act as molecular signals. Indeed, bioactive lipids, such as eicosanoids, fatty acids and cholesterol, are shown to be transferred between cells through EVs [175]. Some examples are the lysophosphatidylcholine which is involved in the maturation of dendritic cells (DCs) and in triggering lymphocyte chemotaxis via the G protein-coupled receptor [222]; the prostaglandins, since vesicle-bound prostaglandins have been reported to induce prostaglandin-dependent intracellular signalling pathways within target cells [223]; the lipid microdomains which are shown to impact Notch signalling inducing cell death in pancreatic tumoral cells [224].

1.4.3. EVs and aging

In the last decade, a growing body of evidence has associated EVs to the cellular senescence and to the aging process. Several studies conducted on young and old subjects pointed out several changes in EVs and EV-associated effects occurring in aging. More in detail, plasma EV concentration seems to decrease with human age, at least from early 30s to late 60s. It has been also reported that monocyte and B cells, but not T cells, internalize plasma EVs and elderlies' B cells uptake more EVs [225,226]. Moreover, in aging EV RNA and protein composition change. For example, older people display a reduction of Galectin-3 in plasma EVs. Galectin-3 is involved in osteoblast maturation and its reduction could be implied in age-related decline of bone formation [227]. In addition, EVs isolated from elderlies' bone marrow are reported to exert an anti-osteogenic effect by suppressing cell proliferation and osteogenic differentiation of bone marrow stromal cells [228]. Furthermore, EVs deriving from aged plasma or endothelial senescent cells are reported to promote the calcification of aortic smooth muscle cells. This effect could be due to an increase of calcium, calcium-binding annexins, and BMP2 content since these factors are implied in vascular calcification. [228,229].

Change in EV secretion

In 2008 Lehmann et al. first highlighted a senescence-associated increase in EV secretion, a feature which appears to be related to senescence and that has been found in several kinds of cells like fibroblasts, epithelial cells, and cancer cells. Accordingly, such characteristic has been observed in different models of senescence, such as those obtained through serial passaging, irradiation, DNA-damaging chemicals, and oncogenic Ras expression [230–232]. In this regard, p53 has been suggested to play a role by triggering genes like TSAP6, Caveolin-1 or the ESCRT-III subunit Chmp4. These last two genes in particular are implied in endosome regulation [233,234]. Alternatively, also members of Rab proteins have been suggested to play a role, since they are increased in senescent cells [235]. The increase in EV secretion could depend on the need from senescent cells to remove harmful molecules [231].

The receptor tyrosine kinase EphA2

In addition to the increase in EV secretion, studies conducted on EVs deriving from senescent cells showed also significant difference in EV content between senescent and non-senescent cells,

implying an alteration of some EV-related factors which can occur in aging or senescence. Such alterations seem to confer to EVs also pro-tumorigenic effects, thus involving EVs both in aging and cancer [232].

In this regard, senescent EVs are reported to be enriched in receptor tyrosine kinase EphA2, which has a pro-proliferative effect by triggering the Erk pathway through the so-called reverse signals in ephrin-expressing cells. This factor is reported to be altered also in different kinds of cancer like breast, ovary, or pancreatic cancer [236,237]. The Expression of EphA2 can increase as a consequence of p53 upregulation, even though in some kinds of senescent cells, such as Ras-induced senescent cells, it does not require this condition. Increase of EphA2 into EVs has been also observed in a model of ROS-induced senescence wherein the PTP1B phosphatase is oxidatively inactivated, in turn allowing EphA2 endosomal internalization, which is the first step before the sorting of EphA2 into EVs [238].

EVs and inflamm-aging

Growing evidence has suggested that during aging EVs could play a key role in the chronic systemic inflammation process and in immunosenescence, which is an age-related impairment of the immune system. Therefore, EVs have been associated at the same time to both aging and inflammation, in other words to inflamm-aging [239,240]. Indeed, EVs deriving from senescent cells have been reported to exert a more pro-inflammatory effect than those deriving from young ones [241]. Accordingly, the EVs of older people contain higher amounts of chemokines like CXCL4 and CXCL7, and HMGB1 (High Mobility Group Box 1), which are factors involved in the inflammation process [242]. Furthermore, given the EV impairment in aging, EVs could be also related to SASP. In addition, as mentioned, SASP and EVs have been reported to be proinflammatory. In this regard NFKB plays a key role since it can modulate SASP and EV cargo at the same time [243,244]. In addition, SASP can modulate immune cells' activity and is able to transmit senescence to bystander cells [245,246].

The role of the DNA

EVs are considered the most common routes of DNA secretion; in particular EVs seem to contain genomic DNA fragments of different sizes which to a large extent does not display relevant sequences [247,248]. Notably, senescent cells show an increased release of DNA into EVs, which is

mostly damaged DNA. Indeed, senescent cells are reported to accumulate γH2AX-cytoplasmic chromatin fragments [231,249]. Accordingly, the inhibition of EV secretion leads the senescent cells to apoptosis or growth arrest via cGAS-STING signalling [231]. cGAS is an intracellular receptor which binds cytosolic DNA and in turn synthesizes cyclic GMP-AMP (cGAMP) from ATP and GTP. Subsequently, cGAMP triggers the endoplasmic reticulum-membrane adaptor STING which in turn activates the inflammation process by inducing transcription factors such as NF-kB, STAT6, and IRF3 through IKK and TBK1 [250]. STING also plays a key role in the induction of senescent phenotype and SASP genes [251–253].

Long non-coding RNA and miRNAs

About the role of EV-associated RNA in aging and inflammation, rising evidence pointed out that EVs can carry long non-coding RNAs and miRNAs implied in the aging and inflammation process [226,246].

More in detail, EVs could be involved in the telomere regulation by modulating telomerase activity [254]. This mechanism seems to be mediated in part by RNA, since EVs are enriched for example in telomeric repeat-containing RNA sequences (TERRA), which indeed are implicated in the repression of telomerase activity [255,256].

In addition, the impairment of telomere integrity, mediated by TRF2 inhibition, can lead to an increase of TERRA levels in cells and EVs [255]. High levels of TERRAs have been observed also in models of replicative senescence [257]. Furthermore, EV-associated TERRAs have been reported to exert pro-inflammatory effects on recipient cells. Nevertheless, TERRAs inside EVs are shorter than those inside cells, therefore their role in telomere modulation is still unclear [255].

Furthermore, EVs are reported to carry a subset of miRNAs which have been implicated in aging, inflammation, and immune regulation at the same time. This group of miRNAs includes relevant inflammamiRs, like miR-19b, -20a, -21, -126, -146a, and -155. These miRNAs are indeed somehow involved in processes like the DNA damage response (DDR), oxidative stress, proteotoxic stress, mitochondrial dysfunction, senescence, inflamm-aging, and nutrient sensing [246]. In addition, these miRNAs display altered levels in senescent cells and/or in serum derived from elderlies, as an example miR-21 and miR-126 increase in aged subjects [258].

2. AIMS

The aim of the present work was the study of the role of EVs secreted by cellular models of senescent-like muscle cells. C2C12 cells were used since they are a well-known *in vitro* model of myogenic cells. Moreover, C2C12 myoblasts provide a validated *ex vivo* model of SC self-renewal. Two different models of *in vitro* senescence of C2C12 cells were explored: the replicative senescence and the oxidative stress induced senescence. The study was first focused on cell characterisation in order to assess the occurrence of senescent-like features. Subsequently, EVs released by senescent-like C2C12 were purified, characterised, and their activity was explored. In particular, the EV-mediated effect was evaluated on low passages C2C12 and RAW264.7 cell lines; this latter was analysed since it is a model of murine macrophages commonly present in the muscle niche.

Finally, the senescent-like C2C12 derived EVs molecular cargo was explored, as well as the signalling pathways triggered in the recipient cells.

3. MATERIALS AND METHODS

3.1. Cell Cultures

RAW264.7 murine macrophages and C2C12 murine adherent myoblasts were cultured in growth medium (GM) consisting in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL), and maintained in a 5% CO_2 atmosphere at 37 °C.

To induce myogenic differentiation of C2C12 myoblasts, when 100% cell confluence was attained, the medium containing 10% FBS was removed, cells were washed twice with phosphate buffered saline (PBS) (8 g/L NaCl, 1.15 g/L Na2HPO4, 0.2 g/L KH2PO4, 0.2 g/L KCl) and medium containing 2% horse serum was added. Alternatively, in order to collect EVs the myogenic differentiation was induced adding a medium containing 2% HS which was previously centrifuged overnight at 4 °C and 110,000 g using a SW28 rotor in a Beckman ultracentrifuge. Then, the supernatant was removed and further centrifuged 70' at 4 °C and 110,000 g using this time a type 90 Ti rotor. The supernatant was carefully removed, passed through a 0,22 µm filter and then added to DMEM.

3.1.1. Stressed C2C12 models

High-passage (HP) C2C12 were attained culturing in GM low-passage (LP) C2C12, which means C2C12 cultured under 15 passages, for over 40 passages and until they showed defects in differentiation, which was assessed by morphological and gene expression analysis.

Oxidative stressed C2C12 (OX) were attained as described by Chen et al. [259]. More in detail when LP C2C12 reached 80% cell confluence the GM was removed, and cells were incubated in GM added with 600 µM H_2O_2, or in serum-free DMEM added with 300 µM H_2O_2, for 2 h. After 2h the medium was removed, and GM was added. Subsequently, cells were incubated 4 days, and then the H_2O_2 treatment was repeated a second time. After that, the following days cells were monitored to evaluate morphological changes.

3.2. SA-β-gal Assay

The activity of the SA-β-galactosidase was evaluated, as described by Chen et al. [259].
When 80% cell confluence was attained, the GM was removed, and cells were washed twice in PBS. Then, cells were fixed with 4% paraformaldehyde/PBS for 5 min at room temperature and washed three times in PBS. At this point, cells were incubated in a freshly prepared staining solution composed as follow: 1 mg/mL X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl. The solution was buffered with a citric acid/sodium phosphate buffer, pH 5.0 or 6.0, which was obtained mixing a 0.1 M citric acid solution with a 0.2 M sodium phosphate solution until pH 5.0 or 6.0 was attained. The pH 6.0 staining solution was used to highlight the activity of the SA-β-galactosidase, while the pH 5.0 staining solution served as a control since it acted by staining the endogen β-galactosidase. The incubation was conducted at 37°C in a humidified chamber overnight. To this end, the incubator was avoided since it was reported that a 5-10% CO_2 environment can interfere with the assay. Subsequently, the blue staining was examined using a light microscope.
Pictures taken with light microscope were then analysed, using the imageJ software, as described by Lozano et al. [260].

3.3. ROS assay

Antioxidant capacity and basal intracellular ROS were analysed performing a DCFH-DA assay as described by Calcabrini et al. [261]. More in detail, cells were seeded in a 24-well plate and, when attained 100% confluence, medium was removed. Cells were washed twice with PBS and incubated with a solution containing, as fluorogenic dye, 20 µM 2',7'-Dichlorofluorescin diacetate (DCFH-DA) for 45 min at 37 °C. Subsequently, the DCFH-DA solution was removed, cells were washed twice with PBS, and 100 µM H_2O_2/PBS, 500 µM H_2O_2/PBS or PBS alone were added. Fluorescence intensity was measured after 10 and 30 min using a Fluoroskan Ascent FL (Thermo Scientific, Milan, Iyaly) set to wavelength of 538 nm as emission and 485 nm as excitation. After the measurement, cells were lysed in 0,1% Triton and total protein contents were determined by the Bradford method.

3.4. Immunofluorescence Assays and Myotube Analysis

Cells were fixed for 15 min at room temperature using 4% formaldehyde/PBS and permeabilized in 0.5% Triton X-100/PBS for 4 min. Expression of MyHC was assayed using the mouse monoclonal antibody against MHC (clone MF20, DSHB) diluted 1:2, incubated for 1 h at 37 °C, and followed by a 30 min incubation at 37 °C with fluorescein-conjugated goat anti-mouse IgG (Biolegend, San Diego, CA, USA) diluted 1:100. Cells were stained with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine) for nuclear visualization and mounted in Mowiol 4-88 (Sigma-Aldrich). Images were acquired with IM50 software (Leica, Wetzlar, Germany) using a DC300F digital camera connected to a Leica microscope. Myogenic index was determined by counting the number of nuclei in differentiated myotubes and expressed as a percentage of the total number of nuclei. Myotube size was estimated measuring the diameter of at least 100 myotubes using ImageJ software. The average diameter per myotube was calculated as the mean of five measurements taken along the length of the myotube.

3.5. Extracellular Vesicle Isolation

Extracellular vesicles were purified by centrifugation for 30 min at 1000g and then for 30’ at 2000g to remove cell debris. Supernatants were centrifuged for 30 min at 10,000g, and then further centrifuged at 15,000g to obtain large EVs pellet. Subsequently, supernatants were centrifuged for 5 h at 110,000g to obtain small EVs pellet. The EV pellets were washed in 2 mL PBS, centrifuged again at 20,000g for 30 min and 110,000g for 70 min to obtain large and small EVs pellets, respectively, and resuspended in 100 µL PBS.

3.6. Density gradient separation

The 110,000g EV pellet was resuspended in a solution of 0.25 M sucrose and 10 mM Tris dissolved in PBS, at 7.5 pH.

A discontinuous iodixanol density gradient was prepared as described by Tauro et al. [262]. More in detail, 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol were made by diluting a stock solution of OptiPrep™ (60% (w/v) aqueous iodixanol from Axis-Shield PoC, Norway) with the

0.25 M sucrose, 10 mM Tris, pH 7.5, solution used to resuspend the EV pellet. The gradient was then formed by adding the 40% iodixanol solution to a polyallomer tube (Beckman Coulter), followed by careful layering the 20%, 10%, and 5% solutions, sequentially. After that, the resuspended EV pellet was overlaid onto the top of the gradient, and the whole was centrifuged at 110,000g for 18 h at 4 °C. Twelve individual 1 mL gradient fractions were collected manually (with increasing density). An aliquot of each fraction was then serially diluted 1:10,000 with water, and the iodixanol concentration determined by absorbance at 244 nm. Finally, the fractions were diluted with 2 mL PBS and centrifuged at 110,000g for 70' at 4 °C, and resuspended in 100 µL PBS.

3.7. Nanoparticle Tracking Analysis (NTA)

EVs were diluted to approximately 1 mL of PBS, loaded into the sample chamber of an LM10 unit (Nanosight, Malvern, UK) and three videos of 30 sec were recorded of each sample. Analysis was performed with NTA 3.1 software (Nanosight) and data are presented as the mean ± SD of the three video recordings. When samples contained high numbers of particles, they were diluted before analysis and the relative concentration was then calculated according to the dilution factor. Control 100 and 400 nm beads were supplied by Malvern Instruments Ltd. (Malvern, UK).

3.8. Transmission Electron Microscopy (TEM)

For a detailed morphological analysis, specimens were processed with transmission electron microscopy observation using the conventional negative staining procedure. EVs isolated from C2C12 culture media were adsorbed to formvar carbon-coated 200 mesh grids (Agar Scientific Ltd., Stansted, UK) for 2 min, and gently washed with filtered PBS. The, EVs on grids were fixed with 2.5% glutaraldehyde for 1 min. The grids were incubated with 2% (w/v) sodium phosphotungstate for 1 min and the liquid excess was removed with filter paper. After negative staining, specimens were observed by means of a Philips CM10 transmission electron microscope at 80 kV.

3.9. Nucleic Acid quantification and DNase1 Digestion

Cells and EVs total RNA and DNA were extracted and the purification was performed using the E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Norcross, GA, USA) and the E.Z.N.A.® Total DNA Kit I (Omega Bio-tek, Norcross, GA, USA), respectively, according to the manufacturer's instructions; to remove contaminant DNA from RNA samples, DNase1 was performed using the E.Z.N.A.® RNase-Free DNase1 Set (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. Total RNA and DNA were quantified using a SpectraMax® QuickDropTM UV-Vis Spectrophotometer. Nuclear and mitochondrial DNA amounts were quantified performing real-time PCR of 36B4 and COX-1 genes, respectively. Real-time PCR amplifications were conducted using Sensi-FAST SYBR Green (Bioline USA Inc., Taunton, MA, USA), with 300 nM primers. The primers used were: 36B4 (36B4-F: 5'-CGACCTGGAAGTCCAACTAC-3' and 36B4-R: 5'-ATCTGCTGCATCTGCTTG-3') and COX-1 (COX-1-F: 5'-TCTACTATTCGGAGCCTGAGC-3' and COX-1-R: 5'-CAAAAGCATGGGCAGTTACGA-3'). Thermocycling was conducted using a LightCycler 480 (Roche, Basel, Switzerland) and started by a 2 min incubation at 95 °C, followed by 40 cycles (95 °C for 5 sec; 60 °C for 5 sec; 72 °C for 10 sec). Each reaction was conducted in duplicate, and melt-curve analysis was used to confirm the specificity of each amplification and lack of primer dimers. Data are presented as absolute expression, multiplied by a 1x10^7 arbitrary factor, and related to total EV protein amount.

3.10. Western Blotting Analysis

Protein extracts were obtained from the organic phase after Qiazol cell lysis following Qiagen User Protocol RY16 May-04. The protein pellet was dissolved in ISOT buffer (8 M urea, 4% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate), 65 mM DTE (1,4-Dithioerythritol), 40 mM Tris base and added with SIGMAFAST™ Protease Inhibitor Cocktail (Sigma-Aldrich, Milan, Italy)) and sonicated for 10 sec on ice. For electrophoresis, samples containing 10–30 µg were mixed with the Laemmli sample buffer 4× (1:4 ratio) and loaded onto 10% SDS–PAGE gels. The proteins were then blotted to a PVDF (Polyvinylidene difluoride) membrane (Thermo). To determine EV surface markers primary antibodies were used against CD63 (1:250 dilution, clone sc-5275 (MX-49.129.5) Santa Cruz, CA, USA), Tsg101 (1:1000 dilution, clone Sigma-Aldrich T5701),

CD81 (1:333 dilution, clone sc-166029 (B-11) Santa Cruz, CA, USA), α-actinin-4 (1:250 dilution, clone sc-3902 (G-4) Santa Cruz, CA, USA), Alix (1:333 dilution, clone sc-53538 (3A9) Santa Cruz, CA, USA).
To analyse cell gene expression primary antibodies were used against myogenin (1:200 dilution, clone F5D Santa Cruz, CA, USA), Notch1 (1:40 dilution, clone bTAN 20 Developmental Studies Hybridoma Bank (DSHB)), MHC (1:200 dilution, clone MF20, DSHB), α-tubulin (1:2000 dilution, T5168, Sigma), PCNA (1:3000 dilution, clone PC10, Millipore), phospho-STING (1:1000 dilution, D8F4W, Cell Signaling), STING (1:1000 dilution, D1V5L, Cell Signaling), phospho-NFKB p65 (1:1000 dilution, 93H1, Cell Signaling), TLR9 (1:1000 dilution, clone 26C593.2, Cayman).
Primary antibodies were incubated overnight at 4 °C followed by washing and the application of secondary HRP-conjugated antibody (Pierce, Waltham, MA, USA), and the immune complexes were visualized using the Clarity and/or Clarity Max (Bio-Rad, Milan, Italy), Chemiluminescence was measured using a BioRad ChemiDoc MP imaging system (BioRad).

3.11. Gene Expression Analysis

Cells total RNA was extracted and the purification was performed using the E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions; to remove contaminant DNA, DNase I digestion (Ambion, Paisley, UK) was performed. cDNA was obtained using the Maxima Reverse Transcriptase kit (Thermo) and PrimeScript RT Reagent Kit (Takara Bio). Real-time PCR amplifications were conducted using Sensi-FAST SYBR Green (Bioline USA Inc., Taunton, MA, USA), with 300 nM primers. The primers used were: Pax7; ID-1 (ID-1-F: 5'-TACTTGGTCTGTCGGAGCAA-3' and ID-1-R: 5'-GATCAAACCCTCTACCCACT-3'); ID-3 (ID-3-F: 5'-CCCTCTCTATCTCTACTCTC-3' and ID-3-R: 5'-GAAGAGGGCTGGGTTAAGAT-3'); IKβ; IL-10 (IL-10-F: 5'-GGTTGCCAAGCCTTATCGTA-3' and IL-10-R: 5'-ACCTGCTCCACTGCCTTGCT-3'); CCND1; POLA1; MYOD; MEF2C; FSTL-1; DESMIN (DESMIN-F: 5'-GTGAAGATGGCCTTGGATGT-3' and DESMIN-R: 5'-GTAGCCTCGCTGACAACCTC-3'); MYF6; FERMT-2 (FERMT-2-F: 5'-GATCACTTTGGAAGGCGGGA-3' and FERMT-2-R: 5'-GCGCGTACTGCTTCTCGTTA-3'); myogenin (MyoG-F: 5'-GCACTGGAGTTCGGTCCCAA-3' and MyoG-R: 5'-TTGTGGGCGTCTGTAGGGTC-3'); TNFα (TNFα-F: 5'-GCTCTTCTGTCTACTGAACTTCGG-3' and TNFα-R: 5'-ATGATCTGAGTGTGAGGGTCTGG-3'); IL-6 (IL-6-F: 5'-CTGGATATAATCAGGAAATTTG-3' and IL-6-R: 5'-AAATCTTTTACCTCTTGGTTGA -3'); IL-1β (IL-1β-F: 5'-TGACGTTCCCATTAGACAACTG-3' and IL-1β-R: 5'-CCGTCTTTCATTACACAGGACA-3'); interferon-1β

(IFN1β) (IFN1β-F: 5'-TCCAAGAAAGGACGAACATTC-3' and IFN1β-R: 5'-TGAGGACATCTCCCACGTCAA-3'); and GAPDH (GAPDH-F: 5'-CACCATCTTCCAGGAGCGAG-3' and GAPDH-R: 5'-CCTTCTCCATGGTGGTGAAGAC-3'). Thermocycling was conducted using a LightCycler 480 (Roche, Basel, Switzerland) and started by a 2 min incubation at 95 °C, followed by 40 cycles (95 °C for 5 sec; 60 °C for 5 sec; 72 °C for 10 sec). Each reaction was conducted in duplicate, and melt-curve analysis was used to confirm the specificity of each amplification and lack of primer dimers. Relative quantification of mRNA expression levels was performed according to the ΔCq method, and the expression levels of GAPDH and S16 were used as a reference [263].

3.12. Cell treatments

3.12.1. EV treatments

EVs, released by cells when attained 5 days since differentiation was induced, were purified from the collected media. The isolated EVs were assayed through NTA, and then resuspended in serum-free DMEM. The medium containing EVs was passed through a 0.22 μm filter. In order to start the treatment, when cells attained a 100% confluence the GM was removed, cells were washed twice in PBS, and the medium containing EVs was added. The incubation was performed for 24h, 48h or 4 days. At the end medium was removed, cells were washed in PBS and resuspended in Qiazol in order to perform cell lysis and in turn the following gene expression analysis.

3.12.2. Treatments with EVs, pDNA and TransIT-X2

When cells attained a 100% confluence, the GM was removed and media containing pDNA alone, pDNA and TransIT-X2, EVs alone, DNase1 pre-treated EVs, DNase1 pre-treated EVs and TransIT-X2, EVs and pDNA, DNase1 pre-treated EVs:pDNA, EVs and pDNA and TransIT. Each condition was designed as described by manufacturer's instructions (TransIT-X2® Dynamic Delivery System, Mirus Bio LLC). Alternatively, the protocol was adjusted with the aim of using EVs. DNase1 pre-treatment was performed using the E.Z.N.A.® RNase-Free DNase1 Set (Omega Bio-tek, Norcross, GA, USA), according to the manufacturer's instructions incubating EVs 30 min RT.

4. RESULTS

4.1. Models of stressed C2C12 display senescence-like traits

4.1.1. Characterisation of high-passage C2C12

In order to study the senescence phenotype in the muscle context, a C2C12 cell line was long-term cultured for over 40 passages. Such high-passage (HP) C2C12 were characterised to assess the onset of cellular senescence features. HP C2C12 cells were compared with a low-passage C2C12 cell line (LP), which was cultured for less than 15 passages. At first, the morphology of undifferentiated myoblasts and differentiated myotubes in HP C2C12, was examined. Differentiated myotubes were evaluated after 5 days since the differentiation process was induced. The analysis, conducted using light microscopy and immunofluorescence, revealed that HP myoblasts resulted shorter (Fig. 1b).

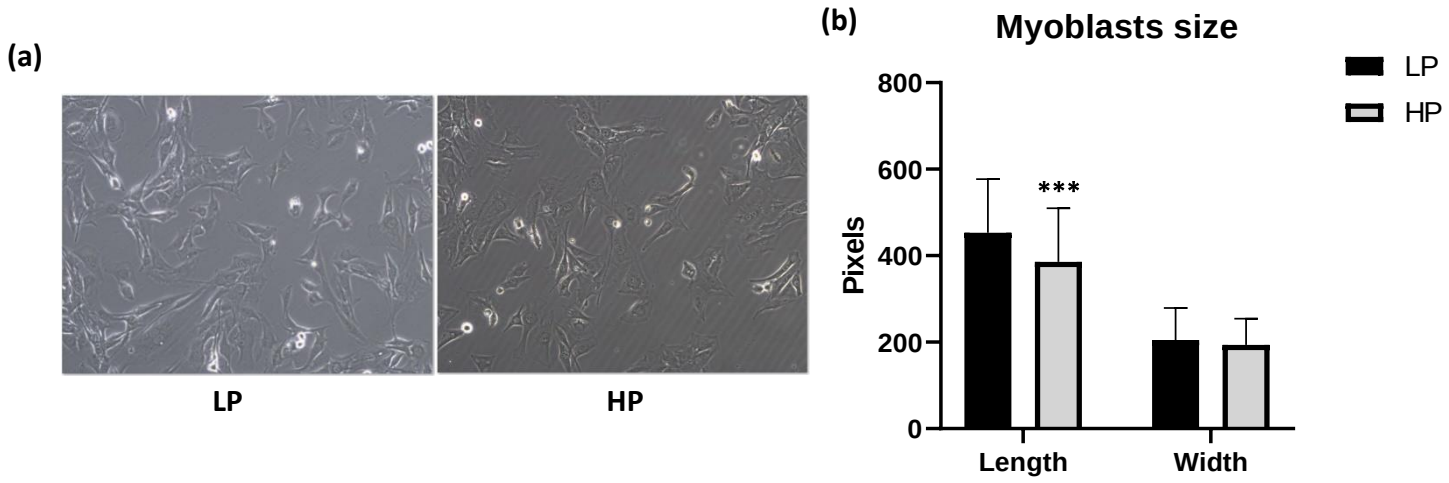


Figure 1. Morphological analysis of LP and HP myoblasts, after they reached an 80% confluence, was conducted through light microscopy (a). Measurement of the average myoblasts' sizes was performed. The results are represented as mean ± SD; *** $p < 0.001$ (b).

Notably, HP myotubes resulted significantly less in number, more disorganized, and thinner (Fig. 2a-b). In addition, the myogenic index, which consists in the ratio between nucleus inside myotubes and those outside, was measured. As a result, HP myotubes displayed a myogenic index significantly lower (Fig. 2c).

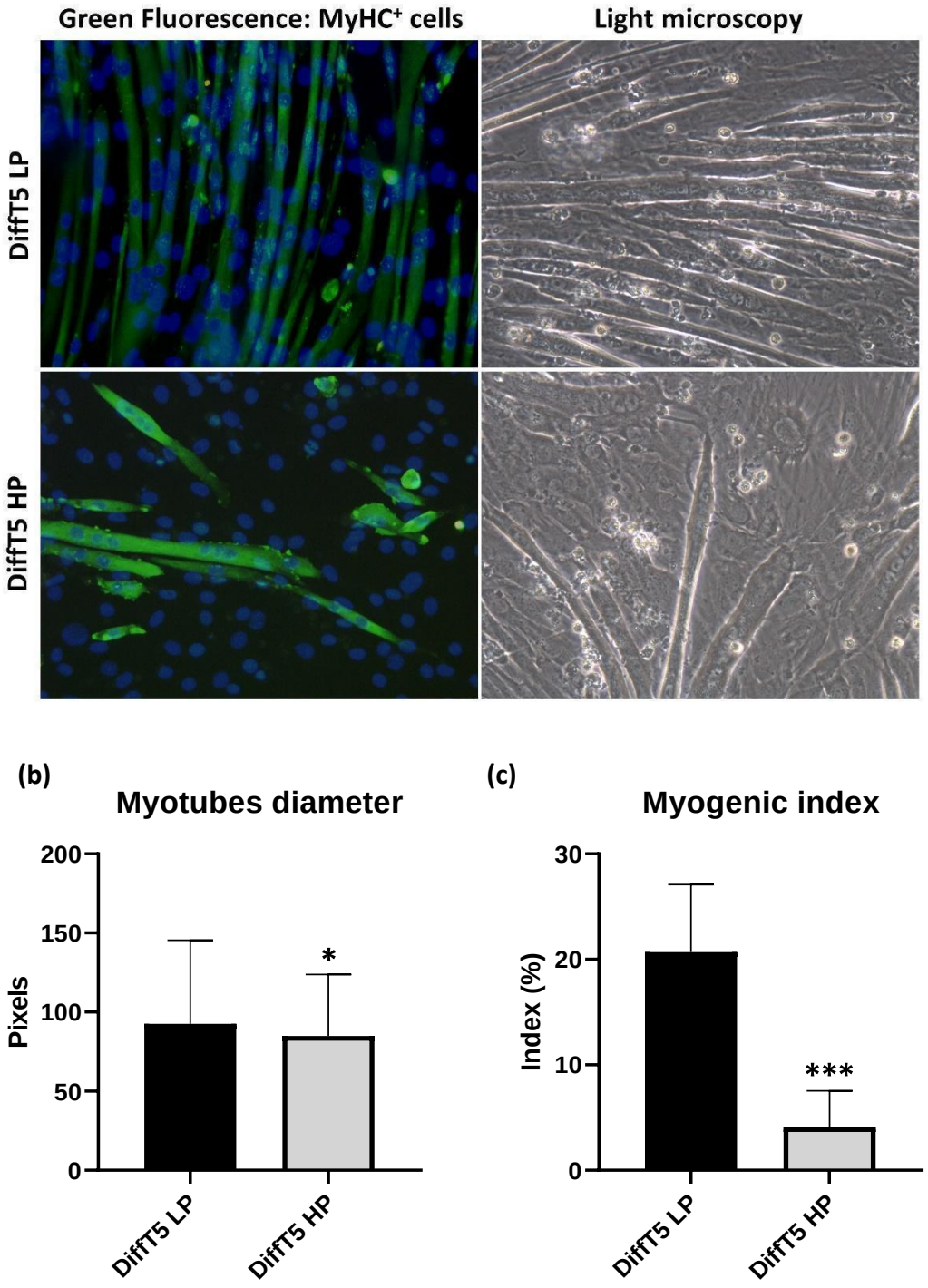


Figure 2. Morphological analysis of LP and HP myotubes, after 5 days since differentiation process was induced, was performed through light and fluorescence microscopy (a). Measurement of myotubes' average diameters was performed; the results are represented as mean ± SD; * $p < 0.05$ (b). The myogenic index was also measured; the results are represented as mean ± SD; *** $p < 0.001$ (c).

In addition, HP myoblasts' growth and death rate were analysed. To this end, growth and mortality of LP and HP myocytes were followed over 3 days. As a result, HP myoblasts displayed a lower growth rate than LP myoblasts (Fig. 3a). On the other hand, HP cells also showed a higher mortality (Fig. 3b). These data might suggest an impairment of HP myoblasts' survival process.

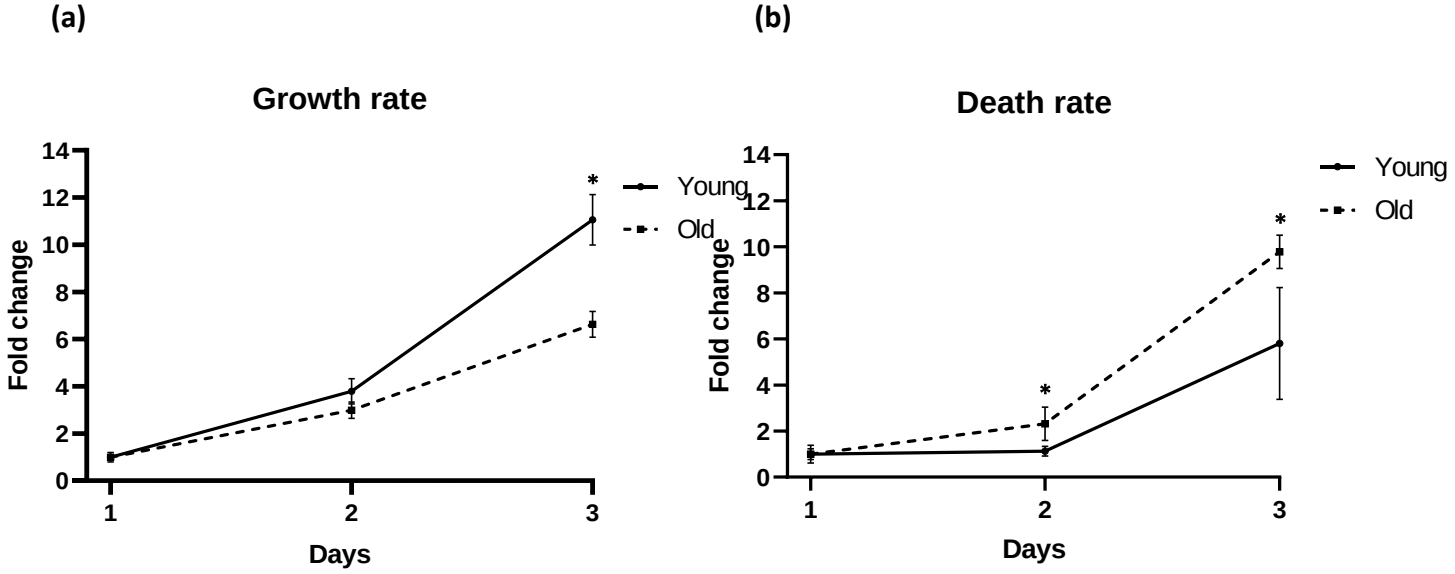


Figure 3. Curves representing growth (a) and death rate (b) of LP and HP myoblasts, obtained analysing the growth of both cell lines over 3 days; The results are represented as mean ± SD; * $p < 0.05$.

At this point, in order to verify the possible alterations associated with the senescent phenotype, HP myocytes' expression profile was explored. To this end, initially RT-PCR analysis was performed. In agreement with the alterations previously observed, HP myocytes displayed an mRNA expression impairment of the myogenesis gene pattern. More in detail, HP cells showed an alteration of almost all the differentiation markers investigated (Fig. 4). Altogether, such impaired genes suggested a delay of HP myoblasts to start the differentiation process. In addition, they also displayed a higher level of POLA1 suggesting that HP differentiating myocytes also delayed the exit from cell cycle (Fig. 4). Interestingly, HP cells also showed a consistent inflammatory level as indicated by the significant higher levels of IL-6 (Fig. 4).

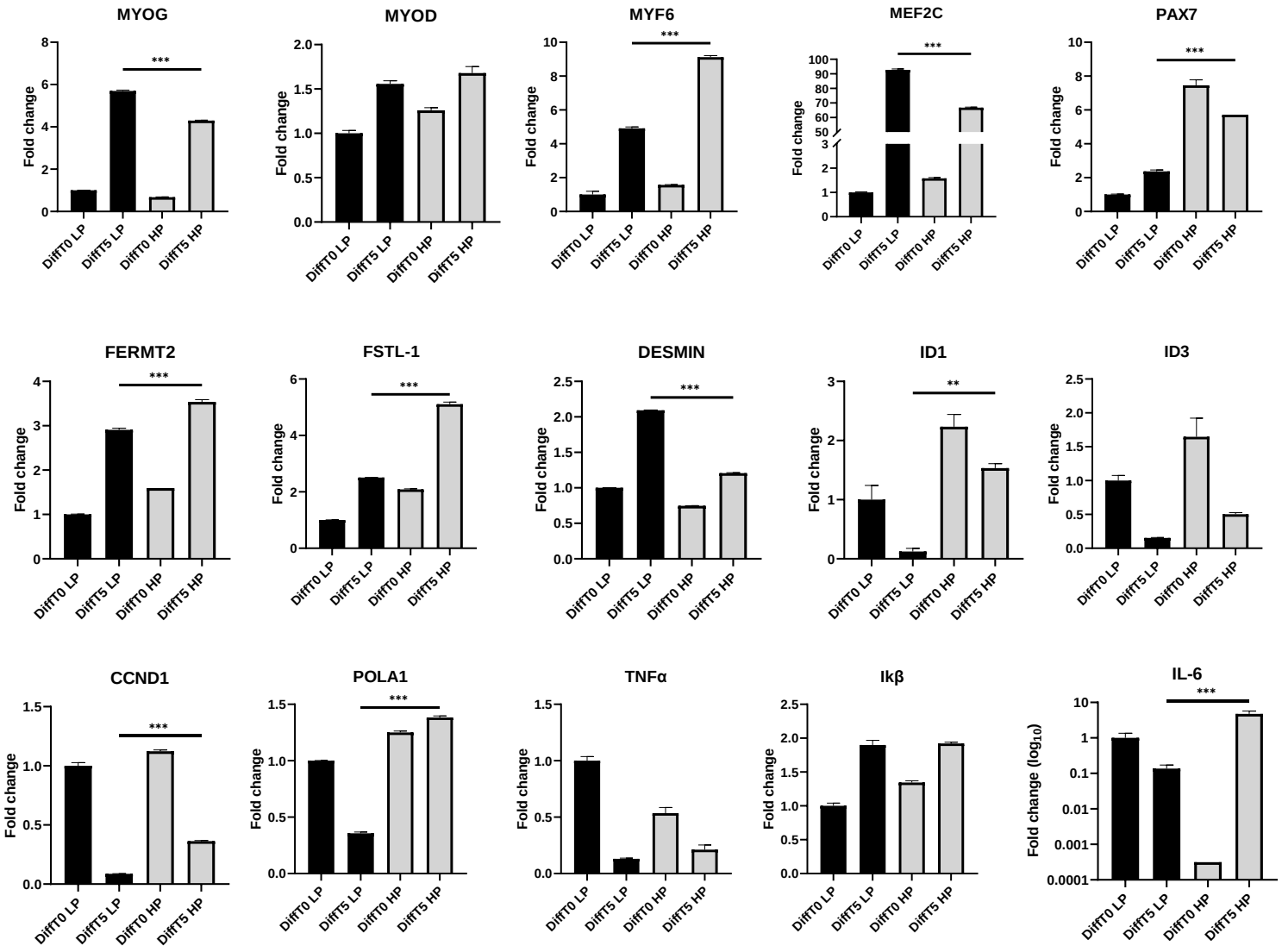


Figure 4. LP and HP myocytes' gene expression were analysed in undifferentiated myoblasts (diffT0) and differentiating myotubes after 5 days since the differentiation process was induced (diffT5). The expression levels of several marker genes indicatives of the myogenic, proliferation and inflammation processes, were evaluated; the results are represented as mean ± SD; ** $p < 0.01$; *** $p < 0.001$.

In order to support RT-PCR results and to further characterise HP C2C12 phenotype as well as investigating which intracellular pathways might be involved in the previously observed alterations, western blot analyses were performed. Moreover, additional intermediate differentiation times were examined. In agreement with RT-PCR data, the obtained results indicated that HP myocytes still showed a proliferative state as suggested by the higher levels of proliferating cell nuclear antigen (PCNA) expression, which is a typical marker of the proliferation process. Moreover, HP cells also displayed a lower expression of the myosin heavy chain (MyHC), a marker which is more expressed in the late phases of differentiation (Fig. 5). In addition, HP cells showed an increased activity of p38 along with a reduced activation of Notch1 (Fig. 5). Altogether, these results at one hand further suggested a delay of HP myocytes to exit the cell cycle and start to differentiate as indicated by the expression of PCNA and MyHC, respectively. On the other hand, the switching state of p38 and Notch1 suggested an impairment of the self-renewal process.

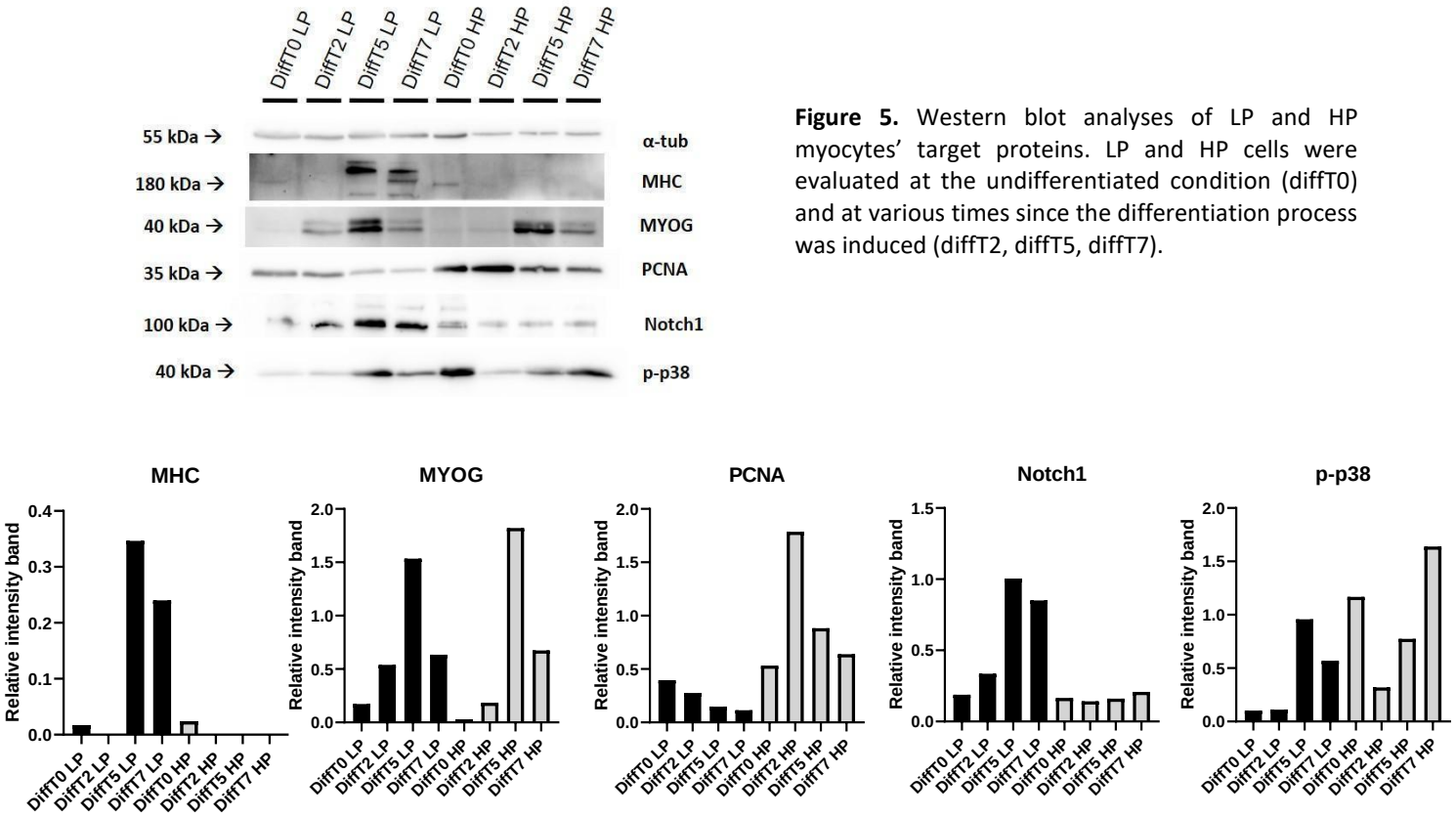


Figure 5. Western blot analyses of LP and HP myocytes' target proteins. LP and HP cells were evaluated at the undifferentiated condition (diffT0) and at various times since the differentiation process was induced (diffT2, diffT5, diffT7).

4.1.2. Characterisation of oxidative stressed C2C12

Afterward, another model of senescence was studied in order to find out any possible similarities with the HP cell model previously examined. The cell line explored was obtained subjecting LP C2C12 cells to an oxidative stress as described by Chen et al. [259]. In particular, two different stimuli were tested: cells were challenged with a medium containing 10% FBS and 600 µM H_2O_2, as reported, but also with a serum-free medium containing 300 µM H_2O_2. Subsequently, as for the HP C2C12 model, such oxidative stressed myocytes (OX) were characterised morphologically as well as concerning gene expression. OX cells' morphology was evaluated using light microscopy, whereas gene expression was explored performing RT-PCR analysis. OX myocytes' morphology was examined in the days following the oxidative insult and throughout the differentiation process. The obtained data were compared both with LP and HP C2C12. The morphological analysis of OX myocytes the days after the oxidative insult indicated that the 300 µM H_2O_2 condition exerted the greatest effect, indeed such cells became more enlarged than HP and LP C2C12, but also than myocytes treated with 600 µM H_2O_2 (Fig. 6). Therefore, the following analyses were focused on the myocytes deriving from the 300 µM H_2O_2 condition.

Surprisingly, in contrast with what observed on HP myotubes, the morphology of OX myotubes appeared similar to that of LP ones. Also, OX myotubes occurred at similar differentiation times (Fig. 7).

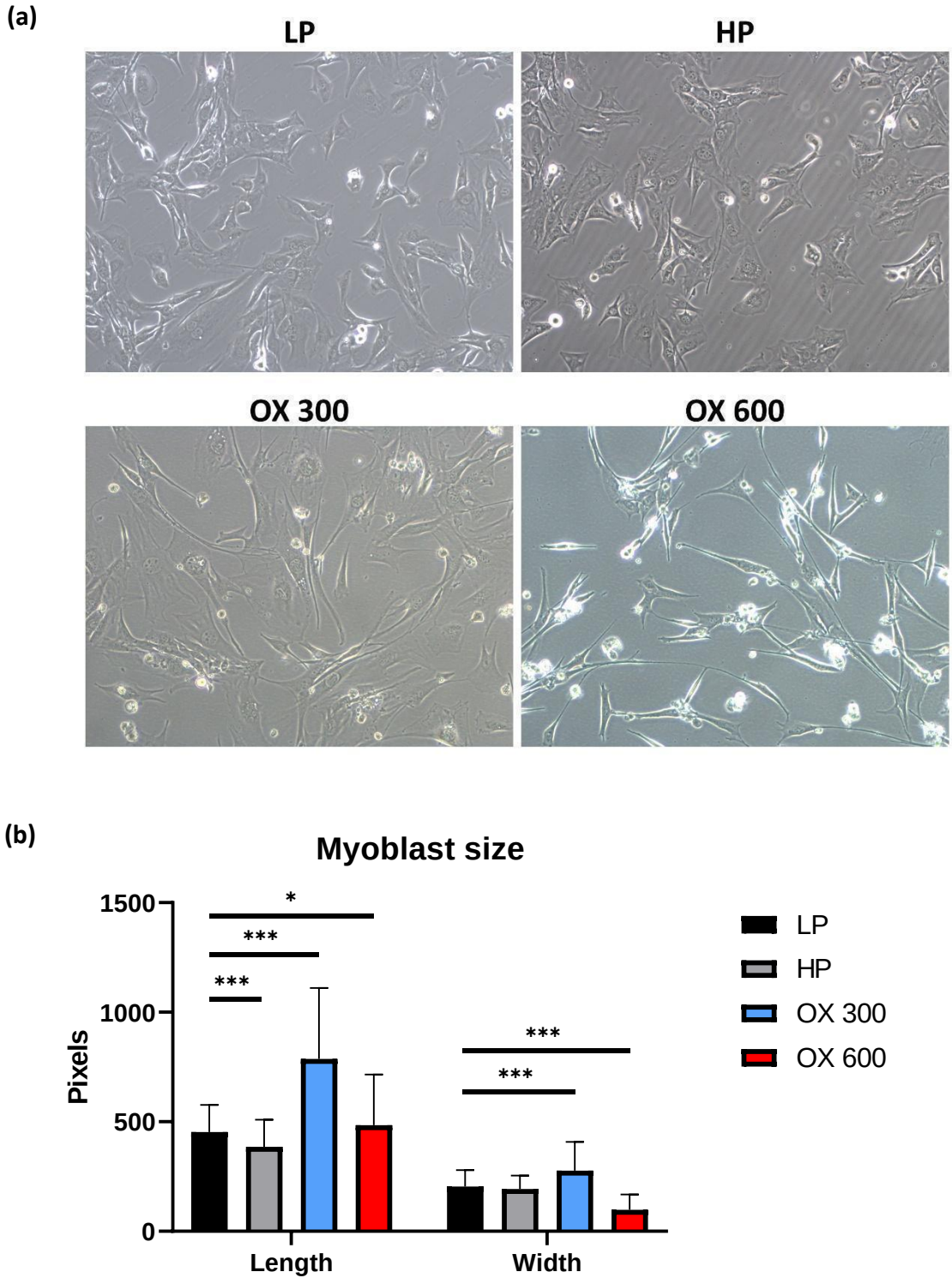


Figure 6. Morphological analysis of LP myoblasts after the induction of the oxidative insult (OX). OX cells' average size was compared with that of LP and HP myoblasts at 80% confluence through light microscopy (a). Measurement of the average myoblasts' sizes was performed; the results are represented as mean ± SD; * $p < 0.05$; *** $p < 0.001$ (b).

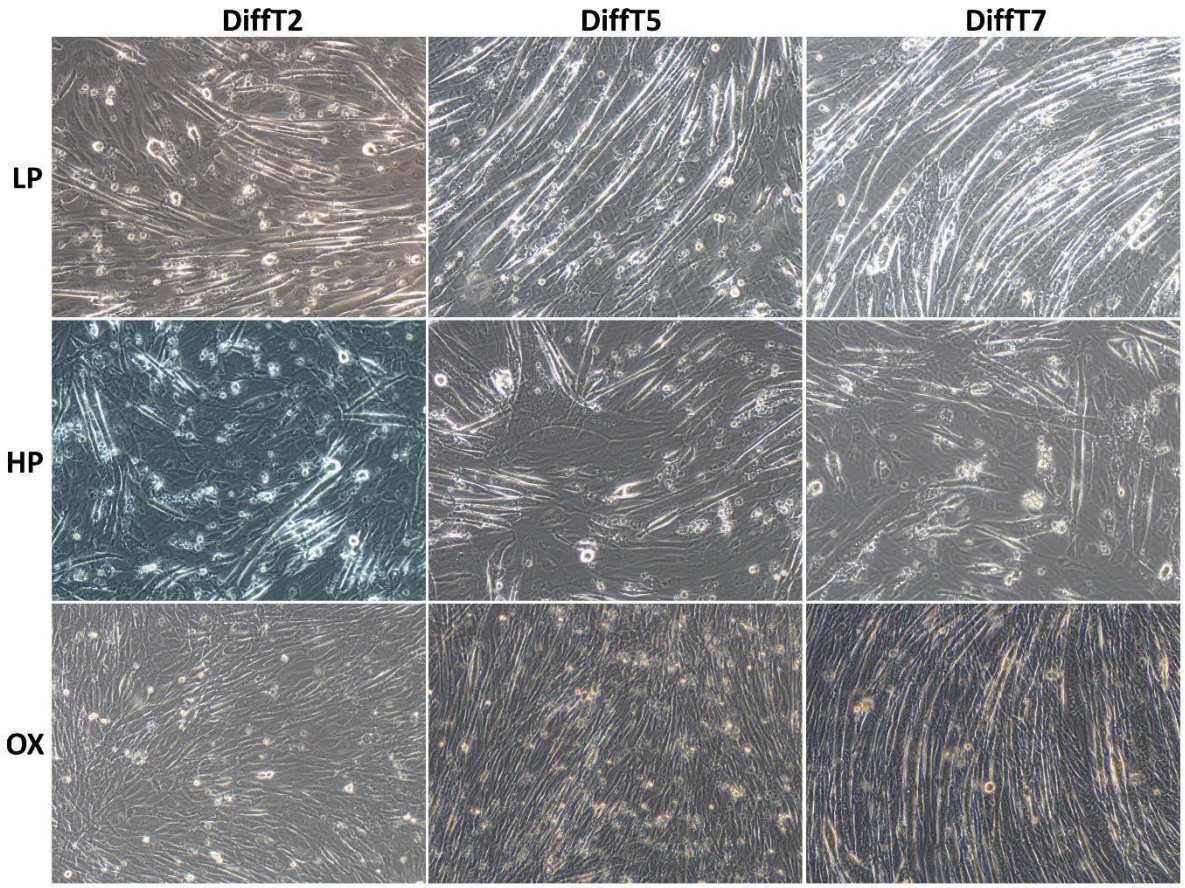


Figure 7. Morphological analysis of LP, HP and OX myotubes, at multiple times since the differentiation process was induced, was performed through light microscopy.

Although morphological analysis has not pointed out significant differences among LP and OX myocytes, gene expression analysis instead suggested an impairment of the differentiation process as shown by the reduced expression levels of MYOG, MEF2C and MYOD. Moreover, in contrast with HP myocytes' expression, OX myocytes did not show a significant difference in the proliferative state. Notably, also OX cells displayed a significantly activated inflammation process as indicated by the high levels of IL-6 expression (Fig. 8). Therefore, the obtained results suggested that HP and OX myocytes effectively displayed some common trait, especially concerning gene expression.

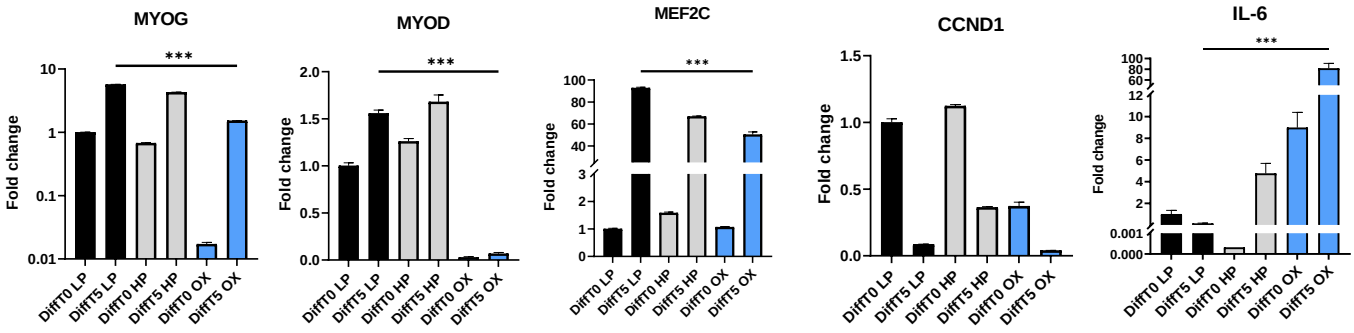


Figure 8. OX myocytes' gene expression was analysed in undifferentiated myoblasts (diffT0) and differentiating myotubes after 5 days since the differentiation process was induced (diffT5). The expression levels of marker genes indicatives of the myogenic, proliferation and inflammation processes, were evaluated; the results are represented as mean ± SD; *** $p < 0.001$.

In addition, in order to highlight any other common trait somehow related to aging, the oxidative state of HP and OX myocytes was examined. To this end, basal amounts of ROS and antioxidant capacity were evaluated and were compared with those of OX myocytes. Antioxidant capacity was tested detecting any modulation of ROS amount after treating cells with two different concentrations of H_2O_2. As a result, all the tested cell lines displayed similar levels of ROS basal level. Interestingly, HP and OX myocytes, but in particular OX cells, displayed higher levels of ROS after H_2O_2 treatment suggesting a possible impairment of the antioxidant capacity (Fig. 9).

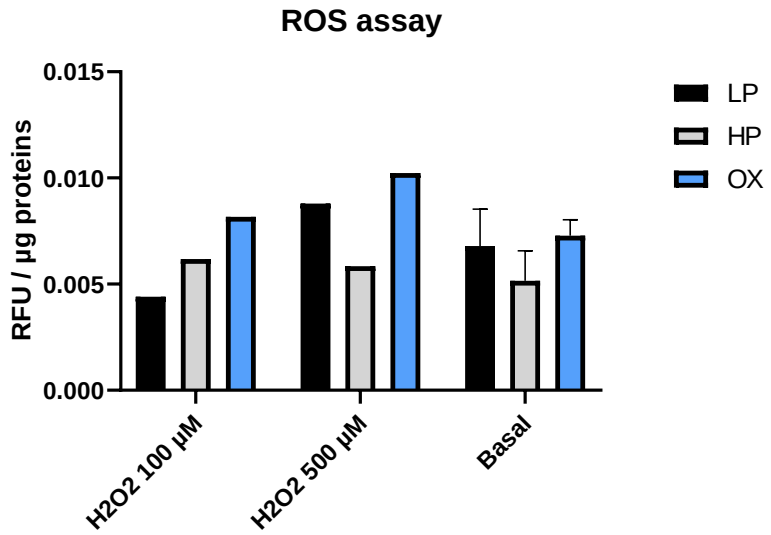


Figure 9. Basal ROS amount and antioxidant capacity, of LP, HP and OX cell lines. Results were obtained performing a DCFH-DA assay in presence/absence of H_2O_2.

Finally, since the studied cell models seemed to display some senescence-associated traits, the modulation of the senescence-associated β-galactosidase (SABG) was tested. To this end, a SABG assay was performed as described by Chen et al. [259]. The obtained pictures of $SABG^+$ cells were analysed as described by Lozano et al. [260]. The assay was tested on LP, HP and OX myoblasts when they reached an 80% confluence. The obtained results were unclear, however HP and OX myocytes seemed to display a slight increasing trend of the SA-β-galactosidase activity compared with LP ones (Fig. 10).

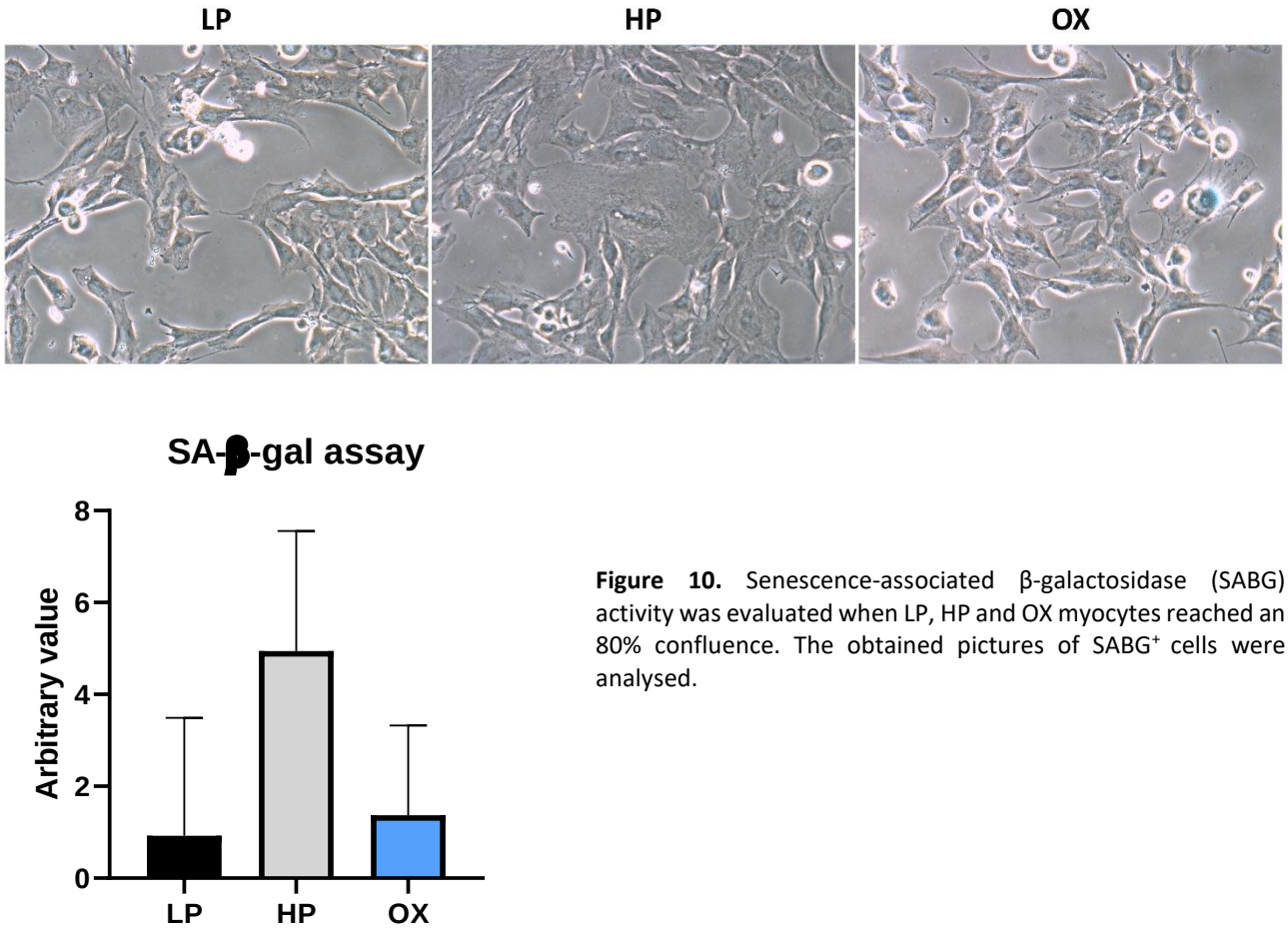


Figure 10. Senescence-associated β-galactosidase (SABG) activity was evaluated when LP, HP and OX myocytes reached an 80% confluence. The obtained pictures of $SABG^{+}$ cells were analysed.

4.2. Features of the EVs secreted by stressed C2C12 models

After the characterisation of the two *in vitro* C2C12 cell models, we purified and characterised the EVs released by such cell lines. Initially, EV secretion of HP and LP myocytes, at different time points upon differentiation induction, was evaluated. To this end, the cell culture media at each time point were collected and subjected to a protocol of serial ultracentrifugation. Therefore, large EVs (lEVs) and small EVs (sEVs) were purified and quantified by nanoparticle tracking analysis (NTA). Preliminary results suggested that HP myocytes released a higher amount of EVs. Indeed, HP myocytes were shown to secrete a higher amount of sEVs, which constitutes a large part of the released EVs, at every differentiation time. On the other hand, HP cells were found to release a lower quantity of lEVs at every differentiation time. NTA analysis also suggested that lEVs deriving from HP myocytes were larger (Fig. 11).

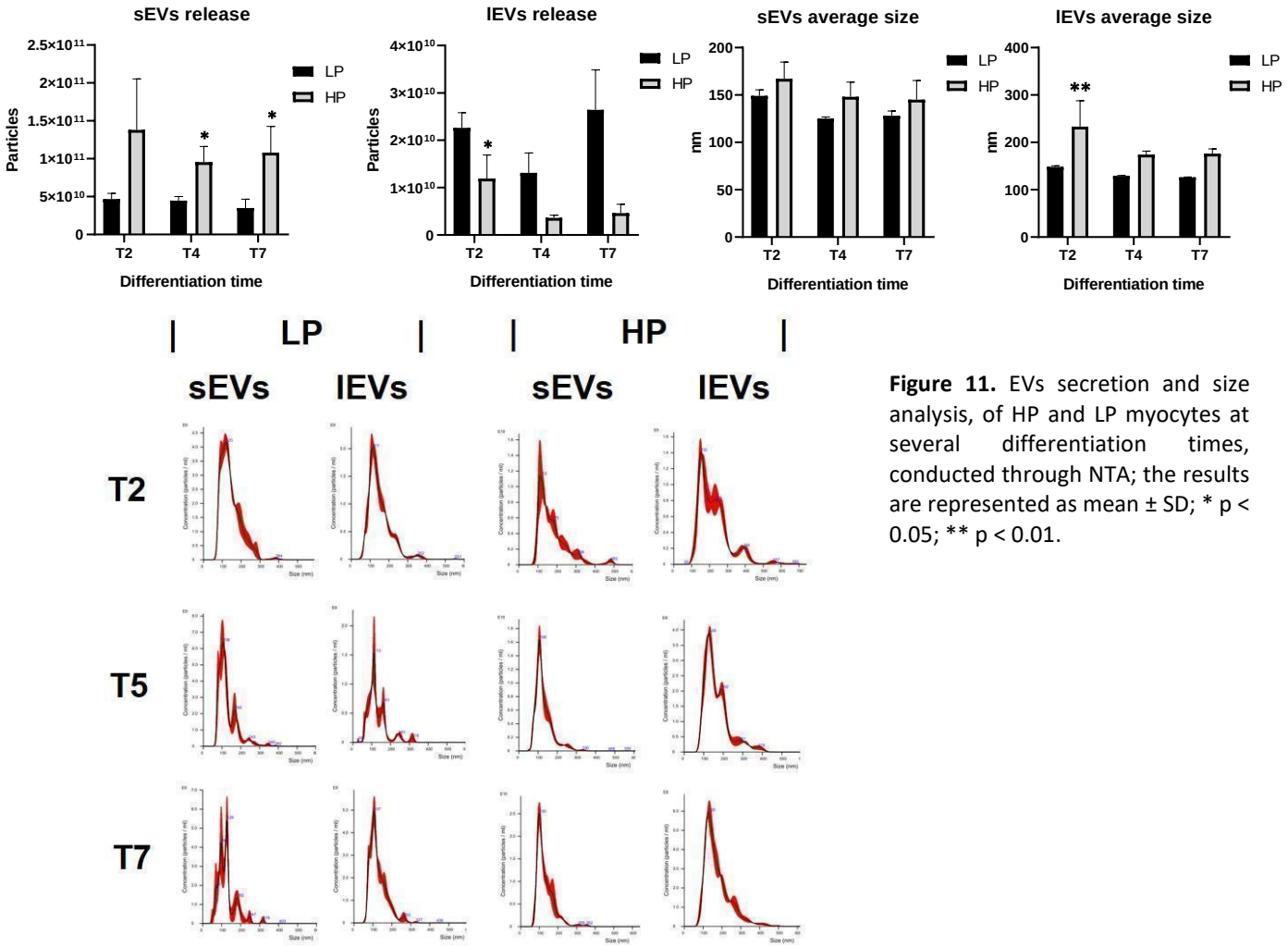


Figure 11. EVs secretion and size analysis, of HP and LP myocytes at several differentiation times, conducted through NTA; the results are represented as mean ± SD; * $p < 0.05$; ** $p < 0.01$.

In order to better characterise the purified EVs, a further purification step was applied.

To this end, EVs released by differentiating myocytes 5 days after differentiation induction, were separated in a discontinuous sucrose and optiprep density gradient. The latter served to reveal the density of each fraction. Each fraction was examined through NTA. The obtained results indicated that total EVs from each cell line showed a pick of particles at fraction 7 corresponding to a density of about 1,08 (Fig. 12a). In addition, since HP myocytes were shown to secrete more EVs, given that most of them were small EVs, such vesicles alone were separated through density gradient. As a result, sEVs deriving from HP cells displayed a double pick: one less dense at about 1,06 and the other denser at about 1,12 (Fig. 12b).

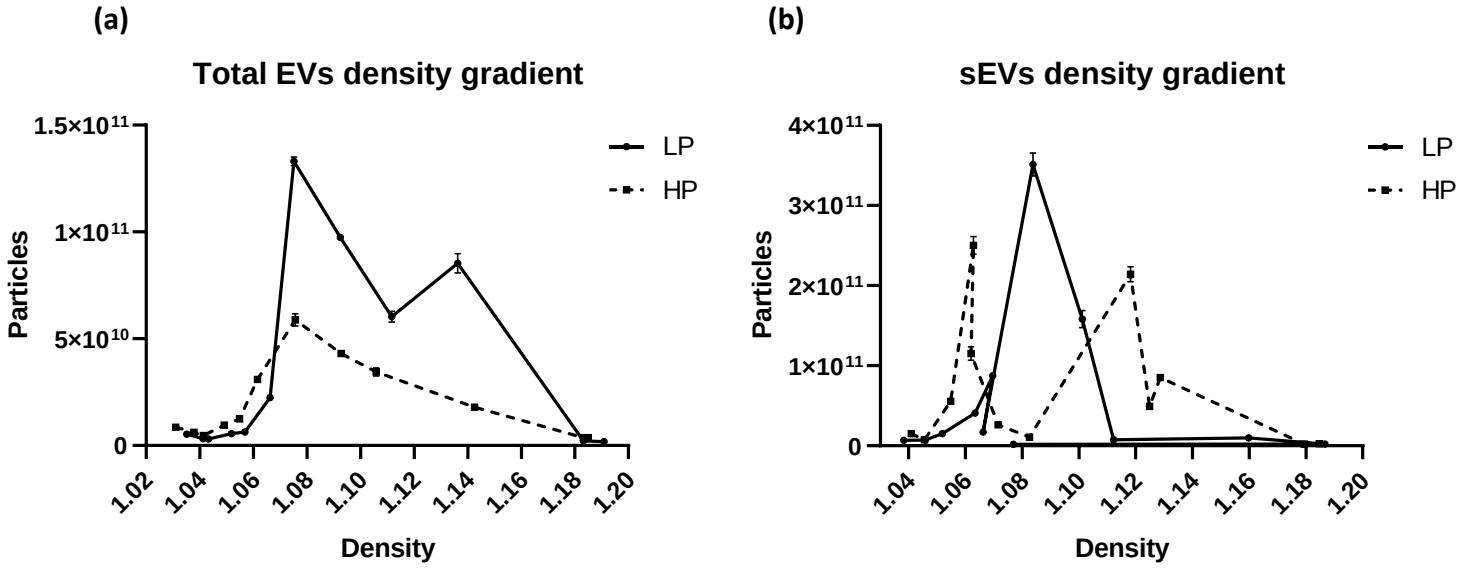


Figure 12. Density analysis of total EVs (a) and sEVs (b) conducted separating corresponding EVs through density gradient. Gradient fractions were evaluated through NTA.

We then proceeded with the characterisation of EVs membrane proteins. Therefore, western blot analyses were performed in order to explore the expression of surface markers such as CD63, Alix, Tsg101 and α-actinin. In addition, given that also EVs released from OX myocytes were purified, this characterisation was extended to them. The studied EVs were obtained from myocytes at several time points after the differentiation process was induced. As a result, all the studied EVs were shown to be positive for at least two of the markers above mentioned (Fig. 13). In particular, both lEVs and sEVs were positive to Tsg101, lEVs were positive to α-actinin, and sEVs to Alix proteins respectively.

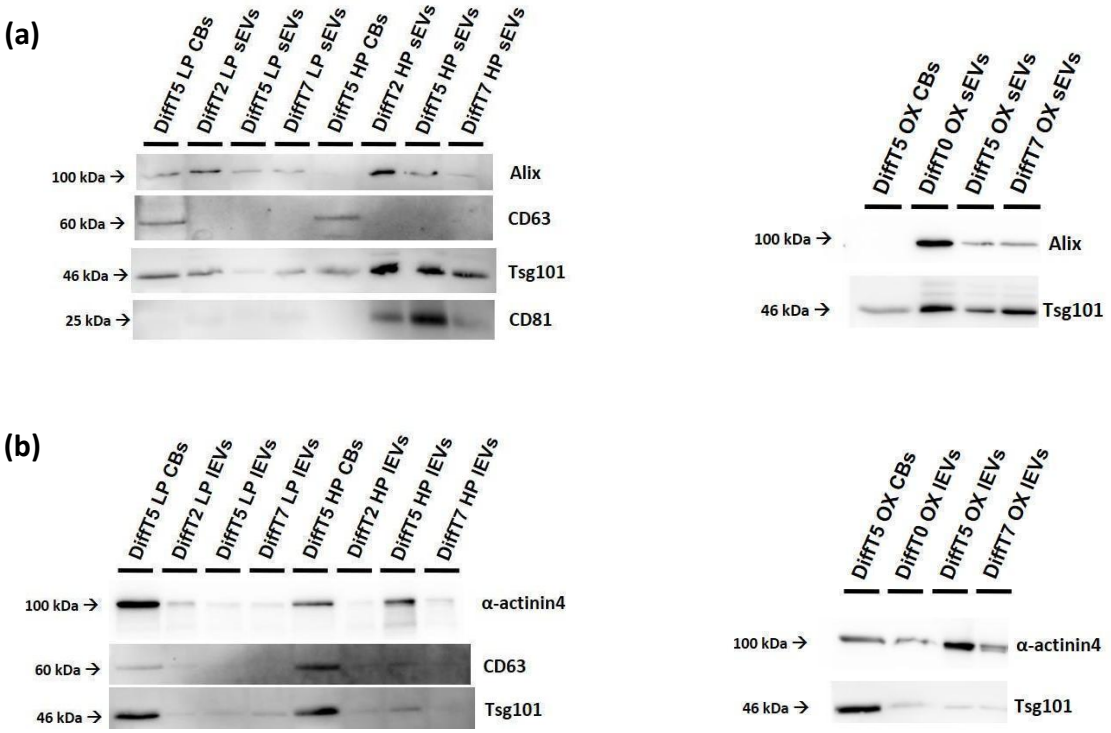


Figure 13. Western blot analysis of the surface markers distinguishing EVs. The performed analyses were conducted on both sEVs (a) and lEVs (b) released by all the cell models studied.

Finally, morphological analysis of both small and large EVs released by differentiating myocytes when reached 5 days after differentiation was induced, was performed through transmission electron microscopy (TEM). As a result, EVs derived by LP and HP cells have not displayed significantly different features (Fig. 14).

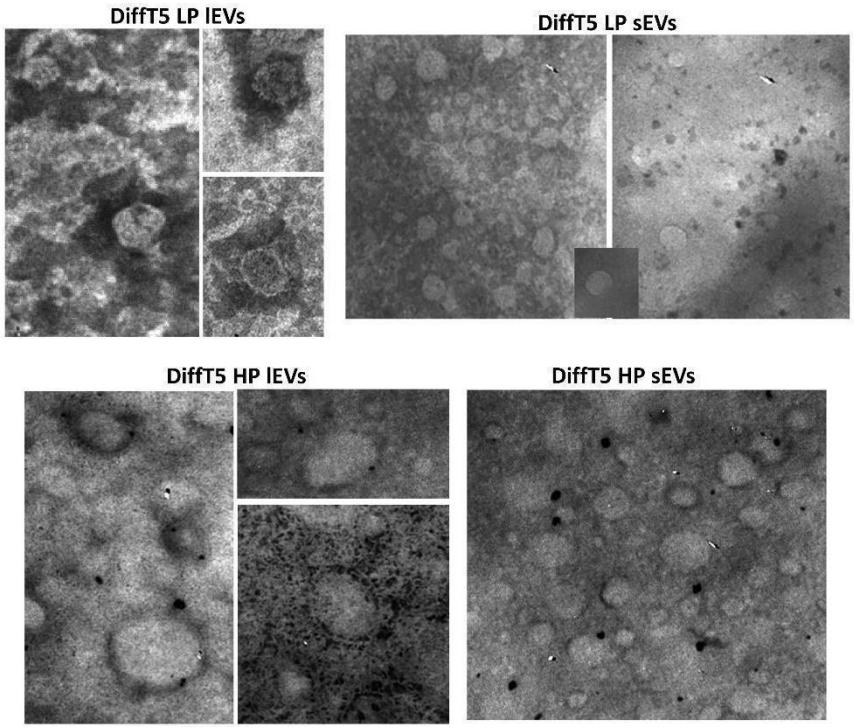


Figure 14. Pictures, obtained through transmission electron microscopy (TEM), of EVs released by differentiating HP and LP myocytes when reached 5 days after the differentiation was induced.

4.3. EVs released by stressed C2C12 induce an inflamed status in C2C12 muscle cells and RAW264.7 macrophages

After having demonstrated that when C2C12 cells lose their ability to properly conduct and complete the myogenic differentiation process, the deregulated myocytes display an altered EVs secretion pattern, it was conceivable that also the EV-associated signals could be impaired. For this reason, we decided to further explore this topic using as EV source HP C2C12 cells and as targets of the senescence-like derived EVs the LP C2C12 and RAW264.7 cells.

The driving hypothesis was to investigate whether senescence-like derived EVs could affect the myogenic differentiation program and/or activate macrophages, therefore exacerbating the muscle inflamed status.

4.3.1. Spread of myocyte inflammation by means of EVs

At first, to shed light on the possible role of EVs in spreading SASP, HP differentiating myocytes were induced to differentiate for 5 days, and the released EVs were collected and used to treat LP C2C12. Subsequently, LP C2C12 were incubated with control EVs (LP-EVs obtained by LP C2C12) and EVs (HP-EVs) derived from HP C2C12 incompetent for a complete myogenic differentiation. Moreover, as the analysis of the EV release during impaired myogenesis pointed out a significant increase in the secretion of small EVs (Fig. 11) and given that most of the secreted EVs were sEVs, we focused on the effect of HP myocytes-derived sEVs was examined.

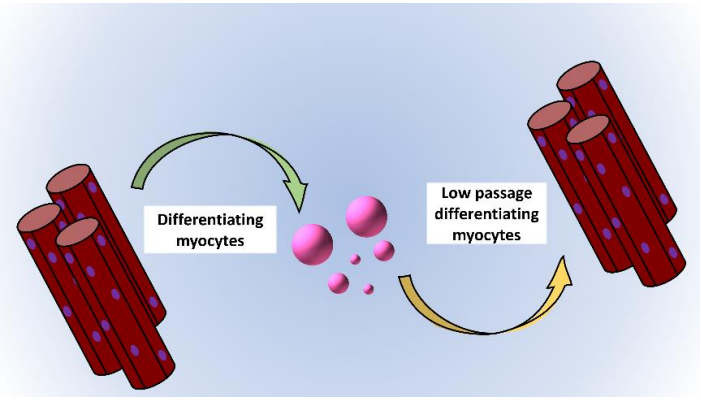


Figure 15. Schematic picture of the EVs treatments conducted on LP C2C12: LP C2C12 were incubated with EVs isolated from both LP and HP C2C12.

In our experimental setup, LP myocytes were treated with EVs at the sub-confluent condition and allowed to start myogenic differentiation for 24h. Cells were collected and their gene expression was analysed through RT-PCR. Notably, the obtained results suggested that sEVs released by HP myocytes induced a significant upregulation of the IL-6 mRNA levels (Fig. 16) suggesting that HP-EVs could have a pro-inflammatory effect.

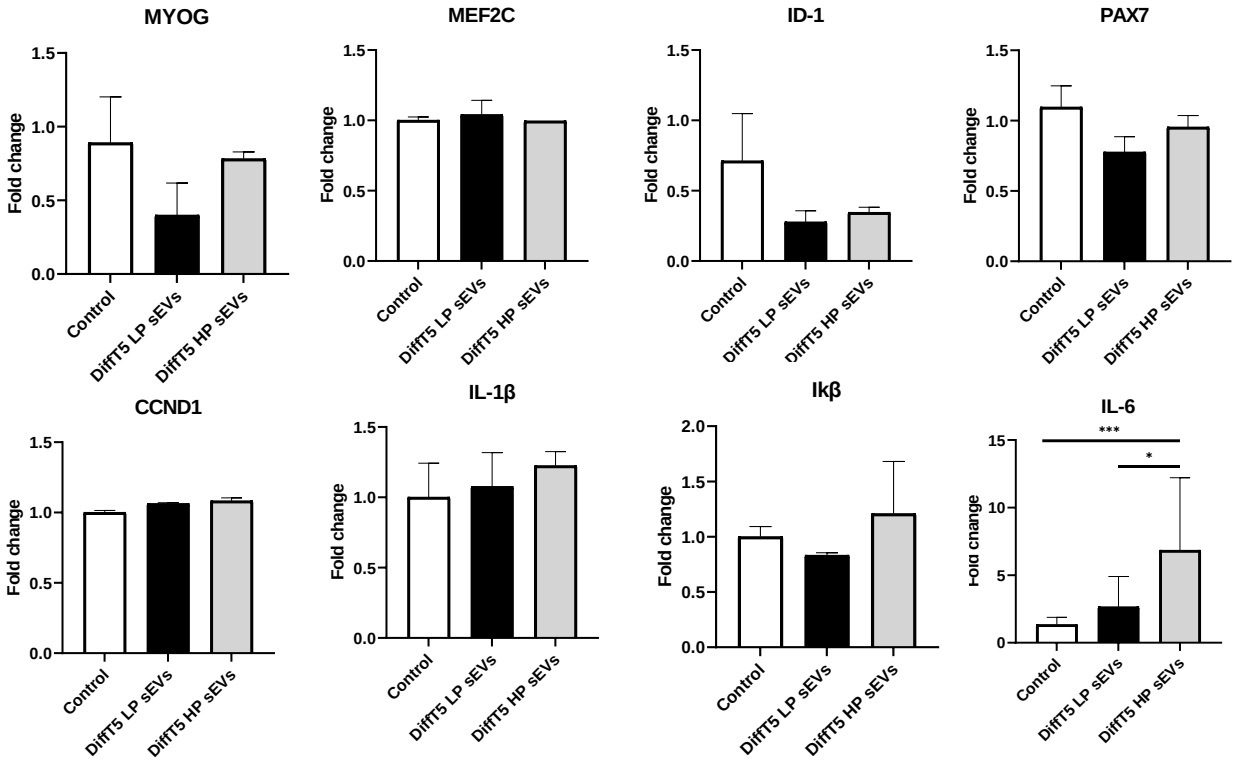


Figure 16. LP C2C12 gene expression analysis following the interaction among sEVs, released by HP myocytes after reaching 5 days since the differentiation was induced, and LP C2C12. The incubation was conducted for 24h. Results were compared with effect induced by corresponding LP cells' EVs; the results are represented as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

The activity of the studied EVs was further investigated exploring possible effects in a wider timeframe. Therefore, LP myocytes were incubated with EVs as previously reported and allowed to differentiate for 4 days. In this case, the obtained results showed no significant difference among the target of interest; altogether these data suggest that the EV treatment induced an acute modulation of inflammatory genes (IL-6), which returned to control levels after 2-3 days (Fig. 17).

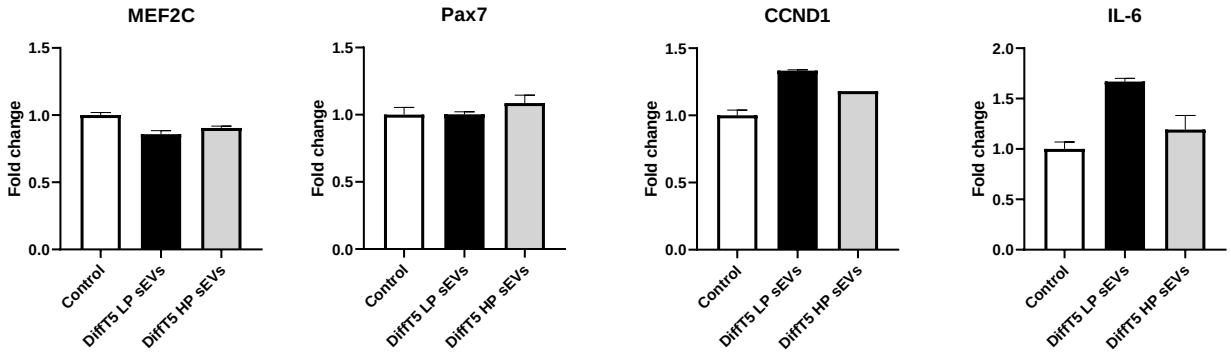


Figure 17. LP C2C12 gene expression analysis following the interaction among sEVs, released by HP myocytes after reaching 5 days since the differentiation was induced, and LP C2C12. The incubation was conducted for 4 days. Results were compared with effects induced by corresponding LP cells' EVs.

Afterwards, we aimed to evaluate whether LP cells' EVs could somehow revert the impairment of the HP myocytes in performing myogenic differentiation.

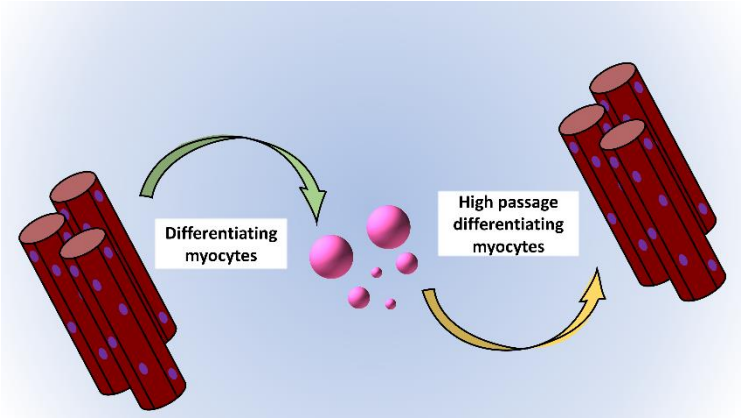


Figure 18. Schematic picture of the EVs treatments conducted on HP C2C12: HP C2C12 were incubated with EVs isolated from both LP and HP C2C12.

Therefore, HP myocytes were stimulated to differentiate in presence of EVs released by Diff-T5 LP myotubes. EV treatment was conducted also performing the 4-day long incubation as previously described. The mRNA expression levels were compared with that induced by corresponding EVs released by HP myocytes. As shown in figure 19 A and B, the acute treatment with EVs was able to exert only an almost significant increase in MEF2C expression levels suggesting a slight improvement of the myogenic differentiation process (Fig. 19a). Likewise, after 4 days of the EV treatment, no significant difference occurred in the mRNA expression of target genes among LP- and HP-derived EVs, with the exception of MEF2C expression which maintained higher levels, similarly to what found after 24h incubation (Fig. 19b).

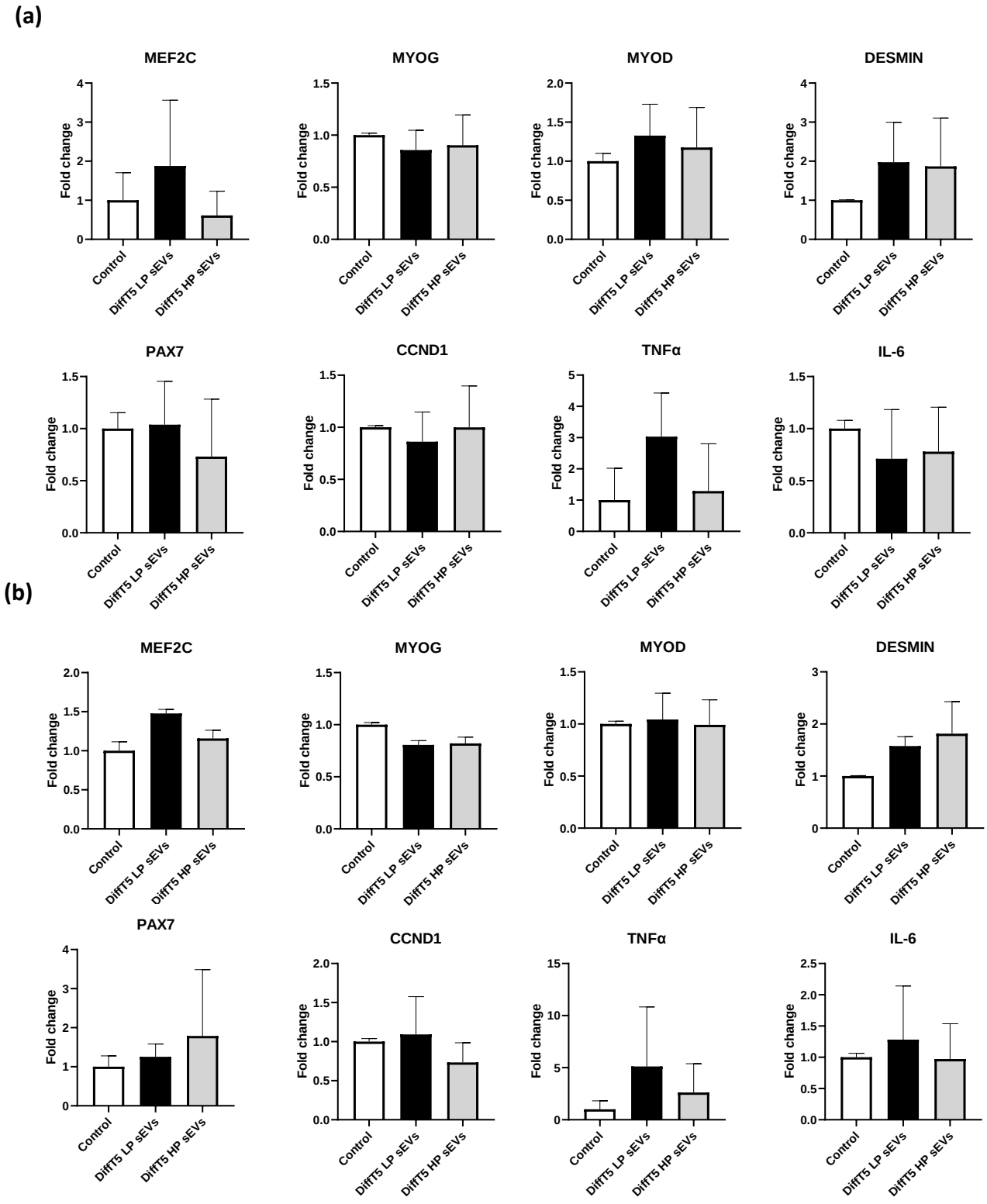


Figure 19. HP C2C12 gene expression analysis following the interaction among sEVs, released by LP myocytes after reaching 5 days since the differentiation was induced, and HP C2C12. The incubation was conducted for both 24h (a) and 4 days (b). Results were compared with the effects induced by corresponding HP cells' EVs.

Altogether, the data reported so far indicate that the greatest effect was induced by HP-derived EVs. In addition, the observed effects were found to occur mostly after 24h since the incubation started. On the other hand, such gene modulation seems to be blunted after 4 days of EV education. Subsequently, the hypothesis of EVs as carriers of pro-inflammatory signals was further investigated using oxidatively-injured muscle cells (OX cells; an alternative model of senescence). Therefore, LP differentiating myocytes were treated with OX cell-derived EVs in order to confirm the results previously reported. Moreover, in this experimental setup the interaction experiments were further implemented extending the study to large EVs. Indeed, initially the analysis was focussed on small EVs since HP myocytes resulted in releasing a higher amount of such EVs, suggesting that they could be the EV subpopulation most implied in transmitting impaired signals. However, EV release analysis on OX myocytes model was not performed. For this reason, the effect of both small and large EVs was explored and compared with that mediated by HP cell-derived EVs. Based on previously reported data, mRNA expression analysis has been performed after 24h from EV supplementation. The obtained results showed that OX-EVs (EVs derived from OX stressed myoblasts) induced IL-6 up-regulatin and MyoG down-regulation (Fig. 20). These results are in line with the effects induced by HP-EVs, in which stress-derived EVs seem to impair the myogenic differentiation process probably by stimulating pro-inflammatory events such as IL-6 production. Notably, in this experimental model, it appears that the main inflammatory activity could be assigned to lEVs subpopulation.

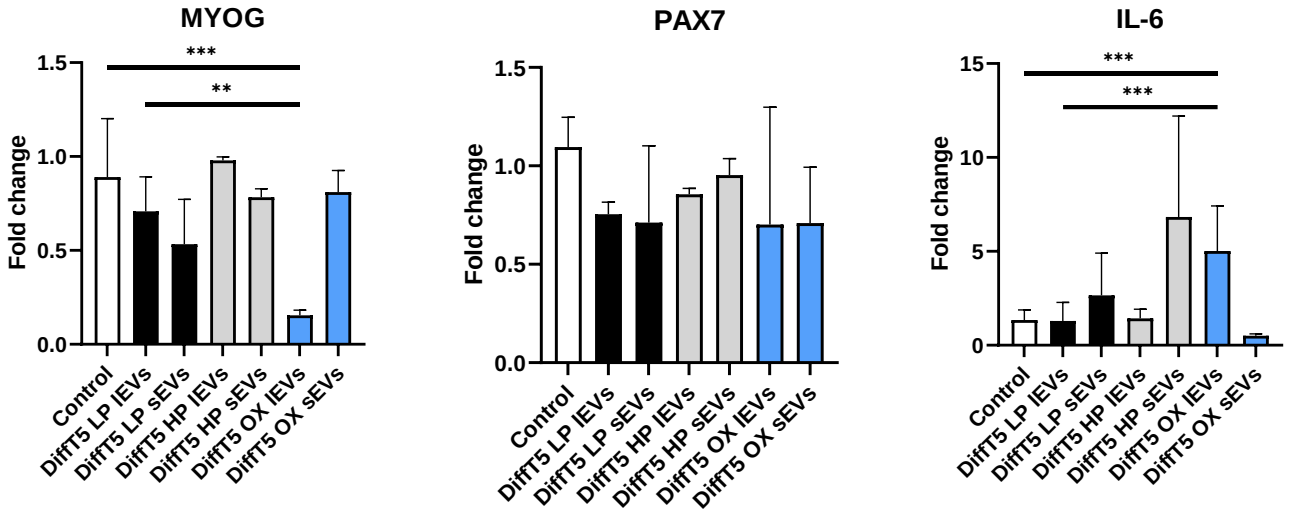
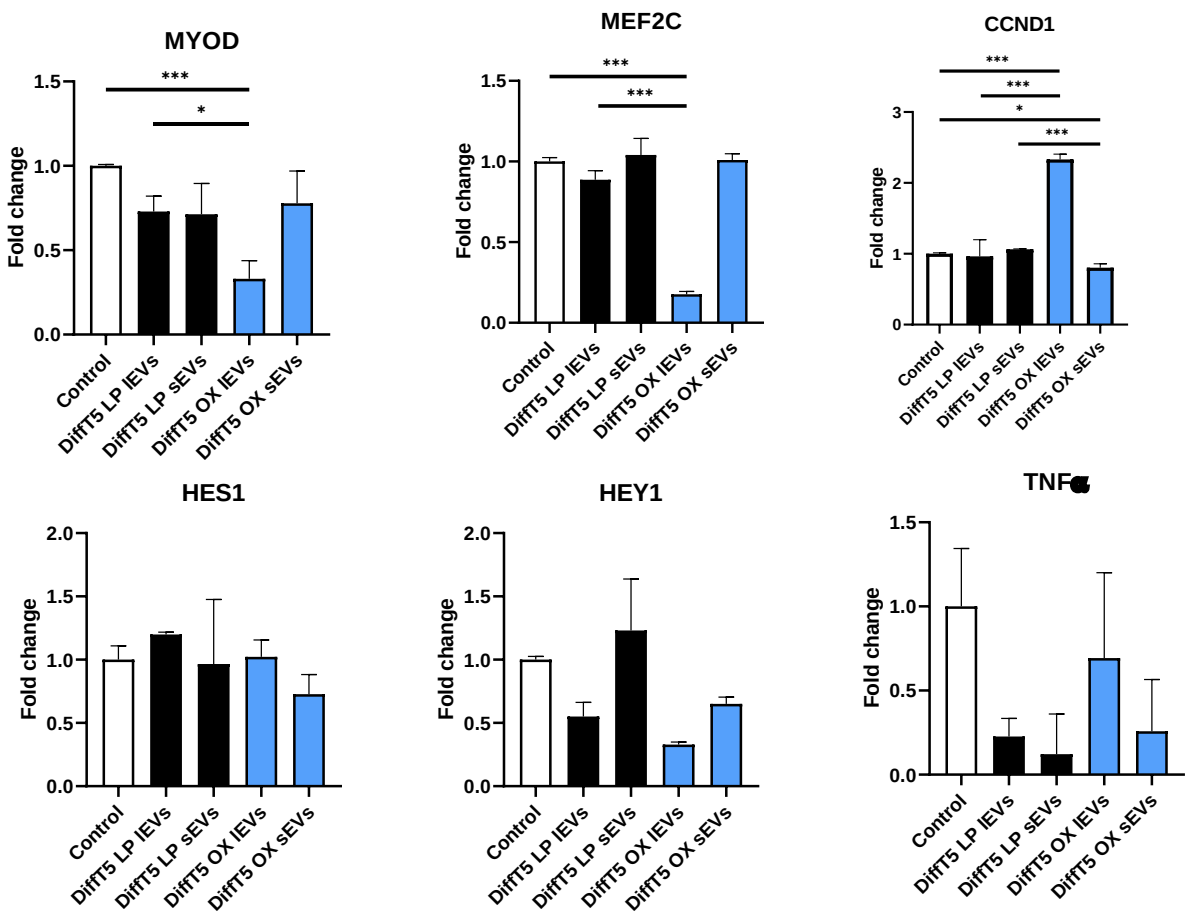


Figure 20. LP C2C12 gene expression analysis following the interaction among EVs, released by HP and OX myocytes after reaching 5 days since the differentiation was induced, and LP C2C12. Results were compared with the effect induced by the corresponding LP and HP cells' EVs; the results are represented as mean ± SD; ** $p < 0.01$; *** $p < 0.001$.

Since EVs released by OX myotubes induced a derangement of myogenic differentiation process, as revealed by MYOG mRNA decrease, the modulation of key genes involved in myotube formation in response to OX-EVs was further evaluated. In addition to MYOG, differentiating myocytes treated with OX-EVs displayed a reduction also in MYOD and MEF2C mRNA levels (Fig. 21a). Western blot analysis confirmed the decrease of MYOG and MyHC proteins in response to OX-EV treatment (Fig. 21b). Notably, we found two quite distinct responses following OX-lEV and -sEV supplementation, indeed western blot and qPCR analyses revealed that OX-lEVs induced MYOD, MYOG, MEF2C and MyHC down-regulation and CCND1 up-regulation, suggesting a slowing myogenic differentiation in favour of proliferation, while OX-sEVs stimulated proliferation without affecting differentiation (as shown by PCNA and MyHC protein levels; see fig. 21B).

(a)



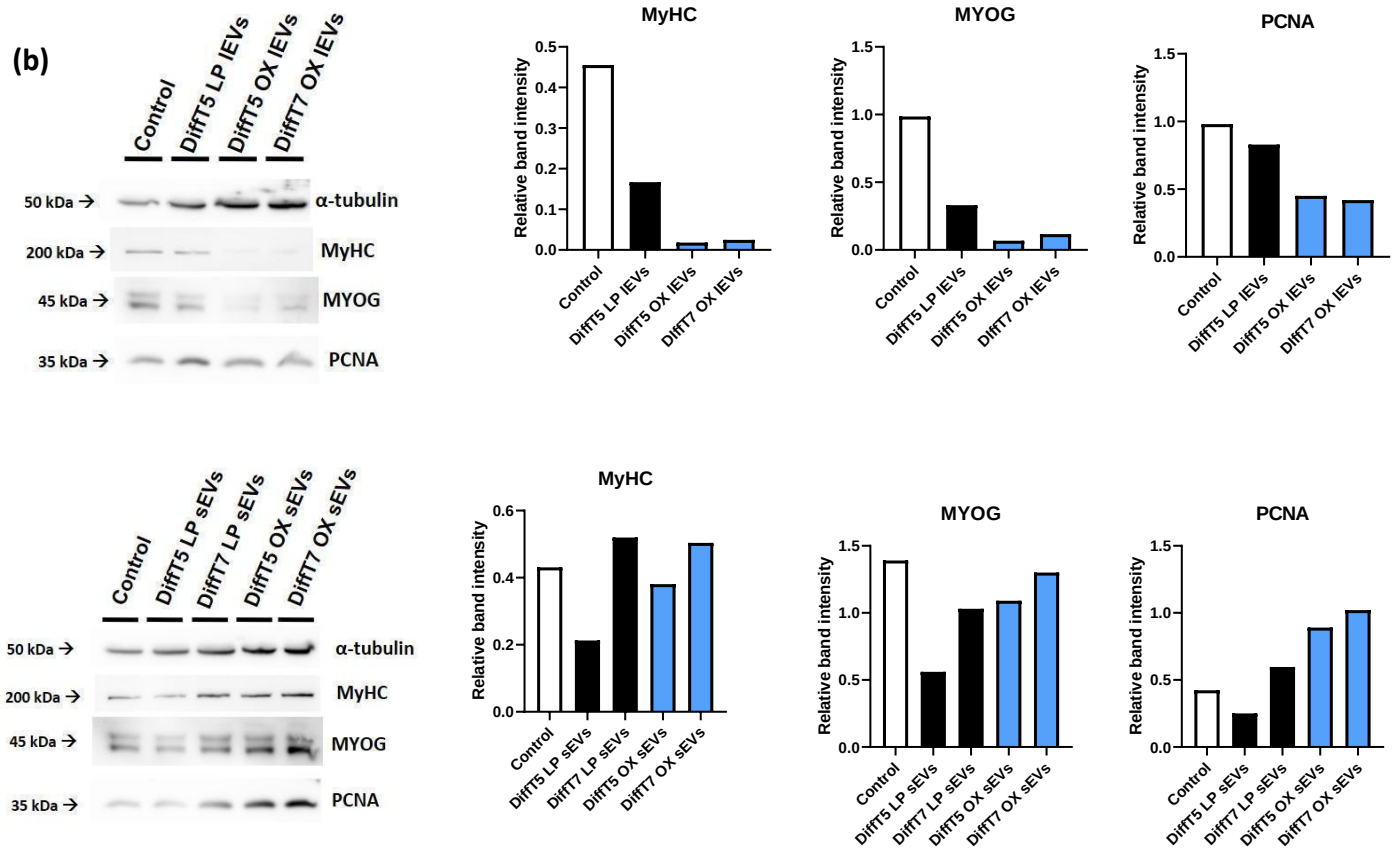
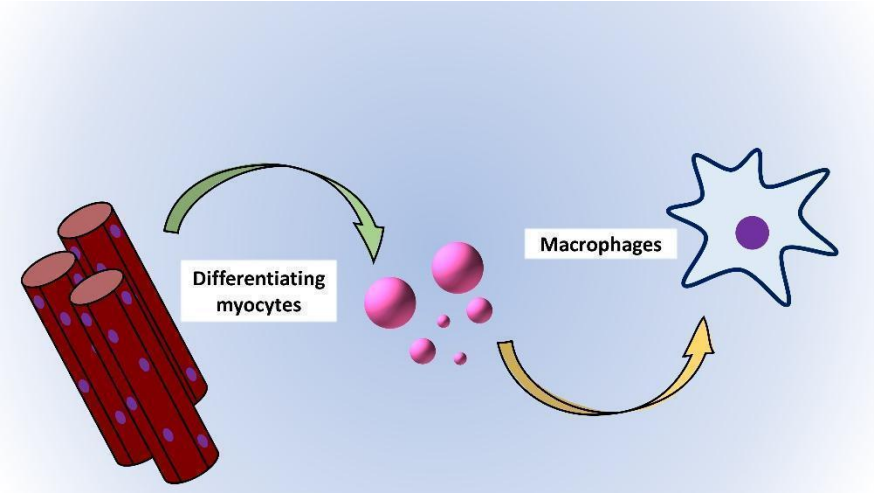


Figure 21. LP C2C12 gene expression analysis following the interaction among EVs, released by OX myocytes after reaching 5 days since the differentiation was induced, and LP C2C12. The results were obtained by performing both RT-PCR (a) and western blot analyses (b). Data were compared with the effect induced by the corresponding LP cells' EVs. The results are represented as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

4.3.2. Stressed-derived EVs activate RAW264.7 cells

The results above described, suggested that the most prominent effect mediated by EVs appeared to be the pro-inflammatory one. Since macrophages are pivotal players in the muscle niche and in muscle inflammation, we wondered whether HP-EVs could activate them.

Figure 22. Scheme representing the EVs treatments conducted on RAW264.7: RAW264.7 cells were incubated with EVs isolated from both LP and HP C2C12.



Indeed, RAW264.7 cells, a well-established model of murine macrophages, were incubated for 24h with a medium containing EVs released by differentiating HP myocytes. After the incubation, cell bodies were collected and gene expression was analysed focusing on the expression of genes involved in the inflammation process. The obtained results indicated that cells, treated with EVs released by HP myocytes, displayed a significant inflamed state as shown by the higher expression levels of IL-6, IL-1β and IFN-1β (Fig. 23). Moreover, the pro-inflammatory effect appeared to be mediated by both sEVs and lEVs, even though sEVs were found to exert a more pronounced effect. On the other hand, no stimulation was found for the anti-inflammatory cytokine IL-10 (Fig. 23).

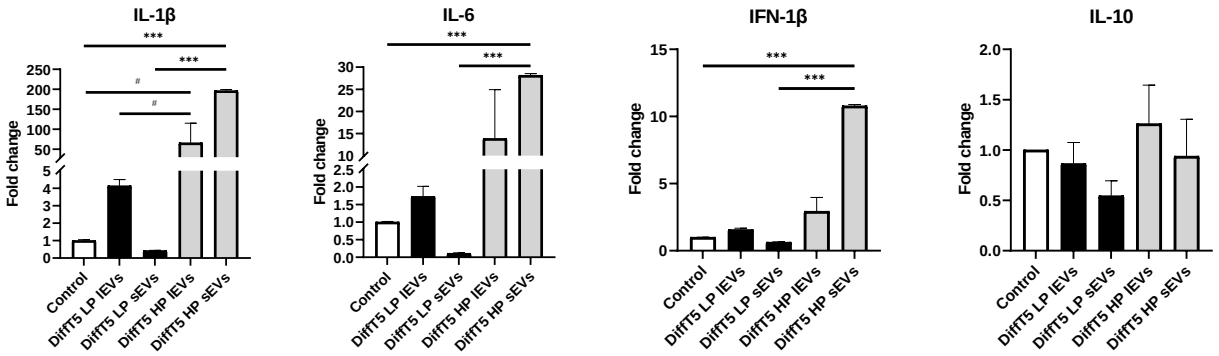


Figure 23. The activity on RAW264.7 cell line, of EVs released by HP myocytes obtained after 5 days since the differentiation process was induced, was analysed. EVs activity was analysed evaluating gene expression; the results are represented as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

4.4. The role of EV cargo in the impairment of recipient cells

4.4.1. HP and OX myocytes release EVs containing high quantities of DNA

Given that EVs released by HP and OX myocytes appeared to induce an impairment in target cells, mainly triggering an inflammation process, the next step was to study which of the molecular components of the EV cargo could be involved in this process. Therefore, EV total proteins and nucleic acids were extracted, purified and assayed. Total nucleic acid payload was normalized versus EV protein amount and then compared among LP, HP and OX conditions. The obtained results showed that EVs released by HP and OX cells contained a higher quantity of total DNA than LP cell-derived EVs (Fig. 24).

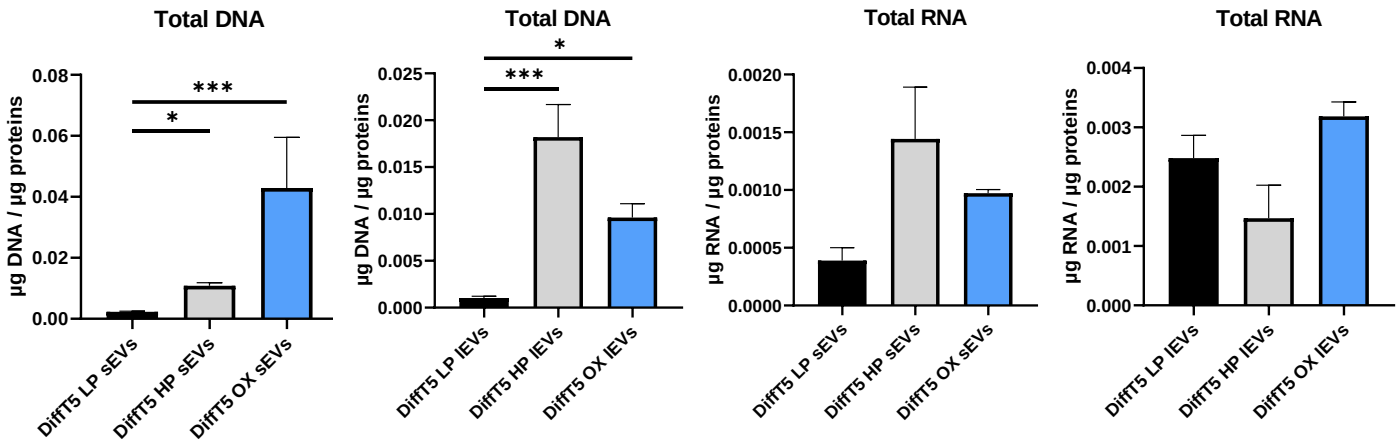


Figure 24. Amount of total nucleic acids content within EVs. The quantity of total DNA and RNA were related to the total amount of EV proteins. EVs were obtained from LP, HP and OX myocytes after 5 days since the differentiation was induced; the results are represented as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

Since the EVs secreted by HP and OX myocytes displayed higher levels of DNA cargo compared with LP cell-derived EVs, it led us to evaluate whether stress-derived EVs carry also mitochondrial DNA (mtDNA), this issue is particularly relevant as mtDNA acts as an inflammatory boost. Therefore, the amount of COX1 (a gene encoded by mtDNA) and 36B4 (a gene encoded by genomic DNA) genes were analysed through RT-PCR. As a result, both HP and OX myocytes' EVs were found to contain a higher amount of nDNA; moreover, EVs released by OX myocytes displayed a higher quantity of mtDNA (Fig. 25).

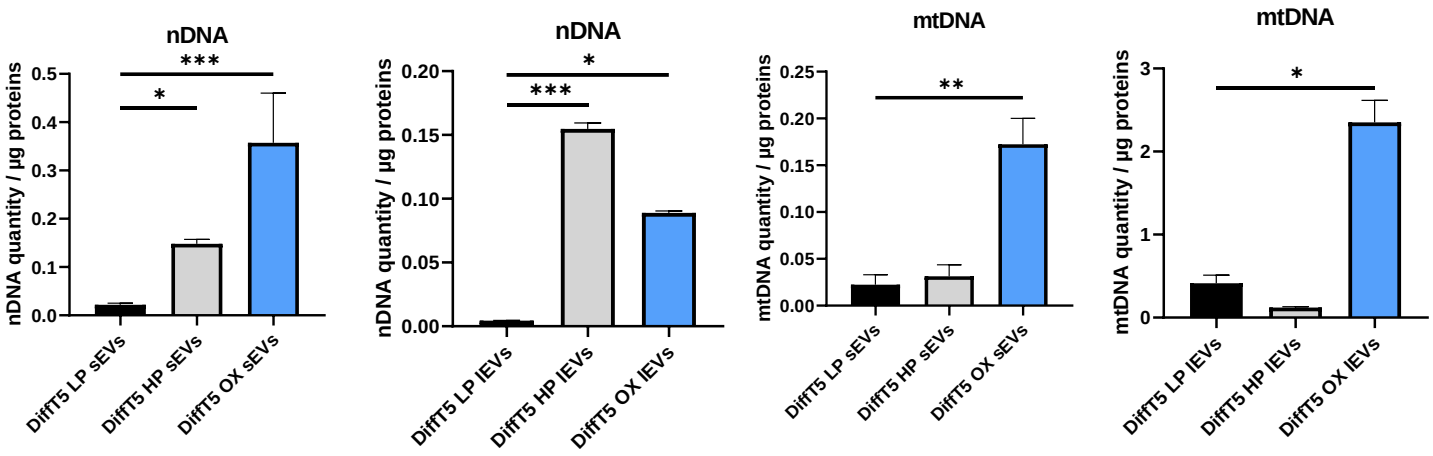


Figure 25. Amount of relative nDNA and mtDNA quantity within EVs. Data were obtained quantifying the expression of 36B4 and COX1, respectively. Gene expression was related to total protein amount. EVs were obtained from LP, HP and OX myocytes after 5 days since the differentiation was induced; the results are represented as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.4.2. The Role of EV-associated DNA in the induction of the inflammation

Study of the signalling pathways activated by EV-DNA in RAW264.7 model

The role of DNA in the EV-mediated impairment of the myogenic differentiation process was further explored. Indeed, the previously reported results indicated that EVs secreted by HP and OX myocytes carried high levels of DNA if compared with EVs from control cells. In addition, such EVs induced an increase of β-interferon expression in RAW264.7 cells further suggesting a possible involvement of DNA in the induction of the inflammatory process. In order to explore this hypothesis RAW264.7 cells were incubated with both LP- and HP-derived EVs. In this experimental setup, some samples were also pre-exposed to chloroquine for 4h at 37 °C, an inhibitor of endosomal TLRs. After the reported incubations, cell bodies were collected and the gene expression was analysed focusing on the expression of genes involved in the inflammatory process, in particular the IFN-1β and IL-1β. The obtained results showed that HP EVs triggered inflammation inducing the increase of both IFN-1β and IL-1β, as noted before. Interestingly, pre-incubation with chloroquine resulted in a decrease of both IFN-1β and IL-1β expression (Fig. 26). Notably, EVs secreted by LP cells did not induce a significant inflammatory response. However, also basal IFN-1β expression appeared down-regulated in the chloroquine treated cells. These data suggested that both HP and LP EVs seem to stimulate the inflammatory process in a similar way. For this reason, the following experiments aimed to shed light on the molecular mechanisms by which EV-associated DNA could trigger IFN-1β and IL-1β gene expression.

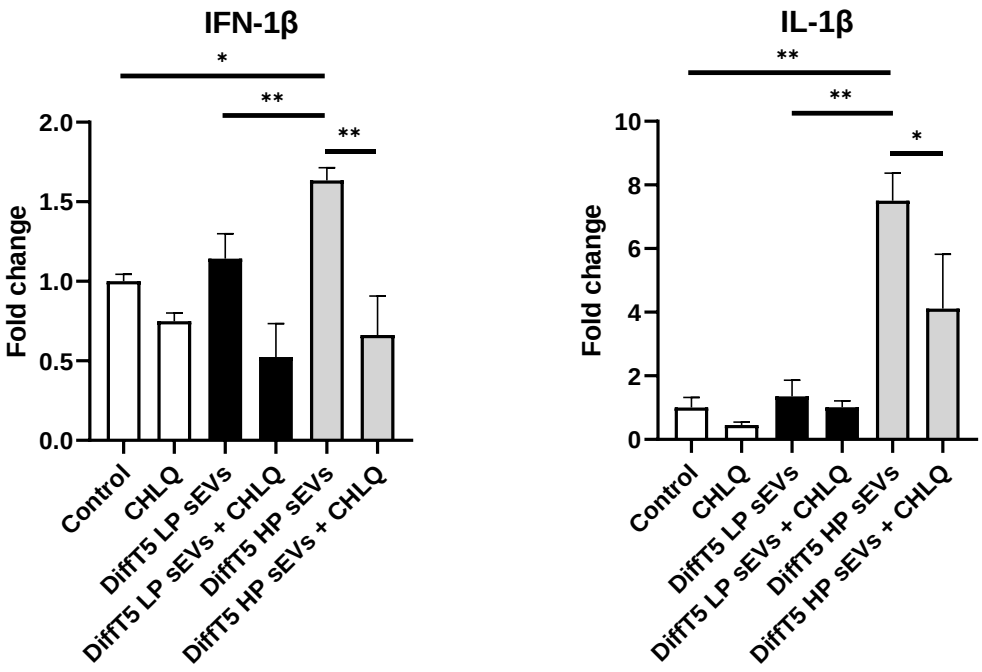


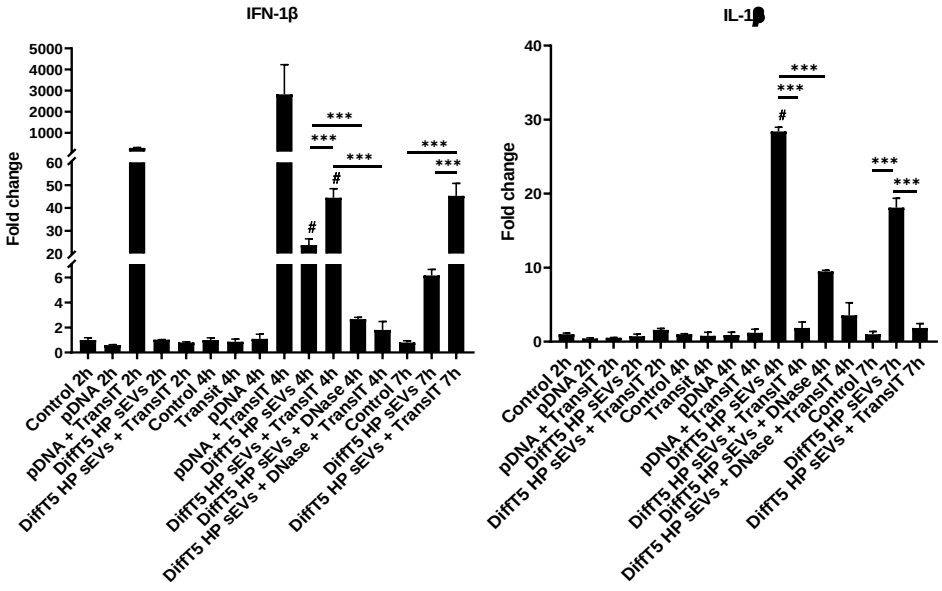
Figure 26. The activity on the RAW264.7 cell line, of EVs released by HP myocytes obtained after 5 days since the differentiation process was induced, was analysed. EVs activity was evaluated in presence/absence of chloroquine. The effect was analysed evaluating gene expression. The results are represented as mean ± SD; * $p < 0.05$; ** $p < 0.01$.

Our data led us to hypothesise that endosomal TLRs activation could play a role in mediating the cytokine up-regulation associated with EV-DNA administration. Therefore, we then tested whether EV-associated DNA triggers also cytosolic DNA sensors. To this end, RAW264.7 cells were incubated with HP EVs and plasmid DNA (pDNA) complexed with TransIT-X2 as positive control. The chemical agent TransIT-X2 promotes cellular membrane crossing thus facilitating pDNA entrance into the cytosol. Moreover, in an attempt to force the fusion of EVs with the plasma membrane we also tried to couple EVs with TransIT-X2 before performing the EV treatment. Lastly, pre-treatment of EVs with DNase1 prior to the administration to target cells, was used as a further control condition. Target cells were incubated with EVs for 2h, 4h and 7h at 37 °C and, subsequently, gene expression analysis was performed on genes associated to DNA-induced inflammation, i.e. IFN-1β and IL-1β. The obtained results showed that the TransIT-X2:pDNA complexes and EVs induced a significant IFN-1β increase at all incubation times and after 4h and 7h of incubation, respectively. (Fig. 27a). Notably, such an increase resulted significantly lower when cells were treated with EVs or TransIT-X2:EVs complexes pre-incubated with DNase1 suggesting that DNA adsorbed on the EV surface could play a role in this process. On the contrary, IL-1β mRNAs showed a different expression pattern in presence of pDNA or EVs. Indeed, neither TransIT-X2:pDNA nor TransIT-X2:EV complexes exerted

any effect on IL-1β mRNA levels, while EVs alone stimulate this pro-inflammatory cytokine. Again, IL-1β up-regulation resulted lower in the DNase1 pre-treatment condition (Fig. 27a). Gene expression analysis was associated to western blot analysis, the latter allowed us to study the activation of STING protein, and key factors related to inflammation. Our data showed that STING was phosphorylated when cells were incubated with TransIT-X2:pDNA complexes (Fig. 27b), suggesting that the IFN-1β expression increase was actually due to cGAS-STING pathway activation. On the other hand, the IFN-1β up-regulation, observed when cells were treated with EVs, was not associated with STING activation (Fig. 27b), further supporting the hypothesis that the EV-mediated effect could be related to the endosomal TLRs pathway.

Regarding the phosphorylation of NF-KB results were unclear, displaying an increase in the cells treated with EVs pre-incubated with DNase1 and only a slight trend to increase in the EV-treated condition (Fig. 27b).

(a)



(b)

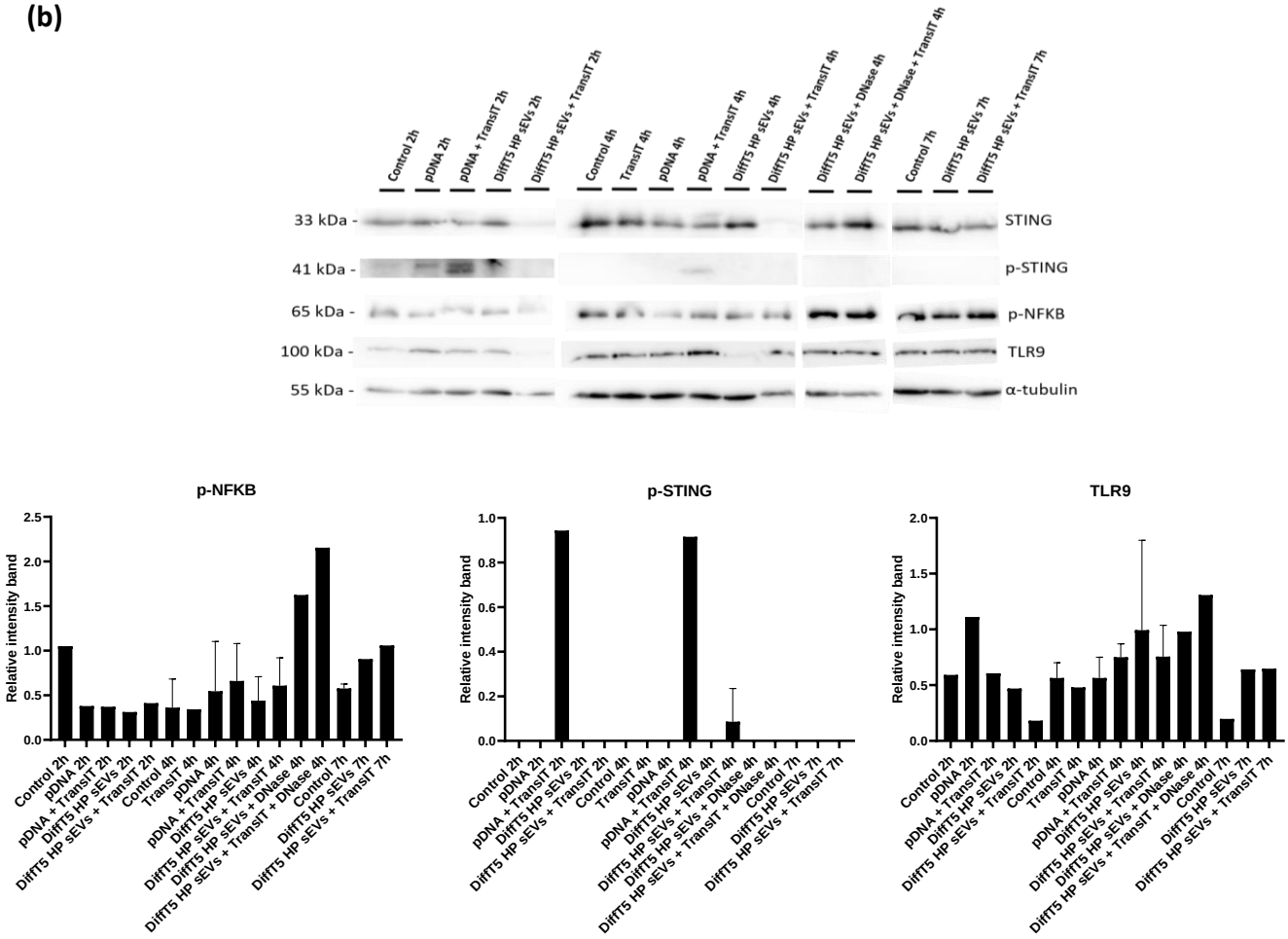


Figure 27. The activity on the RAW264.7 cell line of EVs and DNase1 pre-treated EVs, TransIT-X2:pDNA, TransIT-X2:EVs and DNase1 pre-treated TransIT-X2:EVs complexes, was analysed. The effect was evaluated after 2h, 4h and 7h incubation. The effect was analysed evaluating gene expression through RT-PCR (a) and Western Blot analysis (b). The results are represented as mean ± SD; *** $p < 0.001$; # $p < 0.001$ compared to control 4h. Concerning IFN-1β expression analysis, the condition representing cells' incubation with TransIT-X2:pDNA complexes was excluded from statistical analysis since it resulted clearly higher.

Subsequently, in order to investigate whether EVs can facilitate the entrance of extracellular DNA into a target cell leading to STING activation, EVs were pre-incubated with amounts of pDNA previously demonstrated to activate STING when complexed with the transfection agent TransIT X2. This experiment was designed to evaluate whether DNA adsorbed onto the EV surface can stimulate a pro-inflammatory response.

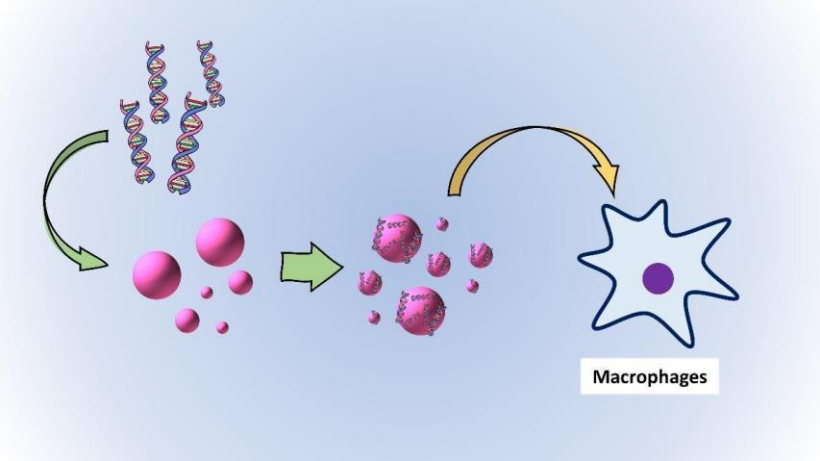


Figure 28. Scheme representing the treatment of RAW264.7 cells with EVs previously incubated with exogenous pDNA.

EVs:pDNA complexes were tested both alone and following the addition of TransIT-X2 or pre-treatment with DNase1. In this experimental condition, cells were incubated for 4h, as previously reported data highlighted that target cells adapted to EV stimulation within a few hours. The obtained data showed that EVs:pDNA complexes induced a slight increase in IFN-1β expression whereas they did not affect IL-1β expression (Fig. 29a). In addition, EVs:pDNA complexes did not stimulate IFN-1β expression after pre-treatment with DNase1, and finally TransIT-X2:EVs:pDNA complexes induced the same increase in IFN-1β expression levels as that induced by EVs:pDNA complexes alone. On the contrary, IL-1β expression did not result to be modulated (Fig. 29a). Concerning the western blot analysis, STING was not phosphorylated following the incubation with the all tested EVs:pDNA complexes (Fig. 29b). Altogether, the obtained results suggest that EVs could have effectively interacted with the exogenous pDNA given that free-pDNA alone did not exert the same effect as when complexed with TransIT-X2 in absence of EVs. In addition, these data could imply that the different levels of IFN-1β expression and STING activation, induced by EVs alone or TransIT-X2:pDNA complexes, may not depend on the DNA amount carried by EVs. In other words EVs may induce lower levels of IFN-1β, and not trigger STING activation, not because of a lower quantity of DNA cargo. Therefore this interpretation could support the hypothesis that EVs and pDNA alone stimulate a different action mechanism.

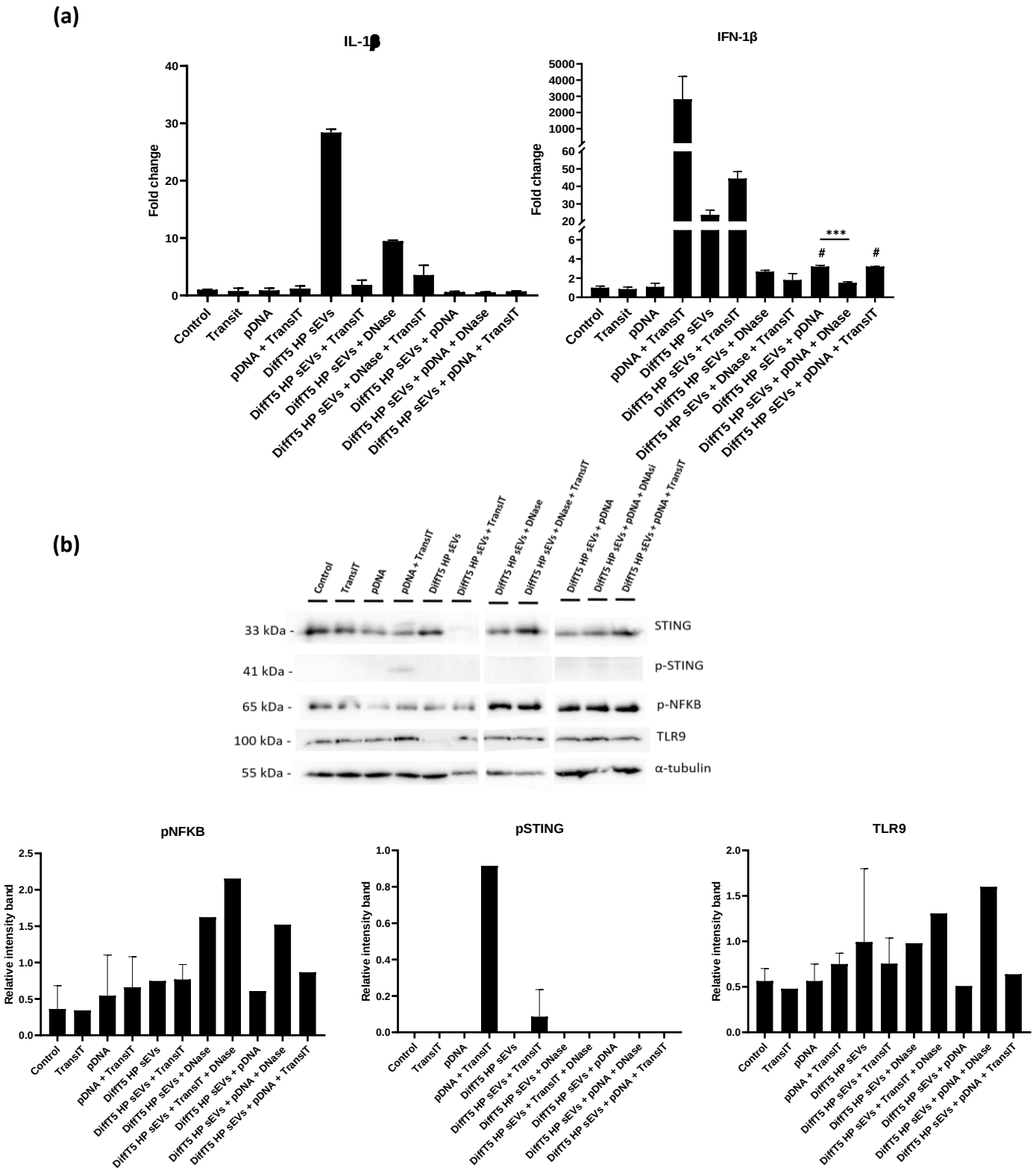


Figure 29. The activity on RAW264.7 cell line of EVs and DNase1 pre-treated EVs, TransIT-X2:pDNA, TransIT-X2:EVs, EVs:pDNA, DNase pre-treated EVs:pDNA and TransIT-X2:EVs:pDNA complexes was analysed. The effect was tested after 4h incubation. The effect was analysed evaluating gene expression through RT-PCR (a) and Western Blot analysis (b). The results are represented as mean ± SD; *** $p < 0.001$; # $p < 0.001$ compared to control. Concerning IFN-1β expression analysis, the condition representing cells' incubation with TransIT-X2:pDNA complexes was excluded from statistical analysis since it resulted clearly higher.

Study of the signalling pathways activated by EV-DNA in C2C12 myocytes

The hypothesized DNA-mediated effect of HP-EVs was further investigated using C2C12 as target cells. To this end differentiating LP C2C12 were incubated with exogenous pDNA, HP-EVs and their combinations with and without DNase1 pre-treatment. Moreover, TransIT-X2:pDNA complexes were used as positive controls. After the incubations, target cells were collected and gene expression of pro-inflammatory cytokines were evaluated. Subsequently, western blot analysis was performed to evaluate STING activation. Gene expression analysis revealed that C2C12 showed an increasing trend of IL-6 expression in all the conditions tested (Fig.26a). On the contrary, IFN-1β expression was induced only when cells were incubated with TransIT-X2:pDNA complexes and at lower extent with TransIT-X2:EVs complexes. In the other conditions, including controls, IFN-1β expression did not occur (Fig. 30a). Furthermore, western blot analysis indicated that, similarly to RAW264.7 treatment, STING resulted to be activated only when cells were stimulated with TransIT-X2:pDNA complexes (Fig. 30b). Interestingly, higher levels of the NFKB phosphorylation were observed when cells were incubated with EVs alone, in agreement with the increase of IL-6 expression, and in presence of TransIT-X2:pDNA complexes (Fig. 30b). Altogether, these data suggest that although C2C12 cells are less responsive to stimuli inducing IFN-1β, HP-EV treatment seems to stimulate NF-KB and consequently IL-6 expression without involving cGAS-STING pathway. Furthermore, EV-induced IL-6 increase and NF-KB phosphorylation were not associated with IFN-1β up-regulation with the exception of the C2C12 cells treated with TransIT-X2:EVs complexes for 7h. However, as previously found, such IFN-1β induction was not coupled with STING activation.

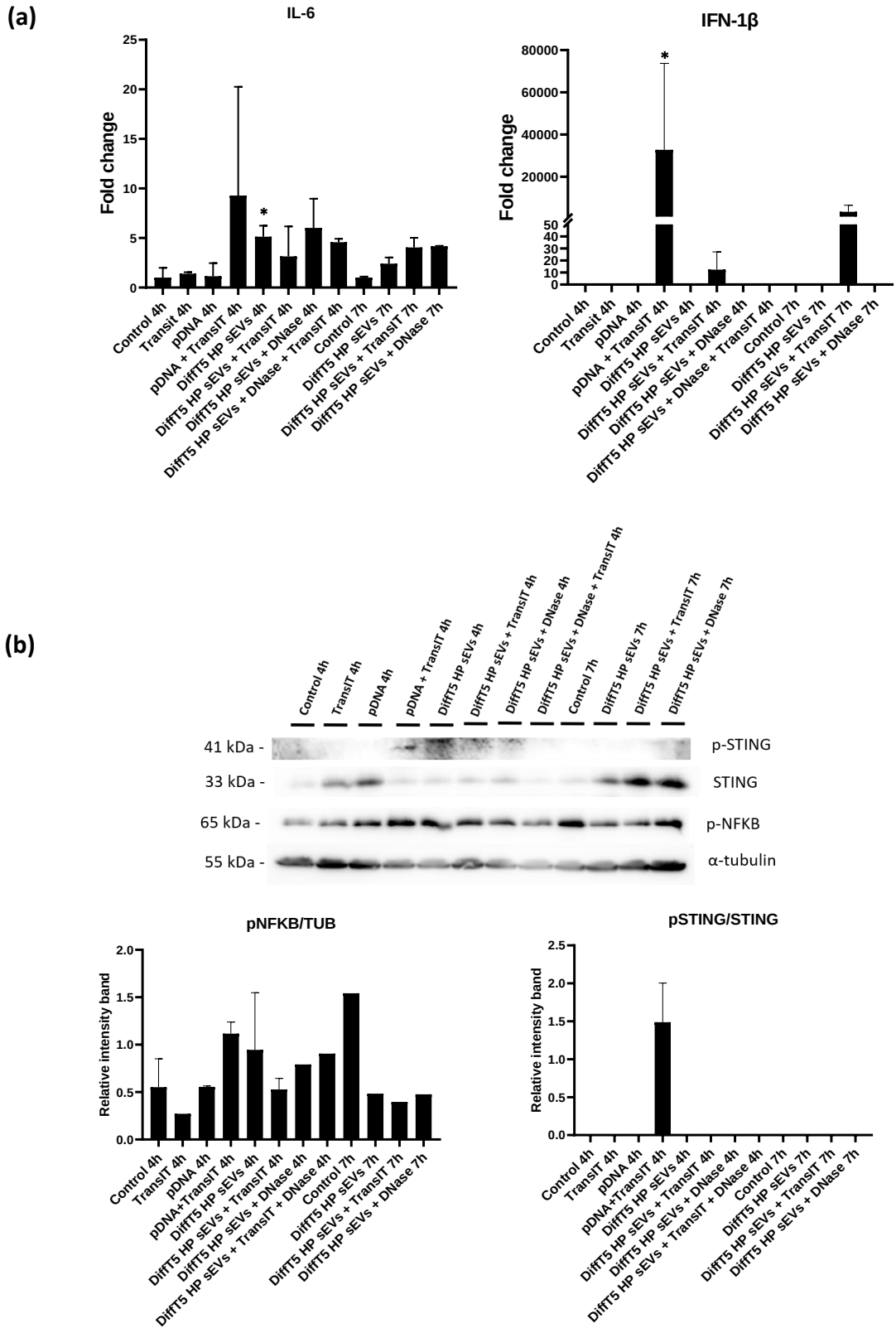


Figure 30. The activity on the C2C12 cell line of EVs and DNase1 pre-treated EVs, TransIT-X2:pDNA, TransIT-X2:EVs was analysed. The effect was evaluated after 4h and 7h incubation. The effect was analysed evaluating gene expression through RT-PCR (a) and Western Blot analysis (b). The results are represented as mean ± SD; * $p < 0.05$ compared to control.

Subsequently, the effect of the EVs:pDNA complexes was tested on myocytes to find out whether non-phagocytic cells show a different behaviour with respect to phagocytic cells such as RAW264.7. Therefore differentiating C2C12 were incubated with EVs, TransIT-X2:pDNA, TransIT-X2:EVs, EVs:pDNA, DNase1 pre-treated EVs:pDNA and TransIT-X2:EVs:pDNA complexes.

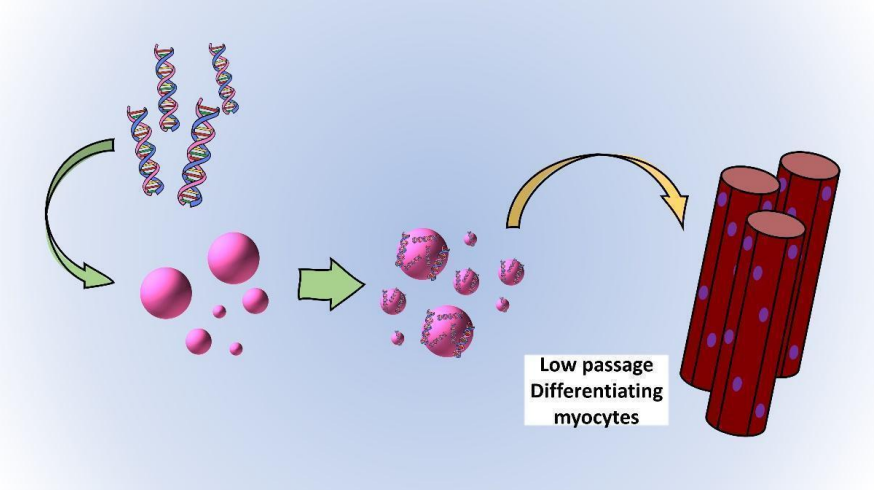


Figure 31. Scheme representing the treatment of LP C2C12 cells with EVs previously incubated with exogenous pDNA.

Target cells were incubated for 4h and then gene expression and western blot analyses were performed to assess the outcomes. Interestingly, EVs-pDNA complexes induced a significant inflammation process, as indicated by the higher levels of IL-6 (Fig. 32a). Such an effect was significantly reduced when EVs:pDNA complexes were pre-treated with DNase1, suggesting that EV surface DNA could have a role in triggering inflammation. Surprisingly, this effect was inhibited by the addiction of TransIT-X2. In agreement with previous data, myocytes did not display IFN-1β expression induction except C2C12 treated with TransIT-X2:pDNA complexes (Fig. 32a). Accordingly, STING was phosphorylated only in this condition (Fig. 32b). By contrast, NFKB was barely activated, with similar phosphorylation levels, only in EVs:pDNA treatment conditions (Fig. 32b). Therefore, these results suggested that EV-associated DNA could play a role also in the C2C12 model even though it was not possible to completely confirm it with IFN-1β expression given that C2C12 appeared less responsive to IFN-1β production. Nevertheless, TransIT-X2:pDNA complexes were able to induce it whereas the same amount, complexed with EVs, have not pointed out a similar result. This data may further support the hypothesis that DNA related to EVs triggers a mechanism different from the cGAS-STING pathway.

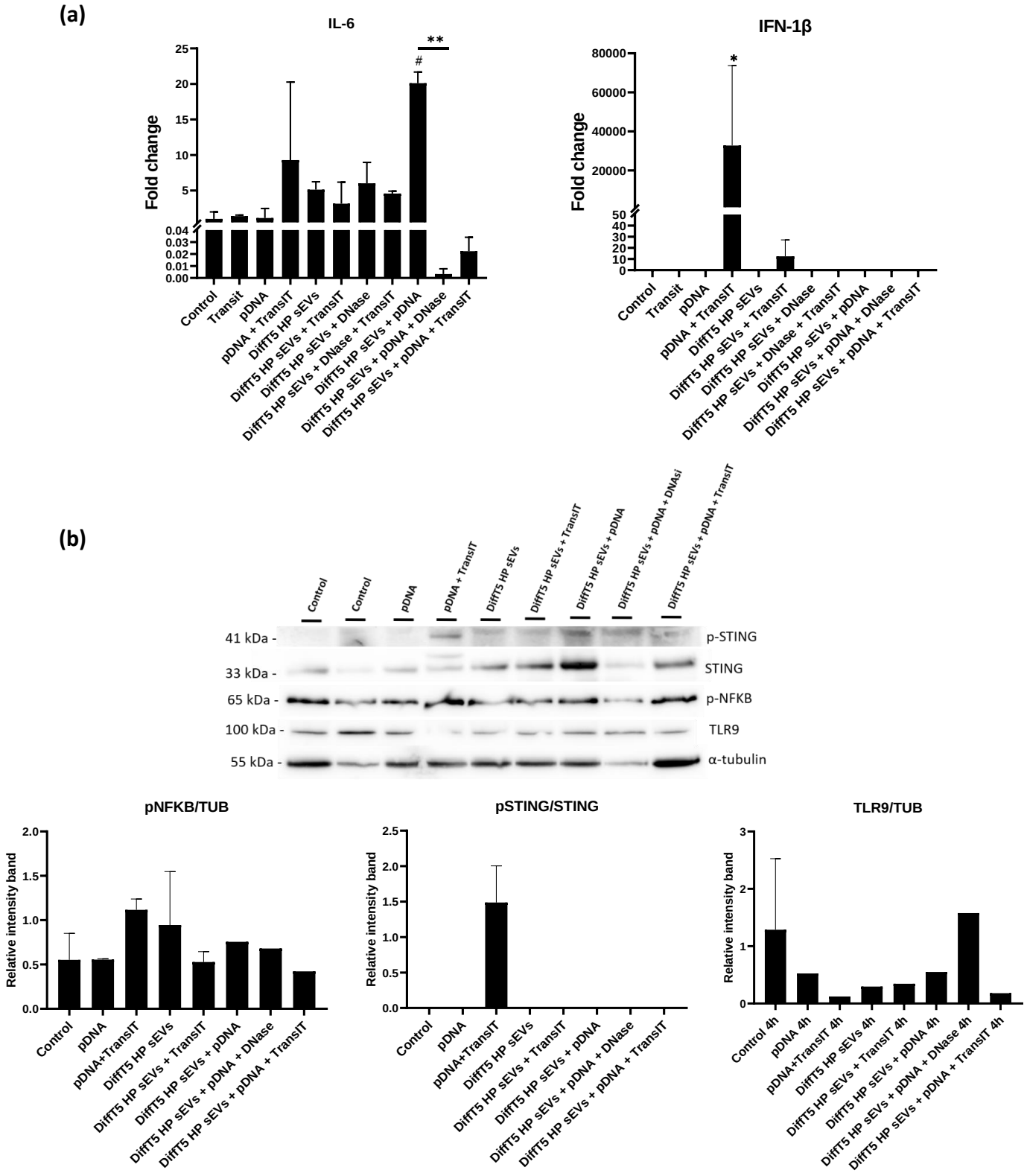


Figure 32. The activity on C2C12 cell line of EVs, TransIT-X2:pDNA, TransIT-X2:EVs, EVs:pDNA, DNase1 pre-treated EVs:pDNA and TransIT-X2:EVs:pDNA complexes were analysed. The effect was evaluated after 4h incubation. The effect was analysed evaluating gene expression through RT-PCR (a) and Western Blot analysis (b). The results are represented as mean ± SD; ** $p < 0.01$; # $p < 0.01$ compared to control.

5. DISCUSSION

5.1. Stressed C2C12 myoblasts display senescence-like traits

Sarcopenia is a geriatric syndrome involving progressive and generalized loss of skeletal muscle mass and strength [65]. Among its features, sarcopenia includes a reduced oxidative capacity and inflammation. Of note, all the alterations associated with muscle aging lead to a histological and morphological impairment of muscle tissue including myofiber atrophy and increased heterogeneity in fiber size [67,264]. These defects in muscle tissue have been reconducted also to a progressive derangement of SCs pool. Indeed, in aging altered intrinsic and extrinsic factors can impair SCs survival and self-renewal leading to apoptosis or senescence [148,158]. Such impairment is also associated with a defective differentiation program and hence to an altered expression profile involving in particular myogenic differentiation-specific genes [150].

Studies on different kinds of cell models were conducted in order to explore the aging process *in vitro*. Among them models of replicative senescence or oxidative stress-induced senescence were extensively studied. The former is a model consisting in cells cultured for many passages, whereas the second is a model in which senescence is induced through an oxidative insult, it has been reported that both lead to telomere attrition [39,265]. On this basis, the aim of the present work was to study the release of EVs in muscle models of replicative senescence and senescence induced by oxidative stress. To this end, C2C12 cells, which are widely used to study the myogenic differentiation process, were cultured for multiple passages or subjected to an oxidative insult. The obtained results suggested that although such cells did not display a clear increase in SA-β-galactosidase activity, a well-known marker of senescence [266], stressed C2C12 showed some of the aging associated features previously mentioned. Indeed, HP myocytes displayed an impairment of the myogenic differentiation process as shown by a reduction in the myogenic fusion index. Additionally, the few formed myotubes were thinner and more disorganized compared with controls (Fig. 2). Moreover, OX myocytes became larger and longer especially during the first days following H_2O_2 treatment (Fig. 6b), this feature was reported to be associated with a senescent phenotype [266]. The observed morphological alterations were supported by the impaired expression of differentiation target genes (Fig. 4, 5, 8). In addition to the myogenic differentiation

delay, HP myocytes showed a bias to cycling also in serum-starved conditions. Indeed, HP myocytes showed high expression levels of POLA1 and PCNA even after differentiation induction (Fig. 4, 5). Moreover, differentiating HP cells, which to a large extent remained myoblasts, also showed a reduced activity of Notch1 along with an increased activity of p38 (Fig. 5). Reduced Notch1 activity could be the result of a defective myogenic program because of the less presence of myotubes. During normal myogenic differentiation, myofibers express Delta1 which in turn activates notch signalling through cell-to-cell contact. On the contrary, it could suggest an impairment of the self-renewal process since a constitutive activation of notch signalling is required for muscle regeneration inducing the proliferation of undifferentiated myoblasts and inhibition of differentiation [138,267]. Moreover, p38 upregulation could suggest an age-associated impairment of the self-renewal process. Indeed, it could indicate an upregulation of p38-MAPK signalling which was related to a persistent SCs activation and to a loss of quiescent SCs pool [268].

Furthermore, growth analysis showed that HP myoblasts displayed a lower growth rate and a higher death rate suggesting that HP cell survival could be altered. Accordingly, in aging the whole of the muscle niche alterations was reported to impact on SCs survival [148,158].

Notably, both HP and OX myocytes resulted significantly inflamed as indicated by the expression level of IL-6 (Fig. 4, 8), which is a pro-inflammatory cytokine largely related to age-associated chronic inflammation [164,269]. Interestingly, some evidence linked inflammation to differentiation and self-renewal process impairment. Indeed, a chronic inflamed state was reported to inhibit differentiation in a C2C12 model through an epigenetically mediated mechanism [270]. Likewise, inflammation was reported to impair self-renewal process altering notch signalling [271].

The next step was the isolation and characterisation of EVs secreted by senescence-like cell models. Therefore, EVs were first purified from the medium of the HP and OX myocytes, which was collected at various times during the differentiation process, through serial ultracentrifugations. Subsequently, the isolated EVs were assayed performing NTA and then characterised to assess particles' identity. The characterisation was conducted evaluating density and surface markers. The obtained results suggested that the purified particles showed to a large extent a density ranging from 1,06 and 1,12 (Fig. 12), which is the typical EV density [169,272]. In addition, EVs were positive to at least two of the well-known EVs surface markers tested. In particular, lEVs were positive to α-actinin and Tsg101, while sEVs to Alix and Tsg101. HP sEVs were also positive to CD81 (Fig. 13) [167,169,272]. Notably, HP myocytes were found to release a higher quantity of EVs which to a large

extent were sEVs (Fig. 11). This data could further support the occurrence in HP cells of senescent-like characteristics since senescent cells were reported to secrete more EVs [230,231].

5.2. EVs released by stressed C2C12 cells induce inflammation in C2C12 muscle cells and RAW264.7 macrophages

Because of the observed impairment of the studied cell models and the associated EVs, it was likely that such alterations could have also affected the signals carried within EVs. During aging both EV secretion and content were reported to change causing EVs mediating several negative effects [227–229]. Notably, very recently EVs were related to the so-called inflamm-aging given that they were associated with both inflammation and aging [239,240]. In line with this view, EVs were found to carry pro-inflammatory signals in aging [241]. Likewise, SASP was reported to exert pro-inflammatory effects through NF-kB activation, which at the same time, was found to be an important regulator of EV cargo [243,244]. SASP was also shown to modulate immune cells' activity and it was suggested to be able to spread senescence to bystander cells [245,246]. Therefore, since EVs induce SASP-like effects, it has been suggested that EVs released from stressed cells could prompt aging. On this basis, the activity of the EVs obtained from different cellular models of myocyte senescence was tested in order to explore whether EVs can be involved in the exchange of some age-associated traits. Therefore, the isolated EVs were used to treat LP C2C12 and RAW264.7, which are representative for non-senescent myocytes and macrophages, respectively [72].

At first, EVs were used to treat LP C2C12. The incubation was conducted for 24h and 4 days, but the most significant results were obtained performing the incubation for 24h. The obtained data indicated that the most relevant effect was the pro-inflammatory one and it was exerted by both HP and OX myocytes' EVs (Fig. 16, 20). Also, OX cells' EVs induced an impairment of the differentiation process (Fig. 20, 21). In addition, the most significant effect mediated by OX cells' EVs was found to be related to lEVs, whereas, concerning HP cells' EVs, sEVs exerted the greatest effect. This interaction experiment was performed also treating HP cells with EVs released by LP C2C12 in order to see whether they induced an amelioration. Indeed, heterochronic parabiosis studies reported that old SCs, when exposed to a young myogenic environment or to young systemic factors, showed an amelioration [155,156]. Therefore, HP cells were incubated for 24h and 4 days with a medium containing EVs released by LP myocytes. The obtained results showed a slight

increase of MEF2C target gene, which occurred also in the 4 days condition, thus suggesting a slight improvement of the differentiation process (Fig. 19).

Afterwards, C2C12 EV activity was tested on RAW264.7, in agreement with what was observed on LP C2C12 treatment, the obtained results indicated that EVs released by HP cells triggered a significant inflamed status as shown by the gene expression increase of all the pro-inflammatory cytokines evaluated. Interestingly, the treated cells also displayed a significant increase of the β-interferon expression (Fig. 23). This result suggested that the inflammation process could be induced by EV-carried signals stimulating intracellular sensors associated with inflammation such as endosomal TLRs, cGAS-cGAMP and MAVS-RIG-I [273].

5.3. Study of the signals associated to EVs released by stressed C2C12 models

Finally, the molecular signals loaded into EVs released by HP and OX myocytes were analysed focusing on the nucleic acids cargo. In particular, the analyses were pointed out on EV-DNA because our data highlighted a clear increase in DNA payload (nuclear and mitochondrial DNA) of the EVs released by stressed cells [247,248]. In addition, senescent cells are found to remove high amounts of DNA by means of EVs which could be to a large extent DNA due to genomic damage [231,249].

Accordingly, HP and OX myocytes' EVs resulted to contain a higher amount of total DNA (Fig. 24). Moreover, the isolated EV-DNA was further analysed quantifying the relative amount of nDNA and mtDNA. HP and OX cells' EVs contained a higher quantity of nDNA, while OX myocytes' EVs also contained a higher amount of mtDNA (Fig. 25). In agreement with this evidence, it has been reported that senescent cells release high amounts of damaged genomic DNA; in fact, they were shown to accumulate γH2AX-cytoplasmic chromatin fragments, which are likely to be removed through EVs [231,249]. On the other hand, the higher amount of mtDNA could be a consequence of a mitochondrial impairment caused by the oxidative damage due to H_2O_2 treatment.. Subsequently, it is conceivable that damaged mtDNA could be secreted through EVs, similarly to nDNA. Indeed, it was reported that mtDNA can be loaded into EVs for example in pathological conditions [220]. These data, along with the previously observed increase of β-interferon induced by HP and OX cells' EVs, further support the involvement of DNA in the EV-mediated impairment of the tested recipient cells, therefore the next step was to investigate how DNA could trigger the inflammation process. As

mentioned above, mounting evidence shows that DNA can induce inflammation differently depending on the intracellular compartment wherein it localizes. In the cytosol, DNA can bind to receptors such as the IFNγ-inducible protein 16 (IFI16, also called p204), cyclic GMP-AMP synthase (cGAS), DEAD-box heli-case 41 (DDX41), Z-DNA-binding protein 1 (ZBP1, also called DAI). These receptors in turn engage the stimulator of IFN genes (STING, also known as TMEM173, MITA, MPYS and ERIS), which is able to activate interferon regulatory factor (IRFs) through the tank binding kinase (TBK), and hence it leads to β-interferon induction. TBK can also trigger the NF-KB signalling pathway that in turn induces the expression of pro-inflammatory cytokines like IL-6 or IL-1β [274–277]. Alternatively, when exogenous DNA localizes in the endosomal compartment it can interact with TLRs, in particular the TLR9 [278]. TLR9 activates a signalling cascade, through MyD88 factor, which in the end leads to IRF phosphorylation, and translocation, and hence to β-interferon induction. Likewise, TLR9, through TBK, can also activate NF-KB signalling [279]. On this basis, in order to identify the signalling pathways triggered by EV-associated DNA, chloroquine pre-treated RAW264.7 cells were incubated with HP and LP myocytes derived EVs. As a matter of fact, chloroquine is an endosomal TLRs inhibitor [280]. In our hands, chloroquine pre-treatment led to a reduction in both IFN-1β and IL-1β expression levels when incubated with HP cells' EVs, suggesting an involvement of endosomal TLRs in the transduction process. Likewise, chloroquine pre-treated cells incubated with LP myocytes' EVs, displayed a similar pro-inflammatory cytokines' induction trend even though HP cells derived EVs exerted the greatest effect (Fig. 26), further suggesting that LP and HP cells' EVs induced the same mechanism. For this reason, only HP myocytes' EV mediated effects were evaluated in the following analyses.

Next, STING signalling pathway was examined. To this end, cells were treated with EVs alone or complexed with a transfectant agent, the TransIT-X2 delivery system. This agent is commonly used in cell transfection with exogenous plasmid DNA (pDNA); indeed, it allows the delivery of exogenous DNA molecules into cytosol. By entering the cytosol, pDNA can also be recognized by DNA cytosolic sensors and in turn activating the inflammation process by triggering STING signalling [276,281]. These experimental conditions were applied also to EVs in order to assess whether, facilitating EV entrance into cytosol, EV-DNA could activate STING. Cells were incubated with both EVs alone and TransIT-X2 complexes, the obtained results were compared with TransIT-X2:pDNA complexes. The obtained outcome suggested that STING was phosphorylated only when cells were treated with TransIT-X2:pDNA complexes, moreover STING activation led to a significant increase in IFN-1β

expression (Fig. 27). Of note in agreement with previous data, EVs induced an IFN-1β increase anyway, supporting the stimulation of a STING-independent mechanism. However, the conjugation of TransIT-X2 to EVs resulted in a higher increase of IFN-1β. A possible explanation for the observed results is that RAW264.7 cells recognize EVs as a "pathogen" and engulf them. Subsequently, EVs are translocated to endosomal compartments where EV-associated DNA engages TLR signalling pathway in turn inducing IFN-1β expression [282].

Moreover, DNase1 treatment was used to investigate whether EV-DNA is adsorbed onto the surface of EVs . Interestingly, IFN-1β expression resulted significantly lower by DNase1 suggesting that surface DNA could play an important role in the EV-mediated effect, however IFN-1β expression increase was not completely abolished therefore suggesting that also DNA within EVs was involved (Fig. 27a). In contrast, IL-1β expression displayed a different pattern compared to IFN-1β, namely it increased only when cells were treated with EVs alone whereas the addiction of TransIT-X2 appeared to inhibit such effect. The same outcome was observed treating cells with TransIT-X2:pDNA complexes.

Subsequently, given the result of incubating cells with DNase1 pre-treated EVs, surface DNA role was further explored. To this end, EVs were incubated with the same amount as that used for the TransIT-X2:pDNA condition. This experiment was conducted to evaluate whether EVs could collect exogenous DNA and therefore if EVs could be involved in the presentation of circulating cell-free DNA. This issue could be noteworthy since during aging nucleic acids, such as DNA in the form of fragmented DNA, have been shown to circulate in the blood and, after being uptaken, it can negatively affect recipient cells by localizing into the nucleus triggering DDR which in turn may also lead to genomic mutations [283]. In addition, circulating DNA has been proposed as a marker of aging given that it can display methylation patterns typically associated with aging and aging-associated diseases like cancer [284]. Alternatively, also circulating oxidized DNA has been proposed as a marker of the chronic stress to which cells are subjected in aging [285].

Moreover, we asked whether EVs failed in inducing STING phosphorylation because of the lower amount of DNA cargo, compared to that used in the TransIT-X2:pDNA condition. Interestingly, the obtained results indicated that pDNA incubated with EVs induced an effect similar to that found for EVs alone, even in presence of TransIT-X2, as indicated by IFN-1β and IL-1β expression levels (Fig. 29a). In addition, in these conditions pDNA did not induce STING phosphorylation (Fig. 29b). In

summary, the obtained results suggest that pDNA may stick onto EVs and travel into the target cell towards endosomal compartments.

Since it has been reported that immunostimulatory DNA in presence of transfectant agents is able to induce IFN-1β expression also in C2C12 despite they are not immune cells [286], STING signalling pathway was analysed also in this cell model. C2C12 cells were subjected to the same experimental conditions, as RAW264.7 cells, in order to find out whether EVs could lead to a similar mechanism in C2C12 cells. As a result, IFN-1β expression in C2C12 cells was clearly detected only when stimulated with TransIT-X2:pDNA complexes. In addition, TransIT-X2:pDNA complexes also induced IL-6 expression increase. Nonetheless, in this case EVs only partially induced IFN-1β expression, which was detected only incubating cells with TransIT-X2:EVs complexes even though they still induced an increase of IL-6 expression (Fig. 30a). Likewise, STING phosphorylation occurred only following TransIT-X2:pDNA complexes incubation (Fig. 30b).

Concerning the effect mediated by EVs:pDNA complexes, they worked in a similar fashion. Indeed, pDNA neither induced IFN-1β expression nor STING phosphorylation in the presence of EVs (Fig. 32). Altogether, these results suggested that C2C12 are less likely to express IFN-1β. Indeed, the only reproducible condition, wherein IFN-1β expression was detected, has been incubating cells with TransIT-X2:pDNA complexes, concurrently with STING activation. On the other hand, EVs did not always induce it. An explanation could be that in C2C12 cells only STING pathway can induce IFN-1β expression efficiently. Therefore, it is still likely that EV uptake occurs through endocytosis and EVs are translocated to the endosomal compartments where EV-DNA could trigger TLR signalling.

6. CONCLUSIONS

The studied EVs released by senescent-like myocytes resulted in exerting a pro-inflammatory activity, indeed accumulating evidence associated EVs to inflamm-aging given that in aging, they have been reported to become more pro-inflammatory, and their content and composition have been reported to change [227–229,239,240].

Among the signals carried by EVs, DNA appeared to be the most likely to be implied in triggering inflammation according to the EV-mediated effects exerted on recipient cells and the higher quantity of DNA detected in senescent-like myocytes' EVs. Indeed, as previously reported, EVs released by senescent cells are found to contain a higher amount of DNA which could be, to a large extent, DNA deriving from genomic damage [231,249]. Furthermore, the obtained results suggested that, both in RAW264.7 and C2C12 cell models, EVs were uptaken by endocytosis and EV-associated DNA may trigger TLR signalling despite, this was not clearly supported by IFN-1β expression in C2C12 cells. Of note, our data also suggested that EVs can work as carriers of circulating DNA. Indeed, DNA, when incubated with EVs, appears to bind onto the EV surface exerting new pro-inflammatory effects. As a matter of fact following EVs:pDNA complexes incubation, cytokine induction was similar to that of EVs incubation alone. These findings suggest a potential involvement for EVs as carriers of circulating cell-free DNA associated with aging.

Concluding, in aging it is conceivable that DNA fragments, due to genomic damage, can induce inflammation through STING signalling, when it localizes into the cytosol, or through TLR9, when it localizes into the endosomes. In addition, the high presence of DNA fragments leads aged myocytes to discard it through EVs which become more pro-inflammatory possibly because of the higher DNA content. Moreover, IL-6 may also recall macrophages in situ. Here, because of the activity of both IL-6 and aged myocytes' EVs, they can be induced to secrete pro-inflammatory cytokines, such as IL-1β, further promoting the inflammation process. In addition, the DNA associated with EVs may induce macrophages to secrete type I interferons, such as IFN-1β. The latter may in turn promote the expression, in recipient cells, of interferon-stimulated genes (ISGs) such as TLRs [287]. Consequently, interferon-stimulated cells, like myocytes, expressing higher levels of TLRs, for example TLR9, could become more sensitive to exogenous DNA and hence they could be more prone to inflammation. This process could be responsible for the loss of muscle stem cells during aging.

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