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**Soluble factors released from activated T-lymphocytes regulate C2C12 myoblast proliferation and cellular signalling, but effects are blunted in the elderly**

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**Keywords:** Myoblasts, proliferation, differentiation, secretome, immune system, lymphocytes

**Running Head:** Young lymphocyte secretomes enhance myoblast regeneration

**Abstract**

The key objective of this work was to investigate the impact of young and old human lymphocyte secretomes on C2C12 myoblasts regeneration. Conditioned media (CMs) were harvested from isolated young and older lymphocytes treated with (activated AC), or without (non-activated NA), anti-CD3/CD28 activators for 4 days. AC conditioned media from older lymphocytes had decreased levels of amphiregulin ( $367 \pm 208$  pg/ml vs.  $904 \pm 323$  pg/ml;  $p=0.018$ ) and IGF-I ( $845 \pm 88$  ng/ml vs.  $1100 \pm 48$  ng/ml;  $p=0.032$ ) compared with younger AC. AC older vs younger lymphocytes had reduced expression of CD25 ( $24.6 \pm 5.5\%$ ;  $p=0.0003$ ) and increased expression of FoxP3 ( $35 \pm 15.7\%$ ;  $p=0.032$ ). Treatment of C2C12 myoblasts with young AC resulted in decreased expression of *MyoD* ( $0.46 \pm 0.12$ ;  $p=0.004$ ) and *Myogenin* ( $0.34 \pm 0.05$ ;  $p=0.010$ ) mRNA, increased activation of MEK1 ( $724 \pm 140$  MFI;  $p=0.001$ ) and ERK1/2 ( $3768 \pm 314$  MFI;  $p=0.001$ ) and a decreased activation of Akt ( $74.5 \pm 4$  MFI;  $p=0.009$ ) and mTOR ( $61.8 \pm 7$  MFI;  $p=0.001$ ) compared with old AC. By contrast, C2C12 myoblasts treated with older AC displayed increased expression of *MyoD* ( $0.7 \pm 0.08$ ;  $p=0.004$ ) and *Myogenin* ( $0.68 \pm 0.05$ ;  $p=0.010$ ) mRNA, decreased phosphorylation of MEK1 and ERK1/2 ( $528 \pm 80$  MFI;  $p=0.008$ , and  $1141 \pm 668$  MFI;  $p=0.001$ , respectively) and increased Akt/mTOR activation ( $171 \pm 35$  MFI;  $p=0.009$ , and  $184 \pm 33$  MFI;  $p=0.001$ , respectively). These data provide new evidence that differences between older and younger lymphocyte secretomes contribute to differential responses of C2C12 myoblasts in culture.

## Introduction

Following damage of skeletal muscle, the repair process involves activation of quiescent satellite cells residing between the sarcolemma and basal lamina (1). The satellite cells transition through stages of proliferation, differentiation and migration to ultimately fuse with pre-existing damaged myofibres in order to repair the injury (2-4). Immune cells play important roles in muscle repair and in older age the processes regulating immune-muscle interactions may be blunted, which in turn can impair muscle regeneration and contribute to the age-related loss of muscle mass. Such defects were demonstrated in older mice, where the reduced rates of satellite cell proliferation and migration in response to muscle trauma led to incomplete regeneration (5, 6) and loss of muscle mass (7). Although intrinsic changes to satellite cells have been implicated in the blunted activation and regenerative potential in old age (8), parabiosis models of muscle ageing provide strong evidence that extrinsic factors within the microenvironment play an important role in adaptation or maladaptation with age (9-11).

The skeletal muscle microenvironment includes soluble factors, such as cytokines and growth factors, produced locally or systemically by different cell types, including those of the innate immune system (12). Factors released by activated T helper -1 (Th1) and T helper -2 (Th2) cells trigger macrophage transition from a pro-inflammatory M1 (13) (occurring early after trauma) to an anti-inflammatory M2 (occurring after ~48hr following trauma) phenotype, ultimately enabling myogenesis in injured rodent skeletal muscle (14). Therefore, any decreased T-cell production (15) and corresponding fall in T-cell numbers in older age (16) may be implicated in the age-related reduced satellite cell activation during muscle regeneration (3). Furthermore, changes in T-cell function with age, irrespective of T-cell number, may also impact upon skeletal muscle satellite cell function.

*In vivo* studies have shown that human lymphocytes helper cells from aged vs younger adults exhibited differential expression in their activation markers (17). Recently, it was also demonstrated that the altered intrinsic T-cell signalling in murine old age affects their cellular function as facilitators of tissue regeneration in skin (18) and skeletal muscle (19). The implication is that factors secreted by activated T-lymphocytes in young individuals may enhance muscle regenerative potential by accelerating myoblast proliferation and migration rates (20). However, the secreted proteins from activated T-lymphocytes in older

people had no discernible effect on myoblast proliferation or migration (20). These studies reveal age-related changes affecting T-lymphocytes impact on the cross talk between immune cells and skeletal muscle satellite cells.

Recent evidence suggests that a specialised sub-population of T-lymphocytes, known as FoxP3+/CD4+/CD25+regulatory T-cells (Treg) exists (21, 22). Interestingly, Treg-stimulated muscle satellite cells showed sustained proliferation and delayed differentiation following injury of mouse muscle (23), marking them as potential candidates in the search for novel regulators of muscle satellite cell activation. Furthermore, activated T-lymphocytes secrete numerous factors to the microenvironment and several are known to influence muscle satellite cell function, including FGF2, IFN- $\gamma$ , TGF- $\beta$ , TNF- $\alpha$  and IL4 (24-26). Given the potential importance of this emerging field, the impact of aged T-lymphocyte secretome on muscle regenerative potential warrants investigation.

The present study progresses previous work (20, 27) and specifically aimed to investigate, for the first time, the C2C12 myoblast cell signalling responses after exposure to T-cell secretomes harvested from young or older adults, and to determine the involvement of selected T-cell-derived growth factors in the C2C12 myoblast proliferation and cellular signalling responses. The novel hypothesis we tested was that IGF-1, *GDF-11* and *amphiregulin* released by activated Treg cells initiate C2C12 myoblast signalling through *myoD*, *myogenin*, Akt/mTOR and MEK-ERK1/2.

## Materials and methods

### Materials

All cell and tissue culture media and supplements were purchased as sterile or were filter sterilised through a 0.20  $\mu$ M filter. Heat-inactivated (hi) foetal bovine serum (FBS) and hi new born calf serum (NCS) were purchased from Gibco (Paisley, Scotland); hi horse serum (HS) was from TCS Biosciences (Corby, England); penstrep (penicillin and streptomycin) and trypsin from Bio Whittaker (Wokingham, England); L-glutamine from BDH (Poole, England), gelatine from Sigma (St. Louis, MO, USA). Plasticware were purchased as sterile from Greiner Bio-one, (Kremsmunster, Austria) unless otherwise stated.

### Isolation and culture human Lymphocytes

Following ethical approval and written, informed consent, whole blood samples were collected from the antecubital vein in the forearm from young healthy (n=22; ages: 18-25) and older (n=21; ages 75-85) men. None of the participants had history of muscular diseases and none were on corticosteroids or immunosuppressant treatments. Lymphocytes were isolated using Ficoll-Paque<sup>PLUS</sup> (GE healthcare Life Science, Buckinghamshire, UK) and macrophages were depleted from the cell preparation by pre-plating for 15 min at 37°C (28). Isolated lymphocytes were cultured at  $1 \times 10^6$  cells/ml in 96 well flat-bottomed plates pre-coated with anti-CD3 (OKT3, 10 µg/ml, eBioscience, UK) as described previously (20, 29). Cells were cultured at 37°C, 5% CO<sub>2</sub> for 4 days in RPMI-1640 containing 10% (v/v) heat-inactivated human AB serum (Thermo, Brown Deer, USA), 1% Penicillin/Streptomycin, and 50 U/ml human recombinant interleukin-2 (rhIL-2, R & D System, Abingdon, UK) in the absence or presence of anti-human CD3 (OKT3, 10 µg/ml, eBioscience, UK) and 5 µg/ml anti-CD28 (BD Pharmingen™, San Diego, CA, USA (20).

#### ***Secretome recovery and conditioned media preparation.***

The lymphocyte secretome was recovered following old and young lymphocyte incubations at 37°C in the presence or absence of activating antibodies (anti-CD3/CD28) for 4 days. To concentrate the secretome, 4 ml lymphocyte secretome was applied to an Ultracel-3 membrane tube (Millipore, Watford, UK). The tubes were centrifuged at 4000 x g for 40 minutes. The concentrated secretomes were used in the preparation of subsequent conditioned media. Two different CMs (activated (AC) Young and old) and (non-activated (NA) young and old) were prepared by reconstituting 200µl of the concentrated secretome with 3.8 ml of differentiation medium DM (DMEM supplemented with 2% horse serum, 1% Penicillin/Streptomycin and 200 nmol/L of L-glutamine (standard muscle cell differentiation medium), as shown in Table 2 below and described previously (20, 27).

#### **Culture of C2C12 myoblasts**

Murine skeletal muscle cells were purchased from ATTC (Rockville, MD, USA) and maintained in a humidified 5% CO<sub>2</sub> environment at 37°C. The cells were grown in growth media (GM) supplemented with DMEM media (Sigma, Gillingham, Dorset, UK), 10% Fetal bovine serum (hi FBS; Thermo Fisher, UK), 10% new born calf serum (hi NCS; Invitrogen,

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GIBCO, Paisley, UK), 1% L-glutamine and 1% of 10,000 U Penicillin/Streptomycin until 80% confluence was attained. Following trypsinisation, C2C12 myoblasts were detached and counted using a haemocytometer in the presence of trypan blue dye (Bio Whittaker, Wokingham, England). Six-well plates were pre-coated with 0.2% gelatine for 5 min at room temperature. Following removal of excess gelatine, C2C12 myoblasts were seeded at  $1 \times 10^5$  cells/ml in GM. On attaining approximately 80% confluency, cells were washed twice with PBS before culturing with CMs. After 4 days of CMs treatment, C2C12 myoblasts were collected to perform the following the studies: Biochemical and morphological studies; cell cycle analysis, gene expression assay qRT-PCR after 4 days, and Luminex xMAP phosphoproteins magnetic bead arrays after 25 min post transfer to CMs.

**Gene expression analysis of *GDF-11*, *Amphiregulin*, *MyoD* and *Myogenin***

Following cell treatments with different CMs, and incubation for 4 days, *GDF-11* and *Amphiregulin* mRNA for lymphocytes and *MyoD/myogenin* mRNA for C2C12 myoblasts were isolated using Trizol® reagent (Thermo, Life Technologies, Paisley, UK). Real time PCR-One step was carried out using the TaqMan® RNA-to CT™ kit (Applied Biosystems, San Jose, CA, USA). The reactions were run on the StepOnePlus™ machine (Thermo, Foster City, CA, USA) as follows: 10 min at 95°C for polymerase activation and 40 cycles of 15 s at 95°C and 60 s at 60°C. Primer sets (Table 1) were obtained from Thermo. The RT-PCR reaction of *GDF-11*, *amphiregulin*, *MyoD* and *myogenin* were performed in parallel with *GAPDH* as control gene. Gene expression levels were calculated using the comparative  $2^{-\Delta\Delta Ct}$  method (30). Results were analysed using StepOne Software v 2.2 (Thermo, Life Technologies, Paisley, UK).

**Cell count and cycle analysis and cell count**

Cell cycle analysis was performed as described previously (31). After 4 days of treatment, C2C12 myoblasts were washed twice with PBS, trypsinised, and fixed with 75% Ethanol at -20°C. After 24 h, cells were washed with PBS and suspended in propidium iodide labelling buffer (50 mg/mL propidium iodide, 0.1 sodium citrate, 20 mg/mL ribonuclease A, and 0.3 Nonidet p-40). The cell cycle events were analysed, using Cell Quest software from FACS

Calibur flow cytometer (BD, Oxford, UK) and cell phase events were identified by ModFit LTv3.0 software (Verity Software House, Topsham, ME, USA). AC (young and old) and NA (young and old) treated cells were counted using haemocytometer in the presence of trypan blue (BioWhittaker, Wokingham, England).

### ***Measurement of CD25/FoxP3 markers in young and old activated lymphocytes***

Anti-CD3/CD28 activated lymphocytes obtained from young and older participants were analysed using flow cytometry. Cells were lysed and re-suspended in 500 µl stain buffer (BD Pharmingen, Oxford, UK) containing 1.0 µg of Anti-CD25 APC-conjugated antibody (Sigma – Aldrich, Poole, UK) and incubated for 30 minutes in the dark (RT). APC-conjugated isotype was used as a control (Sigma, Poole, UK). For Intracellular staining of FoxP3 (BD Pharmingen™, Oxford, UK), cells were fixed with 4% Paraformaldehyde solution for 10 minutes in the dark at RT. Following incubation, cells were washed with 2 ml of Stain Buffer (BD Pharmingen™, Oxford, UK) and centrifuged at 300xg for 10 minutes. Wash buffer was removed, and cells were incubated with permeabilisation buffer (1X BD Perm, San Diego, CA, USA) for 30 minutes in the dark at RT. Following washing, cells were stained with 20 µl of PE-anti-human FoxP3 antibody (BD Pharmingen™, Oxford, UK). Following 30 min incubation at RT protected from light, the cells were centrifuged at 300xg for 10 min. The supernatant was discarded; single-cell suspensions were prepared in 500 µl of sheath fluid solution for each tube and transferred to the FACS tubes prior to measurement by a FACS Calibur flow cytometer.

### ***Measurement of IGF-I secreted in young and old secretomes***

A Luminex MILLIPLEX MAP Human IGF-I, Magnetic Bead Panel (EMD Millipore, MA, USA) was used according to the manufacturer's protocols to measure IGF-I. Briefly, a volume of 25 µl of beads suspension was added to all wells containing 25 µl of standards and controls and 25 µl of sample. The plates were sealed and incubated overnight on a plate shaker at 4°C. Wells were washed 3 times in wash buffer. A volume of 50 µl of detection antibody was added to each well and the plate incubated for 1 hour at RT. Streptavidin-Phycoerythrin was added to each well and incubated for 30 min at RT. All wells were washed 3 times and 100 µl of sheath fluid was added prior to reading on the Luminex 200™.



***Measurement of amphiregulin in secretome of young and old lymphocytes***

A human amphiregulin enzyme-linked immunosorbent assay (ELISA) (Abcam, UK) was performed for the quantitative measurement of Amphiregulin in young and older secretomes. Standards were prepared according to manufacturer's recommendations. Samples and standards were added to their pre-coated amphiregulin antibody wells and incubated overnight with gentle shaking at 4°C. Following incubation, the plate was washed with 1X wash solution and 1X biotinylated Amphiregulin detection antibody was added to all wells. The plate was incubated for 1 hour at RT with gentle mixing. Following washing, 1X HRP-Streptavidin solution was added to all wells. The plate was sealed and incubated for 45 minutes at RT with gentle mixing, prior to washing and incubation with TMB one-step substrate reagent at RT for 30 minutes in the dark with gentle mixing. The plate was read at 450 nm using a Bio-Tek ELISA plate reader (Winooski, VT, USA) immediately after adding 50µl of stop solution to each well.

***Determination of MEK-ERK1/2 and Akt/mTOR***

Initial experiments were undertaken to establish the optimal activation time. For this purpose, MILLIPLEX MAP Akt/mTOR Phosphoprotein Magnetic Bead was used to test at 5, 15, 25 & 60 min and it revealed 25 min was optimal for activation. In preparation for multiplex analysis, C2C12 myoblasts were treated with older or younger AC or NA for 25 min, prior to addition of ice cold cell signalling lysing buffer (Watford, Millipore, UK) and storage at -80°C. Samples were thawed, centrifuged and the supernatants collected for protein determination using the Pierce<sup>TM</sup> BCA Protein Assay (Thermo Fisher Scientific, USA). Samples were diluted to the final concentration of 10µg/ml with assay buffer (Milliplex<sup>®</sup> Map Assay Buffer; Watford, Millipore, UK). Samples were run in triplicate on a 96-well filter plate pre-treated with wash buffer and 1x antibody-coupled beads. Samples and beads were incubated overnight in the dark at RT. Plates were vacuum filtered and washed. Detection antibodies were added and the plate was incubated for 1hr at RT. Following washing, Streptavidin-Phycoerythrin was added to each well. Following incubation, beads were then re-suspended in buffer prior to reading on a Luminex 200<sup>®</sup>. Multiplex analysis was performed to measure the Mean Fluorescent Intensity (MFI) of phosphorylated Akt/mTOR and MEK/ERK using Luminex 200<sup>®</sup> reader (Watford, Millipore, UK).

**C2C12 myoblasts treatment with IGF-IR inhibitor (OSI-906)**

Optimal concentration of OSI-906 was carried out using 0.1, 0.3, 0.5, and 0.8 $\mu$ M, and it was shown that 0.3 $\mu$ M was the optimal concentration and therefore used in the current study. On attaining 70 % confluency, cell monolayers were washed twice with PBS and treated with reconstituted IGF-IR inhibitor (IGF-1R; OSI-906; Selleckchem, UK) at 0.3 $\mu$ M for 30 min in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Following OSI-906 treatment, C2C12 myoblasts were washed with PBS and cultured with either young AC or DM. After 4 days incubation, cells were collected for counting using haemocytometer in the presence of trypan blue (BioWhittaker, Wokingham, England). For determination of phosphorylated levels of Akt or mTOR, following 30 min incubation with IGF-IR inhibitor, cells were treated 25 min with either young AC or DM. Phosphorylated levels of Akt and mTOR were measured using Luminex multiplex as described above. Cell cycle analysis was performed as described previously (20). The cell cycle events were analysed, using Cell Quest software from FACS Calibur flow cytometer (BD, Oxford, UK) and cell phase events were identified by ModFit LT v3.0 software.

**Statistical analysis**

All experiments were repeated three times in triplicate, unless otherwise stated, and were analysed using GraphPad software version 5.0 (La Jolla, CA). Repeated measures ANOVA was used to compare effects between Age (young and old secretomes), as well as Activation Status (activated and non-activated secretomes) and their interactions. A significant effect of Age indicates a difference between young and old, independent of Activation Status. A significant effect of Activation Status indicates differences between activated and non-activated secretomes, independent of Age. A significant interaction between Age and Activation Status indicates that the difference between non-activated and activated secretomes for old is different from that of non-activated and activated secretomes for young. Where effects between all groups were directly compared (young, old, activated, non-activated and control conditions), one-way ANOVA was used, followed by Bonferroni post-test analysis. Results are presented as mean  $\pm$  standard deviation ( $\pm$ SD).

Values of  $p < 0.05$  were considered statistically significant, and significance is indicated on figures using \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## Results

### ***CD25 and FoxP3 expression in Young and Old T-lymphocytes***

CD25 and FoxP3 expression levels in young/old and activated/non-activated lymphocytes were determined as markers of Treg status. Under non-stimulated conditions, there was no significant difference in basal expression levels of CD25 between young and old lymphocytes. However, activation of lymphocytes increased expression of CD25 ( $p < 0.001$ ) and the increase in CD25 in response to activation was 3-fold greater for young than it was for old, as indicated by the significant interaction between age and activation status (Fig 1A,  $p < 0.001$ ).

Positive CD25 cells (as shown in Fig. 1A) were further analysed to determine the expression of FoxP3 in young and old CD25 positive cells. Basal expression levels of FoxP3 did not differ significantly between non-activated young and non-activated old lymphocytes (Fig 1B). Activating the lymphocytes increased FoxP3 expression ( $p = 0.047$ ). The significant interaction between age and activation status revealed that the increase in CD25 expression from non-activated to activated states was greater for old lymphocytes than it was for young ( $p = 0.036$ ). FoxP3 in old activated lymphocytes was 35% of all cells, compared with 15% of cells in young activated lymphocytes.

### ***Amphiregulin and GDF-11 gene expression in young and old T-lymphocyte colonies***

Amphiregulin is known to accelerate skeletal muscle repair and regeneration (32). Therefore, the expression level of Amphiregulin in young and old T-lymphocytes was determined. Basal expression levels of amphiregulin did not differ significantly between non-activated young and old cells ( $p = 0.079$ ; Fig 2A). However, activation caused a 2-fold increase in amphiregulin expression in young, which was absent in the old (age x activation interaction:  $p = 0.006$ ).

GDF11 is reportedly increased with ageing (33) and is believed to inhibit myogenesis of the chick limb (34), as well as decrease satellite cell proliferation and attenuate skeletal muscle regeneration in aged mice (35). To evaluate whether GDF11 expression differed between

older and young T-lymphocytes, the expression level was measured. Basal expression levels did not differ significantly between non-activated younger and older cells and expression levels did not change following lymphocyte activation (Fig. 2B).

#### **Amphiregulin and IGF-I protein concentration in Young and Old T-lymphocyte secretomes**

Treg cells produce and respond to amphiregulin; therefore, its expression was also assessed. There was no difference in amphiregulin concentrations between non-activated older and younger cells (Fig 3A). Following lymphocyte activation, there was a significant increase in amphiregulin secretion ( $p=0.011$ ; Fig 3A). There was no interaction between age and activation status, suggesting that both older and younger activated lymphocytes can increase amphiregulin production following lymphocyte activation. However, amphiregulin protein concentration was nevertheless two-fold higher in activated young compared with activated old lymphocyte secretomes ( $p=0.014$ ).

It is well established that IGF-I has not only a mitogenic effect on muscle satellite cells (36) but also a potent survival and differentiation role (37). To date, no research has determined whether IGF-I secreted from T-lymphocytes has an influencing role in skeletal muscle satellite cell proliferation and whether it is down-regulated in older T-lymphocytes, potentially culminating in blunted satellite cell function. There was no difference in IGF-I concentration between non-activated old and young cells (Fig 3B). Following lymphocyte activation, both older and younger lymphocytes increased secretion of IGF-I ( $p=0.013$ ; Fig 3B).

#### **Gene expression analysis of *MyoD* and *Myogenin* for C2C12 myoblasts treated with CMs.**

Having ascertained that young and older lymphocytes respond differently to activation by anti-CD3/CD28, the next step was to determine whether these differences could influence C2C12 myoblast behaviour. There was no difference in expression levels of *MyoD* or *myogenin* in the presence of non-activated control (NA) (Fig. 4A and B). However, myoblasts exposed to activated lymphocyte secretomes decreased *MyoD* expression ( $p=0.029$ ). The decrease was greater for myoblasts exposed to young AC compared to older AC secretomes (age x activation status  $p=0.007$ ). Similarly, there was a significant effect of activation status on *myogenin* expression ( $p<0.001$ ) and an interaction between age and

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activation status for *myogenin* expression ( $p=0.005$ ), once again reflecting a greater reduction in response to activated CM (compared with non-activated) from younger compared with older lymphocytes.

***Cell Count and cell cycle analysis for C2C12 myoblasts treated with CMs.***

Having preliminary data which suggest that secretomes from younger compared with older activated lymphocytes attenuates C2C12 myoblast fusion, the next step was to analyse cell cycle progression. Flow cytometric analyses of treated C2C12 myoblasts revealed that entry into S phase by myoblasts was not different in the presence of non-activated secretomes from older compared with younger lymphocytes (Fig 5A). There was, however, a significant effect of activation ( $p<0.001$ ) and an interaction between age and activation status ( $p<0.001$ ): a higher percentage of C2C12 myoblasts treated with activated younger lymphocytes ( $P<0.001$ ) were in S phase compared with those treated with activated secretomes from older lymphocytes (Fig. 5A). This increase in S phase in C2C12 myoblasts treated with the younger vs. older activated secretomes also culminated in a significant increase ( $P<0.001$ ) in cell number (Fig. 5B).

***MEK1-ERK 1/2 signalling pathways are induced in response to Young AC but effects are attenuated in response to Old AC.***

Given the different myoblast proliferation responses to activated young compared with old lymphocyte secretomes, experiments were undertaken to examine the impact on MEK-ERK1/2 activity in C2C12 myoblasts. As with FLOW and cell counting studies, the Mean Fluorescent Intensity (MFI) of pMEK and pERK were not different between the control conditions young and old non-activated CMs (Figs 6A and 6B). However, both MEK1 ( $p=0.010$ ) and Erk1/2 ( $p=0.002$ ) phosphorylation increased with exposure to younger, but not older AC.

***AKT/mTOR signalling pathways are suppressed in response to younger compared with older CM.***

Having determined an increase in MEK/Erk activation in response to young vs old activated lymphocyte secretomes, the impact on myoblast Akt/mTOR activation was investigated. As

for most analyses, the MFI of pAkt and pmTOR were not different in C2C12 myoblasts following treatment with younger vs. older non-activated lymphocyte CMs (Figs 7A and 7B). There was a significant interaction between age and activation status for both pAkt ( $p=0.005$ ) and pmTOR ( $p=0.023$ ), revealing that the younger AC decreased, whereas older AC increased pAkt and pmTOR in C2C12 myoblasts.

***The proliferation of young AC-treated C2C12 cells was partially attenuated by the IGF-1-R inhibitor OSI-906***

To investigate whether the increase in IGF-I may impact on the increased proliferation in C2C12 cells treated with younger vs. older AC, the IGF-1R was inhibited prior to treatment with young AC, where proliferation was increased.

C2C12 myoblasts proliferation was significantly higher in the presence of young AC compared with the control conditions of DM+OSI 906 and DM alone ( $p<0.001$  and  $p<0.001$ ; Fig 8A), confirming no direct influence of the OSI 906 alone on C2C12 proliferation. The addition of OSI 906 to young AC treated C2C12 myoblasts reduced proliferation compared with young AC alone ( $p<0.001$ ). However, cell numbers were still significantly higher in young AC plus OSI 906 compared with both control conditions. FLOW cytometric analyses confirmed the FLOW data, with % C2C12 myoblasts in the S phase of the cell cycle significantly reduced in the presence of OSI-906 plus young AC as compared with young AC alone ( $p<0.01$ ; Fig 8B).

***Determining the AKT/mTOR signalling pathway within C2C12 myoblasts treated with the IGF-1R inhibitor OSI-906***

pAkt ( $p=0.072$ ) and pmTOR ( $p=0.056$ ) changes were not statistically significant following co-incubation of C2C12 myoblasts with OSI-906 inhibitor plus young AC as compared with young AC alone (Fig 9). The level of pAkt and mTOR in OSI-906 plus young AC remained significantly lower than the control conditions of OSI-906 plus DM and DM alone (all  $p<0.01$ ; Fig. 9).

## Discussion

An immortalised murine myoblast cell line (C2C12) was used to demonstrate that soluble factors released by human T cells of young adults regulate myoblast proliferation, increased myoblast MEK and ERK1/2, and decreased levels of phosphorylated Akt and mTOR. However, the factors released by T-cells of older adults had lesser effects on myoblasts compared with the young T cells. These novel findings suggest that age-dependent changes to the profile or efficacy of secreted factors from activated T-cells subsequently impact on the skeletal muscle regenerative potential.

The T cells in the basal state, without any activating agents, showed no age-dependent differences in FoxP3 and CD25 phenotypes, but age-dependent differences became evident when T cells were activated using anti-CD3/CD28. It is reported that CD25 positive cells produce IL-2 upon T-cell receptor (TCR) stimulation (38) and this agrees with our findings that young activated lymphocytes expressed higher CD25 than old activated lymphocytes. Activated T cells of young adults expanded with proportionally greater increases in Treg cells expressing CD25 (a component of IL-2 receptor and clonal expansion) compared with FoxP3 (a master suppressor protein of Treg function). Conversely, activated T cells from older adults expanded with more FoxP3 expression than CD25 which is in line with previous reports that ageing of the immune system (human and mice) is associated with increased Treg cells expression of FoxP3 and decreased CD25 expression (Reviewed in (39)). It is clear that the difference between young and older T-cells lies in the response to activating agents, which is known to decrease with ageing (40-42).

Recently, the blunted muscle repair of aged mice was attributed in part to lower infiltration of Treg cells compared to young mice (19). In the present work, there was a relatively greater expansion of FoxP3-positive cells in old compared with young activated T-cells, so it seems that expansion *per se* of the Treg FoxP3-positive cells is proportionally linked to the myoblast response. In humans and mice, factors released from the older Treg FoxP3<sup>high</sup> CD25<sup>low</sup> cells (these cells have suppressed function with restricted proliferation) were found to secrete a different profile of soluble factors from the young FoxP3<sup>low</sup> CD25<sup>high</sup> (these cells induce proliferation) (43, 44). This helps to explain why, in our study, the C2C12 myoblasts cultured with young activated T-lymphocytes secretome exhibit significant proliferation and

associated cellular signalling responses, but effects were attenuated in myoblasts treated with old activated lymphocytes secretomes.

The secretomes of non-activated T-cells from young or older donors had no discernible additional effect on C2C12 myoblast behaviour. However, the secretomes of activated T-cells did influence myoblast behaviour, although this effect was dependent on the age of the participants donating the T-cells. Levels of MyoD (a marker of myogenic lineage) and myogenin (a marker of differentiation (45)) decreased after exposure to activated T-cell secretomes, with the decrease being greater after treatment with young compared with old activated T-cell secretomes. The C2C12 pAkt and pmTOR levels increased in cells treated with old T-cell secretomes, but not after treatment with the young T-cell secretomes. Conversely, the %S phase as well as ERK1/2 and MEK1 were higher in C2C12 myoblasts treated with young secretomes than older secretomes. These findings are in line with previous studies that identified higher pAkt and mTOR levels in differentiating myoblasts, whereas MEK1 and ERK1/2 were previously shown to be higher in proliferating myoblasts (31, 46, 47). This finding also extends previous work showing effects of young T-cells on myoblast responses (27) and differential effects of old compared with young T-cells on myoblast responses (20). Castiglioni et al (23) cultured satellite cells with activated T-cells isolated from 10 weeks old mice and found that the T-cells induced greater proliferation of myoblasts and attenuated myoblast differentiation compared to non-activated T-cells cultured with myoblasts. These findings taken together suggest that activated T-cells release factors that can influence C2C12 myoblast expansion and myogenic differentiation, and changes in T-cell function with ageing may contribute to impaired skeletal muscle regeneration.

The profile of secreted factors from activated young T-cells may differ from that of activated older T-cells and a potential candidate for the age-dependent differential regulation of myoblasts is IGF-1. IGF-1 is best known as a growth factor secreted by skeletal muscles to regulate their growth, differentiation and repair. Although less well documented, IGF-I is also secreted by T cells (48, 49). Our results add to this literature by showing that the release of IGF-1 from T cells is dependent upon the age of the T-cell donor. We observed 30% higher IGF-1 levels secreted by young activated T-cells compared to old. High IGF-1 concentrations *in vitro* favours proliferation, whereas lower IGF-1



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concentrations promote differentiation (50). These findings support a role for IGF-1 as a candidate regulator of the observed effects of T cell secretomes on C2C12 myoblasts. However, neutralising IGF-1 using anti-IGF-1R antibodies reduced, but did not entirely diminish, the pro-proliferative and cellular signalling effects of the secretome on myoblasts. It is clear that factors in addition to IGF-1 contribute to the myoblast responses.

Another possible candidate identified from the literature is GDF-11. This molecule is similar in structure to myostatin, a potent negative regulator of muscle growth. GDF-11 was reported to increase with ageing and contribute to decreases in satellite cell proliferation and skeletal muscle regeneration in aged mice (35). However, our data did not reveal any preferential influence of ageing on GDF-11 expression from T-cells. A third possible growth factor released by T-cells is amphiregulin, which was previously linked to muscle regeneration (51). The results presented here provide the first evidence, to our knowledge, that amphiregulin expressed by young activated T-cells was higher compared to old activated T-cells. This was associated with lower proportion of Treg FoxP3 in young activated T-cells that tended to correspond to higher amphiregulin production. Another possible explanation for the above finding could be that the suppression of CD25 expression in older adults could lead to low production of amphiregulin, which is known as inducer factor for Treg FoxP3 (52). Previous studies showed that IGF-1 and amphiregulin function synergistically to promote cell proliferation through activation of the MAPK/ERK pathway (53). This is in line with the higher MAPK and ERK1/2 phosphorylated protein levels in myoblasts treated with young activated T-cell secretomes compared to old.

Taken together, our novel findings suggest that as yet unidentified cytokines and/or growth factors present in the young activated T cell secretomes can induce a sustained proliferation via low expression of *MyoD* and *myogenin* and activation of MEK-ERK1/2 signaling pathways. While proteins present in the old secretomes have little effect, C2C12 myoblasts treated with old secretomes progress towards differentiation with increased levels of *MyoD* and *Myogenin* and activation of the Akt/mTOR signaling pathway.

**Conclusion**

Ageing is associated with intrinsic changes of T-lymphocytes, and consequently changes in T cell amphiregulin, CD25, FoxP3 and IGF-1 production which are involved in effective C2C12

myoblast proliferation through *MyoD/Myogenin* expression and activation of the MEK-ERK1/2. The age-dependent changes to T cells contributed to attenuated C2C12 myoblast proliferation. These observations provide new insights into the potential regenerative role of activated lymphocytes in muscle wasting conditions. However, further studies are required to investigate the potential role of amphiregulin and Treg FoxP3 cells in muscle wasting.

### Conflict of Interests

The authors declare that they have no conflict of interest.

### Acknowledgements

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**Tables****Table (1): Predesigned primers for human lymphocytes.**

<b>Genes</b>	<b>Assay ID</b>	<b>Reference Sequence number</b>	<b>Amplicon length (bp)</b>
Amphiregulin	Hs00956669	NM-001657.2	66
GDF-11	Hs00195156	NM-005811.3	64
MyoD	Mm00440387-m1	NM-010866.2	86
Myogenin	Mm00446194-m1	NM-031189.2	68
GAPDH	Mm99999915-g1	NM-001289726.1	70

**Table (2):** The ingredients of designed control conditions for C2C12 culture.

Conditioned Media (CM)	Components
Activated AC (Young and Old)	Concentrated secretomes obtained from activated young or older anti-CD3/CD28-lymphocytes plus DM (1:20).
Non-activated NA (Young and Old) (negative control)	Concentrated secretomes obtained from younger or older non-activated lymphocytes plus DM (1:20).

**Figure legends.**

**Figure 1: Percentage of positive CD25 and FoxP3 in Young and Old lymphocytes.** Lymphocytes were cultured for 4 days with or without the activators anti-CD3/anti-CD28. Cells were stained with APC-anti-human CD25 antibody (A) and PE-anti-human CD28 antibody (B). **A.** CD25 expression was significantly higher in activated lymphocytes compared with non-activated (\*\*\*,  $p<0.001$ ) and the increase with activation was greater in young than old (\*\*\*,  $p<0.001$ ). **B.** FoxP3 was higher in activated lymphocytes compared with non-activated (\*;  $p<0.05$ ) and the increase with activation was greater in old than young (\*;  $p<0.05$ ). Data represent means  $\pm$  SD (n=5 samples of each age group) of each young (22 $\pm$ 3years) and old (80 $\pm$ 5 years) in duplicates. NA: Non-activated; AC: Activated.

**Figure 2: Amphiregulin and GDF-11 gene expression in young and old lymphocytes.** Lymphocytes were cultured for 4 days with or without the activators anti-CD3/anti-CD28. **A.** Amphiregulin mRNA expression levels increased in activated compared with non-activated lymphocytes of young, but not for old (interaction between age and activation status: \*\*,  $p<0.01$ ). **B.** GDF-11 mRNA expression levels did not differ significantly between Young and

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Old or activated vs non-activated cells. All values are represented as a fold change to young non-activated lymphocytes and presented as mean  $\pm$  SD (n= 5s samples of each age group) in triplicates. NA: Non-activated; AC: Activated.

**Figure 3: Amphiregulin and IGF-I concentration in secretomes of young and old lymphocytes.** Lymphocytes were cultured for 4 days with or without the activators anti-CD3/anti-CD28. **A.** The concentration of Amphiregulin was higher in activated compared with non-activated secretomes ( $p<0.05$ ). **B.** The concentration of IGF-1 was higher in activated compared with non-activated secretomes ( $p<0.05$ ). Data represent means  $\pm$  SD (n=6 samples of each of young aged (18-25 years) and old (78-86 years) in triplicates. AC: Activated and NA: Non-activated.

**Figure 4: RT-PCR analysis of *MyoD* and *myogenin* mRNA gene expression.** C2C12 myoblasts were treated with young or old (activated lymphocytes: **AC**) or non-activated lymphocytes: (**NA**) for 4 days before RNA collection. **A.** Expression of *MyoD* was lower in C2C12 cells exposed to AC compared with NA conditioned media ( $p<0.05$ ) and the decrease in AC young was greater than that for AC old ( $p<0.01$ ). **B.** Expression of *Myogenin* was lower in C2C12 cells exposed to activated compared with non-activated secretomes ( $p<0.001$ ) and the decrease in AC young was greater than that for AC old ( $p<0.01$ ). All values are represented as fold difference compared with the control calibrator (NA young and old) and represent the means  $\pm$  SD (n=5 samples of each age group) in triplicates.

**Figure 5: Cell Cycle and Cell Count for C2C12 myoblasts treated with conditioned media (CM).** C2C12 myoblasts were treated with young or old (activated lymphocytes: AC) or non-activated lymphocyte (NA) for 4 days before cell cycle analysis and cell count. **A:** %S phase was significantly lower in activated compared with non-activated CM ( $p<0.001$ ). **B:** Cell count increased in activated compared with non-activated young CM ( $p<0.0001$ ) and this increase with activation was greater for young than for old ( $p<0.001$ ). Data represent the means  $\pm$  SD (n=5) in triplicates.

**Figure 6: Expression levels of phosphorylated MEK1 and ERK1/2 in C2C12 myoblasts treated with different CMs.** C2C12 myoblasts were cultured with different conditioned media AC (young or old) or NA (young or old) for 25 min. **A:** MFI levels of C2C12 pMERK1 was significantly higher when adding conditioned media from activated compared with non-activated lymphocyte secretomes ( $p < 0.05$ ). **B.** MFI levels of C2C12 pERK1/2 was significantly higher when adding conditioned media from activated compared with non-activated lymphocyte secretomes ( $p < 0.01$ ). Data represent means  $\pm$  SD ( $n=5$  samples of each age group) in triplicates.

**Figure 7: Phosphorylated levels of pAkt and pmTOR in C2C12 myoblasts treated with different CMs.** C2C12 myoblasts were cultured with different conditioned media AC (young or old) or NA (young or old) for 25 min. **A.** MFI Levels of pAkt decreased in young but increased in old after exposure to activated lymphocyte secretomes (interaction effect:  $p < 0.01$ ). **B.** MFI Levels of pmTOR decreased in young but increased in old after exposure to activated lymphocyte secretomes (interaction effect:  $p < 0.05$ ). Data represent means  $\pm$  SD ( $n=4$ ) in triplicates.

**Figure 8: Effects of IGF-IR OSI-906 inhibitor on C2C12 myoblasts cultured with Young AC conditioned media.** C2C12 myoblasts were treated with or without IGF-IR inhibitor (OSI-906) for 30 min, washed then were cultured with conditioned media from young AC or differentiation media (DM) for 4 days. **A:** Cell count was significantly higher in young AC treated C2C12 myoblasts compared with all other conditions (all  $p < 0.001$ ). **B:** % S phase was significantly higher in young AC treated C2C12 myoblasts compared with all other conditions (all  $p < 0.01$ ). Data are shown as means  $\pm$  SD ( $n=4$ ) in triplicates. DM: Differentiation Media.

**Figure 9: The level of phosphorylated Akt and mTOR in C2C12 myoblasts.** C2C12 myoblasts were treated with or without IGF-IR inhibitor (OSI-906) for 30 min, washed then treated for 25 min with conditioned media from young AC or differentiation media (DM). **A:** AC young + OSI-906 was significantly lower than DM + OSI-906 and DM alone (both  $p < 0.01$ ). **B:** AC



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young + OSI-906 was significantly lower than DM + OSI-906 and DM alone (both  $p < 0.01$ ).

Data represent the means  $\pm$  SD (n=4) in triplicates.

Figures

Fig 1A

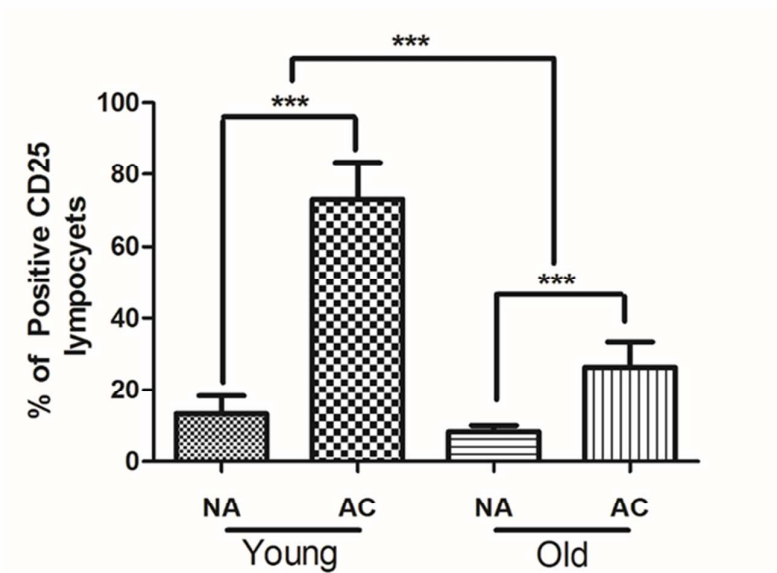
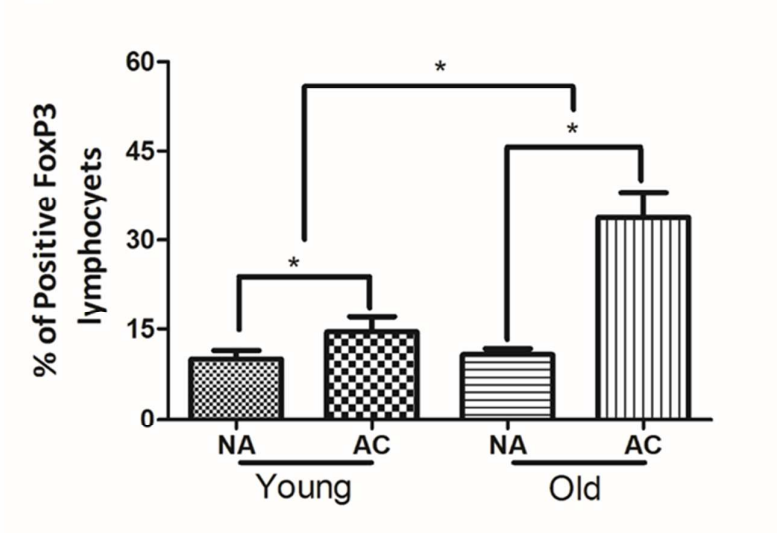


Fig 1B



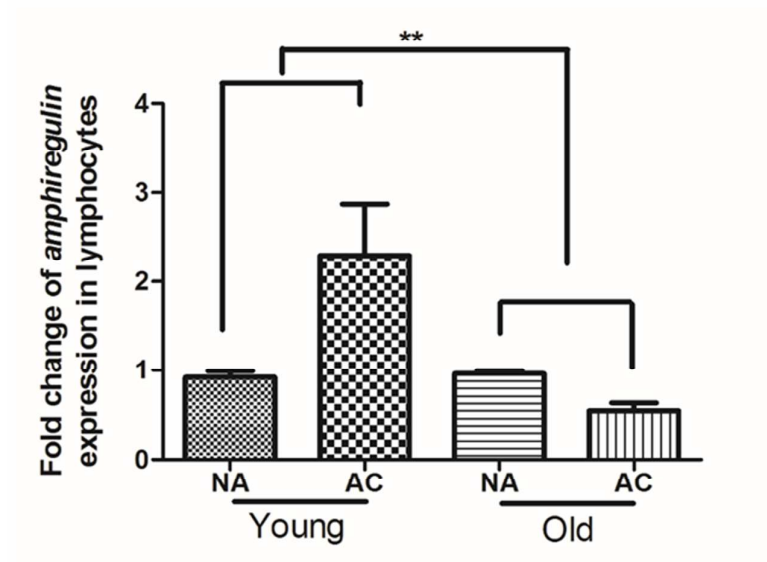
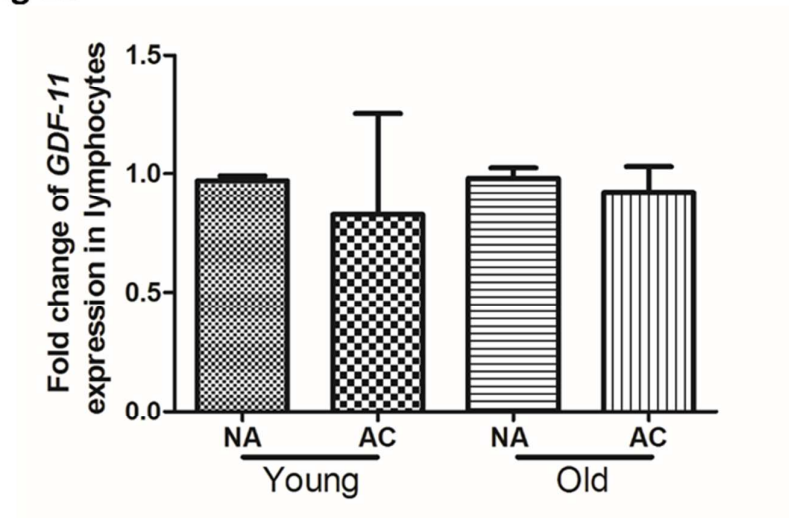
**Fig 2A****Fig 2B**

Fig 3A

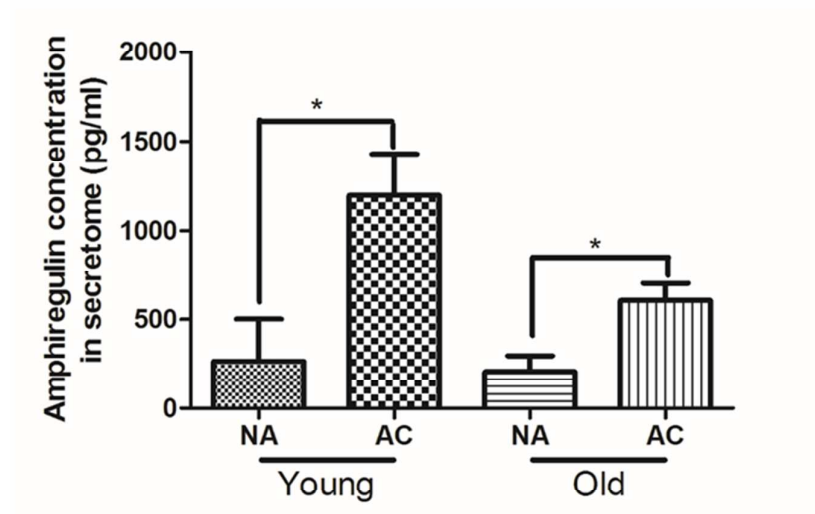


Fig 3B

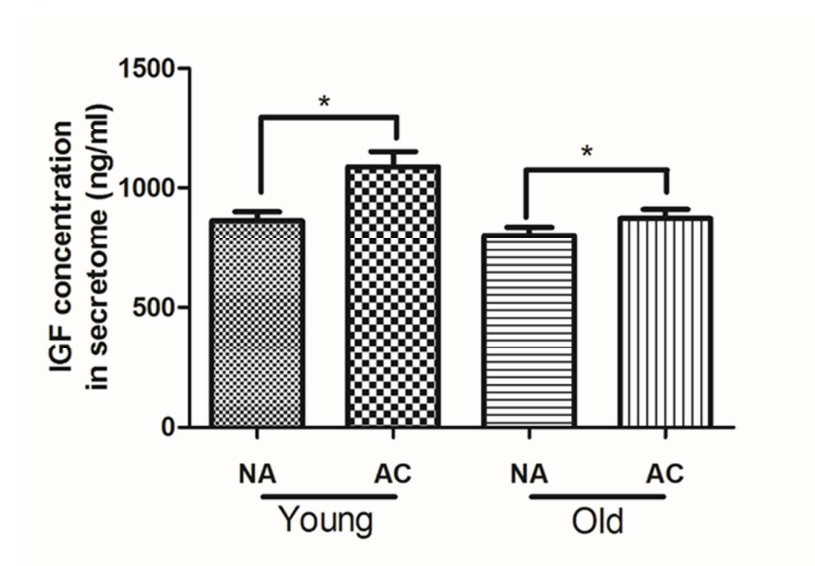


Fig 4A

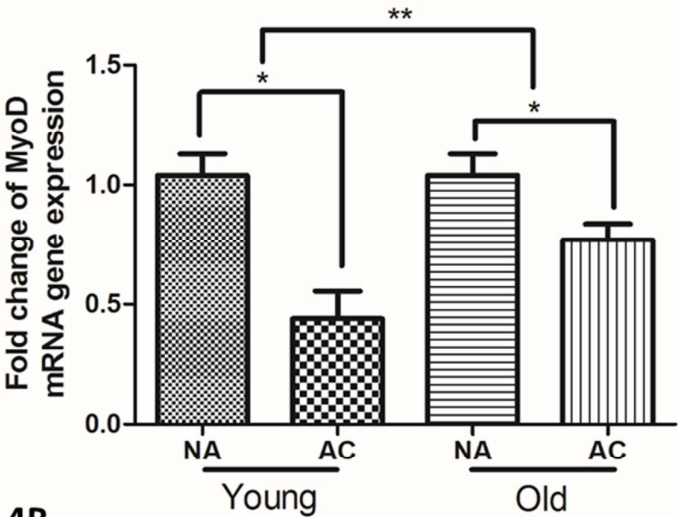


Fig 4B

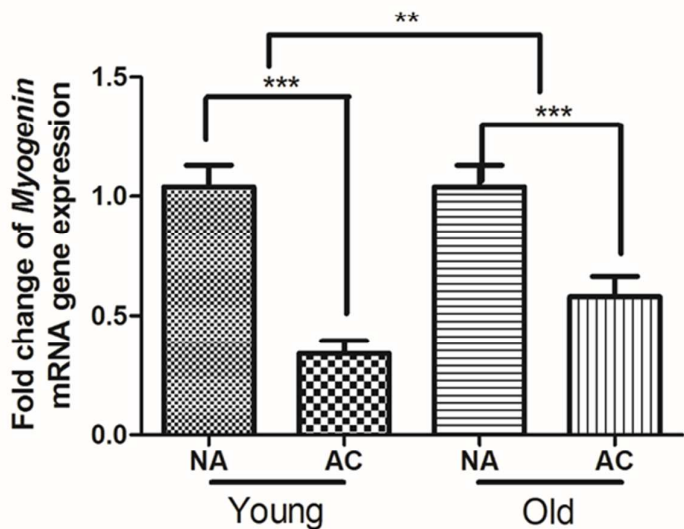


Fig 5A

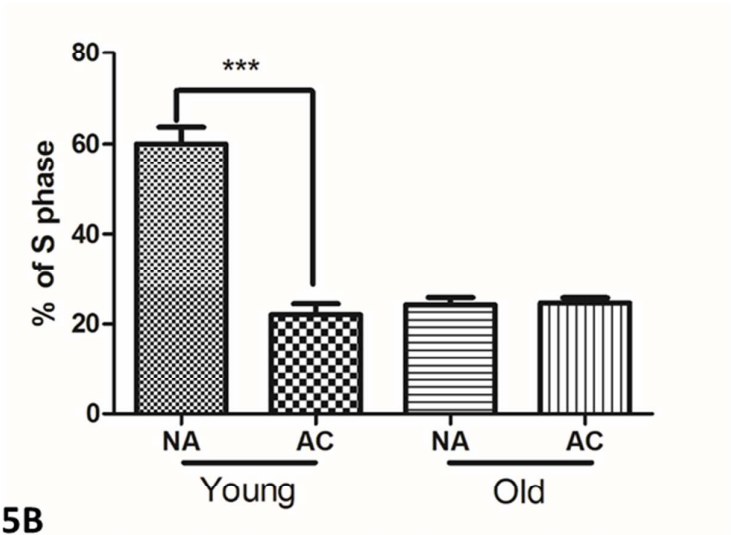
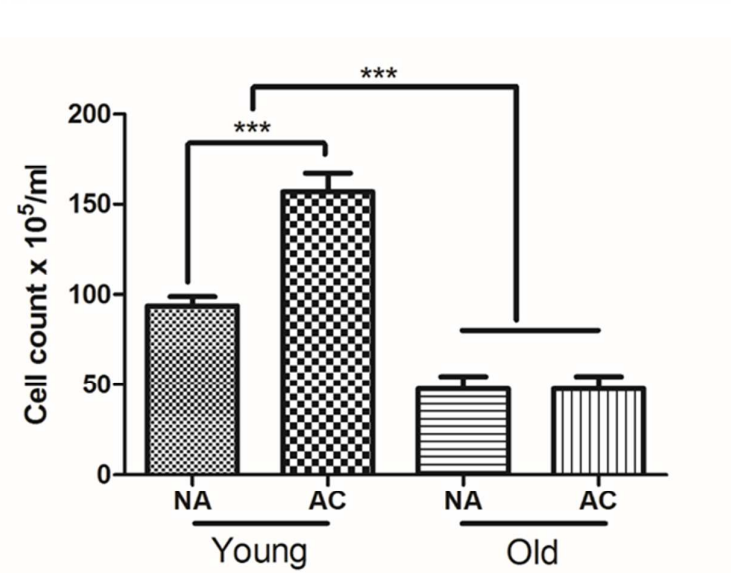


Fig 5B



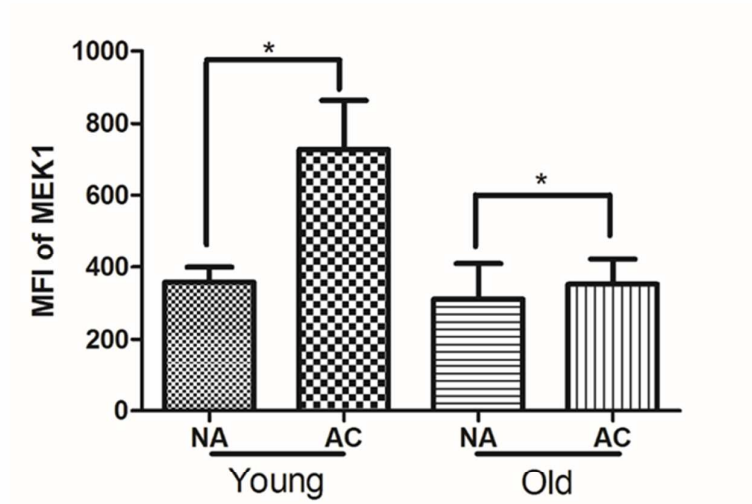
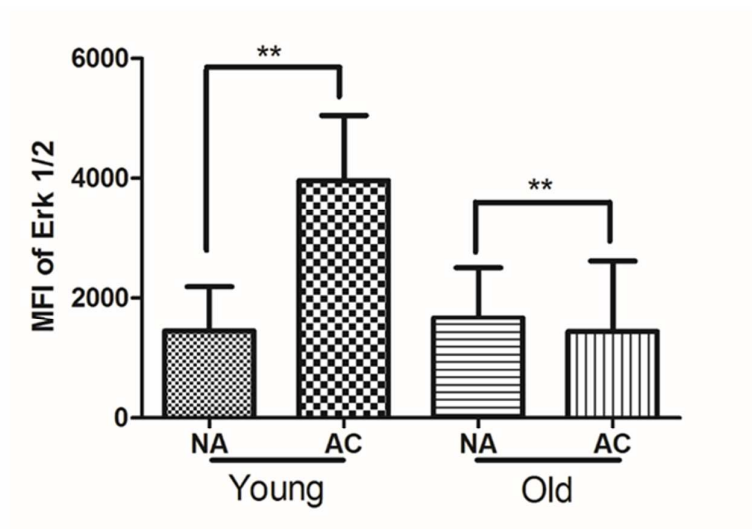
**Fig 6A****Fig 6B**

Fig 7A

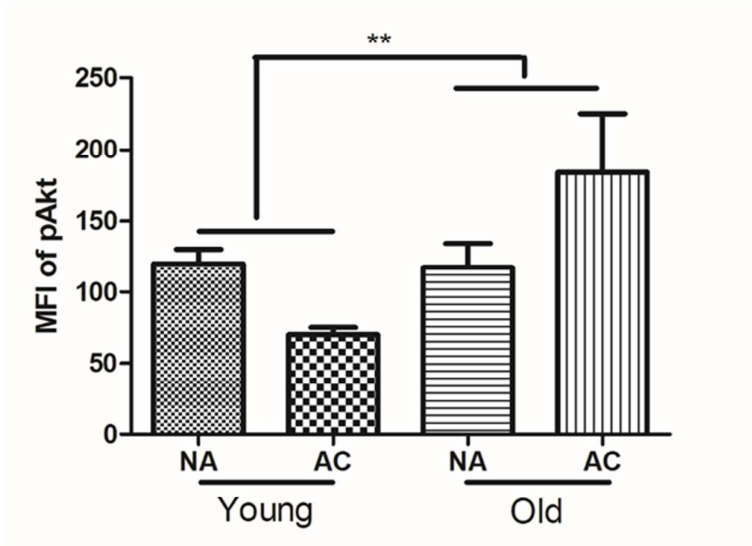
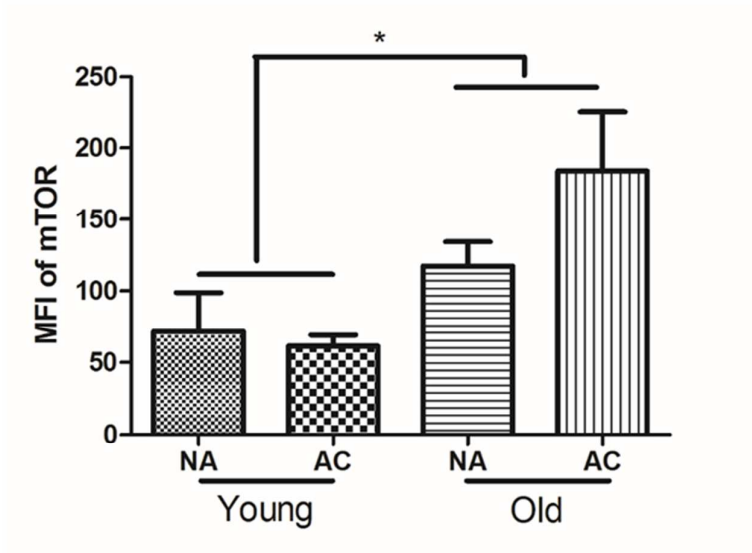


Fig 7B





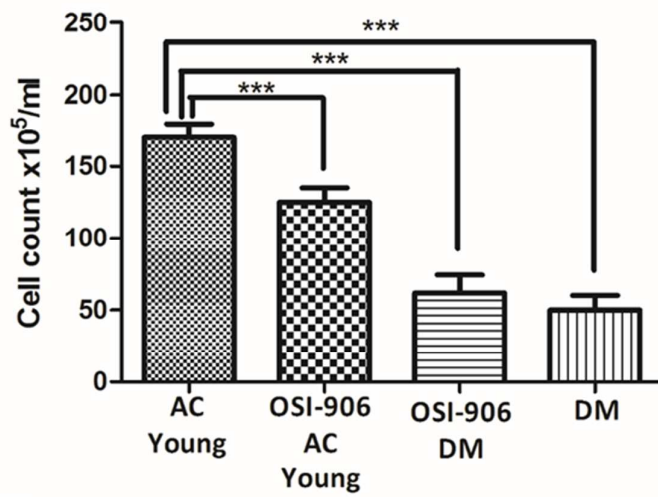
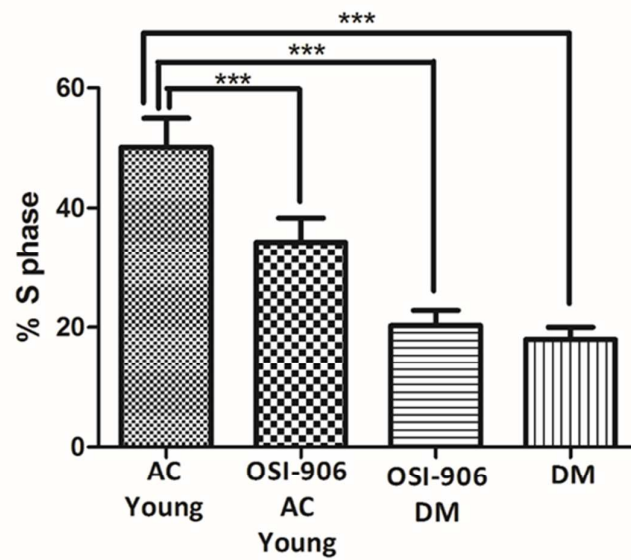
**Fig 8A****Fig 8B**

Fig 9A

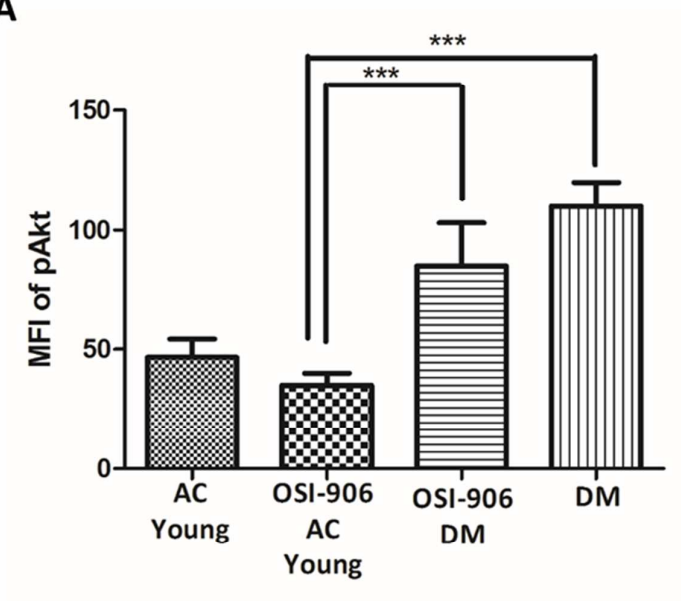


Fig 9B

